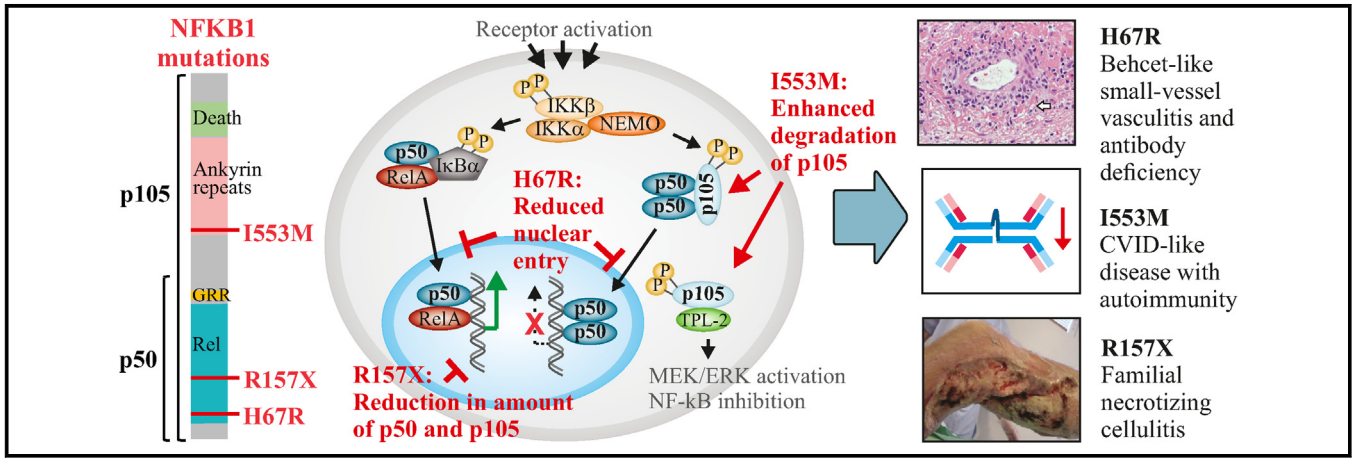
Damaging heterozygous mutations in *NFKB1* lead to diverse immunologic phenotypes

Meri Kaustio, MSc,[a]* Emma Haapaniemi, MD, PhD,[b,c]* Helka Göös, MSc,[d]* Timo Hautala, MD, PhD,[e] Giljun Park, PhD,[f] Jaana Syrjänen, MD, PhD,[g] Elisabet Einarsdottir, PhD,[b,c,t] Biswajyoti Sahu, PhD,[h] Sanna Kilpinen, MD, PhD,[i] Samuli Rounioja, MD, PhD,[j,k] Christopher L. Fogarty, MSc,[b,l,m] Virpi Glumoff, PhD,[n] Petri Kulmala, MD, PhD,[n,o] Shintaro Katayama, PhD,[c] Fitsum Tamene, MSc,[d] Luca Trotta, MSc,[a] Ekaterina Morgunova, PhD,[c] Kaarel Krjutškov, PhD,[b,c,p] Katariina Nurmi, PhD,[q] Kari Eklund, MD, PhD,[q] Anssi Lagerstedt, MD, PhD,[j] Merja Helminen, MD, PhD,[k] Timi Martelius, MD, PhD,[r] Satu Mustjoki, MD, PhD,[f,s] Jussi Taipale, PhD,[c] Janna Saarela, MD, PhD,[a]‡ Juha Kere, MD, PhD,[b,c,t]‡ Markku Varjosalo, PhD,[d]‡ and Mikko Seppänen, MD, PhD[r,u]‡

Helsinki, Oulu, Tampere, and Jyväskylä, Finland; Stockholm, Sweden; and Tartu, Estonia

GRAPHICAL ABSTRACT



Background: The nuclear factor κ light-chain enhancer of activated B cells (NF-κB) signaling pathway is a key regulator of immune responses. Accordingly, mutations in several NF-κB pathway genes cause immunodeficiency.

Objective: We sought to identify the cause of disease in 3 unrelated Finnish kindreds with variable symptoms of immunodeficiency and autoinflammation.

Methods: We applied genetic linkage analysis and next-generation sequencing and functional analyses of *NFKB1* and its mutated alleles.

Results: In all affected subjects we detected novel heterozygous variants in *NFKB1*, encoding for p50/p105. Symptoms in variant carriers differed depending on the mutation. Patients harboring a p.I553M variant presented with antibody deficiency, infection susceptibility, and multiorgan autoimmunity. Patients with a p.H67R substitution had antibody deficiency and experienced autoinflammatory episodes, including aphthae, gastrointestinal disease, febrile attacks, and small-vessel vasculitis characteristic of Behçet disease. Patients with a p.R157X stop-gain experienced hyperinflammatory responses to surgery and showed enhanced inflammasome activation. In functional analyses the p.R157X variant caused proteasome-dependent degradation of both the truncated and wild-type proteins, leading to a dramatic loss of p50/p105. The p.H67R variant reduced nuclear entry of p50 and showed decreased transcriptional activity in luciferase reporter assays. The p.I553M mutation in turn showed no change in p50 function but exhibited reduced p105 phosphorylation and stability. Affinity purification mass spectrometry also demonstrated that both missense variants led to altered protein-protein interactions.

Conclusion: Our findings broaden the scope of phenotypes caused by mutations in *NFKB1* and suggest that a subset of

From [a]the Institute for Molecular Medicine Finland, [d]the Institute of Biotechnology, [f]Hematology Research Unit Helsinki, Department of Clinical Chemistry and Hematology, [h]the Research Programs Unit, Genome-scale Biology Program, [m]the Research Programs Unit, Diabetes and Obesity, and [t]the Research Programs Unit, Molecular Neurology, University of Helsinki; [b]the Folkhälsan Institute of Genetics, Helsinki; [c]the Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm; [e]the Department of Internal Medicine, Oulu University Hospital; [g]the Department of Internal Medicine, [j]Fimlab Laboratories, and [k]the Tampere Center for Child Health Research, Tampere University Hospital; [i]the Department of Internal Medicine, Jyväskylä Central Hospital; [l]Abdominal Center Nephrology, [q]the Department of Rheumatology, [r]the Adult Immunodeficiency Unit, Infectious Diseases, Inflammation Center, and [u]the Rare Diseases Center, Children's Hospital, University of Helsinki and Helsinki University Hospital; [n]the Research Unit of Biomedicine, University of Oulu; [o]the Research Unit for Pediatrics, Pediatric Neurology, Pediatric Surgery, Child Psychiatry, Dermatology, Clinical Genetics, Obstetrics and Gynecology, Otorhinolaryngology and Ophthalmology (PEDEGO) and MRC Oulu, Oulu University Hospital and University of Oulu; [p]the Competence Centre on Health Technologies, Tartu; and [s]the Comprehensive Cancer Center, Helsinki University Central Hospital.

*These authors contributed equally to this work.

‡These authors contributed equally to this work.

autoinflammatory diseases, such as Behçet disease, can be caused by rare monogenic variants in genes of the NF-κB pathway. (J Allergy Clin Immunol 2017;140:782-96.)

Key words: *Nuclear factor κ light-chain enhancer of activated B cells, hypogammaglobulinemia, autoinflammation, Behçet disease,* NFKB1*, p50, p105, B cell*

The nuclear factor κ light-chain enhancer of activated B cells (NF-κB) pathway regulates many cellular processes, such as proliferation, apoptosis, stress responses, inflammation, ectodermal development, and immune responses.[1,2] As such, NF-κB signaling plays a key role in inflammatory diseases, and mutations in several NF-κB components cause primary immunodeficiency or ectodermal dysplasia.[3-11]

The NF-κB transcription factor family consists of 5 Rel proteins, p50/p105, p52/p100, RelA, RelB, and c-Rel, which dimerize with each other and drive or inhibit gene expression in the nucleus.[12] The canonical NF-κB pathway is triggered by microbial products or the cytokines IL-1β and TNF and progresses through phosphorylation-dependent degradation of NF-κB inhibitor α (IκBα). IκBα phosphorylation is mediated by the inhibitor of κB kinase (IKK) complex, which includes IKKα and IKKβ and the regulatory protein NF-κB essential modulator (NEMO). This releases RelA- and c-Rel–containing dimers to enter the nucleus and drive transcription of proinflammatory genes.[13]

NFKB1 encodes for p105, which is processed by the proteasome to generate the p50 transcription factor. p50 can heterodimerize with RelA or c-Rel and activate canonical NF-κB signaling or form homodimers that function as repressors of proinflammatory gene expression.[14,15] The full-length p105 inhibits NF-κB signaling by binding to and inhibiting nuclear entry of RelA, c-Rel, and p50 through ankyrin repeats in the C-terminal half of the protein.[16,17]

Recently, haploinsufficiency of p50 was shown to cause antibody deficiency.[3] Common variants in *NFKB1* also associate with inflammatory bowel disease and Behçet disease, a vasculitis of largely unknown cause characterized by recurrent oral and genital aphthous ulcers, uveitis, and skin lesions.[18-22] Here we describe heterozygous *NFKB1* mutations (H67R, p.R157X, and I553M) in 3 Finnish kindreds that variably display dominantly segregating antibody deficiency, recurrent infections, and autoinflammatory features, including Behçet-like disease and hyperinflammatory reactions. Our results show that disease can ensue from dysregulation of NF-κB signaling caused by mutations affecting either p50 or p105.

Abbreviations used
IκBα: NF-κB inhibitor α
IKK: Inhibitor of κB kinase
NEMO: NF-κB essential modulator
NF-κB: Nuclear factor κ light-chain enhancer of activated B cells
NLRP3: NLR family pyrin domain containing 3
WB: Western blotting
WT: Wild-type

METHODS

Study participants

The study was conducted in accordance with the principles of the Helsinki Declaration and was approved by the Helsinki University Central Hospital Ethics Committee. Written informed consent was obtained from patients and healthy control subjects.

DNA extraction and genetic analysis

Genomic DNA was extracted from EDTA blood samples by using the Qiagen FlexiGene DNA kit (Qiagen, Hilden, Germany). HLA-B*51 typing was performed in an accredited (European Federation for Immunogenetics) histocompatibility testing laboratory, the Finnish Red Cross Blood Service.

Supported by the Academy of Finland (nos. 288475 and 294173), the Sigrid Juselius Foundation, the Emil Aaltonen Foundation, the Finnish Medical Foundation, the Jane and Aatos Erkko Foundation, the Alma and K.A. Snellman Foundation, Biocentrum Helsinki, the Instrumentarium Science Foundation, the Finnish Cancer Institute, the Foundation for Pediatric Research, the Biomedicum Helsinki Foundation, the Integrative Life Science Doctoral Program (ILS) and Doctoral Programme in Biomedicine of the University of Helsinki, Karolinska Institutet Distinguished Professor Award, the Strategic Research Program for Diabetes Funding at Karolinska Institutet, and the Swedish Research Council.

Disclosure of potential conflict of interest: M. Kaustio has received a grant from Biomedicum Helsinki Foundation and travel support from Keystone Symposia. J. Syrjänen has consultant arrangements with Merck Sharp & Dohme, BMS, and Gilead; has received payment for lectures from Gilead, GlaxoSmithKline, Merck Sharp & Dohme, and UCB Pharma; has received royalties from the Finnish Medical Society Duodecim; has received payment for educational presentations from Kandidattikustannus Oy; and has received travel support from Gilead, Octapharma, Merck Sharp & Dohme, and GlaxoSmithKline. P. Kulmala is employed by the University of Oulu and Oulu University Hospital and has received grants from the Pediatric Research Foundation and the Alma and K.A. Snellman Foundation. K. Krjutškov is employed by Competence Centre on Health Technologies. K. Nurmi is employed by the Helsinki Rheumatic Diseases and Inflammation Research Group. K. Eklund has consultant arrangements with Abbvie and Pfizer; has received a grant from Pfizer; has received payment for lectures from Novartis, BMS, and Pfizer; and has received travel support from BMS. A. Lagerstedt is employed by Fimlab Laboraties and Tampere University Hospital. T. Martelius has received a grant from Sanquin, has received payment for lectures from Merck Sharp and Dohme and CSL Behring, and has received travel support from Octapharma, CSL Behring, Sanquin, Gilead, and Merck Sharp and Dohme. S. Mustjoki has received grants from the Instrumentarium Science Foundation, Academy of Finland, Sigrid Juselius Foundation, the Finnish Cancer Institute, Pfizer, Ariad, BMS, and Novartis; has consultant arrangements with Novartis, BMS, and Pfizer; and has received payment for lectures from BMS, Novartis, and Pfizer. J. Saarela has received a grant from Sanofi/Genzyme, has received payment for lectures from Roche, and has received travel support from Sanofi/Genzyme. J. Kere has received grants from the Academy of Finland (nos. 288475 and 294173), the Sigrid Juselius Foundation, the Emil Aaltonen Foundation, the Finnish Medical Foundation, the Jane and Aatos Erkko Foundation, the Alma and K.A. Snellman Foundation, Biocentrum Helsinki, the Instrumentarium Science Foundation, the Finnish Cancer Institute, the Foundation for Pediatric Research, the Biomedicum Helsinki Foundation, the Integrative Life Science Doctoral Program (ILS) and Doctoral Programme in Biomedicine of University of Helsinki, a Karolinska Institutet Distinguished Professor Award, the Strategic Research Program for Diabetes funding at Karolinska Institutet, and the Swedish Research Council. M. Seppänen has received a grant from the Finnish Medical Foundation, has received travel support from CSL Behring and Octapharma, and has received payment for lectures from Octapharma. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication April 11, 2016; Revised September 2, 2016; Accepted for publication October 7, 2016.

Available online January 21, 2017.

Corresponding author: Juha Kere, MD, PhD, Department of Biosciences and Nutrition, Novum, Hälsovägen 7, SE-141 83 Huddinge, Sweden. E-mail: juha.kere@ki.se.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2016.10.054

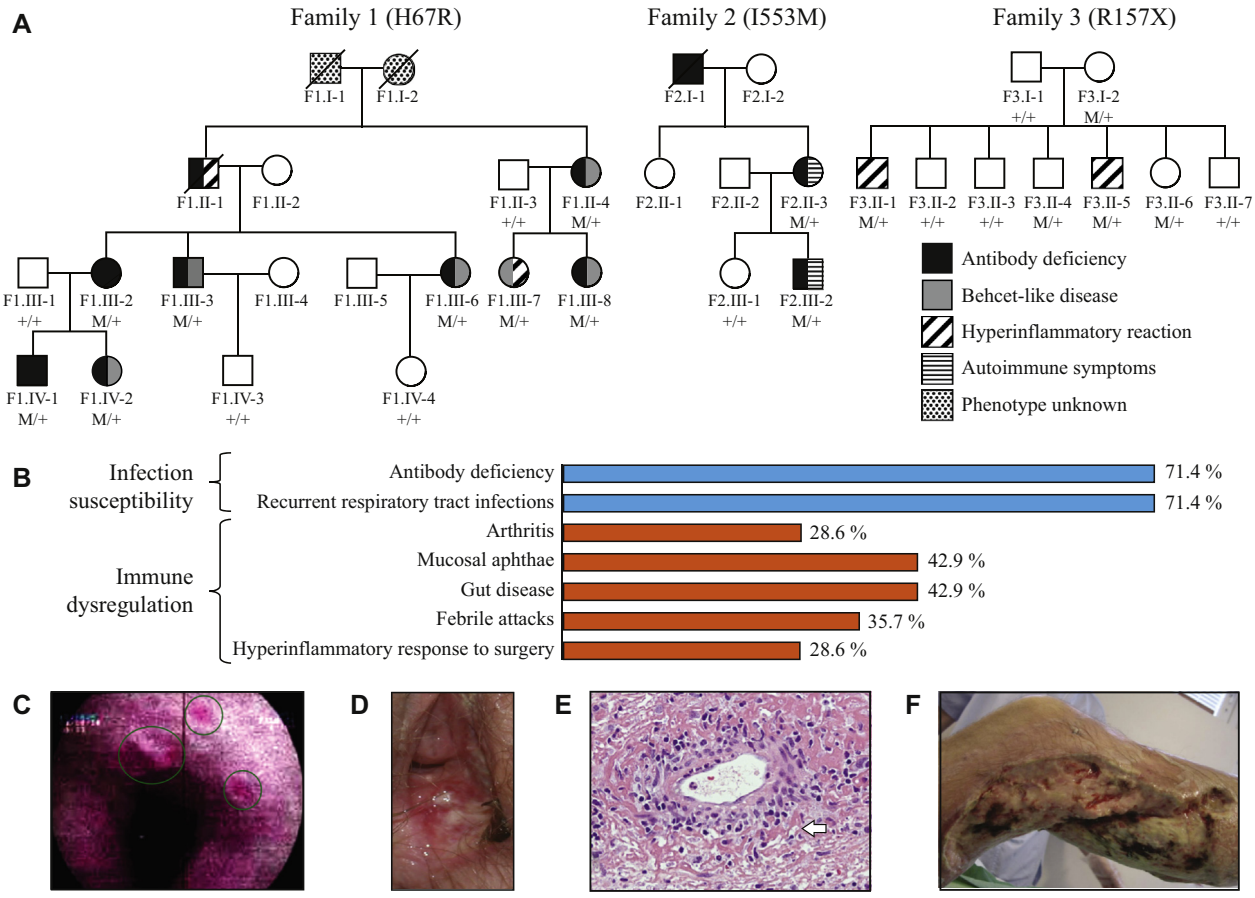


FIG 1. Phenotypic characteristics of families with newly identified *NFKB1* mutations. **A,** Pedigrees of the studied families with *NFKB1* mutations. Patients with clinical diagnosis are highlighted. *Subject with a low IgG level but without a clinical diagnosis potentially caused by young age. +/+, Absence of mutation; *M*/+, presence of the familial *NFKB1* mutation. **B,** Summary of common clinical findings in the 3 families with *NFKB1* mutations. **C,** Multiple esophageal aphthae *(circled)* in patient F1.III-3. **D** and **E,** Genital aphthae (Fig 1, *D*) and arteriole (Fig 1, *E*) with fibrinoid necrosis and lymphocytic infiltrate, suggesting small-vessel vasculitis *(white arrow)* in patient F1.III-8. Pictures were provided by the Dermatology and Venereology Outpatient Clinic, University Hospital of Tampere. **F,** Tissue damage in patient F3.II-1 after inflammatory reaction to surgery.

Generation and processing of sequencing libraries, read mapping, variant calling, genome annotation, and variant filtering, as well as genotyping and linkage analysis, are described elsewhere[23] or in the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org. Mutations and their expression were verified from genomic and cDNA by means of capillary sequencing. Primers (Sigma-Aldrich, St Louis, Mo) are listed in Table E1 in this article's Online Repository at www.jacionline.org.

Computational modeling

Modeling of mutant amino acid residue effects was performed with the Coot single-mutation option (version 0.8.1, http://scripts.iucr.org/cgi-bin/paper?S0907444904019158)[24] and PyMol (Schrödinger). Crystallographic images were downloaded from the RCSB Protein Data Bank (www.rcsb.org).[25]

Experiments on patient-derived cells

PBMCs were isolated from patients' blood by using Ficoll-Paque gradient centrifugation. Whole protein was extracted by means of cell lysis in RIPA buffer, and expression of p50/p105 and RelA was detected by means of Western blotting (WB) with anti-p50/p105 and RelA antibodies (nos. 3035 and 8242, respectively; Cell Signaling Technology, Danvers, Mass). Glyceraldehyde-3-phosphate dehydrogenase antibody (no. 258; Thermo Fisher Scientific, Waltham, Mass) was used as a loading control. IRDye 800CW and 680LT secondary antibodies were used for signal detection with the Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, Neb). Lymphocyte immunophenotyping, inflammasome activation, and ELISAs are described in the Methods section in this article's Online Repository.

Generation of mutation constructs and cell lines

The wild-type (WT), H67R, and I553M mutant *NFKB1* full-length coding sequences were commercially cloned into a Gateway-compatible entry-vector (GenScript, Piscataway, NJ). R157X and A156S mutations were generated by using PCR from the WT template. For transient and stable expression, constructs were subcloned into the pcDNA-DEST40 expression Vector (Thermo Fisher Scientific) and pTO_HA_StrepIII_N_GW_FRT (SH-tag) or pTO_Myc_BirA_N_GW_FRT (BioID) vectors, respectively.[26] Constructs were transfected into Flp-In T-REx 293 cells to generate tetracycline-inducible isogenic cell lines (Life Technologies, Grand Island, NY).[27] Construct expression on tetracycline induction (2 μg/mL) was confirmed by means of mass spectrometry and WB with anti-p50/p105 and anti-hemagglutinin (HA.11; Covance, Princeton, NJ) antibodies. α-Tubulin antibody (ab7291; Abcam, Cambridge, United Kingdom) was used as a loading control.

Proteasome inhibition

Tetracycline-induced (2 μg/mL for 20 hours) WT and R157X-p50/p105-Flp-In T-REx 293 cells were treated with the proteasome inhibitor MG132 (474790; Merck Millipore, Billerica, Mass) for 4 hours in 0, 5, 10, or 20 μmol/L concentrations. Cells were lysed, and p50/p105 levels were assessed by using WB.

Luciferase reporter assays

Tetracycline-treated p50/p105-Flp-In cells were transfected (on 96-well plates in triplicate) with a p50:RelA-responsive firefly luciferase reporter plasmid[28] and a constitutively expressed pRL-TK *Renilla* luciferase control reporter using FuGENE HD (Promega, Madison, Wis). For transient transfections, HEK293 cells were cotransfected with the reporters and pcDNA-DEST40-p50/p105 vectors. Signaling was activated with TNF (10 or 25 ng/mL for 16 hours). Luminescence was measured with the Dual-Glo Luciferase Assay System (Promega) and PHERAstar Plate Reader (BMG Labtech, Ortenberg, Germany). The NF-κB/control plasmid signal ratio was calculated to account for differences in cell numbers, and these data were normalized to the uninduced WT sample. Results were pooled from 2 to 4 repeats of each assay. The *t* test was used to calculate *P* values.

Immunofluorescence assay

The p50/p105-Flp-In cell lines were seeded on coverslips coated with rat-tail collagen (Thermo Fisher Scientific). Tetracycline-treated cells were induced with TNF (10 or 25 ng/mL for 40 minutes), fixed in paraformaldehyde, and blocked with 1% BSA-PBS (Sigma-Aldrich). The HA.11 antibody was used for detection of tagged p50/p105 with anti-mouse Alexa Fluor 488 (A-11029, Life Technologies) secondary antibody. Nuclei were stained with 4′-6-diamidino-2-phenylindole (D9542-5MG, Sigma-Aldrich). Imaging was performed at ×40 magnification with the Axio Imager.Z2 (Zeiss, Oberkochen, Germany) and the Nuance multispectral imaging system FX (PerkinElmer, Waltham, Mass). ImageJ software (National Institutes of Health, Bethesda, Md) was used for quantification of signal intensities.[29] Three hundred to 600 cells per condition were analyzed.

Affinity purification, BioID, mass spectrometry, and protein quantification

For each single-step Strep-tag affinity purification and BioID[30] experiment, approximately 5×10^7 cells (2 biological and technical replicates) from the p50/p105-Flp-In cells were induced with tetracycline (2 μg/mL for 24 hours) with or without TNF (10 ng/mL for 90 minutes). Cysteine bonds were reduced with 5 mmol/L Tris(2-carboxyethyl)phosphine and alkylated with 10 mmol/L iodoacetamide, and proteins were digested to peptides with sequencing-grade trypsin (Promega). Peptides were purified with C-18 MicroSpin Columns (The Nest Group, Southborough, Mass). After vacuum concentration, dried samples were dissolved in 30 μL of buffer A.[27] Mass spectrometric analyses of samples were performed with a 60-minute linear gradient on an Orbitrap Elite ETD Hybrid Mass Spectrometer coupled to the EASY-nLC II System (Thermo Scientific). Proteins were identified and quantified by using the Andromeda search engine and MaxQuant proteomics software.[31,32] Raw data were searched against the human component of the UniProtKB-database (release 2014_11). Results were filtered to a maximum false discovery rate of 0.05. For phosphopeptide identification, phosphorylation (Ser, Thr, and Tyr) was included as a variable modification in MaxQuant/Andromeda. Filtering parameters for 2 accepted phosphopeptides (QMGYTEAIEVIQAASSPVK modified at serine 893 and TTSQAHSLPLSPASTR with 1 or 2 phosphorylations at serines 907 and/or 903) and a more detailed description of the analyses are found in the Methods section in this article's Online Repository.

RESULTS

Identification of *NFKB1* variants in 3 families with immunodeficiency and autoinflammation

We studied a cohort of unsolved primary immunodeficiency cases representing a broad spectrum of immunologic phenotypes with next-generation sequencing methods to identify the causal genes. Independent genetic analyses revealed novel heterozygous *NFKB1* variants as the most likely cause of disease in 3 families from this cohort.

Family 1 included 9 affected subjects who presented with recurrent respiratory tract infections and progressive B-cell dysfunction marked by hypogammaglobulinemia, poor antibody response to anti-pneumococcal vaccines, or decreased switched memory B-cell counts (Fig 1, *A* and *B*; Table I; and see Tables E2-E5 in this article's Online Repository at www.jacionline.org). Recurrent episodes of aphthous mucositis in the upper gastrointestinal and genital areas, which were sometimes accompanied by abdominal pain, monoarthritis, or fever with increased inflammatory markers (peripheral blood leukocytes, $>10 \times 10^6$ cells/mL; C-reactive protein, >100 mg/L), were frequent (Fig 1, *C* and *D*, and see Fig E2 in this article's Online Repository at www.jacionline.org). Lesional biopsy specimens from 2 family members revealed lymphocytic small-vessel vasculitis (Fig 1, *E*), supporting a clinical diagnosis of Behçet disease. In 2 affected family members routine surgical procedures led to a hyperinflammatory state consisting of fever and excessive inflammation in the wound area.

We performed genotyping and linkage analysis on 10 subjects from this family to identify regions of interest, followed by whole-genome sequencing of 2 subjects (F1.III-3 and F1.III-8) to pinpoint the causative variant. Nonparametric linkage (NPL) analysis revealed no statistically significant linkage peaks, but NPL scores of 1.78 to 1.80 were observed on 5 loci on chromosomes 2, 4, 5, 10, and 19 (see Fig E3 in this article's Online Repository at www.jacionline.org). Haplotype analysis within the 2 largest peaks identified a 15.4-Mb haplotype between the single nucleotide polymorphisms rs6811317 and rs4403120 on chromosome 4 and a large haplotype from single nucleotide polymorphism rs1171096 to the telomere on 19q cosegregating with the phenotype. In these regions only 1 novel and heterozygous variant negatively affecting a conserved residue was identified at residue 67 in the DNA-binding domain of p50 (*NFKB1*, NM_003998: c.A667G/p.H67R; Fig 2). The histidine residue in the WT protein is predicted to interact with DNA through a water molecule, and its transition to arginine likely abolishes this interaction. Sanger sequencing verified the presence of the mutation in all affected subjects in the family (see Fig E4 in this article's Online Repository at www.jacionline.org).

In family 2 mother and son (F2.II-3 and F2.III-2 in Fig 1) presented with recurrent respiratory tract and other severe infections, hypogammaglobulinemia, and poor antibody responses to polysaccharide vaccines. Both patients had inflammatory gastrointestinal disease, and the mother experienced multiorgan autoimmunity with thyroiditis, enteropathy, spondyloarthropathy, and urticaria. The mother's father had succumbed to similar symptoms before the study. We performed whole-exome sequencing on patient F2.II-3 and filtered the data for novel heterozygous variants affecting conserved residues. Of 10 surviving variants (see Table E6 in this article's Online Repository at www.jacionline.org), a variant in *NFKB1* (NM_003998: c.C1659G/p.I553M) was the most attractive based on known protein functions.[33,34] The variant localizes to the ankyrin repeat region at the C-terminal half of p105 and was predicted to affect its posttranslational processing and protein-protein interactions (Fig 2). Sanger sequencing confirmed the presence of the variant in the affected son of the proband.

TABLE I. Clinical characteristics of study participants

	F1.II-1	F1.II-4	F1.III-2	F1.III-3	F1.III-6	F1.III-7
Current age (y)	Deceased at age 39	55	40	37	30	29
Sex	M	F	F	M	F	F
Mutation status	NA	H67R	H67R	H67R	H67R	H67R
Infection susceptibility						
Infections	URTI	URTI	—	URTI	URTI	URTI
Antibody deficiency	ND	IgG hypogammaglobulinemia SAD (dg at age 44 y)	IgG subclass deficiency SAD (dg at age 25 y)	IgG hypogamm-aglobulinemia SAD (dg at age 26 y)	IgG subclass deficiency SAD (dg at age 25 y)	—
Switched memory B cells* (reference, 6.5-29.2)	—	5↓	2.3↓	1.9↓	2.3↓	3.5↓
Immunoglobulin replacement therapy	−	+	−	+	+	−
Immune dysregulation						
Febrile attacks	+	−	−	+	−	+
Complex aphthae	ND	Mouth, genitalia	—	Esophagus	Mouth	Mouth, genitalia
Arthritis	—	Recurrent monoarthritis	—	—	—	Recurrent monoarthritis
Gut disease	ND	Periodic abdominal pain, chronic idiopathic diarrhea	—	—	—	Periodic abdominal pain
Other	ERCP pancreatitis	—	Benign kidney tumor	Rudimentary left kidney	—	Keratitis, hyper-inflammatory response to tooth excision

dg, Diagnosis; *F*, female; *M*, male; *ND*, not detected; *RTI*, respiratory tract infections, including recurrent pneumonia; *SAD*, specific antibody deficiency; *URTI*, upper respiratory tract infections.
*$CD27^+IgD^-IgM^-$ shown as a percentage of total B-cell count. See the Methods section in this article's Online Repository for detailed laboratory values.

Two affected brothers in family 3 (F3.II-1 and F3.II-5) had postoperative deep necrotizing cellulitis with abscesses, fever, neutrophilia, and increased inflammatory markers that required prolonged intensive care and multiple surgical revisions (Fig 1, *A*, *B*, and *F*, and see Fig E2). No pathogenic bacteria could be cultured from the affected sites. Although these patients' switched memory B-cell counts were less than the reference range, they had normal immunoglobulin levels and did not show increased susceptibility to infection. Analysis of their whole-exome sequencing data identified a novel stop-gain variant in *NFKB1* (NM_003998: c.C936T/p.R157X). Targeted Sanger sequencing detected this variant also in 2 asymptomatic siblings and their mother.

Because of Behçet-like symptoms in family 1, all symptomatic *NFKB1* mutation carriers were genotyped for the Behçet syndrome–associated HLA B*51:01 allele.[22] Several subjects in family 1 (F1.III-2, F1.IV-1, F1.IV-2, F1.III-3, and F1.III-8) were found to carry this allele, but carriership did not cosegregate with Behçet-like symptoms, leaving the significance of this allele to the phenotype unclear.

Immunologic findings of the *NFKB1* mutation carriers

The participants underwent extensive blood immunophenotyping, which showed rather wide interindividual variation (see Tables E2-E5). However, most patients exhibited chronic mild leukocytosis (9-10 × 10^9/L), with the affected p.R157X carriers presenting with very high counts (50-60 × 10^9/L, with the increase being mostly neutrophils) during inflammatory episodes. Patients from the first 2 families (p.H67R and p.I553M) were hypogammaglobulinemic or presented with IgG subclass deficiency; additionally, 8 of 9 tested subjects had nonprotective responses to pneumococcal polysaccharide. Most patients, even the p.R157X carriers lacking clinical antibody deficiency, had low switched memory B-cell counts, with a corresponding increase in naive B-cell counts. On examination of T-cell subsets, we noted low $CD4^+$ effector memory T-cell counts and effector memory RA T-cell counts ($CCR7^-CD45RA^-$ and $CCR7^-CD45RA^+$, respectively), as well as relatively low T_H17 memory subsets ($CCR6^+CXCR3^-CD45RA^-$) in several patients. In addition, some (4/8) of the tested patients had moderately increased IFN-γ and TNF secretion on T-cell stimulation.

The R157X mutant causes depletion of p50/p105 and excessive production of IL-1β

The effect of *NFKB1* mutations on p50/p105 expression was estimated by using WB of PBMC-derived protein extracts from patients and age/sex-matched control subjects. Protein amounts in patients with missense mutations were comparable with those in control subjects, but patients with the R157X mutation showed significant depletion of p50, p105, and RelA (Fig 3, *A*, and see Fig E5, *A* and *B*, in this article's Online Repository at

TABLE I. Continued

F1.III-8	F1.IV-1	F1.IV-2	F2.I-1	F2.II-3	F2.III-2	F3.II-1	F3.II-5
25	10	7	Deceased at age 78	61	32	62	56
F	M	F	M	F	M	M	M
H67R	H67R	H67R	NA	I553M	I553M	R157X	R157X
URTI	—	URTI	RTI	RTI	RTI	—	—
IgG hypogamm-aglobulinemia SAD (dg at age 13 y)	IgG hypogamm-aglobulinemia (dg at age 10 y)	Hypogamma-globulinemia (dg at age 3 y)	Hypogamm-aglobulinemia	Hypogamma-globulinemia SAD (dg at age 36 y)	SAD (dg at age 18 y)	—	—
8	1.7↓	1.7↓	—	9.9	2.6↓	5.4↓	3.8↓
+	−	−	−	+	−	−	−
+	−	+	−	−	−	−	−
Mouth, genitalia	—	Mouth, esophagus	—	—	—	—	—
Recurrent monoarthritis	—	—	—	Spondyloarthropathy oligoarthritis	—	—	—
Periodic abdominal pain, microscopic colitis	—	Periodic abdominal pain	—	Chronic idiopathic diarrhea	Coeliac disease	—	—
			—	Asthma, autoimmune hypothyroiditis	Asthma	Postoperative necrotizing cellulitis	Postoperative necrotizing cellulitis

www.jacionline.org). ELISA for active p50 and RelA on patients' PBMC-derived nuclear protein produced similar results (see Fig E5, *C* and *D*).

Because NF-κB is involved in regulation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome and excessive inflammasome activation is involved in the pathogenesis of several autoinflammatory diseases, we compared inflammasome activation in LPS-primed macrophages derived from patients and healthy control subjects. After ATP-induced inflammasome activation, macrophages from patients with the R157X mutation displayed significantly increased secretion of IL-1β, whereas those with the H67R mutation showed a trend toward decreased IL-1β secretion (Fig 3, *B*). In LPS-induced R157X macrophages, quantitative real-time RT-PCR revealed higher expression of pro–IL-1β compared with that seen in control subjects also at the mRNA level, whereas no difference in *NLRP3* expression was observed (see Fig E6 in this article's Online Repository at www.jacionline.org).

To study the effects of the mutations further, we constructed Flp-In T-REx 293 cell lines stably expressing WT or mutant p50/p105 under a tetracycline-inducible promoter. Equal expression of WT and missense constructs was confirmed by using WB (Fig 3, *C*), whereas expression of the truncated R157X mutant protein was only detected in transiently transfected cells after a long exposure time (see Fig E7 in this article's Online Repository at www.jacionline.org). Despite this, overexpression of this construct caused a marked reduction in endogenous p50 and p105, an effect alleviated by treatment with proteasome inhibitors (Fig 3, *D* and *E*, and see Fig E8 in this article's Online Repository at www.jacionline.org). In contrast, expression of the A156Sfs-NFKB1 mutant published by Fliegauf et al[3] affected only the level of endogenous p105 and not that of p50.

H67R and R157X mutants show reduced NF-κB activation

The p50/p105-Flp-In cell lines were transiently transfected with an NF-κB–responsive firefly luciferase reporter and a constitutively active *Renilla* luciferase control reporter to test the effect of the *NFKB1* mutations on NF-κB transcriptional activity and to control for cell viability, respectively. Reporter activities were measured after induction of signaling with TNF. Overexpression of the WT and I553M constructs increased NF-κB reporter activity comparably, whereas overexpression of the H67R mutant did not increase reporter activity to greater than the signal from the endogenous protein, indicating loss of function (Fig 3, *F*). As expected, overexpression of the R157X stop-gain mutant also did not increase signaling. Transient transfections of WT, H67R, and I553M expression constructs into HEK293 cells produced similar results but, interestingly, also decreased cell viability compared with the WT construct (see Fig E9 in this article's Online Repository at www.jacionline.org).

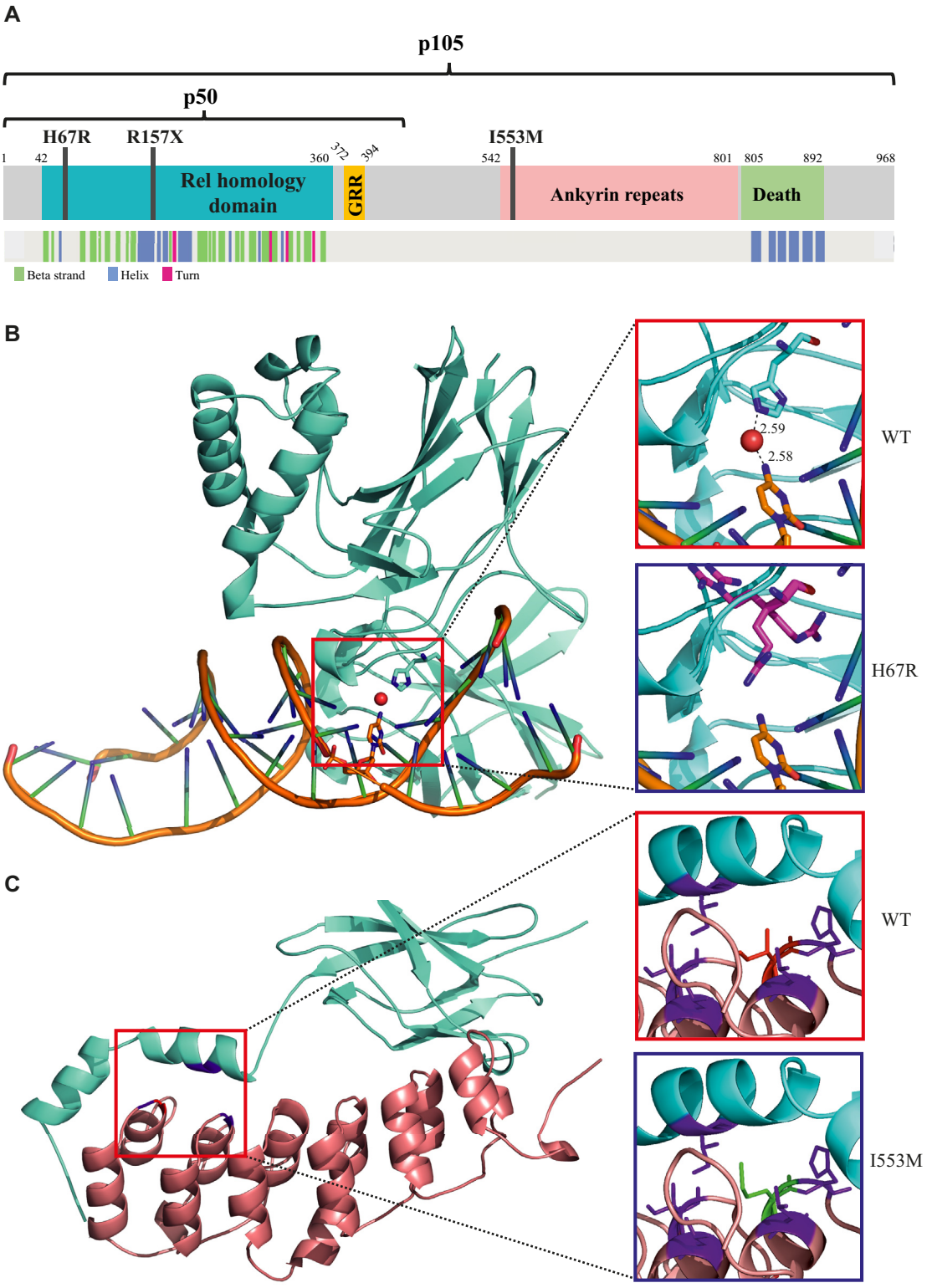


FIG 2. p50/p105 protein structure and structural representation of mutated sites. **A,** Schematic illustration of p50/p105 structure with corresponding protein domains *(up)* and the fold *(below)*. Mutated residues are shown with *vertical bars*. **B,** Crystallographic structure of p50 (figure based on Protein Data Bank entry 1SVC, residues 62-352). Normally, the H67 residue interacts with DNA through a water molecule *(red ball)*. The mutant 67R has 4 different conformations (shown in magenta) that affect the distance of the residue from DNA. One of them reaches DNA directly, removing the mediator water molecule. **C,** Crystallographic representation of p105 before cleavage (reconstructed from Protein Data Bank entries 1NFI and 1SVC). The I553M mutation (green) affects the hydrophobic contact surfaces of the ankyrin repeat domain (violet). For both Fig 2, *B* and *C*, a close up of the WT state is featured in the *red box*, and the mutated state is featured in the *blue box*.

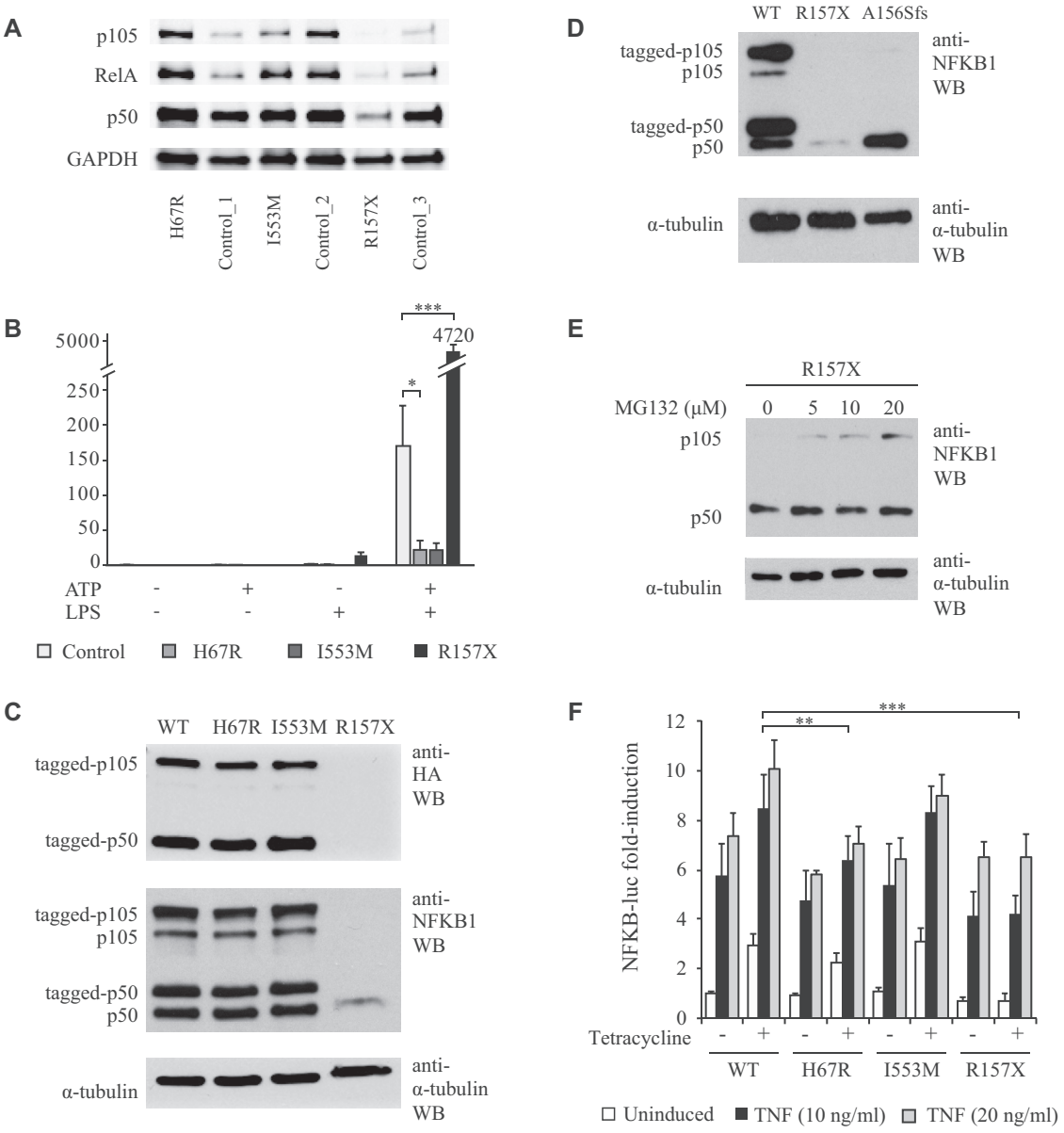


FIG 3. H67R, I553M and R157X mutations affect p50/p105 function. **A,** Expression of p50, p105, and RelA in patients' PBMCs was studied by using WB with anti-p50/p105 and anti-RelA antibodies. Samples are from patients F1.III-8 (H67R), F2.II-3 (I553M), and F3.II-5 (R157X) are shown. **B,** NLRP3 inflammasome activation was studied in unprimed or LPS-primed patient-derived macrophages activated with ATP. Results were pooled from 2 to 3 affected carriers per *NFKB1* mutation: F1.III-2, F1.III-7, and F1.III-8 for H67R; F2.II-3 and F2.III-2 for I553M; and F3.II-1 and F3.II-5 for R157X. **C** and **D,** Expression of p50 and p105 in Flp-In T-REx 293 cell lines was verified by using anti-HA and anti-p50/p105 antibodies. **E,** WB with anti-p50/p105 antibody showing p50/p105 levels of the R157X mutant Flp-In T-REx 293 cell line after 4 hours of proteasome inhibition with MG132. Because of a low level of p50 in samples, a long exposure time was used. **F,** Activities of WT and the H67R and I553M p50 mutants were analyzed by using an NF-κB–responsive luciferase reporter with or without pathway activation by TNF in the Flp-In T-REx 293 cell lines. N = 9. $*P < .05$, $**P < .001$, and $***P < .0001$.

NFKB1 mutants exhibit altered protein-protein interactions

The p50/p105-Flp-In cell lines were used to map protein-protein interactions of mutant and WT p50/p105 by means of affinity purification mass spectrometry (Fig 4 and see Fig E10 and Table E7 in this article's Online Repository at www.jacionline.org). This revealed 17 high-confidence protein-protein interactions, of which 2, Myc-associated zinc finger protein (MAZ), and IlvB-like protein (ILVBL), were previously undescribed. All interactions were verified by using BioID, which detected an additional transient signal transducer and activator of transcription 3 interaction. Both missense mutants showed an altered interaction profile both without and with TNF stimulation. Notably, in unstimulated cells we observed decreased affinity between the H67R mutant

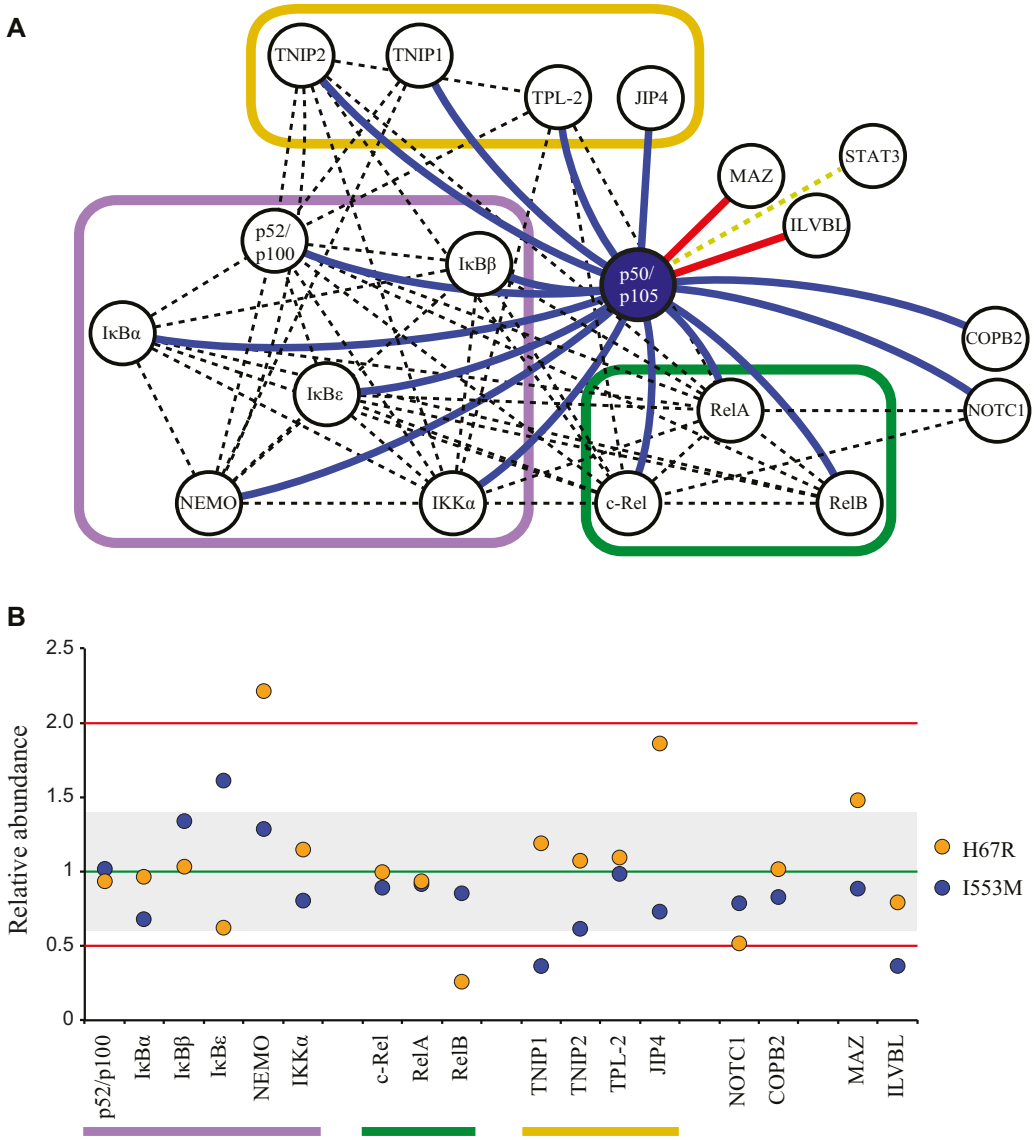


FIG 4. Mutant p50/p105 shows quantitative changes in protein-protein interactions. **A,** High-confidence p50/p105 protein-protein interactome from affinity purification mass spectrometry. All of the interactions are shared by the WT and the missense mutants. The bait p50/p105 is shown in blue, and interacting proteins are shown in white. Known p50/p105 interactions (PINA2 database) are marked with *blue edges*, novel interactions are marked with *red edges*, and known prey-prey interactions are marked with a *black dashed line*. A complementary BioID analysis validated all interactions and also detected a transient interaction with signal transducer and activator of transcription 3 (*STAT3*; *yellow dashed line*). Proteins regulating p50/p105 activity are shown in a *purple box*, and class II NF-κB family members (c-Rel, RelA, and RelB) with a c-terminal transactivation domain are shown in a *green box*. The additional p50/p105-interacting proteins TNFAIP3 interacting protein 1 and 2 (TNIP1 and TNIP2), tumor progression locus 2 (TPL-2), and C-Jun-amino-terminal kinase-interacting protein 4 (JIP-4), which are linked to both NF-κB and mitogen-activated protein kinase signaling, are shown in a *yellow box*. **B,** Quantitative changes in the interacting prey protein abundances between mutants and the WT p50/p105. The interaction abundances (n = 4) of the 2 mutants are compared with those of the WT (n = 4). Fold changes within ±40% are shown in *gray shading*, and a 2-fold change is indicated by a *red line*.

and RelB (0.26) and between the I553M mutant and TNIP1 (0.37). Moreover, we detected increased affinity between the I553M mutant and IκBε (1.61) and between the H67R mutant and NEMO (2.2). As expected, activation of the NF-κB pathway with TNF resulted in loss of interactions between p50/p105 and the upstream proteins IκBα, NEMO, and IKKα both in WT and the missense mutants (see Fig E11 in this article's Online Repository at www.jacionline.org). Although interaction of p50/p105 with RelB increased in response to pathway activation in WT (1.63) and the H67R mutant (1.28), no change in RelB affinity was observed with the I553M mutant. With the R157X mutant, similar to A156Sfs-NFKB1 mutant, only very few peptides of the protein were present, and only an interaction with MAZ was detected (see Table E8 in this article's Online Repository at www.jacionline.org). From PBMCs of patients with R157X mutation, only N-terminal peptides were detected from antibody-based

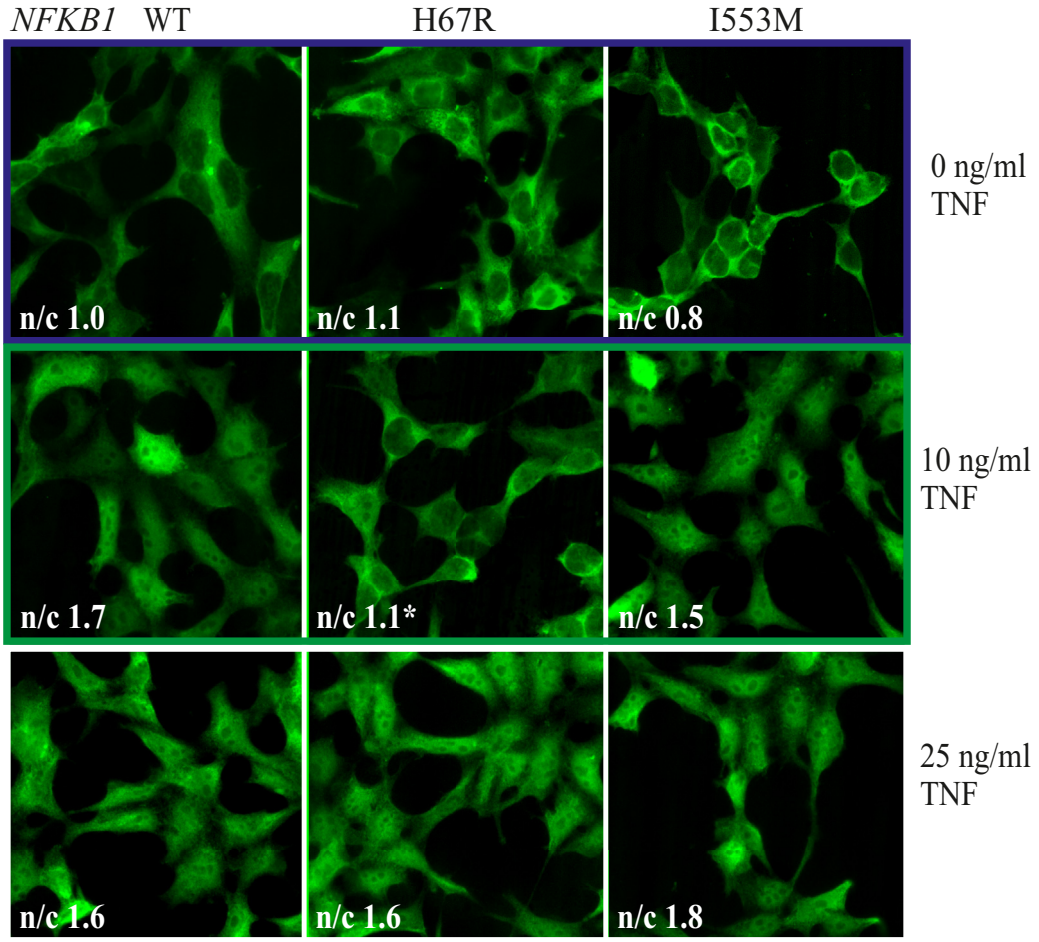


FIG 5. The H67R mutation reduces nuclear entry of p50 in response to TNF. Flp-In T-REx 293 cell lines expressing WT or mutant p50/p105 were treated with 0, 10, or 25 ng/mL TNF for 40 minutes and stained with an anti-HA antibody. The ratio of nuclear versus cytoplasmic signal *(n/c)* was calculated from imaged cells and normalized with the uninduced WT sample to calculate the median fold change in nuclear localization in response to treatment. TNF (10 ng/mL)–induced nuclear entry of p50 in the WT and I553M lines but not in the H67R line (marked by an *asterisk*).

p50/p105 purifications (see Fig E12 in this article's Online Repository at www.jacionline.org).

H67R mutant displays reduced nuclear localization

We asked whether increased binding between NEMO and the H67R mutant would affect p50 nuclear localization. By using the p50/p105-Flp-In cell lines, we performed immunofluorescence staining and microscopy after induction with TNF (10 or 25 ng/mL for 40 minutes). An anti-hemagglutinin antibody was used to visualize only tagged proteins. Although addition of 25 ng/mL TNF caused a 1.5- to 2-fold increase in nuclear localization in all cell lines, 10 ng/mL TNF induced nuclear localization of the WT and I553M proteins, but not of the H67R mutant, suggesting a reduction in efficiency of nuclear localization by this mutant (Fig 5).

I553M mutation alters posttranslational processing of p105

To study whether the I553M mutation affects the stability of p105, we performed a TNF titration on the mutant cell lines (Fig 6, *A*). In unstimulated conditions, p105 and p50 were expressed equally. However, with increasing TNF concentrations, the amount of I553M-p105 decreased more than that of WT or H67R-p105. Because degradation of p105 during pathway induction by TNF is mediated by phosphorylation,[35,36] we tested whether the I553M mutation alters p105 phosphorylation.

To assess this, we identified and quantified phosphopeptides from the affinity purification mass spectrometry data of WT and mutant p50/p105 using the Andromeda search engine combined with MaxQuant. Two peptides of p105 with a total of 3 serine residues (S893, S903, and S907) were identified and quantified in all replicates (see Table E9 in this article's Online Repository at www.jacionline.org). The I553M mutation caused a significant decrease in S893 and S907 phosphorylation (Fig 6, *B*). The first peptide (amino acids 878-896) was phosphorylated at serine 893, and the second peptide (amino acids 897-912) was found to be either singly phosphorylated at serine 907 or double phosphorylated at serines 907 and 903. Compared with the WT protein, the phosphorylation status of both S893 and S907 was significantly decreased (with a relative amount of phosphorylated to unphosphorylated peptide at 0.65 and 0.67, respectively) in the I553M mutant.

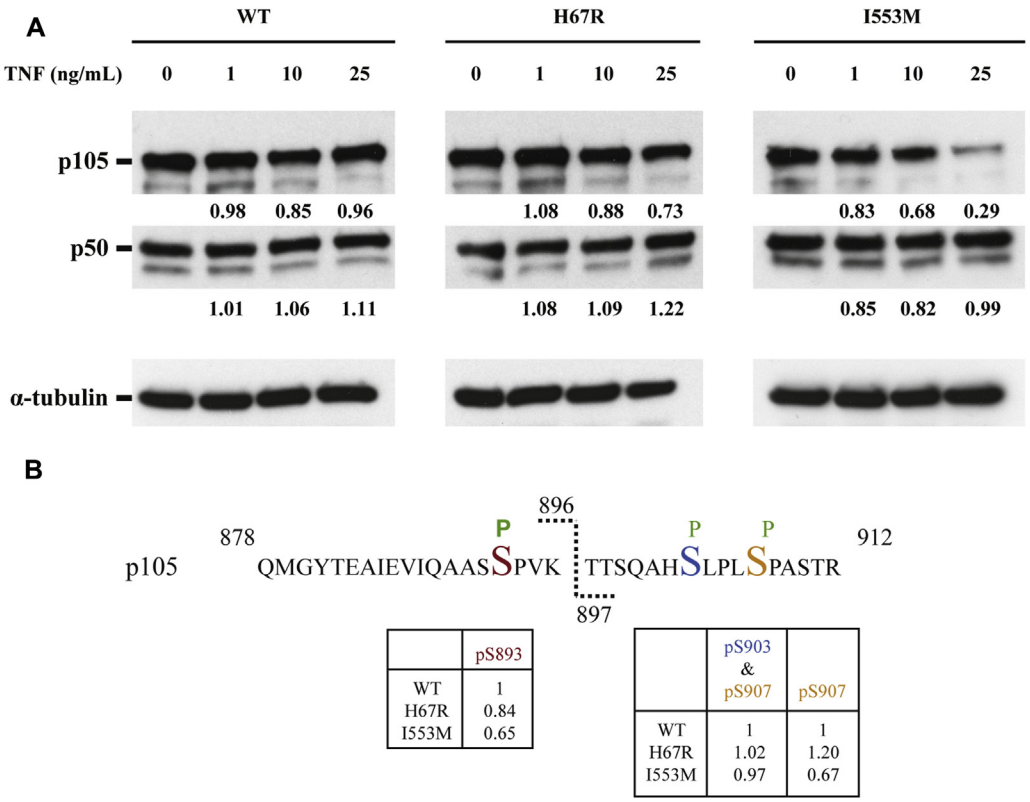


FIG 6. The I553M variant affects the stability and phosphorylation of p105. **A,** WT and mutant (H67R and I553M) p50/p105-expressing cells were treated with TNF gradient and analyzed by means of WB with anti-p50/p105 antibody to study the effect of TNF induction on p105 stability. α-Tubulin antibody was used as a loading control. **B,** Differences in phosphorylation levels of 2 phosphopeptides (878-896 and 897-912) from the C-terminus of p105 identified by mass spectrometric analyses. *Values shown in boxes* denote the ratio of phosphorylated to unphosphorylated peptide normalized to WT.

DISCUSSION

In this study we identified heterozygous *NFKB1* mutations in 3 families presenting with varying combinations of antibody deficiency, Behçet-like disease, and life-threatening postoperative complications of autoinflammatory origin. Furthermore, we showed that these mutations functionally affect the NF-κB subunits p50 and p105.

Haploinsufficiency of p50 was recently shown to cause common variable immunodeficiency, but none of the previously reported cases had autoinflammatory symptoms.[3,37] However, an immunodysregulatory phenotype is well demonstrated in p50/p105-deficient mouse models. *NFKB1*$^{-/-}$ mice have multiorgan autoimmunity and show increased IL-6 production, activation of autoreactive CD8^{+} T cells, and defective maturation of immunoregulatory natural T lymphocytes.[38-42] Similar to some of our patients, the mice produce increased amounts of the proinflammatory cytokines IFN-γ and TNF.[43-45] p50 regulates TNF production, and TNF controls intestinal inflammation and epithelial cell turnover in the colon.[44,46,47] Hence increased TNF production might contribute to the gastrointestinal symptoms in our patients. TNF-induced apoptosis and necrosis could also contribute to inflammatory symptoms by compromising mucosal barrier function and increasing tissue degeneration at inflamed sites.[48-50] Defects in other molecules of the NF-κB signaling pathway also lead to inflammation in human subjects: NEMO deficiency can cause systemic inflammation, enterocolitis, and Behçet disease.[51-54] Moreover, haploinsufficiency of A20, a negative regulator of NF-κB signaling, causes Behçet-like symptoms, and IκBα mutations can present with noninfectious inflammation.[9,55] Hence autoinflammatory symptoms in patients with *NFKB1* mutations are not surprising.

Phenotypes of patients with the now reported *NFKB1* mutations varied between subjects, but variable disease severity has also been reported by others.[37] Genetic factors, such as common variants in immunologically relevant genes, and environmental effects, such as pathogens or immunomodulatory treatments, are likely partially responsible for these differences. Patient age can also factor in because our young patients had only mild disease, and most affected carriers were asymptomatic as children. Indeed, animal age affects the phenotype and immune cell numbers also in *NFKB1*$^{-/-}$ mice.[38]

In addition, we detected molecular differences between the now identified and previously described *NFKB1* mutations.[3] In our cell model the R157X mutant induced degradation of the endogenous p50/p105 through a proteasome-dependent mechanism, whereas the A156Sfs mutant did not. The binding of mutant R157X protein to nascent WT p50/p105 could lead to misfolding and degradation of both peptides because dimerization of p50 and p105 is required for their stabilization and folding.[56] Despite the severe insufficiency of p50/p105, only some of the R157X variant carriers had hyperinflammatory reactions, and none had antibody

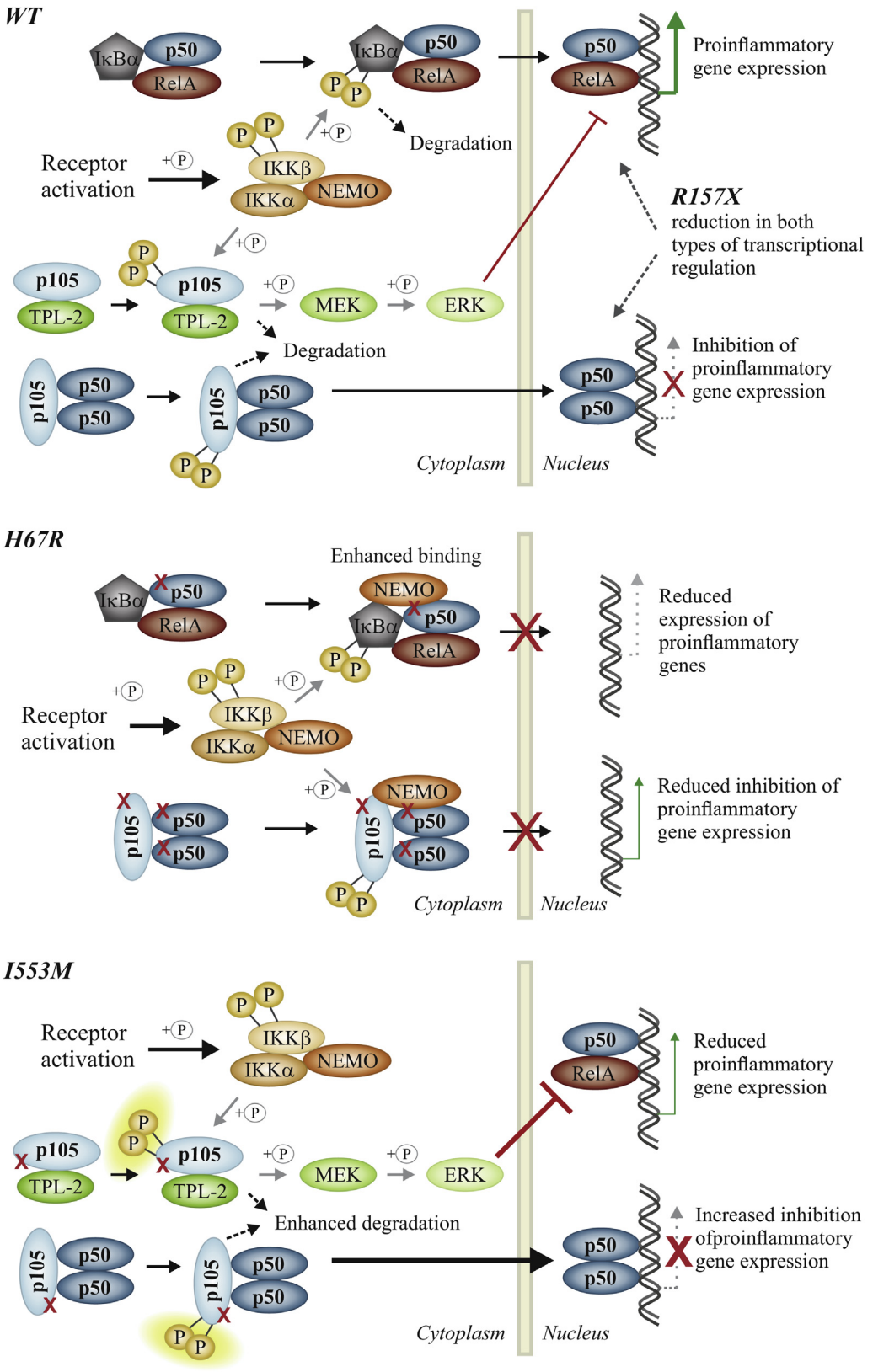


FIG 7. Suggested mechanisms of signaling disturbance by *NFKB1* missense mutations. In normal conditions p50 forms both proinflammatory heterodimers and anti-inflammatory homodimers. The R157X mutation reduces the amount and the H67R mutation reduces the activity of both types of dimers, likely dysregulating both initiation and termination of proinflammatory gene transcription. The I553M mutant might increase p50 concentration in the nucleus because p105 is an important regulator of p50 homodimers. In addition, reduction in p105 can increase signaling through extracellular signal-regulated kinase *(ERK)* because p105 regulates mitogen-activated protein kinase *(MAPK)*/ERK signaling through inhibition of tumor progression locus 2. The inhibited MAPK-ERK pathway downregulates overall NF-κB signaling.

deficiency. Although p50 is necessary for immunoglobulin diversification in mice,[33,57] compensatory mechanisms might exist to maintain normal antibody production in cases of p50 insufficiency in human subjects.

The R157X mutation carriers had life-threatening postoperative complications. Macrophages derived from these patients exhibited increased inflammasome activation and IL-1β secretion. Priming of the NLRP3 inflammasome in macrophages is mediated by NF-κB, but the NF-κB/p62/mitophagy pathway can also restrict the secretion of IL-1β and consequently attenuate sterile inflammation.[58,59] Hence p50 deficiency in macrophages can promote IL-1β production and lead to tissue inflammation. In addition, increased neutrophil recruitment was detected both in *NFKB1*$^{-/-}$ mice and in carriers of the R157X mutation during inflammatory episodes, which exacerbates disease and contributes to tissue damage.[60-62]

The H67R mutation impaired p50 transcriptional activity. p50 is bound by NEMO, which keeps p50 in the cytoplasm and releases it to enter the nucleus on NF-κB activation. Our data suggest increased binding between the mutant p50 and NEMO, resulting in impairment of p50 nuclear entry. The disease could be caused by a diminished pool of transcriptionally active p50 heterodimers (Fig 7), as has been seen in previously reported cases of p50 haploinsufficiency. However, autoinflammatory symptoms could also be due to reduced numbers of p50:p50 homodimers, which normally curb inflammatory reactions by repressing inflammatory and inducing anti-inflammatory gene expression.[15,63,64]

Patients with the I553M mutation had common variable immunodeficiency, phenotypically resembling Fliegauf et al's cohort.[3] In contrast to their data, we did not detect changes in p50 quantity or activity. Instead, we observed decreased phosphorylation and increased degradation of I553M-p105. p105 degradation is induced by TNF and leads to release of p50 and other NF-κB transcription factors. Thus a decrease in p105 can affect activation or termination of downstream signaling.[65] In mice loss of p105 is sufficient to cause inflammation and infection susceptibility.[34] Additionally, we detected altered protein interaction profiles between WT and mutant p105. Loss of interaction between I553M-p105 and IκBε on the NF-κB pathway activation might contribute to the phenotype because hematopoietic IκBε regulates chronic inflammation.[66] Both I553M and H67R mutants also showed reduced interactions with RelB. Expression of RelB is activated by canonical NF-κB signaling, and the lost interaction might merely indicate reduced canonical pathway activation in mutant cells.[67] In response to activation of the nonclassical NF-κB pathway, RelB dimerizes with p52 encoded by *NFKB2*, mutations that cause common variable immunodeficiency.[6] By contrast, functions of p50:RelB dimers are largely uncharted, but in dendritic cells and macrophages they regulate expression and activation of certain anti-inflammatory genes.[68-70]

In conclusion, we broaden the phenotype of *NFKB1* mutations, showing that certain patients can have autoinflammatory disease in combination with antibody deficiency. Increased production of TNF and IL-1β during autoinflammatory episodes suggests a potential therapeutic venue for IL-1 and TNF inhibitors in severe cases. Moreover, we show that mutations affecting only p105 are sufficient to cause hypogammaglobulinemia and autoimmunity. These results highlight the importance of p50/p105 for proper immune function and control of inflammation.

We thank the personnel at the Hematology Research Unit Helsinki and Institute for Molecular Medicine Finland (FIMM) for their expert clinical and technical assistance. Exome sequencing and analysis of the primary sequencing data were conducted in the Technology Centre of the Institute for Molecular Medicine Finland (FIMM). Image analysis of immunofluorescence experiments was aided by Teijo Pellinen at FIMM.

Key messages

- **In addition to antibody deficiency, mutations in *NFKB1* cause autoinflammatory symptoms resembling Behçet disease and inflammatory gastrointestinal diseases and might lead to severe postoperative complications.**
- **Mutations that disrupt the function of either p50 or p105 can dysregulate NF-κB signaling and result in autosomal dominant disease.**
- **The autoinflammatory symptoms are mediated by IL-1β–dependent and possibly TNF-dependent mechanisms, suggesting a potential therapeutic venue for IL-1 and TNF inhibitors in severe cases.**

REFERENCES

1. Karin M, Lin A. NF-kappa B at the crossroads of life and death. Nat Immunol 2002;3:221-7.
2. Vallabhapurapu S, Karin M. Regulation and function of NF-kappa B transcription factors in the immune system. Annu Rev Immunol 2009;27:693-733.
3. Fliegauf M, Bryant VL, Frede N, Slade C, Woon S, Lehnert K, et al. Haploinsufficiency of the NF-kappa B1 subunit p50 in common variable immunodeficiency. Am J Hum Genet 2015;97:389-403.
4. Courtois G, Smahi A, Reichenbach J, Doffinger R, Cancrini C, Bonnet M, et al. A hypermorphic I kappa B alpha mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency. J Clin Invest 2003;112:1108-15.
5. Picard C, Puel A, Bonnet M, Ku CL, Bustamante J, Yang K, et al. Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science 2003;299:2076-9.
6. Chen K, Coonrod EM, Kumanovics A, Franks ZF, Durtschi JD, Margraf RL, et al. Germline mutations in NFKB2 implicate the noncanonical NF-kappa B pathway in the pathogenesis of common variable immunodeficiency. Am J Hum Genet 2013;93:812-24.
7. Pannicke U, Baumann B, Fuchs S, Henneke P, Rensing-Ehl A, Rizzi M, et al. Deficiency of innate and acquired immunity caused by an IKBKB mutation. N Engl J Med 2013;369:2504-14.
8. Willmann KL, Klaver S, Dogu F, Santos-Valente E, Garncarz W, Bilic I, et al. Biallelic loss-of-function mutation in NIK causes a primary immunodeficiency with multifaceted aberrant lymphoid immunity. Nat Commun 2014;5:5360.
9. Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y, et al. Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. Nat Genet 2016;48:67.
10. Tak P, Firestein G. NF-kappaa B: key role in inflammatory diseases. J Clin Invest 2001;107:7-11.
11. D'Assante R, Fusco A, Palamaro L, Giardino G, Gallo V, Cirillo E, et al. Unraveling the link between ectodermal disorders and primary immunodeficiencies. Int Rev Immunol 2016;35:25-38.
12. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa-B. Annu Rev Cell Biol 1994;10:405-55.
13. Gilmore TD. Introduction to NF-kappa B: players, pathways, perspectives. Oncogene 2006;25:6680-4.
14. Watanabe N, Iwamura T, Shinoda T, Fujita T. Regulation of NFKB1 proteins by the candidate oncoprotein BCL-3: generation of NF-kappa B homodimers from the cytoplasmic pool of p50-p105 and nuclear translocation. EMBO J 1997;16:3609-20.
15. Grundstrom S, Anderson P, Scheipers P, Sundstedt A. Bcl-3 and NF kappa B p50-p50 homodimers act as transcriptional repressors in tolerant CD4(+) T cells. J Biol Chem 2004;279:8460-8.
16. Hatada EN, Nieters A, Wulczyn FG, Naumann M, Meyer R, Nucifora G, et al. The ankyrin repeat domains of the NF-kappa-B precursor P105 and the

protooncogene bcl-3 act as specific inhibitors of NF-kappa-B DNA-binding. Proc Natl Acad Sci U S A 1992;89:2489-93.
17. Savinova OV, Hoffmann A, Ghosh G. The Nfkb1 and Nfkb2 proteins p105 and p100 function as the core of high-molecular-weight heterogeneous complexes. Mol Cell 2009;34:591-602.
18. Karban AS, Okazaki T, Panhuysen CIM, Gallegos T, Potter JJ, Bailey-Wilson JE, et al. Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. Hum Mol Genet 2004;13:35-45.
19. Todaro M, Zerilli M, Triolo G, Iovino F, Patti M, Accardo-Palumbo A, et al. NF-kappa B protects behqet's disease T cells against CD95-induced apoptosis up-regulating antiapoptotic proteins. Arthritis Rheum 2005;52:2179-91.
20. Hung Y, Wu C, Ou T, Lin C, Li R, Lin Y, et al. I kappa B alpha promoter polymorphisms in patients with Behcet's disease. Dis Markers 2010;28:55-62.
21. Yenmis G, Oner T, Cam C, Koc A, Kucuk OS, Yakicier MC, et al. Association of NFKB1 and NFKBIA polymorphisms in relation to susceptibility of Behcet's disease. Scand J Immunol 2015;81:81-6.
22. Yazici H, Fresko I, Yurdakul S. Behcet's syndrome: Disease manifestations, management, and advances in treatment. Nat Clin Pract Rheumatol 2007;3:148-55.
23. Haapaniemi EM, Kaustio M, Rajala HLM, van Adrichem AJ, Kainulainen L, Glumoff V, et al. Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. Blood 2015;125:639-48.
24. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallog 2004;60:2126-32.
25. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The protein data bank. Nucleic Acids Res 2000;28:235-42.
26. Varjosalo M, Keskitalo S, Van Drogen A, Nurkkala H, Vichalkovski A, Aebersold R, et al. The protein interaction landscape of the human CMGC kinase group. Cell Rep 2013;3:1306-20.
27. Turunen M, Spaeth JM, Keskitalo S, Park MJ, Kivioja T, Clark AD, et al. Uterine leiomyoma-linked MED12 mutations disrupt mediator-associated CDK activity. Cell Rep 2014;7:654-60.
28. Galang C, GarciaRamirez J, Solski P, Westwick J, Der C, Neznanov N, et al. Oncogenic neu/ErbB-2 increases ets, AP-1, and NF-kappa B-dependent gene expression, and inhibiting ets activation blocks neu-mediated cellular transformation. J Biol Chem 1996;271:7992-8.
29. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671-5.
30. Roux KJ, Kim DI, Burke B. BioID: a screen for protein-protein interactions. Curr Protoc Protein Sci 2013;74:Unit 19.23.
31. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 2008;26:1367-72.
32. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: A peptide search engine integrated into the MaxQuant environment. J Proteome Res 2011;10:1794-805.
33. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the P50 subunit of nf-kappa-B leads to multifocal defects in immune-responses. Cell 1995;80:321-30.
34. Ishikawa H, Claudio E, Dambach D, Raventos-Suarez C, Ryan C, Bravo R. Chronic inflammation and susceptibility to bacterial infections in mice lacking the polypeptide (p)105 precursor (NF-kappa B1) but expressing p50. J Exp Med 1998;187:985-96.
35. Fujimoto K, Yasuda H, Sato Y, Yamamoto K. A role for phosphorylation in the proteolytic processing of the human nf-kappa-B1 precursor. Gene 1995;165:183-9.
36. Demarchi F, Bertoli C, Sandy P, Schneider C. Glycogen synthase kinase-3 beta regulates NF-kappa B1/p105 stability. J Biol Chem 2003;278:39583-90.
37. Boztug H, Hirschmugl T, Holter W, Lakatos K, Kager L, Trapin D, et al. NF-kappa B1 haploinsufficiency causing immunodeficiency and EBV-driven lymphoproliferation. J Clin Immunol 2016;36:533-40.
38. de Valle E, Grigoriadis G, O'Reilly LA, Willis SN, Maxwell MJ, Corcoran LM, et al. NF kappa B1 is essential to prevent the development of multiorgan autoimmunity by limiting IL-6 production in follicular B cells. J Exp Med 2016;213:621-41.
39. Stanic A, Bezbradica J, Park J, Matsuki N, Mora A, Van Kaer L, et al. NF-kappa B controls cell fate specification, survival, and molecular differentiation of immunoregulatory natural T lymphocytes. J Immunol 2004;172:2265-73.
40. Artis D, Kane C, Fiore J, Zaph C, Shapira S, Joyce K, et al. Dendritic cell-intrinsic expression of NF-kappa B1 is required to promote optimal Th2 cell differentiation. J Immunol 2005;174:7154-9.
41. Dissanayake D, Hall H, Berg-Brown N, Elford AR, Hamilton SR, Murakami K, et al. Nuclear factor-kappa B1 controls the functional maturation of dendritic cells and prevents the activation of autoreactive T cells. Nat Med 2011;17:1663-7.
42. Gugasyan R, Horat E, Kinkel SA, Ross F, Grigoriadis G, Gray D, et al. The NF-kappa B1 transcription factor prevents the intrathymic development of CD8 T cells with memory properties. EMBO J 2012;31:692-706.
43. Bohuslav J, Kravchenko V, Parry G, Erlich J, Gerondakis S, Mackman N, et al. Regulation of an essential innate immune response by the p50 subunit of NF-kappa B. J Clin Invest 1998;102:1645-52.
44. Inan M, Tolmacheva V, Wang Q, Rosenberg D, Giardina C. Transcription factor NF-kappa B participates in regulation of epithelial cell turnover in the colon. Am J Physiol Gastrointest Liver Physiol 2000;279:G1282-91.
45. Gadjeva M, Tomczak M, Zhang M, Wang Y, Dull K, Rogers A, et al. A role for NF-kappa B subunits p50 and p65 in the inhibition of lipopolysaccharide-induced shock. J Immunol 2004;173:5786-93.
46. Artis D, Shapira S, Mason N, Speirs K, Goldschmidt M, Caamano J, et al. Differential requirement for NF-kappa B family members in control of helminth infection and intestinal inflammation. J Immunol 2002;169:4481-7.
47. Zheng S, Abraham C. NF-kappa B1 inhibits NOD2-induced cytokine secretion through ATF3-dependent mechanisms. Mol Cell Biol 2013;33:4857-71.
48. Gadjeva M, Wang Y, Horwitz BH. NF-kappa B p50 and p65 subunits control intestinal homeostasis. Eur J Immunol 2007;37:2509-17.
49. Dannappel M, Vlantis K, Kumari S, Polykratis A, Kim C, Wachsmuth L, et al. RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. Nature 2014;513:90.
50. Takahashi N, Vereecke L, Bertrand MJM, Duprez L, Berger SB, Divert T, et al. RIPK1 ensures intestinal homeostasis by protecting the epithelium against apoptosis. Nature 2014;513:95.
51. Takada H, Nomura A, Ishimura M, Ichiyama M, Ohga S, Hara T. NEMO mutation as a cause of familial occurrence of Behcet's disease in female patients. Clin Genet 2010;78:575-9.
52. Klemann C, Pannicke U, Morris-Rosendahl DJ, Vlantis K, Rizzi M, Uhlig H, et al. Transplantation from a symptomatic carrier sister restores host defenses but does not prevent colitis in NEMO deficiency. Clin Immunol 2016;164:52-6.
53. Zilberman-Rudenko J, Shawver LM, Wessel AW, Luo Y, Pelletier M, Tsai WL, et al. Recruitment of A20 by the C-terminal domain of NEMO suppresses NF-kappa B activation and autoinflammatory disease. Proc Natl Acad Sci U S A 2016;113:1612-7.
54. Cheng LE, Kanwar B, Tcheurekdjian H, Grenert JP, Muskat M, Heyman MB, et al. Persistent systemic inflammation and atypical enterocolitis in patients with NEMO syndrome. Clin Immunol 2009;132:124-31.
55. Yoshioka T, Nishikomori R, Hara J, Okada K, Hashii Y, Okafuji I, et al. Autosomal dominant anhidrotic ectodermal dysplasia with immunodeficiency caused by a novel NFKBIA mutation, p.Ser36Tyr, presents with mild ectodermal dysplasia and non-infectious systemic inflammation. J Clin Immunol 2013;33:1165-74.
56. Lin L, DeMartino G, Greene W. Cotranslational dimerization of the rel homology domain of NF-kappa B1 generates p50-p105 heterodimers and is required for effective p50 production. EMBO J 2000;19:4712-22.
57. Snapper C, Zelazowski P, Rosas F, Kehry M, Tian M, Baltimore D, et al. B cells from p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line C-H transcription, and ig class switching. J Immunol 1996;156:183-91.
58. Greten FR, Arkan MC, Bollrath J, Hsu L, Goode J, Miething C, et al. NF-kappa B is a negative regulator of IL-1 beta secretion as revealed by genetic and pharmacological inhibition of IKK beta. Cell 2007;130:918-31.
59. Zhong Z, Umemura A, Sanchez-Lopez E, Liang S, Shalapour S, Wong J, et al. NF-kappa B restricts inflammasome activation via elimination of damaged mitochondria. Cell 2016;164:896-910.
60. Oakley F, Mann J, Nailard S, Smart D, Mungalsingh N, Constandinou C, et al. Nuclear factor-kappa beta 1 (p50) limits the inflammatory and fibrogenic responses to chronic injury. Am J Pathol 2005;166:695-708.
61. Han W, Joo M, Everhart MB, Christman JW, Yull FE, Blackwell TS. Myeloid cells control termination of lung inflammation through the NF-kappa B pathway. Am J Physiol Lung Cell Mol Physiol 2009;296:L320-7.
62. Song L, Woermann S, Ai J, Neuhoefer P, Lesina M, Diakopoulos KN, et al. BCL3 reduces the sterile inflammatory response in pancreatic and biliary tissues. Gastroenterology 2016;150:499.
63. Collins PE, Kiely PA, Carmody RJ. Inhibition of transcription by B cell leukemia 3 (bcl-3) protein requires interaction with nuclear factor kappa B (NF-kappa B) p50. J Biol Chem 2014;289:7059-67.
64. Cao S, Zhang X, Edwards JP, Mosser DM. NF-kappa B1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. J Biol Chem 2006;281:26041-50.
65. Janssens S, Pulendran B, Lambrecht BN. Emerging functions of the unfolded protein response in immunity. Nat Immunol 2014;15:910-9.

66. Patel MN, Bernard WG, Milev NB, Cawthorn WP, Figg N, Hart D, et al. Hematopoietic IKBKE limits the chronicity of inflammasome priming and metaflammation. Proc Natl Acad Sci U S A 2015;112:506-11.
67. Bren G, Solan N, Miyoshi H, Pennington K, Pobst L, Paya C. Transcription of the RelB gene is regulated by NF-kappa B. Oncogene 2001;20:7722-33.
68. Shih VF, Davis-Turak J, Macal M, Huang JQ, Ponomarenko J, Kearns JD, et al. Control of RelB during dendritic cell activation integrates canonical and noncanonical NF-kappa B pathways. Nat Immunol 2012; 13:1162.
69. Gasparini C, Foxwell BM, Feldmann M. RelB/p50 regulates TNF production in LPS-stimulated dendritic cells and macrophages. Cytokine 2013;61:736-40.
70. Bhardwaj R, Yester JW, Singh SK, Biswas DD, Surace MJ, Waters MR, et al. RelB/p50 complexes regulate cytokine-induced YKL-40 expression. J Immunol 2015;194:2862-70.