Biallelic hypomorphic mutations in a linear deubiquitinase define otulipenia, an early-onset autoinflammatory disease

Qing Zhou[a,1], Xiaomin Yu[b,1], Erkan Demirkaya[c], Natalie Deuitch[a], Deborah Stone[a], Wanxia Li Tsai[d], Hye Sun Kuehn[e], Hongying Wang[a], Dan Yang[f], Yong Hwan Park[a], Amanda K. Ombrello[a], Mary Blake[d], Tina Romeo[a], Elaine F. Remmers[a], Jae Jin Chae[a], James C. Mullikin[g], Ferhat Güzel[c], Joshua D. Milner[b], Manfred Boehm[f], Sergio D. Rosenzweig[e], Massimo Gadina[d], Steven B. Welch[h], Seza Özen[i], Rezan Topaloglu[i], Mario Abinun[j], Daniel L. Kastner[a,2], and Ivona Aksentijevich[a,2]

[a]Inflammatory Disease Section, National Human Genome Research Institute, Bethesda, MD 20892; [b]Genetics and Pathogenesis of Allergy Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892; [c]Familial Mediterranean Fever Arthritis Vasculitis and Orphan Disease Research Center, Institute of Health Sciences, R&D Center, Gulhane Military Medical Academy, Ankara 06018, Turkey; [d]Translational Immunology Section, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892; [e]Department of Laboratory Medicine, National Institutes of Health Clinical Center, Bethesda, MD 20892; [f]Laboratory of Cardiovascular Regenerative Medicine, National Heart, Lung, and Blood Institute, Bethesda, MD 20892; [g]National Institutes of Health Intramural Sequencing Center, National Human Genome Research Institute, Rockville, MD 20852; [h]Heart of England National Health Service Foundation Trust, Birmingham B9 5ST, United Kingdom; [i]Department of Pediatric Nephrology and Rheumatology, Faculty of Medicine, Hacettepe University, Ankara 06100, Turkey; and [j]Institute of Cellular Medicine, Newcastle University, Newcastle NE2 4HH, United Kingdom

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Systemic autoinflammatory diseases are caused by mutations in genes that function in innate immunity. Here, we report an autoinflammatory disease caused by loss-of-function mutations in *OTULIN* (*FAM105B*), encoding a deubiquitinase with linear linkage specificity. We identified two missense and one frameshift mutations in one Pakistani and two Turkish families with four affected patients. Patients presented with neonatal-onset fever, neutrophilic dermatitis/panniculitis, and failure to thrive, but without obvious primary immunodeficiency. HEK293 cells transfected with mutated *OTULIN* had decreased enzyme activity relative to cells transfected with WT *OTULIN,* and showed a substantial defect in the linear deubiquitination of target molecules. Stimulated patients' fibroblasts and peripheral blood mononuclear cells showed evidence for increased signaling in the canonical NF-κB pathway and accumulated linear ubiquitin aggregates. Levels of proinflammatory cytokines were significantly increased in the supernatants of stimulated primary cells and serum samples. This discovery adds to the emerging spectrum of human diseases caused by defects in the ubiquitin pathway and suggests a role for targeted cytokine therapies.

OTULIN | linear deubiquitinase | NF-κB pathway | autoinflammatory disease | cytokines

Posttranslational modifications by ubiquitination are important for the regulation of many signaling complexes (1). Linear ubiquitin chains, also known as Met1-linked chains, are generated by the linear ubiquitin assembly complex (LUBAC) (2). LUBAC-mediated Met1 ubiquitination is critical for regulation of immune signaling and cell death (3). Absence of LUBAC attenuates NF-κB signaling and patients with loss-of-function mutations in LUBAC present with paradoxical features of susceptibility to infection and systemic inflammation, the latter due to increased responsiveness to IL-1β in monocytes (3–5). OTULIN and CYLD are deubiquitinases (DUBs) that cleave Met1-linked chains (6). Although OTULIN functions exclusively as a Met1 deubiquitinase (7, 8), CYLD may also hydrolyze Lys63-linked ubiquitin (9). OTULIN is an evolutionarily highly conserved protein, and in mice complete deficiency is embryonically lethal (8). Recently, we reported patients with heterozygous germline mutations in *TNFAIP3*/A20 (10), which has DUB activity for K63-linked polyubiquitin chains. Both OTULIN and A20 are important gatekeepers of innate immunity (7, 11).

Results

Identification of Loss-of-Function Mutations in OTULIN in Three Patients. Using a combination of exome sequencing and candidate gene screening, we identified three homozygous mutations in the *OTULIN*/*FAM105B* gene in unrelated families of Pakistani and Turkish descent (Fig. 1, Fig. S1, Table 1, and Tables S1 and S2). Unaffected parents and siblings were carriers for the respective mutations. None of the mutations was reported in public databases or detected in 1,630 Turkish healthy controls. Two missense mutations, p.Leu272Pro and p.Tyr244Cys, are predicted to be deleterious by multiple algorithms (Table S3) and affect highly conserved amino acid residues (Fig. S2*A*). Similar to A20 disease-causing mutations (10), all three OTULIN

Significance

We describe a human disease linked to mutations in the linear deubiquitinase (DUB) OTULIN, which functions as a Met1-specific DUB to remove linear polyubiquitin chains that are assembled by the linear ubiquitin assembly complex (LUBAC). OTULIN has a role in regulating Wnt and innate immune signaling complexes. Hydrolysis of Met1-linked ubiquitin chains attenuates inflammatory signals in the NF-κB and ASC-mediated pathways. OTULIN-deficient patients have excessive linear ubiquitination of target proteins, such as NEMO, RIPK1, TNFR1, and ASC, leading to severe inflammation. Cytokine inhibitors have been efficient in suppressing constitutive inflammation in these patients. This study, together with the identification of haploinsufficiency of A20 (HA20), suggests a category of human inflammatory diseases, diseases of dysregulated ubiquitination.

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[1]Q.Z. and X.Y. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: kastnerd@mail.nih.gov or aksentii@mail.nih.gov.

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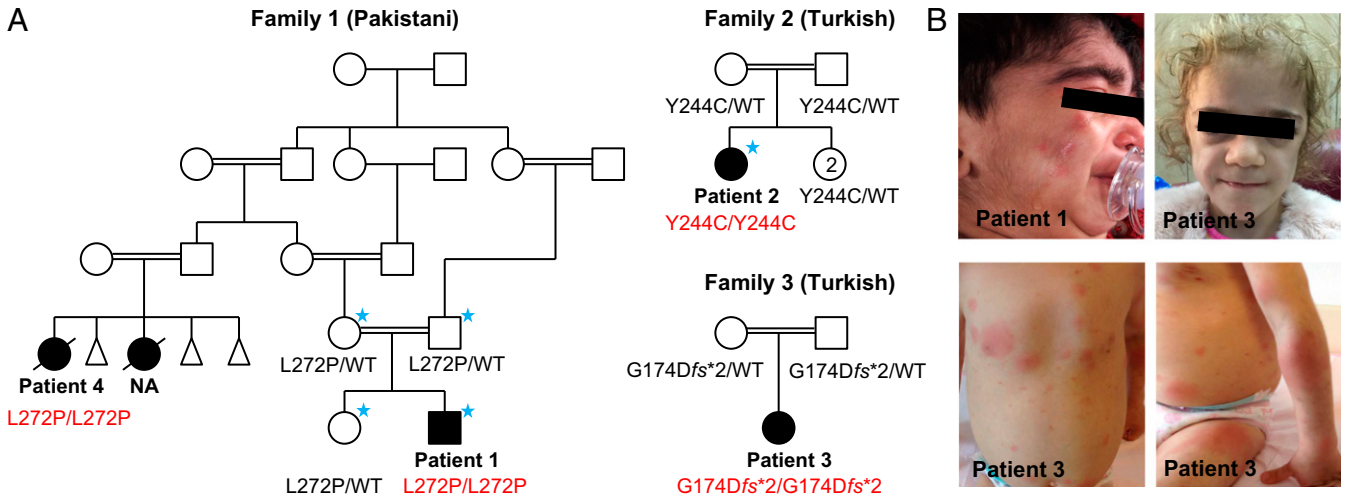

Fig. 1. Mutations in OTULIN cause severe early-onset systemic autoinflammatory disease. (*A*) Pedigrees and the identified genotypes in three families with mutations in *OTULIN*. WT indicates wild-type *OTULIN* alleles. The individuals selected for exome sequencing are marked with blue asterisks. NA: an affected cousin of patient 1 had similar disease, but her DNA sample was not available for this study. (*B*) Clinical manifestations of three patients with otulipenia. Top two panels show facial features of the patients, including cushingoid appearance (*Left*) and prominent fat loss (lipodystrophy) (*Right*). Bottom two panels show erythematous skin lesions and subcutaneous nodules.

mutations are located in the ovarian tumor (OTU) domain (Fig. S2*B*). The p.Gly174Aspfs*2 mutation introduces a premature stop codon, and mutant truncated protein was detectable by overexpression in HEK293 cells (Fig. 2*A*) but not in the patient's fibroblasts (Fig. 2*B*, patient 3). The missense mutations likely affect the S1 site of the linear ubiquitin-binding domain (Fig. S2*C*). They reduce OTULIN protein stability (Fig. 2*B*) and may result in the instability of the LUBAC complex subunits, SHARPIN and HOIP (Fig. 2*B*).

Clinical Manifestations of OTULIN Deficiency. Patient 1, from a large consanguineous family, was born prematurely and soon after birth presented with fever and rash (Fig. 1 and Table S4). Two of his first cousins died from a similar disease in early childhood. Only one DNA sample was available for genotyping and was found to have the same homozygous mutation as patient 1. Other findings included failure to thrive, joint swelling, lipodystrophy, and diarrhea. Treatment with an IL-1β inhibitor (anakinra) was not steroid sparing; however, within 1 mo of starting a TNF inhibitor (infliximab) at the age of 3 y, his fevers and rash subsided. Eight years after initiation of the treatment, he is normal size for his age and fully functional (Fig. S3). Patient 2 presented at the age of 4.5 mo with prolonged fevers and pustular, scarring rashes. Skin biopsy revealed panniculitis and neutrophilic dermatosis. Initially, she responded to treatment with steroids, and subsequently symptoms improved on treatment with anakinra (Table S4). Patient 3 presented with neonatal-onset fever and prominent cutaneous lesions including an erythematous rash with painful skin nodules (Fig. 1*B*). Her skin biopsy showed a predominantly septal panniculitis with vasculitis of small and medium-sized blood vessels. Other manifestations included arthralgia, progressive lipodystrophy, and developmental delay. Her disease is partially controlled with a TNF inhibitor (etanercept), but she is still steroid dependent (Table S4). Patients did not have clear evidence for primary immunodeficiency and suffered from infections related to use of immunosuppressive therapies. Patients 1 and 3 had normal to high levels of T, B, and natural killer cells (Table S5), Ig levels were normal to high, and IgA was elevated in the two patients. T- and B-cell proliferative responses were normal (Fig. S4*A*). Patients had adequate specific antibody responses to vaccines or natural infections when tested.

Mutations Do Not Disrupt OTULIN Interaction with LUBAC. OTULIN is a 352-residue protein that consists of an N-terminal LUBAC-binding domain and a C-terminal ovarian tumor (OTU) domain (Fig. S2*B*). OTULIN interacts with the PUB domain of HOIP, and their interaction is required for the recruitment of OTULIN to the TNF receptor complex (12). The three mutations do not disrupt the OTULIN interaction with LUBAC, and mutant proteins maintain the intact N-terminal domain necessary for LUBAC interaction (Fig. 2*A*).

Increased NF-κB Signaling in OTULIN-Deficient Cells. OTULIN restricts NF-κB signaling activity (7). We performed NF-κB luciferase assays to study the function of mutant OTULIN proteins in human embryonic kidney (HEK) 293 cells. Overexpressed mutant OTULIN plasmid p.Leu272Pro and p.Gly174Aspfs*2 failed to restrain NF-κB activity compared with WT OTULIN (Fig. 2*C* and Fig. S5*A*).

Table 1. ***OTULIN*** **mutations identified in three consanguineous families**

Family	Ancestry	Nucleotide alteration[†]	cDNA alteration[‡]	Amino acid alteration	Domain	ExAC	Turkish population	Software prediction[§]	Conservation[¶]
1	Pakistani	chr5: 14690368T>C	c.815T>C	p.Leu272Pro	OTU	0/122,972	0/3,260	Damaging	Conserved
2	Turkish	chr5: 14690284A>G	c.731A>G	p.Tyr244Cys	OTU	0/122,972	0/3,260	Damaging	Conserved
3	Turkish	chr5: 14687678delC	c.517delC	p.Gly174Asp*fs**2	OTU	0/122,972	0/3,260	/	/

[†]Genome reference: GRCh37 (hg19).
[‡]cDNA reference: NM_138348.4.
[§]SIFT, PolyPhen2, LRT, Mutation Taster, and CADD.
[¶]GERP, SiPhy 29 way, and CLUSTALW.

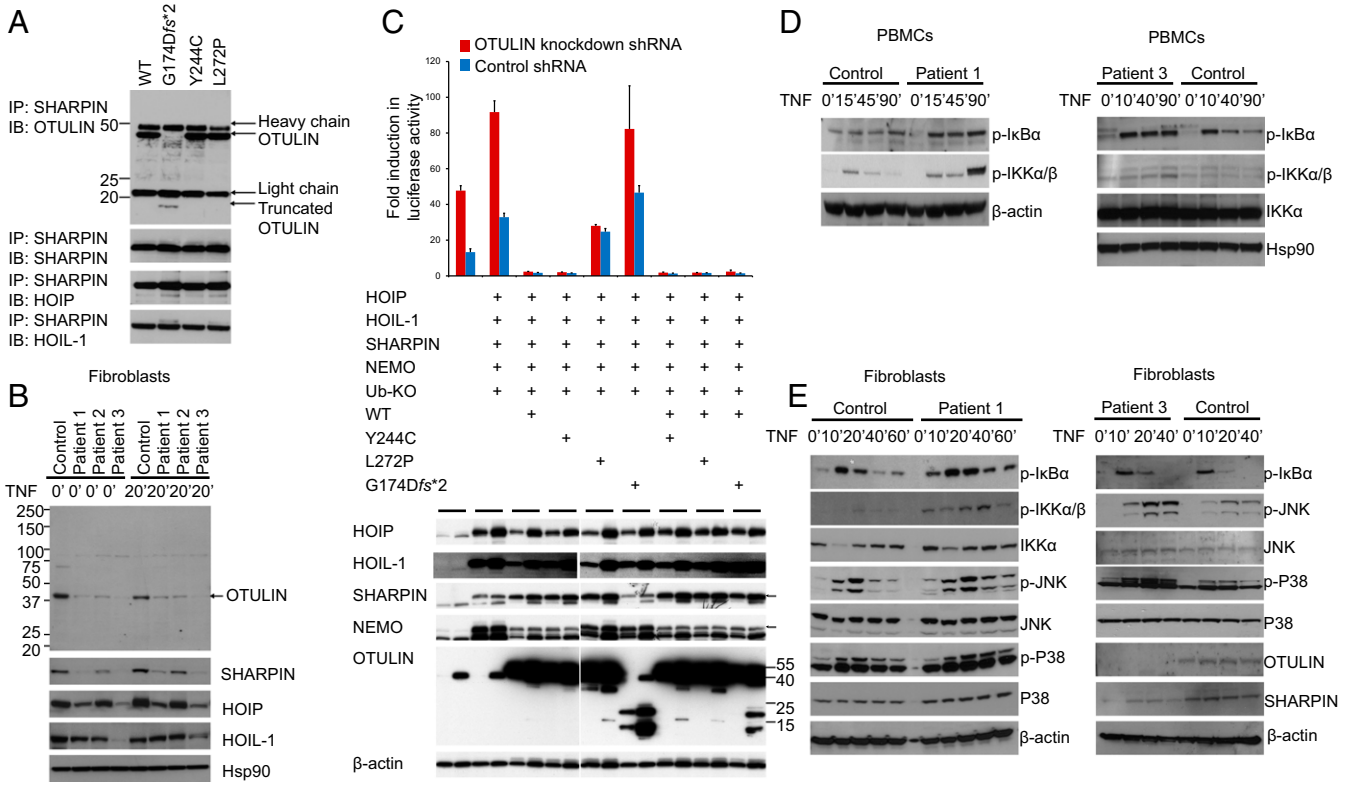

Fig. 2. Induced NF-κB activity in cells with mutant OTULIN. (*A*) OTULIN mutants do not disrupt interaction with LUBAC. HEK293 cells were transiently transfected with plasmids encoding the LUBAC components (SHARPIN, HOIP, and HOIL-1), and WT or mutant OTULIN plasmids. Whole-cell lysates were collected 36 h later, and subjected to immunoprecipitation with antibodies against SHARPIN. The precipitates were then immunoblotted with OTULIN, SHARPIN, HOIP, and HOIL-1. (*B*) OTULIN and LUBAC complex expression in patients' fibroblasts. Whole-cell lysates from the OTULIN-deficient patients and one healthy donor were immunoblotted with antibodies for OTULIN, SHARPIN, HOIP, HOIL-1, and Hsp90. (*C*) NF-κB luciferase assay in transiently transfected HEK293 cells with endogenous OTULIN down-regulated by shRNA. OTULIN mutants p.L272P and p.G174Dfs*2 do not inhibit LUBAC-induced NF-κB activation in HEK293 cells transfected with firefly NF-κB reporter plasmid, a *Renilla* luciferase control vector, and expression plasmids for WT or mutant OTULIN, together with LUBAC (SHARPIN, HOIL-1, HOIP), Ub-KO (ubiquitin mutant with all lysines mutated to arginines, which only forms linear polyubiquitin chains), and LUBAC linear ubiquitination substrate NEMO. Results are plotted as firefly normalized to *Renilla* luciferase activity to account for variance in transfection efficiency and cell number. One representative result of three independent experiments is shown. Values are reported as the means of technical triplicates ± SEM. (*Lower*) Whole-cell lysates from transfected cells were immunoblotted with antibodies for HOIP, HOIL-1, SHARPIN, NEMO, OTULIN, and β-actin. (*D*) PBMCs from OTULIN-deficient patients showed increased levels of phosphorylated IκBα and phosphorylated IKKα/IKKβ compared with a healthy control. Whole-cell lysates from TNF-stimulated PBMCs were immunoblotted for respective target proteins. (*E*) Stimulated fibroblasts from OTULIN-deficient patients sustained increased levels of phosphorylated IκBα, increased phosphorylated IKKα/IKKβ, and increased phosphorylated JNK and P38. Fibroblasts from patients 1 and 3 were stimulated with TNF for the time periods indicated. Whole-cell lysates were immunoblotted for respective target proteins. Two healthy individuals' fibroblasts served as controls.

Mutant p.Tyr244Cys plasmid suppressed the LUBAC-induced NF-κB activity similar to WT OTULIN. These results indicate that p. Leu272Pro and p.Gly174Aspfs*2 mutations affect the OTULIN enzyme activity, whereas the p.Tyr244Cys mutation retains sufficient residual OTULIN activity in the overexpression experiment, or may affect the protein function in a different manner. We then studied the activity of the NF-κB pathway in stimulated patients' fibroblasts and peripheral blood mononuclear cells (PBMCs) (Fig. 2 *D* and *E*, and Fig. S5*B*). Sequential phosphorylation of IKKs and IκBα are essential steps in activation of the canonical NF-κB pathway (13). Patient-derived mononuclear leukocytes and fibroblasts sustained higher levels of phosphorylated IKKα/IKKβ and IκBα, and showed increased phosphorylation of P38 and JNK MAP kinases compared with healthy controls. These results demonstrate enhanced signaling of the NF-κB and MAPK pathways in OTULIN-deficient patients. Our data also suggest that NF-κB activation was not affected in patient's lymphocytes in the context of T-cell receptor and B-cell receptor stimulation (Fig. S4 *B* and *C*).

Defect in the Deubiquitinase Function of Mutant OTULIN Proteins. OTULIN cleaves Met1-linked linear polyubiquitin chains from target substrates, such as NEMO (IKKγ), RIPK1, ASC, and TNFR1 to restrict signaling activation and propagation (7, 14, 15). To investigate the effect of OTULIN mutations on its deubiquitinase function, we cotransfected WT and mutant OTULIN plasmids into HEK293 cells along with plasmids encoding the LUBAC subunits, mono specific-ubiquitin plasmid, and each of the OTULIN substrates NEMO (Fig. 3*A*), RIPK1 (Fig. 3*B*), ASC (Fig. 3*C*), and TNFR1 (Fig. 3*D*). Cells transfected with mutant p.Leu272Pro and p.Gly174Aspfs*2 proteins showed substantial defects in deubiquitination of the target substrate as indicated by accumulated high–molecular-weight linear-ubiquitin aggregates (Fig. 3 *A*–*D*). The defect in RIPK1 deubiquitination was more noticeable in cells transfected with WT monoubiquitin plasmid (Fig. 3*B*) than in cells transfected with the mutant monoubiquitin plasmid (Ub-KO), which can only form linear ubiquitin chains (Fig. S5*C*). RIPK1 and TNFR1 require the assembly of K63 and linear polyubiquitin chains for proper signaling activity, and they are subject to deubiquitination by A20 and OTULIN (14). The K63 ubiquitination of RIPK1 was not affected by the presence of mutant OTULIN proteins (Fig. 3*B*, second panel). Cells transfected with p.Tyr244Cys

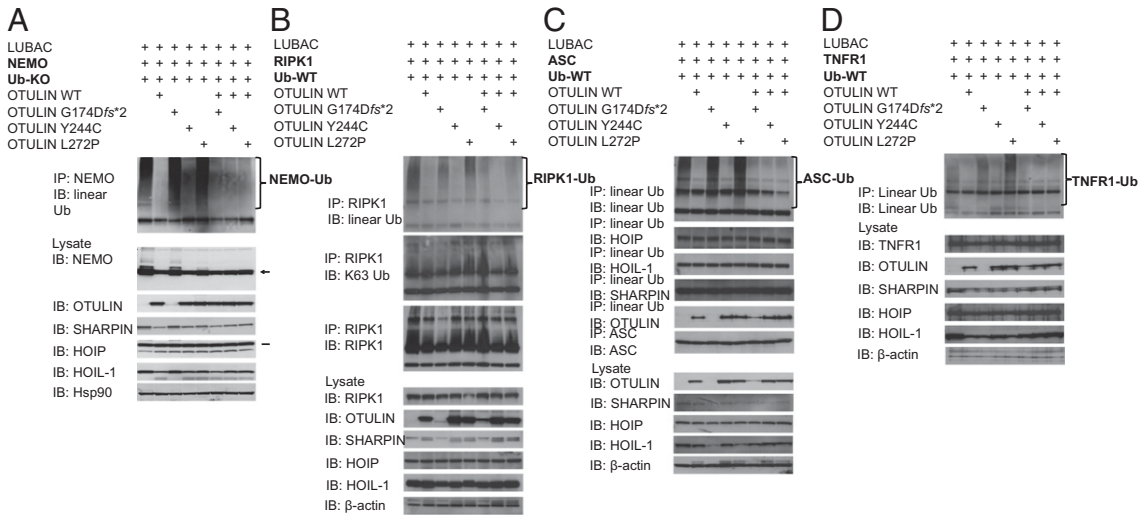

Fig. 3. Mutant OTULIN plasmids show impaired deubiquitinase function in transfected HEK293 cells. (*A*–*D*) OTULIN mutants failed to deubiquitinate Met1-linked linear ubiquitin chains from molecules targeted by LUBAC. HEK293 cells were transiently transfected with expression plasmids for one of the OTULIN target proteins, including NEMO (*A*), RIPK1 (*B*), ASC (*C*), or TNFR1 (*D*) together with plasmids for the LUBAC (SHARPIN, HOIL-1, and HOIP), Ub plasmids (Ub-KO: ubiquitin mutant with all lysines mutated to arginines, which only forms linear polyubiquitin chains; Ub-WT: WT ubiquitin, which forms linear polyubiquitin chains and other polyubiquitin chains, such as K48-Ub, K63-Ub chains), and WT or mutant OTULIN plasmids. Whole-cell lysates were subjected to immunoprecipitation with antibodies against NEMO (*A*), RIPK1 (*B*), ASC (*C*), and linear ubiquitination chain (*C* and *D*). High–molecular-weight (HMW) ubiquitin aggregates (*Top*) were detected by immunoblotting the precipitates with linear Ub antibody (*A*–*D*). Cell lysates were also blotted with antibody against NEMO, RIPK1, TNFR1, ASC, HOIP, SHARPIN, HOIL-1, OTULIN, and Hsp90 or β-actin.

plasmid showed only mild, if any, defect compared with the other two mutant proteins (Fig. 3 *A*–*D*). The in vitro-observed defect in DUB activity of mutant proteins was rescued by cotransfection with WT OTULIN (Fig. 3 *A*–*D*).

Increased Linear Ubiquitination in Patients' PBMCs and Fibroblasts. Consistent with the data from overexpressed mutant proteins, TNF- or IL-1β–stimulated OTULIN-deficient primary patients' cells showed accumulation of linear-ubiquitinated NEMO (Fig. 4 *A* and *C*),

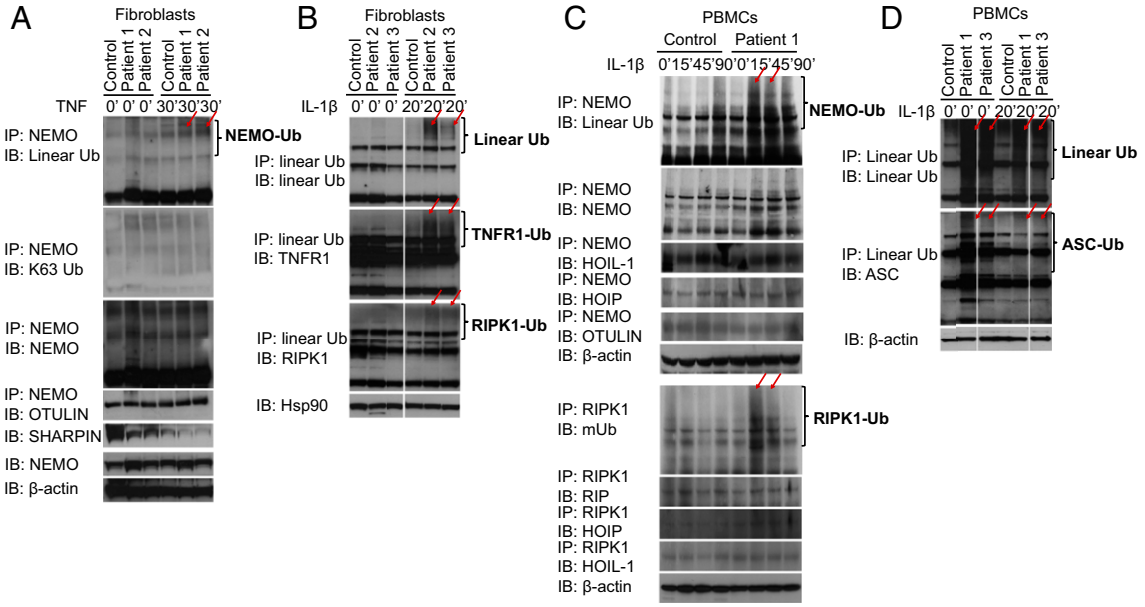

Fig. 4. Patient-derived PBMCs and fibroblasts lose their ability to deubiquitinate Met1-linked linear ubiquitin chains. Whole-cell lysates were immunoprecipitated with antibodies for NEMO (*A* and *C*), linear Ub (*B* and *D*), and RIPK1 (*C*), and the precipitates were blotted with antibodies against linear ubiquitin. The precipitates were also blotted with antibodies against NEMO, TNFR1, RIPK1, K63-linked ubiquitin, HOIP, HOIL-1, or OTULIN, and the cell lysates were blotted with antibodies against Hsp90 or β-actin. Red arrows point to the differences for comparison. (*A*) Patients' TNF-stimulated fibroblasts showed increased abundance and molecular weight of linear-ubiquitinated NEMO as a result of the impaired OTULIN deubiquitinase activity. K63-ubiquitinated NEMO is mainly unaffected (second panel). Fibroblasts from patient 1, patient 2, and a healthy control were stimulated with TNF for 30 min. (*B*) Patients' IL-1β–stimulated fibroblasts showed increased abundance of linear ubiquitinated TNFR1 and RIPK1 and accumulation of high-molecular linear-ubiquitin chains. Fibroblasts from patient 2, patient 3, and a healthy control were stimulated with IL-1β for 20 min. (*C* and *D*) Patients' IL-1β–stimulated PBMCs showed increased linear ubiquitination of NEMO (*C*, *Upper*), RIPK1 (*C*, *Lower*), and ASC (*D*, *Lower*), and accumulation of high-molecular linear-ubiquitin chains (*D*, *Upper*). PBMCs from patients 1 and 3, and a healthy control were stimulated with IL-1β for the indicated time. mUB is a mouse monoclonal antibody that is not linear Ub specific (*C*).

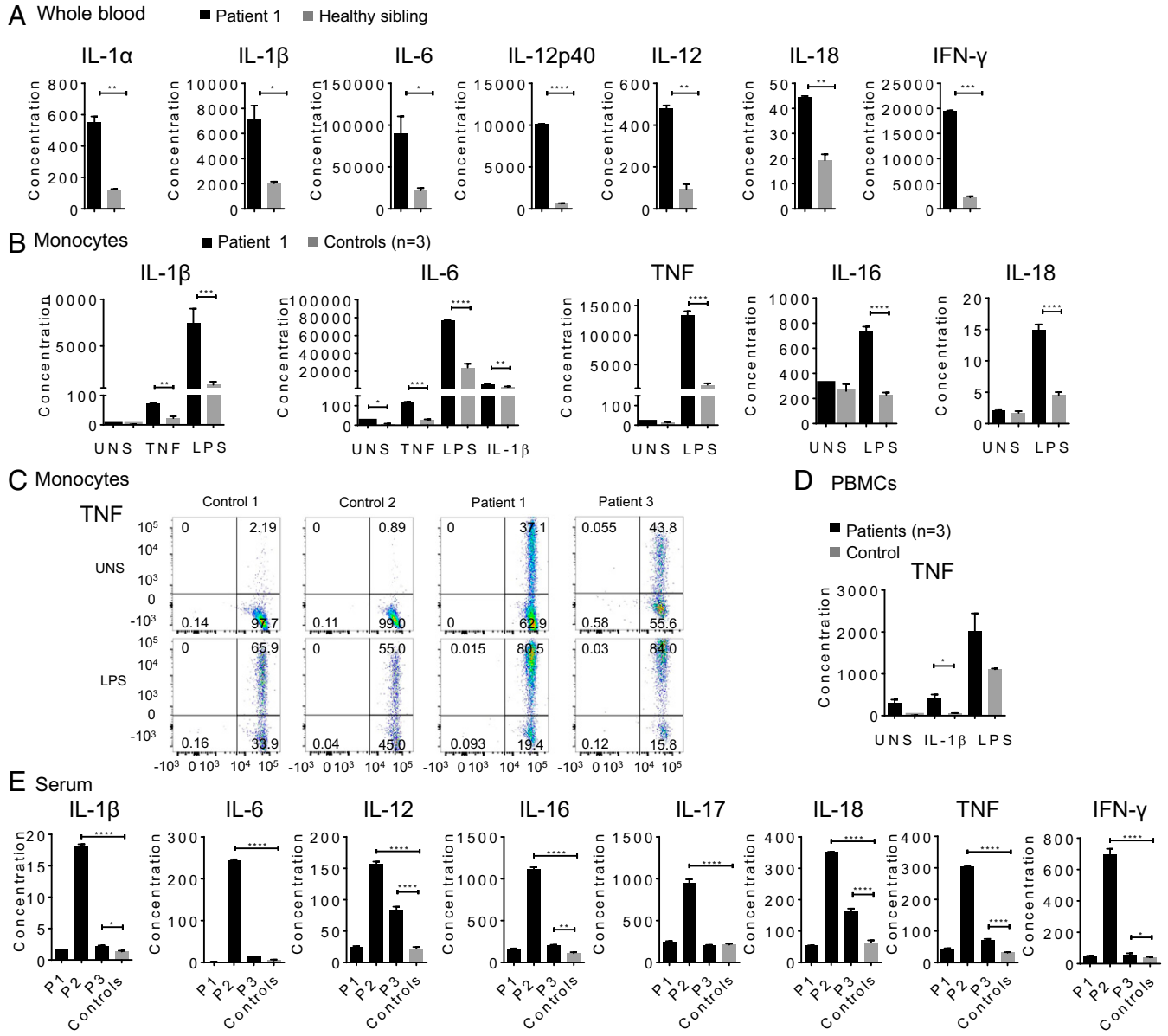

Fig. 5. Patient-derived immune cells display a strong inflammatory signature. Cytokine profiles are compared for OTULIN-deficient patients and healthy controls. Cytokine concentration shown in *y* axis is in picograms per milliliter. Values are represented as means ± SEM. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$. (*A*) Whole-blood samples from patient 1 and his unaffected sibling were stimulated with bacterial LPS (1 μg/mL) for 24 h. A total of 48 cytokines or growth factors listed in *SI Materials and Methods* were assayed in duplicates. (*B*) Cytokine levels from the supernatant of stimulated purified monocytes from patient 1 compared with three healthy controls. Cells were unstimulated, TNF stimulated (20 ng/mL), LPS stimulated (1 μg/mL), or IL-1β stimulated (10 ng/mL) for 48 h. A total of 48 cytokines or growth factors were assayed; however, only the most significant results are shown. Samples were assayed in duplicates. (*C*) Intracellular staining of TNF in monocytes from patient 1 and patient 3 compared with two healthy controls before stimulation and following LPS stimulation (1 μg/mL). PBMCs from patient 2 were not available due to sample limitation. (*D*) TNF levels in the supernatants of PBMCs derived from three patients and one healthy control, at the basal level and after stimulation with IL-1β (10 ng/mL), and LPS (1 μg/mL). Samples were assayed in triplicates. (*E*) Serum cytokine levels from 3 patients and 12 healthy controls. Patient 1 (P1) has been treated with the TNF inhibitor, infliximab, and had no evidence of active disease at the time of sampling. Patient 2 (P2) has been treated with the IL-1 inhibitor, anakinra, and had active disease at the time of sampling. Patient 3 (P3) has been treated with the TNF inhibitor, etanercept, and still had some symptoms at the time of sampling. Patients' samples were assayed in triplicates.

TNFR1 (Fig. 4*B*), RIPK1 (Fig. 4 *B* and *C*), and ASC (Fig. 4*D*), and accumulation of high-molecular linear Ub aggregates (Fig. 4 *B* and *D*) compared with healthy controls. In ex vivo experiments with cells from patient 2, who carries the p.Tyr244Cys mutation, we noted an increase in the linear ubiquitinated NEMO, TNFR1, RIPK1 (Fig. 4 *A* and *B*), and accumulation of high-molecular linear Ub aggregates (Fig. 4*B*). This ex vivo experiment supports the strong genetic data for pathogenicity of the p.Tyr244Cys mutation identified in patient 2. The combination of in vitro and ex vivo experiments provides compelling evidence that loss-of-function mutations in OTULIN result in increased linear ubiquitination of signaling molecules and lead to enhanced TNFR1, NF-κB, and ASC-dependent inflammation.

A Strong Inflammatory Signature in OTULIN-Deficient Patients' Cells. Stimulated patient whole-blood samples showed an increased production of IL-1β, IL-6, IL-12, IL-18, and IFN-γ in response to

LPS, and increased levels of multiple cytokines and chemokines in response to staphylococcal enterotoxin B stimulation (Fig. 5*A* and Fig. S6*A*). Purified patients' monocytes had significantly higher secretion of IL-1β, IL-6, IL-16, IL-18, and TNF in response to LPS, TNF, or IL-1β stimulation relative to cells from healthy controls (Fig. 5*B*). Intracellular staining for TNF and IL-6 from patient 1 and 3 was higher at basal levels in monocytes (Fig. 5*C* and Fig. S6*B*), T cells (Fig. S6*C*), and dendritic cells (Fig. S6*D*) than in controls. Fig. 5*D* represents the average of TNF responses assayed on the three patients individually. Cytokine profiling in serum samples was consistent with disease activity. Patient 2, who had the most active disease at the time of sampling, had the highest levels of proinflammatory cytokines. Patients 1 and 3 had less active disease phenotypes at the time of sampling and substantially lower cytokine levels (Table S4 and Fig. 5*E*). Transcriptome profiling of patient whole-blood samples and stimulated fibroblasts showed similar results. Patients 2 and 3 displayed strong inflammatory signatures enriched for NF-κB, Jak-STAT, and TNF signaling (Fig. S7 *A* and *B*). In contrast, patient 1, whose disease was both clinically and biochemically inactive at the time of visit, had a transcriptome profile similar to controls. These data provide evidence that a malfunction in linear deubiquitination leads to an up-regulation in cytokine production and that the disease is amenable to targeted anticytokine treatment.

Discussion

We describe a recessively inherited autoinflammatory disease caused by excessive linear ubiquitination in innate immune signaling pathways, which we denote as "otulipenia." We show that OTULIN deficiency leads to increased linear ubiquitination of target proteins, which is associated with enhanced NF-κB activity, increased TNFR1 signaling, and NLRP3 inflammasome activity. The phenotype is very severe and potentially lethal if left untreated.

This is a report of a human disease caused by excessive linear ubiquitination. Conversely, patients with LUBAC deficiency have impaired linear ubiquitination of the same target molecules, which leads to immunodeficiency due to decreased NF-κB activity in fibroblasts, and a concomitant inflammatory phenotype due to hyperresponsiveness to IL-1β in monocytes (4). These latter studies demonstrate cell type-specific functions of the LUBAC subunits HOIP and HOIL-1. No human disease has yet been linked to SHARPIN deficiency. In contrast, OTULIN-deficient patients have a broader constitutive inflammatory phenotype in fibroblasts and monocytes and no overt primary immunodeficiency. Heterozygote carriers of these mutations are asymptomatic, which suggests that OTULIN expression levels do not appear to be critical for immune homeostasis. The importance of the linear ubiquitin pathway in the regulation of innate immune responses has been demonstrated in murine models. Mice deficient in LUBAC subunits have variable degrees of inflammation, from a mild phenotype in HOIL-1–deficient mice (16) to more severe inflammation and dermatitis in SHARPIN KO (2, 17) to defective vascularization and embryonic lethality in HOIP KO (18). Consistent with the essential function of OTULIN in regulation of multiple signaling pathways, OTULIN-deficient mice (*gumby*/*gumby*) are embryonic lethal due to vascular and neuronal defects caused by dysregulation in canonical Wnt signaling (8).

This is the second report of human germline mutations in a deubiquitinase protein leading to an inflammatory phenotype, the first being mutations in DUB A20 (10). In contrast, deficiency of another deubiquitinase CYLD, which hydrolyzes both Met1 and K63 ubiquitin chains, leads to cylindromatosis (19). Although A20 and OTULIN have roles in attenuating common signaling pathways, patients with otulipenia have a more severe inflammatory phenotype than patients with A20 haploinsufficiency (HA20) likely for two reasons: (*i*) OTULIN has a unique and nonredundant function in regulation of the linear ubiquitin pathway, and (*ii*) patients with otulipenia have a more profound protein deficiency than patients with HA20, who still retain 50% of nonmutant A20 protein. The discoveries of otulipenia, HA20, and LUBAC deficiencies demonstrate a complex interplay between LUBAC and deubiquitinases in controlling immune signaling complexes.

Materials and Methods

Patients. We studied three patients in this report. All subjects and their family members were enrolled in an Institutional Review Board-approved protocol and provided written informed consent. Samples from patient 1 were available for all experiments, whereas samples from patients 2 and 3 were limited. More detailed information is reported in *SI Materials and Methods*.

Genetic and Functional Analysis. We performed whole-exome sequencing in patients 1 and 2 and their family members, candidate-gene sequencing in patient 3 and her parents, and mutation-specific genotyping in 1,630 DNA samples from the Turkish population. To study protein function, we used short hairpin RNA (shRNA) knockdowns in 293 cells and NF-κB luciferase assay, and Met1-linked linear polyubiquitin deubiquitination assay in 293 cells. Immunoprecipitation and immunoblotting, flow cytometry, Nanostring, intracellular cytokine staining, and cytokine profiling were performed on samples from the patients and healthy controls. *SI Materials and Methods* describes the methods used for all these procedures.

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