Immunodeficiency, autoinflammation and amylopectinosis in humans with inherited HOIL-1 and LUBAC deficiency

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We report the clinical description and molecular dissection of a new fatal human inherited disorder characterized by chronic autoinflammation, invasive bacterial infections and muscular amylopectinosis. Patients from two kindreds carried biallelic loss-of-expression and loss-of-function mutations in HOIL1 (RBCK1), a component of the linear ubiquitination chain assembly complex (LUBAC). These mutations resulted in impairment of LUBAC stability. NF-κB activation in response to interleukin 1β (IL-1β) was compromised in the patients’ fibroblasts. By contrast, the patients’ mononuclear leukocytes, particularly monocytes, were hyper-responsive to IL-1β. The consequences of human HOIL-1 and LUBAC deficiencies for IL-1β responses thus differed between cell types, consistent with the unique association of autoinflammation and immunodeficiency in these patients. These data suggest that LUBAC regulates NF-κB-dependent IL-1β responses differently in different cell types.

Autoinflammatory disorders are characterized by hyperactivation of the immune system in the absence of autoantibodies and self-reactive T cells1. The genetic dissection of several autoinflammatory disorders has implicated two proinflammatory cytokines in these conditions: IL-1β and tumor necrosis factor (TNF)2,3. Autosomal-dominant TNF receptor–associated periodic fever (TRAPS) was the first genetic etiology of autoinflammatory disorders to be identified. It is caused by mutations in TNFRSF1A, which result in the enhanced activation of MAP kinases and the secretion of proinflammatory cytokines in response to lipopolysaccharide (LPS)3. Inflammasomopathies include autosomal-dominant cryopyrin-associated periodic fever (CAPS), also known as chronic infantile neurological cutaneous and articular syndrome (CINCA) or neonatal-onset multisystem inflammatory disease (NOMID), Muckle-Wells syndrome (MWS) and familial cold autoinflammatory syndrome (FCAS), which is caused by mutation of NLRP3 (also known as CIAS1), autosomal recessive familial Mediterranean fever (FMF), caused by mutation of MEFV, autosomal recessive mevalonate kinase (MVK) deficiency, caused by mutation of MVK, autosomal recessive deficiency of the IL-1 receptor antagonist (DIRA), caused by mutation of IL1RN, and autosomal dominant pyogenic arthritis pyoderma gangrenosum and acne (PAPA) syndrome, caused by mutation of PSTPIP1. The clinical phenotypes of these diseases result largely from enhanced IL-1β activity2. All these autoinflammatory disorders are associated with recurrent episodes of...

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fever, skin rash and aseptic organ inflammation, and may be complicated by metabolic abnormalities (for example, 30% of patients with TRAPS develop amyloidosis)\(^1,^3\). Patients with these diseases are not generally prone to unusual infectious diseases with the exception of NF-κB-deficient patients reported recently\(^4\).

In sharp contrast, inborn errors that decrease TNF-mediated and IL-1β-mediated immunity are associated with severe infectious diseases in the context of impaired inflammation. Inborn errors of NF-kB-mediated immunity are associated with impaired cellular responses to various stimuli, including TNF and IL-1β; these diseases include X-linked recessive and autosomal dominant anhidrotic ectodermal dysplasia with immunodeficiency owing to hypomorphic mutations of the NF-kB essential modulator (NEMO), also known as IKBK\(\alpha\) gene and hypermorphic mutations of the IKBa (also known as NFKB\(\alpha\)) gene, respectively\(^5\)–\(^8\). Defects restricted to the pathway involving Toll-like receptor (TLR) and IL-1 receptor (IL-1R), collectively defined as the Toll-like and interleukin 1 receptor (TIR) pathway, which affect the NF-kB, IRF3 and MAPK signaling molecules, include autosomal-recessive IRAK-4 and autosomal-recessive MyD88 deficiencies\(^9\)–\(^11\). Patients with these two deficiencies are prone to the development of life-threatening pyogenic bacterial diseases\(^11\). The range of infections is much broader in patients with NEMO and 1K\(\beta\)T mutations\(^11\). A characteristic of these four inborn errors of immunity is that clinical and biological signs of inflammation are absent or delayed during infectious episodes, although they may reach normal levels during prolonged infection\(^11\). For example, the induction of IL-6–dependent C-reactive protein (CRP) is impaired in these patients\(^1,^11\).

We report here the description and investigation of three patients from two unrelated families displaying a paradoxical clinical phenotype combining autoinflammatory syndrome and pyogenic bacterial diseases. These patients also developed muscular amylopectinosis, consisting of intracellular glycogen inclusions, complicated by myopathy and cardiomyopathy, which have never previously been associated with any inborn error of immunity. These patients carry loss-of-function mutations in HOIL1 (also known as RBCK1), a component of the linear ubiquitination chain assembly complex (LUBAC). This E3 ligase complex, which adds head-to-tail linear polyubiquitin chains to substrate proteins, has been implicated in NF-kB\(^\alpha\) signaling\(^16\)–\(^17\).

RESULTS

**HOIL1 germ-line mutations in patients from two kindreds**

The first kindred investigated (kindred A, French) was not consanguineous, but we nonetheless hypothesized that the two sisters (patients 1 and 2; P1 and P2) suffered from an autosomal-recessive disorder (Fig. 1a; case reports in Supplementary Note and Supplementary Fig. 1). We set out to decipher the underlying genetic defect by two genome-wide approaches: (i) use of a genome-wide human high-density single-nucleotide polymorphism (SNP) array (genome-wide investigations) to search for large genetic lesions, including copy-number variations (CNV) in particular and (ii) a whole-exome sequencing approach to search for small genetic lesions, including coding-gene variations in particular\(^18\)–\(^20\). We identified no homozygous candidate lesion by either approach, suggesting that the two patients might be compound heterozygous. We therefore searched for heterozygous lesions in the same gene by genome-wide investigations and whole-exome sequencing. In both patients, we found a single-copy loss of 31.799 kilobases (kb) on chromosome 20p.13, encompassing the three last exons of TRIB3 and the first four exons of HOIL1 (Fig. 1b). This deletion resulted from a genomic rearrangement owing to nonallelic homologous recombination between intron 1 of TRIB3 and intron 4 of HOIL1 (named TRIB3\(\alpha\)–HOIL1\(\gamma\); Supplementary Fig. 2a,b). The lesion was inherited from the mother and was not transmitted to the healthy siblings. We identified no other mutation in TRIB3 by whole-exome sequencing or Sanger sequencing. By contrast, whole-exome sequencing and Sanger sequencing both showed that the two patients were heterozygous for the paternally derived nonsense p.Q185X (c.553C>T) mutation in exon 5 of HOIL1 (Fig. 1c).
The second kindred investigated (kindred B, Italian) is consanguineous. The search for large genetic lesions by genome-wide investigations was not informative (Fig. 1a; case report in Supplementary Note). By contrast, we identified a homozygous deletion of two nucleotides, cytosine and thymine at positions 121 and 122 (c.121_122delCT) in exon 2 of HOIL1 in the genome of patient 3 (P3) by whole-exome sequencing and confirmed this by Sanger sequencing. This deletion resulted in a frame shift and a premature stop codon (p.L41fsX7; Fig. 1d). Genome-wide linkage and homozygosity mapping showed that the HOIL1 gene was located in a chromosomal region linked to the disease (data not shown). Both parents and one healthy sibling were heterozygous for the mutation. The three HOIL1 variants found in the two kindreds were not found in public databases (US National Center for Biotechnology Information dbSNP, University of California Santa Cruz genome browser and 1000 genomes) or in our own genome-wide investigation and whole-exome sequencing databases of 124 and 621 individuals, respectively. These variants were also absent from the data for the 392 individuals of the Centre d’Etude du Polymorphisme Humain–Human Genome Diversity Project (CEPH-HGDP) panel tested, suggesting that they are not irrelevant polymorphisms. HOIL1 encodes hemoxidized iron-regulatory protein 2 ubiquitin ligase 1 (HOIL1). HOIL1 is one of the components of the LUBAC, an E3 ligase complex that adds head-to-tail linear polyubiquitin chains to substrate proteins. The large deletion in HOIL1 in P3 was predicted to result in the deletion of all functional domains of HOIL1. The nonsense mutation in P1 and P2 was predicted to result in premature truncation in the novel zinc finger (NZF) domain of HOIL1. The Ubl domain is required for LUBAC formation and linear ubiquitination. Collectively, these genetic data suggest that P1 and P2 from kindred A and P3 from kindred B are homozygous, for rare deleterious alleles of HOIL1.

**Impaired HOIL1 expression in the patients’ cells**

We assayed TRIB3 and HOIL1 mRNA by reverse transcription–quantitative PCR (RT-qPCR) in SV40-immortalized fibroblasts from patients and controls. The amounts of TRIB3 mRNA were normal in the patients’ cells (data not shown), whereas the amounts of HOIL1 mRNA were one-third to one-quarter those in the controls (Fig. 2a). Similarly, TRIB3 protein was present in the patients’ cells (Fig. 2b), whereas HOIL1 was undetectable by immunoblotting with antibodies to N-terminal (Fig. 2b) or C-terminal part of the protein (data not shown). HOIL1 was also not detectable in Epstein-Barr virus (EBV)-immortalized B cell lines derived from P2 (data not shown). LUBAC is thought to consist of three protein subunits: HOIL1, SHANK-associated RH domain-interacting protein (SHARPIN) and HOIL1-1 interaction protein (HOIP, also known as RNF31). We thus assessed the abundance of these three components of LUBAC in fibroblasts. In cells from the three patients, SHARPIN protein amounts were about 50% lower than those in control cells and HOIP was almost undetectable (Fig. 2c). It was therefore not possible to coimmunoprecipitate SHARPIN with HOIP in cells from P1 (Fig. 2d).

No lesion in HOIP or SHARPIN was detected by genome-wide investigations and whole-exome sequencing in the three patients. We then used fibroblasts from P1 and P2 to investigate whether the expression of a wild-type HOIL1 allele rescued LUBAC expression: HOIL1 production was restored in the patients’ cells by stable transfection with wild-type HOIL1 (Fig. 2e). This expression of HOIL1 also restored expression of the other two LUBAC components, HOIP and SHARPIN, to the amount observed in healthy controls (Fig. 2e).

These findings demonstrate that HOIL1 is required for the overall stability of the LUBAC. Cells from the two compound heterozygous patients (P1 and P2) and from the patient homozygous for HOIL1 mutations (P3) therefore displayed HOIL1 deficiency, resulting in a large decrease in the amounts of the other two molecules normally present in the LUBAC.

**Impaired NF-κB activation in fibroblasts and B cells**

It has been suggested that, at least on some cell types, the LUBAC regulates activation of the canonical NF-κB pathway, which has a key role in inflammatory and immune responses. We therefore assessed the functionality of the canonical NF-κB pathway in SV40-immortalized fibroblasts from the patients. The lack of HOIL1 in the patients’ cells led to lower levels of IKK kinase phosphorylation, slower IkBα degradation and a decrease in NEMO ubiquitination in response to stimulation with either TNF or IL-1β (Fig. 3a,b). By contrast, JNK phosphorylation in response to both stimuli was normal in the patients’ fibroblasts (Fig. 3a,b). The lower amounts...
of NF-κB activation in the patients' cells were associated with lower NF-κB transcriptional activity, as assessed by NF-κB reporter assays (Supplementary Fig. 3a). This impairment of the canonical NF-κB pathway led to weak induction of the NF-κB target genes IL6 and TNFAIP3 (also known as A20), which are normally induced by TNF or IL-1β (Supplementary Fig. 3b). Consistent with these RT-qPCR data, IL-6 production, as assessed by enzyme-linked immunosorbent assay (ELISA), in response to TNF or IL-1β treatment was weak in cells from the patients (Fig. 3c and Supplementary Fig. 4a,b), and the response to IL-1β treatment was more severely impaired than that to TNF treatment. The induction of the NF-κB target gene products, cellular inhibitors of apoptosis, was weaker in cells from P2 than in those from controls, and in response to stimulation with TNF we observed higher amounts of caspase-3 cleavage compared to the amounts in controls (Supplementary Fig. 4c). These observations indicated that HOIL-1–deficient cells were more susceptible than controls to TNF-induced apoptosis. Introduction of wild-type HOIL-1 into fibroblasts from P1 restored IKK kinase phosphorylation, IκBα degradation and IL-6 secretion in response to both TNF and IL-1β treatment (Fig. 3d–f). This confirmed that the HOIL-1 mutant alleles in the patients' fibroblasts were responsible for the impairment of the NF-κB response to both TNF and IL-1β. Analysis of IL-6 production in the patients' fibroblasts after stimulation with TLR2, TLR6 or TLR4 agonists indicated that these TLR pathways were also partially affected (Supplementary Fig. 4d). IFN-β production after stimulation with the TLR3 agonist poly(I:C) was abolished in fibroblasts from the patients, whereas after stimulation of the RNA helicase RIG-I with 7SK-AS or poly(I:C) plus Lipofectamine resulted in normal IFN-β production (Supplementary Fig. 4e). Likewise, stimulation with R-848 (TLR7 and TLR8 agonist) did not induce TNF production in the patients' EBV-immortalized B cells (Supplementary Fig. 5a). Finally, IKK phosphorylation and IκBα degradation in response to both IL-1β and CD40L were abolished in EBV-immortalized B cells from P2 (Supplementary Fig. 5b,c). Overall, NF-κB responses to all tested agonists of TNF receptor (TNFR), IL-1R and TLR family members were impaired in fibroblasts and EBV-immortalized B cells from patients with HOIL-1 deficiency.

**Impaired NEMO recruitment to cytokine receptors**

We investigated the molecular mechanisms underlying the defects in NF-κB activation in HOIL-1–deficient fibroblasts. It has been suggested that LUBAC regulates NF-κB activation by stabilizing the recruitment of the NEMO-IKK complex to the cytokine-activated receptor. We therefore investigated the consequences of HOIL-1 deficiency for NEMO recruitment to the TNF and IL-1 receptor signaling complexes (RSCs). In control cells, NEMO was rapidly and transiently recruited to the RSCs, and we observed a clear enrichment in ubiquitinated NEMO species (Fig. 4a,b). However, in cells from the HOIL-1–deficient patients, less NEMO was recruited to the TNF RSC (particularly for the ubiquitinated NEMO species; Fig. 4a), and NEMO recruitment to the IL-1 RSC was almost entirely abolished (Fig. 4b). SHARPIN recruitment to the RSCs was also reduced, especially in response to IL-1β (data not shown). We then investigated the association between NEMO and the polyubiquitinated...
Figure 4  Impaired recruitment of NEMO to cytokine receptors in the patients’ fibroblasts. (a) Immunoblotting of total NEMO (top left) and TNFR (bottom) in whole-cell extracts from Flag-TNF–stimulated cells, and of TNF RSC–associated NEMO (right; cells were stimulated with Flag-TNF and lysed, and TNF RSC was purified on Flag-affinity resin). [Ub]-NEMO indicates the ubiquitinated species of NEMO. (b) Same as in a, except that Flag–IL-1β was used to stimulate the cells and to isolate the IL-1 RSC. IL-1R in whole-cell extracts is shown as a control (bottom left). (c) Immunoprecipitation with anti–NEMO (NEMO IP) of whole-cell lysates from control or patient (P1) fibroblasts treated with biotinylated TNF and subsequently analyzed by immunoblotting for RIP1, NEMO and IKKβ (bottom three gels). TNF RSC–associated RIP1, HOIL-1 (arrow) and SHARPIN were evaluated by streptavidin (strept) pull-down followed by immunoblotting (top three gels). (d) Immunoprecipitation of IRAK-1 (top) or NEMO (bottom) from lysates of IL-1β–treated fibroblasts and subsequent immunoblot analysis for IRAK-1 and NEMO as indicated. Data are representative of three experiments.

Genome-wide impact of HOIL-1 deficiency in fibroblasts

The above findings are generally consistent with the high incidence of opportunistic infections in the HOIL-1–deficient patients, which was similar to that of patients with impaired IL-1R–dependent and/or TNFR–dependent NF-κB immunity. However, unlike patients with these other immunodeficiencies, HOIL-1–deficient patients also presented with autoinflammation and amylopectinosis. We therefore investigated the effects of HOIL-1 deficiency on TNF and IL-1β responses, by analyzing the genome-wide transcriptional profiles (47,231 probes) of primary fibroblasts from four healthy controls, three HOIL-1–deficient patients (P1, P2 and P3), one MYD88-deficient patient and one NEMO-deficient (IKBKG-deficient) fetus. In control fibroblasts, 544 and 2,208 transcripts were regulated by IL-1β after 2 h and 6 h of stimulation, respectively, and 456 and 1,614 transcripts were regulated by TNF at the same time points. NEMO-deficient cells did not respond to either stimulus, whereas, as expected, MyD88-deficient cells responded to TNF but not to IL-1β. By contrast, HOIL-1–deficient cells were poorly responsive to IL-1β, as assessed after 2 h and 6 h; the response to TNF was almost identical to that of control cells after 2 h, but was weaker than that of control cells after 6 h. A more detailed analysis showed that control fibroblasts responded to IL-1β and TNF by the rapid induction of inflammatory cytokines, chemokines and cell-surface receptors (for example, CCL5, CCL8, CXCL1, CXCL2, CXCL10, CXCR7, IL1β and TNFAIP3 (also known as A20) genes). The upregulation of these transcripts after 6 h of IL-1β stimulation was generally blunted in patients with HOIL-1 deficiency, although there were a few notable exceptions (for example, CCL2, CXCL1, IL8 and IL32). Thus, HOIL-1 deficiency in human fibroblast cells results in the impaired expression of NF-κB target genes in response to IL-1β and, to a lesser extent, TNF. The genome-wide transcriptome analysis data for fibroblasts were therefore consistent with the in vitro investigations described above and, in particular, with the receptor-recruitment data (Fig. 4a.b): HOIL-1 deficiency and NEMO deficiency had many similarities, with only a few differences in terms of the fibroblast response to IL-1β, whereas the responses to TNF were more discordant.

Genome-wide impact of HOIL-1 deficiency in leucocytes

We studied the basis of autoinflammation in the patients further, by investigating their leukocytes ex vivo. We first investigated the consequences of HOIL-1 deficiency, by analyzing the genome-wide transcriptional profiles of whole blood cells from P1 (without acute phase and free of infection) in the absence of stimulation. We compared the transcriptional profile of P1 with those of 41 healthy age-matched children and other patients with various genetically determined autoinflammatory disorders, including CAPS disorders (CINCA (2 patients) and MWS (5 patients) and MVK deficiency (2 patients). Blood cells from P1 had a unique gene expression profile (Fig. 6a and Supplementary Fig. 6a), with 2,900 transcripts upregulated or downregulated by a factor of at least two with respect to healthy controls. Transcripts encoding HOIL-1, IFN-γ and several members of the TNF family including CD30L (also known as TNFSF8) and APRIL (also known as TNFSF13) were downregulated by a factor of at least two. Transcripts encoding the proinflammatory cytokines IL-6, IL-6R and IL-6ST were upregulated in the patient’s blood ex vivo (Supplementary Fig. 6b). Genes belonging to networks involved in cell death, cell cycle, cell signaling, the inflammatory response, immune cell trafficking and carbohydrate metabolism were identified as being dysregulated in the patient. Using a pre-established framework of transcriptionally
co-regulated transcripts, we demonstrated a distinct upregulation of erythroid lineagerelated and ubiquitination-related transcripts that differentiated the pattern of gene expression in the blood cells from this patient from those of patients with NLRP3 and MVK mutations (Supplementary Fig. 6a). We also retrospectively studied the concentrations of inflammatory markers and cytokines in plasma samples collected from P1 and P2 at various ages. Consistent with the clinical phenotype, background concentrations of CRP and IL-6 were high, increasing further during each inflammatory episode (Fig. 6b). The concentrations of other proinflammatory cytokines, including IL-8, TNF and IL-1β, and of some anti-inflammatory cytokines, notably of the IL-1R antagonist, were also high (Fig. 6b and data not shown). Using ELISA, we then studied the production of cytokines by whole blood cells from P1 and P2 in response to stimulation (IL-1β, TLR agonists, two heat-killed pneumococcus strains and TNF). IL-1β and agonists of TLR1 and TLR2 induced IL-6 production in both patients more strongly than in healthy controls, whereas IL-10 production in response to treatment with TNF was abolished (Fig. 6c,d and Supplementary Fig. 6c). Therefore, by contrast to the phenotype seen with fibroblasts, leukocytes from these two HOIL-1–deficient patients appeared to be both constitutively hyperinflammatory and hyperresponsive to IL-1β (but probably not TNF) ex vivo; this may explain, in part, the patients’ autoinflammatory syndrome in vivo.

Hyperactivation of HOIL-1–deficient mononuclear leukocytes

We further investigated the inflammatory phenotype observed in whole blood cells from HOIL-1–deficient patients. We analyzed

Figure 6 Whole-blood analysis reveals a new hyperinflammatory disorder in HOIL-1–deficient patients. (a) Transcriptional profiles of whole blood from HOIL-1–deficient patient (P1), two patients with CINCA, five patients with MWS and two MVK-deficient patients. Comparison of the transcriptional profile of P1 with those of 41 healthy, age-matched children and other patients with CAPS (2 CINCA, 5 MWS and 2 MVK patients). P1 presented a distinct pattern of gene expression in the blood, with 2,900 transcripts upregulated or downregulated by a factor of more than two with respect to healthy controls. Red, blue and yellow indicate a relative increase, decrease and no change in expression, respectively. (b) Quantification of IL-1RA (IL-1 antagonist), TNF, IL-6 and CRP in plasma samples from healthy donors, P1 and P2 taken at various ages. Individuals with CRP concentrations above 3 mg/ml were considered to have an inflammatory condition. (c) IL-6 secretion by whole-blood cells from 30 healthy donors and HOIL-1-deficient patients (P1, P2), treated with IL-1β for 48 h. Error bars, s.e.m. (n = 2); ***P < 0.001. (d) IL-10 secretion by whole-blood cells from healthy donors and HOIL-1–deficient patients (P1 and P2) and the heterozygous parents (I.1 and I.2), activated by incubation with TNF or PMA plus ionomycin (PMA+iono) for 48 h. nd, not detectable. Error bars, s.e.m. (n = 2).
Each dot corresponds to one probe in one individual. The number of probes per gene is 1 for three healthy controls, three HOIL-1–deficient patients (P1, P2 and P3) and two MyD88–deficient patients, extracted from the microarray data. Median gene expression in HOIL-1–deficient or MyD88-deficient patients (relative gene expression) was plotted against the median gene expression sorted by ascending order of fold induction in the healthy controls (two of whom were age-matched). Each dot corresponds to one probe. (b) Levels of IL6, IL8, MIP1A, MIP1B and IL1B mRNA in three healthy controls, three HOIL-1–deficient patients (P1, P2 and P3) and two MyD88–deficient patients, extracted from the microarray data. Each dot corresponds to one probe in one individual. The number of probes per gene is 1 for IL6 and IL1B, 2 for MIP1B and IL8 and 4 for MIP1A. The nonparametric Mann-Whitney test was used for statistical analysis. Error bars, s.e.m., n = 3 samples (P1, P2 and P3); **P < 0.1, ***P < 0.05, ****P < 0.005. (c) Cytokine production in CD14+ cell subsets determined by intracellular staining of stimulated PBMCs. PBMCs from P2, seven healthy controls and one IRAK-4–deficient patient were stimulated with TNF, IL-1β or LPS. NS, not stimulated. The secretory pathways were concomitantly blocked with monensin and brefeldin A. Twelve hours after stimulation, cells were immunolabeled with antibodies to CD3, CD14 and CD19, and then permeabilized to assess the production of IL-6, MIP-1α and IL-8. Cells were analyzed by flow cytometry. Monocytes were gated on CD14+CD3− and CD14+CD19+ cells (one sample). Error bars, s.e.m. (n = 7 samples).

**Figure 7** HOIL-1–deficient monocytes display hyperproduction of IL-6 upon IL-1β stimulation. (a) Transcriptional profile of leukocytes from P1, P2 and P3 after 2 and 6 h of stimulation by TNF or IL-1β. Median gene expression in HOIL-1–deficient or MyD88-deficient patients (relative gene expression) was plotted against the median gene expression sorted by ascending order of fold induction in the healthy controls (two of whom were age-matched). Each dot corresponds to one probe. (b) Levels of IL6, IL8, MIP1A, MIP1B and IL1B mRNA in three healthy controls, three HOIL-1–deficient patients (P1, P2 and P3) and two MyD88–deficient patients, extracted from the microarray data. Each dot corresponds to one probe in one individual. The number of probes per gene is 1 for IL6 and IL1B, 2 for MIP1B and IL8 and 4 for MIP1A. The nonparametric Mann-Whitney test was used for statistical analysis. Error bars, s.e.m., n = 3 samples (P1, P2 and P3); **P < 0.1, ***P < 0.05, ****P < 0.005. (c) Cytokine production in CD14+ cell subsets determined by intracellular staining of stimulated PBMCs. PBMCs from P2, seven healthy controls and one IRAK-4–deficient patient were stimulated with TNF, IL-1β or LPS. NS, not stimulated. The secretory pathways were concomitantly blocked with monensin and brefeldin A. Twelve hours after stimulation, cells were immunolabeled with antibodies to CD3, CD14 and CD19, and then permeabilized to assess the production of IL-6, MIP-1α and IL-8. Cells were analyzed by flow cytometry. Monocytes were gated on CD14+CD3− and CD14+CD19+ cells (one sample). Error bars, s.e.m. (n = 7 samples).

Discussion

We identified autosomal-recessive HOIL-1 deficiency as the cause of a new clinical entity combining invasive pyogenic bacterial infections, systemic autoinflammation and amylopectin-like deposits in muscle. HOIL-1, SHARPIN and HOIP are the three components of LUBAC, and their restoration after HOIL-1 expression suggests that LUBAC is a ternary complex. HOIP, the catalytic center of LUBAC, was almost undetectable in fibroblasts and B cells from HOIL-1–deficient patients, suggesting that these patients are LUBAC-deficient. LUBAC is involved in the NF-κB pathway and conjugates linear polyubiquitin chains onto specific lysine residues of NEMO. HOIL-1–deficient human fibroblasts displayed impaired NF-κB activation, resulting in impaired NF-κB–driven gene transcription and cytokine production in response to TNF and IL-1β, consistent with data in mouse Hoil1 (Rbck1) knockdown or knockout cells. However, TNF-induced and IL-1β–induced JNK activity was normal in HOIL-1–deficient human fibroblasts, whereas TNF-induced JNK activity was enhanced in Hoil1−/− mouse embryonic fibroblasts. Overall, our results demonstrate that human HOIL-1 and LUBAC are required for TNF-induced and IL-1β–induced NF-κB responses, at least in fibroblasts.

LUBAC facilitates the recruitment of the NEMO-IKK complex to cytokine receptors, in at least some cell types. We showed that...
the recruitment of human NEMO to TNF RSCs and IL-1 RSCs was compromised in HOIL-1–deficient fibroblasts. LUBAC-mediated linear ubiquitination may facilitate the activation of IKK kinases; presumably, the attachment of linear ubiquitin chains to NEMO and IRAK-4 or MyD88 mutations, are also prone to pyogenic bacterial infections6,7,9–11,14. The infectious phenotype of MyD88-deficient and IRAK-4 or MyD88 mutations, are also prone to pyogenic bacterial infections6,7,9–11,14. The infectious phenotype of MyD88-deficient and IRAK-4–deficient patients is narrower than that of most patients with NEMO or IkBα mutations6,7,9,11,14. It may not be coincidental that IRAK-4–deficient and MyD88-deficient and HOIL-1–deficient patients have in common a profound lack of response to TIRs and predisposition to pyogenic bacterial diseases. One of the HOIL-1–deficient patients (P3) had a persistent cytomegalovirus infection, but it is unclear whether this was a consequence of HOIL-1 deficiency. Unlike most IRAK-4–deficient and MyD88-deficient patients, patients bearing mutations in NEMO and HOIL1 are deficient in memory B cells, and their antibody response to pneumococcal glycan is impaired. Two HOIL-1–deficient patients (P1 and P2) died before hematopoietic stem cell transplantation (HSCT) and the third (P3) died too soon after HSCT for any firm conclusions to be drawn about a possible hematopoietic origin of the predisposition to infection, as in patients with NEMO and IkBα mutations14. Similarly, Hoil1−/− and spontaneous Sharpin-deficient (also known as Sharpin–chronic proliferative dermatitis (cpdm)) mice are uninformative on this issue, as neither has been challenged with pathogens16,22–24,29.

The susceptibility of these patients to invasive pyogenic bacterial disease is probably due to impaired NF-κB–dependent responses to at least some key members of the TIR and TNFR families in fibroblasts and, possibly, other cell types. Indeed, patients with inborn errors of NF-κB–mediated or TIR-mediated immunity, carrying IkBα, NEMO, IRAK-4 or MyD88 mutations, are also prone to pyogenic bacterial infections6,7,9–11,14. The infectious phenotype of MyD88-deficient and IRAK-4–deficient patients is narrower than that of most patients with NEMO or IkBα mutations6,7,9,11,14. It may not be coincidental that IRAK-4–deficient and MyD88-deficient and HOIL-1–deficient patients have in common a profound lack of response to TIRs and predisposition to pyogenic bacterial diseases. One of the HOIL-1–deficient patients (P3) had a persistent cytomegalovirus infection, but it is unclear whether this was a consequence of HOIL-1 deficiency. Unlike most IRAK-4–deficient and MyD88-deficient patients, patients bearing mutations in NEMO and HOIL1 are deficient in memory B cells, and their antibody response to pneumococcal glycan is impaired. Two HOIL-1–deficient patients (P1 and P2) died before hematopoietic stem cell transplantation (HSCT) and the third (P3) died too soon after HSCT for any firm conclusions to be drawn about a possible hematopoietic origin of the predisposition to infection, as in patients with NEMO and IkBα mutations14. Similarly, Hoil1−/− and spontaneous Sharpin-deficient (also known as Sharpin–chronic proliferative dermatitis (cpdm)) mice are uninformative on this issue, as neither has been challenged with pathogens16,22–24,29.

The HOIL-1–deficient patients displayed no amyloidosis, but had amylopectinosis, in the form of intracellular glycogen inclusions in their muscles. Amylopectinosis has not been reported in Sharpin–cpdm and Hoil1−/− mice16,22–24,32. Several genetic etiologies of human amylopectinosis have been reported35–41. HOIL-1 deficiency is a new cause of amylopectinosis involving unknown mechanisms probably independent of IL-1, TNF and NF-κB, as amylopectinosis is not seen in other disorders associated with enhanced or impaired NF-κB immunity6–8,14. In conclusion, the patients described here displayed a unique combination of clinical phenotypes, highlighting the important and complex role of the LUBAC in inflammation, immunity and metabolism in humans. The paradoxical association of autoinflammation and immunodeficiency probably results from an imbalance between cellular responses, to NF-κB–dependent proinflammatory stimuli at least, mediated by TIRs and TNFRs in particular. The molecular basis of the immunodeficiency may be impaired responses to inflammatory stimuli in fibroblasts and possibly other nonhematopoietic cell types, whereas the autoinflammation may result from enhanced responses to IL-1β in leukocytes, including monocytes in particular.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: GSE40752 (microarray data).

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
B.B., E.L., S.G., A.A., L.I., G.T.-N. and M.C. performed experiments. C. Prando, A.A. and D.V. performed genetic analysis. F.B., M.D., E.M., D. Bonnet, P.Q., L.D.N. and C. Picard provided all the clinical data for the patients. D. Bogunovic., D.M., M.H., F.A. and H.W.V. provided reagents and suggestions. C.B. and C. Picard performed immunological explorations. C.R., F.F. and J.-C.F. performed histological analysis. E.L., Z.X., V. B. and J.-C.F. performed transcriptome analysis. A.I., J.-L.C. and C. Picard coordinated the study, and B.B., E.L., C. Prando, VP, D.C., L.D.N., A.P., A.I., J.-L.C. and C. Picard wrote the manuscript. All authors...
discussed the results and commented on the manuscript. B.B., E.L. and C. Prando equally contributed as first authors. S.G., E.L. and A.A. equally contributed as second authors. V.P., D.C., L.D.N. and A.P. equally contributed as second to last authors. A.I., J.L.C. and C. Picard equally contributed as last authors.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Masters, S.L., Simon, A., Akseintijevich, I. & Kastner, D.L. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease. Annu. Rev. Immunol. 27, 621–668 (2009).
2. Akseintijevich, I. et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. N. Engl. J. Med. 360, 2426–2437 (2009).
3. McDermott, M.F. et al. Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell 97, 133–144 (1999).
4. Ombrello, M.J. et al. Cold urticaria, immunodeficiency, and autoimmunity related to PLAG2 deletions. N. Engl. J. Med. 366, 330–338 (2012).
5. Puel, A., Picard, C., Ku, C.L., Smahi, A. & Casanova, J.L. Inherited disorders of NF-κB-mediated immunity in man. Curr. Opin. Immunol. 16, 34–41 (2004).
6. Doffinger, R. et al. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-κB signaling. Nat. Genet. 27, 277–285 (2001).
7. Courtois, G. et al. A hypermorphic lBxα mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency. J. Clin. Invest. 112, 1108–1115 (2003).
8. Casanova, J.L., Abel, L. & Quintana-Murci, L. Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. Annu. Rev. Immunol. 29, 447–491 (2011).
9. von Bernuth, H. et al. Pyogenic bacterial infections in humans with MyD88 deficiency. Science 321, 691–696 (2009).
10. Picard, C. et al. Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science 299, 2076–2079 (2003).
11. Picard, C. et al. Clinical features and outcome of patients with IRAK-4 and MyD88 deficiency. Medicine 89, 403–425 (2010).
12. Casanova, J.L. & Abel, L. Inborn errors of immunity to infection: the rule rather than the exception. J. Exp. Med. 202, 197–201 (2005).
13. Casanova, J.L. & Abel, L. Primary immunodeficiencies: a field in its infancy. Science 317, 617–619 (2007).
14. Picard, C., Casanova, J.L. & Puel, A. Infectious diseases in patients with IRAK-4, MyD88, NEMO, or lBxα deficiency. Clin. Microbiol. Rev. 24, 490–497 (2011).
15. von Bernuth, H. et al. Septicemia without sepsis: inherited disorders of nuclear factor-κB-mediated inflammation. Clin. Infect. Dis. 41 (suppl. 7), S436–S439 (2005).
16. Tokunaga, F. et al. Involvement of linear polyubiquitylation of NEMO in NF-κB activation. Nat. Cell Biol. 11, 123–132 (2009).
17. Iwas, K. & Tokunaga, F. Linear polyubiquitylation: a new regulator of NF-κB activation. EMBO Rep. 10, 706–713 (2009).
18. Vissers, L.E., Veltman, J.A., van Kessel, A.G. & Brunner, H.G. Identification of disease genes by whole genome CGH arrays. Hum. Mol. Genet. 14, R215–R223 (2005).
19. Byun, M. et al. Whole-exome sequencing-based discovery of STIM1 deficiency in a child with fatal classic Kaposi sarcoma. J. Clin. Investig. 120, 2307–2312 (2010).
20. Bolze, A. et al. Whole-exome sequencing-based discovery of human FADD deficiency. Am. J. Hum. Genet. 87, 873–891 (2010).
21. Kirisako, T. et al. A ubiquitin ligase complex assembles linear polyubiquitin chains. EMBO J. 25, 4877–4887 (2006).
22. Ikeda, F. et al. SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. Nature 471, 637–641 (2011).
23. Gerlach, B. et al. Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 471, 591–596 (2011).
24. Tokunaga, F. et al. SHARPIN is a component of the NF-κB-activating linear ubiquitin chain assembly complex. Nature 471, 633–636 (2011).
25. Tatematsu, K. et al. Transcriptional activity of RBCK1 protein (RBCC protein interacting with PKC1): requirement of RING-finger and B-Box motifs and regulation by protein kinases. Biochem. Biophys. Res. Commun. 247, 392–396 (1998).
26. Tian, Y. et al. RBCK1 negatively regulates tumor necrosis factor- and interleukin-1-triggered NF-κB activation by targeting TAB2/3 for degradation. J. Biol. Chem. 282, 16776–16782 (2007).
27. Haas, T.L. et al. Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction. Mol. Cell 36, 831–844 (2009).
28. Hostager, B.S., Kashiwada, M., Colgan, J.D. & Rothman, P.B. HOIL-1L interacting protein (HOIP) is essential for CD40 signaling. PLoS ONE 6, e23061 (2011).
29. Zakt, D.E. et al. Systems analysis identifies an essential role for SHANK-associated RH domain-interacting protein (SHARPIN) in macrophage Toll-like receptor 2 (TLR2) responses. Proc. Natl. Acad. Sci. USA 108, 11536–11541 (2011).
30. Picard, C., Puel, A., Bustamante, J., Ku, C.L. & Casanova, J.L. Primary immunodeficiencies associated with pneumococcal disease. Curr. Opin. Allergy Clin. Immunol. 12, 167–173 (2012).
31. Seymour, R.E. et al. Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis. Genes Immun. 8, 416–421 (2007).
32. HogenEsch, H., Janke, S., Boggess, D. & Sundberg, J.P. Absence of Peyer’s patches and abnormal lymphoid architecture in chronic proliferative dermatitis (cpdm/cpdm) mice. J. Immunol. 162, 3890–3896 (1999).
33. HogenEsch, H. et al. Increased expression of type 2 cytokines in chronic proliferative dermatitis (cpdm) mutant mice and resolution of inflammation following treatment with IL-12. Eur. J. Immunol. 31, 734–742 (2001).
34. Liang, Y., Seymour, R.E. & Sundberg, J.P. Inhibition of NF-kappab signaling retards eosinophilic dermatitis in SHARPIN-deficient mice. J. Invest. Dermatol. 131, 141–149 (2011).
35. Moses, S.W. & Parvari, R. The variable presentations of glycosen storage disease type IV: a review of clinical, enzymatic and molecular studies. Curr. Mol. Med. 2, 177–188 (2002).
36. Bruno, C. et al. Clinical and genetic heterogeneity of branching enzyme deficiency (glycosen type IV). Neurology 63, 1053–1058 (2004).
37. Pelissier, J.F., de Barys, T., Bill, J., Serratrice, G. & Toga, M. Polysaccharide (amylopectin-like) storage myopathy histochemical ultrastructural and biochemical studies. Acta Neuropathol. Suppl. 7, 292–296 (1981).
38. Ewer, R. et al. [Glycosen-type IV as a rare cause of cardiomyopathy—report of a successful heart transplantation.] Z. Kardiol. 88, 850–856 (1999).
39. Vernet, S., Rubio, T., Heredia, M., Rodriguez de Cordoba, S. & Sanz, P. Increased endoplasmic reticulum stress and decreased protranslational function in lafora disease models lacking the phosphatase laforin. PLoS ONE 4, e5907 (2009).
40. Lesca, G. et al. Novel mutations in EPM2A and NHLRC1 widen the spectrum of Lafora disease. Epilepsia 51, 1691–1698 (2010).
41. Monaghan, T.S. & Delanty, N. Lafora disease: epidemiology, pathophysiology and management. CNS Drugs 24, 549–561 (2010).
Genetic analysis. Massively parallel sequencing. Genomic DNA extracted from the patient’s peripheral blood cells was sheared with a Covaris S2 Ultrasonicator. An adaptor-ligated library was prepared with the Paired-End Sample Prep kit V1 (Illumina). Exome capture was performed with the SureSelect Human All Exon kit (Agilent Technologies). Single-end sequencing was performed on an Illumina Genome Analyzer IIx, generating 72-base reads.

Sequence alignment, variant calling and annotation. Sequences were aligned with the human genome reference sequence (hg19 build), using BWA aligner. Downstream processing was carried out with the Genome analysis toolkit (GATK)43, SAMTools44 and Picard Tools (http://picard.sourceforge.net). Substitution and indel calls were identified with a GATK UnifiedGenotyper and a GATK IndelGenotyperV2, respectively. All calls with a read coverage ≤20 were filtered out. All the variants were annotated with the GATK Genomic Annotator.

Genome-wide human single-nucleotide polymorphism array. Genomic DNA was isolated from the peripheral blood of two patients, a healthy sister and parents, by the phenol-chloroform method. The GeneChip Genome-Wide Human SNP Array 6.0 (Affymetrix) oligonucleotide-based array was used. Data were analyzed with Affymetrix Genotyping Console 4.0 software and the Affymetrix Genotyping Console Browser. The break points of the deletion on chromosome 20 were determined by PCR, with primers binding intron 1 and a GATK UnifiedGenotyper and a GATK IndelGenotyperV2, respectively. All calls with a read coverage ≤20 were filtered out. All the variants were annotated with the GATK Genomic Annotator.

Cell lines, immortalization and complementation. Control and patient-derived fibroblasts were immortalized by transfection with a plasmid expressing SV40 large T antigen. Immortalized cell lines were grown in DMEM (Gibco) supplemented with 10% FCS (Gibco). NEMO−/− fibroblasts45 were provided by A. Smahi (INSERM U781, Necker hospital, Paris Cité Sorbonne University). HOIL1 mRNA was reverse-transcribed, amplified and inserted into the retroviral vector pMSCV (Clontech). Infectious viral particles were produced by cotransfecting GP2-293 packaging cells with pSV-G and pMSCV-HOIL1- or an empty vector (Clontech). Viral particles were collected 48–72 h after transfection and used to infect SV40-immortalized fibroblasts. Infected cells were selected with 0.4 µg/ml puromycin (Invitrogen).

Antibodies and reagents. The antibodies used for detection on immunoblots were anti–HOIL-1 N-ter (provided by H. Walczak, Imperial College London)47, anti–HOIL-1 C-ter (provided by K. Iwai, Osaka University)47, anti–TRIB3 (2488-1, Epitomics), anti–HOIP (PAB6229, Abnova), anti–NEMO (sc-8330, Santa Cruz and #611306, BD), anti–SHARPIN (14626-1-AP, ProteinTech), anti–IKKα (sc12102, Santa Cruz), anti–IKKγ (sc-557, Santa Cruz), anti–IκBα (sc-1777, sc-8838 and sc-5288, Santa Cruz), anti–IκBβ (sc-166058, BD), anti–IL-1R1 (40-465, Millipore), anti–TNF-R1 (H-5, Santa Cruz), anti–c-FLIP (sc-4490, R&D Systems), anti–cleaved caspase-3 (Asp175) (9661, Cell Signaling), anti–β-tubulin (T4026, Sigma–Aldrich) and anti–GAPDH (sc-81545, Santa Cruz). Species-specific secondary antibodies coupled to horseradish peroxidase were obtained from Vector Laboratories or Amersham-Pharmacia.

Cell lysis, immunoprecipitation and immunoblotting. Cells were lysed in a buffer containing 30 mM Tris–HCl pH 7.5, 120 mM NaCl, 2 mM KCl, 1% Triton X-100 and 2 mM EDTA supplemented with protease and phosphatase inhibitors (Complete and Phostop, Roche). For immunoprecipitations, antibodies (5 µg) were added to 1 mg of total protein extract and incubated overnight at 4 °C. Protein A or protein G agarose beads (Sigma–Aldrich) were added to the samples, which were then incubated for 1 h at 4 °C. For experiments involving Flag-TNF and Flag–IL1-β treatments, cleared supernatants were incubated with anti-Flag M2 affinity gel (Sigma–Aldrich) for 2 h at 4 °C. For experiments involving biotinylated TNF treatments, cleared supernatants were incubated with streptavidin–agarose (Sigma–Aldrich) for 2 h at 4 °C. In all cases, beads were washed three times with lysis buffer and resuspended in Laemmli buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Immuno reactive proteins were visualized by chemiluminescence. The relative amounts of HOIP and SHARPIN were determined with Quantity One software (Bio-Rad). For NF-κB–dependent reporter assays, SV40-immortalized fibroblasts were transiently transfected with the NK-κB–dependent firefly luciferase plasmid pGL4.32 (Promega) and the Renilla luciferase plasmid as an internal control, in the presence of Lipofectamine LTX reagent (Invitrogen). After 24 h, cells were stimulated with TNF or IL-1β for 4 h and luciferase activities were assessed with the dual luciferase assay kit (Promega).

Gene expression and microarray analysis. RNA was extracted from SV40-immortalized fibroblasts with Trizol (Invitrogen) and reverse-transcribed (2 µg) with the SuperScript III first-strand synthesis kit (Invitrogen). HOIL1, TRIB3, A20 and IL6 mRNAs were quantified by RT-qPCR in a TaqMan Gene Expression Assay, with normalization against GUSB probes.

Data acquisition. Whole blood cells from patients and healthy donors were collected and total RNA was extracted using the MagMAX 96 Blood RNA Isolation Kit (Applied Biosystems). Primary fibroblasts and PBMCs from patients and healthy donors were stimulated with IL-1β or TNF for 2 or 6 h. Total RNA was isolated from fibroblasts and PBMCs with the RNeasy Mini Kit (Qiagen). RNA integrity was assessed on an Agilent 2100 Bioanalyzer. Biotinylated cDNA targets were prepared with the Illumina TotalPrep RNA Amplification Kit (Ambion) and hybridized to Illumina human HT-12 Expression Microarray Chips.

Data preprocessing. For the analysis, the background was subtracted and the raw signal values were extracted with Beadstudio v2 software (Illumina) and scaled by quantile normalization. The minimum intensity was set to 10, and all the intensity values were log2-transformed. Only the probes called as present in at least one sample (P < 0.01) were retained for downstream analysis (n = 26,607).

Data analysis. Raw data were first subjected to background subtraction and scaled to the average with Genome studio software (Illumina). They were then analyzed with Genespring software (Agilent). The minimum intensity was set to 10, and the intensity values were log2-transformed. For the analysis of ex vivo whole-blood samples, all samples were normalized with respect to the median intensity for control samples. After filtering out non-expressing probes, at least a twofold difference in expression between patient 1 and 41 healthy controls was observed for 2,900 transcripts. A pre-established set of transcriptional modules was used as a framework for microarray data analysis. The approach used to construct this framework has been described elsewhere46. Genes with coordinated expression within or across nine human disease data sets were selected in multiple rounds of clique and paraclique clustering, to form a transcriptional framework module. The percentage of transcripts significantly upregulated or downregulated with respect to healthy controls (P < 0.05, assuming equal variance) in each module was visualized on module maps. For the analysis of primary fibroblasts, transcripts without detectable expression changes (difference in normalized expression of less than twofold in either direction, in all samples) or absent from the data set (as defined by an Illumina detection P value greater than 0.01 in all samples) were filtered out, leaving 13,693 transcripts for downstream analysis. Stimulated samples from each donor were normalized with respect to their nonstimulated reference sample. We identified the genes differentially regulated upon IL-1β or TNF stimulation in healthy donors on the basis of transcripts displaying a change in signal intensity of at least twofold (up- or downregulation) with respect to the nonstimulated control. We then investigated the expression of these genes in samples from the patients.

Cell stimulation, ELISA and FACS analysis. Cells were stimulated with the following cytokines and agonists: TNF, biotinylated-TNF and IL-1β (R&D Systems), Flag–TNF and Flag–IL1-β (Alexis Biochemicals), Mega–CD40L (Enzo), LPS (Sigma–Aldrich), phosphol–12-myristate–acetate (Sigma–Aldrich), ionomycin (Sigma–Aldrich) and CpG-B (Invivogen). For cytokine production
by whole blood cells, blood was first mixed with an equal volume of RPMI, left untreated for 48 h and then treated with cytokines or agonists for the indicated time. IL-6 and IL-10 secretion levels were determined with ELISA kits (M9316 and M1910, Sanquin).

Frozen PBMCs were washed twice with FCS-containing medium and used to seed 48-well plates (10⁶ cells/well). They were cultured for 4–6 h and then stimulated with TNF (20 ng/ml), IL-1β (10 ng/ml), LPS (10 ng/ml) or PMA (10⁻⁹ M) + ionomycin (10⁻⁷ M) + CpG (100 µg/ml). Concomitantly to stimulation, protein transport was blocked with GolgiStop and GolgiPlug (BD). Cells were stained with anti-CD3 (BD, #560366), anti-CD19 (BD, #340951), anti-CD14 (BD, #557742), anti-CD56 (Biolegends, #318331) antibodies for cellular phenotyping and with Aqua Live/Dead (Invitrogen) to exclude dead cells. After fixation and permeabilization (BD, #554722), the cells were stained with antibodies to IL6 (BD, #561441), MIP-1α (BD, #554730), MIP-1β (BD, #560565), IL-1β (BD, #340515), IL-8 (BD, #511410) and IFN-γ (BD, #559326). Stained PBMCs were captured by flow cytometry with a BD LSRII flow cytometer and FACS Diva software. The data were analyzed with FlowJo (Tree Star).

**Histology and immunohistochemistry.** Samples from the gut, heart and skeletal muscles of patients and healthy controls were studied. All cases were reviewed by two expert pathologists to confirm diagnosis. Formalin-fixed paraffin-embedded tissue blocks were cut into 4-µm sections that were stained with hematein-eosin and periodic acid-Schiff (PAS) reagent before and after diastase digestion. Images were acquired with a Leica DM LB2 microscope at a magnification of ×400 and a Leica DFC 280 camera. Slides were digitized with a Hamamatsu Nanozoomer.

**Statistics.** GraphPad Prism software was used to conduct unpaired, two-tailed Student’s t-tests for sample analysis or one-way ANOVA followed by Tukey’s multiple comparison test. Values of P < 0.05 were considered significant.

42. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754–1760 (2009).
43. McKenna, A. *et al.* The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* **20**, 1297–1303 (2010).
44. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
45. Smahi, A. *et al.* Genomic rearrangement in NEMO impairs NF-κB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature* **405**, 466–472 (2000).
46. Chaussabel, D. *et al.* A modular analysis framework for blood genomics studies: application to systemic lupus erythematosus. *Immunity* **29**, 150–164 (2008).