

Characterization of Crohn disease in X-linked inhibitor of apoptosis-deficient male patients and female symptomatic carriers

Claire Aguilar, MD,^{a,b} Christelle Lenoir, MS,^{a,b} Nathalie Lambert, AS,^c Bernadette Bègue, MS,^{b,d} Nicole Brousse, MD,^e Danielle Canoni, MD,^e Dominique Berrebi, MD, PhD,^{f,g,h} Maryline Roy, MS,^{g,h} Stéphane Gérart, ME, PhD,^{a,b} Helen Chapel, MA, MD,ⁱ Tobias Schwerd, MD,^j Laurent Siproudhis, MD, PhD,^k Michela Schäppi, MD, PhD,^l Ali Al-Ahmari, MD,^m Masaaki Mori, MD, PhD,ⁿ Akiko Yamaide, MD,^o Lionel Galicier, MD,^p Bénédicte Neven, MD, PhD,^q John Routes, MD,^r Holm H. Uhlig, MD, PhD,^s Sibylle Koletzko, MD,^j Smita Patel, MD, PhD,ⁱ Hirokazu Kanegane, MD, PhD,^t Capucine Picard, MD, PhD,^{a,b,c,u} Alain Fischer, MD, PhD,^{a,b,q,*} Nadine Cerf Bensussan, MD, PhD,^{b,d,*} Frank Ruemmele, MD, PhD,^{b,d,v,*} Jean-Pierre Hugot, MD, PhD,^{g,h,w,*} and Sylvain Latour, PhD^{a,b} *Paris and Rennes, France, Oxford, United Kingdom, Munich, Germany, Geneva, Switzerland, Riyadh, Saudi Arabia, Yokohama, Chiba, and Toyama, Japan, and Milwaukee, Wis*

Background: Crohn disease is an inflammatory bowel disease (IBD) with a complex mode of inheritance. Although nucleotide binding and oligomerization domain containing 2 (*NOD2*) is the strongest risk factor, the cause of Crohn disease remains unknown in the majority of the cases. X-linked inhibitor of apoptosis (*XIAP*) deficiency causes X-linked lymphoproliferative syndrome type 2. IBD has been reported in some *XIAP*-deficient patients.

Objective: We characterize the IBD affecting a large cohort of patients with mutations in *XIAP* and examine the possible pathophysiologic mechanisms.

Methods: We performed a phenotypical and histologic analysis of the IBD affecting 17 patients with hemizygous mutations in *XIAP*, including 3 patients identified by screening 83 patients with pediatric-onset IBD. The X chromosome inactivation was

analyzed in female carriers of heterozygous *XIAP* mutations, including 2 adults with IBD. The functional consequences of *XIAP* deficiency were analyzed.

Results: Clinical presentation and histology of IBD in patients with *XIAP* deficiency overlapped with those of patients with Crohn disease. The age at onset was variable (from 3 months to 41 years), and IBD was severe and difficult to treat. In 2 patients hematopoietic stem cell transplantation fully restored intestinal homeostasis. Monocytes of patients had impaired *NOD2*-mediated IL-8 and monocyte chemoattractant protein 1 (MCP-1) production, as well as IL-10, in response to *NOD2* and Toll-like receptor 2/4 costimulation. Nucleotide binding and oligomerization domain containing 1 (*NOD1*)-mediated IL-6 and IL-8 production was defective in fibroblasts from *XIAP*-deficient patients. The 2 heterozygous female carriers of *XIAP*

From ^athe Laboratory of Lymphocyte Activation and EBV Susceptibility, ^dthe Laboratory of Interactions of the Intestinal Epithelium and the Immune System, and ^ethe Laboratory of Human Genetics of Infectious Diseases, INSERM UMR 1163, Hospital Necker-Enfants Malades, Paris; ^bUniversity Paris Descartes Sorbonne Paris Cité, Institut Imagine, Paris; ^cthe Study Center for Primary Immunodeficiencies (CEDI), ^ethe Pathology Department, ^fthe Department of Immunology and Haematology, and ^vthe Pediatric Gastroenterology Unit, Hospital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris (APHP), Paris; ^gthe Pathology Department, Hospital Robert Debré, APHP, Paris; ^hINSERM UMR 843, Hospital Bichat, Paris; ⁱUniversity Paris-Diderot, Sorbonne Paris Cité, Paris; ^jthe Primary Immunodeficiency Unit, Oxford NIHR Biomedical Research Centre, University of Oxford; ^kUniversity of Munich Medical Center, Dr von Hauner Children's Hospital, Munich; ^lthe Department of Hepato-Gastroenterology, University Hospital of Rennes; ^mthe Pediatrics Center, Clinique des Grangettes and Medical University Center, Geneva; ⁿthe Department of Pediatric Hematology/Oncology, King Faisal Specialist Hospital & Research Center, Alfaisal University, Riyadh; ^othe Department of Pediatrics, City University Medical Center, Yokohama; ^pthe Division of Allergy and Rheumatology, Chiba Children's Hospital; ^qthe Department of Clinical Immunology, Hospital Saint-Louis, APHP, Paris; ^rthe Division of Allergy and Clinical Immunology, Department of Pediatrics, Children's Research Institute, Medical College of Wisconsin, Milwaukee; ^sthe Translational Gastroenterology Unit and Children's Hospital, University of Oxford; ^tthe Department of Pediatrics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama; ^uthe Pediatric Gastroenterology Unit, Hospital Necker-Enfants Malades, APHP, Paris; and ^wthe Pediatric Gastroenterology Unit Hospital Robert Debré, APHP, Paris.

*These authors equally contributed to this study.

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Corresponding author: Sylvain Latour, PhD, Imagine-Institut des maladies génétiques, INSERM UMR 1163, Laboratory of Lymphocyte Activation and EBV Susceptibility, Hôpital Necker Enfants-Malades, 24 boulevard du Montparnasse, Paris 75015 Cedex, France. E-mail: sylvain.latour@inserm.fr.

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mutations with IBD displayed abnormal expression of the *XIAP* mutated allele, resulting in impaired activation of the NOD2 pathway.

Conclusion: IBD in patients with *XIAP* deficiency is similar to Crohn disease and is associated with defective NOD2 function in monocytes. Importantly, we report that it is not restricted to male patients because we identified 2 symptomatic female heterozygous carriers of *XIAP* mutations. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Inflammatory bowel disease, Crohn disease, X-linked inhibitor of apoptosis immunodeficiency, NOD receptors, monocytes

Inflammatory bowel diseases (IBDs) include Crohn disease and ulcerative colitis, which are lifelong disorders with prevalences of up to 0.1% in Western populations. In the past 10 years, genome-wide association studies have identified up to 163 IBD susceptibility loci,¹⁻³ many of which are involved in the immune system, pointing out the relationship between disturbance of the immune system and IBD. However, causal links between most of the identified variants and IBD have remained difficult to establish, and the conferred risks are often very low. IBD is also a clinical trait associated with several primary immune deficiencies, including chronic granulomatous disease. The recent discovery of mutations in *IL10* and its receptors (*IL10RA* and *IL10RB*) in patients with very early-onset intestinal pathology has confirmed that IBD can be the result of a monogenic disease.^{4,5} Investigations of these Mendelian disorders have provided several important clues into the mechanisms controlling intestinal homeostasis in human subjects.

X-linked lymphoproliferative disease type 2 (XLP-2) is caused by mutations in the gene encoding the X-linked inhibitor of apoptosis protein (*XIAP*). XLP-2 is a primary immunodeficiency that affects young boys who frequently have hemophagocytic lymphohistiocytosis (HLH) syndrome in association with EBV infection and recurrent splenomegaly. Patients with XLP-2 can also have IBD (17% in our previous report), and *XIAP* deficiency had been proposed to be a Mendelian cause of IBD.⁶⁻¹⁰ In some patients IBD was found to be the initial clinical manifestation.^{8,9}

The protein *XIAP* possesses an antiapoptotic activity and also activates nuclear factor κ B (NF- κ B).^{11,12} Recently, *XIAP* was found to be essential for the signal transduction of nucleotide binding and oligomerization domain containing 1 (NOD1) and nucleotide binding and oligomerization domain containing 2 (NOD2), 2 intracellular pattern recognition receptors involved in immune innate host defense.^{8,13} NOD receptors recognize peptidoglycan products derived from the bacterial cell wall. *XIAP* is required for NOD receptor signaling through its interaction with the receptor-interacting protein 2 kinase (RIP2 kinase), which allows recruitment of the linear ubiquitin chain assembly complex and in turn activates NF- κ B signaling and cytokine production.¹⁴⁻¹⁶ Importantly, *NOD2* is the strongest susceptibility gene associated with Crohn disease but not with ulcerative colitis, although it only accounts for 2.5% to 5% of the total genetic variance of Crohn disease.^{17,18} The 3 most frequent risk alleles identified in *NOD2* are coding missense or frameshift mutations that affect NOD2 ligand recognition and functions. The molecular link between *XIAP* and NOD2 prompted us to further explore and characterize the relationship between *XIAP* deficiency and IBD. Our results show that IBD seen in patients with *XIAP* deficiency is a frequent phenotype clinically and histologically

Abbreviations used

HLH:	Hemophagocytic lymphohistiocytosis
HSCT:	Allogeneic hematopoietic stem cell transplantation
IBD:	Inflammatory bowel disease
MCP-1:	Monocyte chemoattractant protein 1
MDP:	Muramyl dipeptide
NF- κ B:	Nuclear factor κ B
NOD1:	Nucleotide binding and oligomerization domain containing 1
NOD2:	Nucleotide binding and oligomerization domain containing 2
TLR:	Toll-like receptor
<i>XIAP</i> :	X-linked inhibitor of apoptosis
XLP-2:	X-linked lymphoproliferative syndrome type 2

indistinguishable from the IBD found in patients with Crohn disease. Importantly, 2 symptomatic female carriers of *XIAP* mutations were identified, showing abnormal skewed X-inactivation toward the mutated allele. In patients we observed that NOD2-mediated cytokine production by monocytes is impaired.

METHODS

Details of the materials and methods used in this study are provided in the Methods section in this article's Online Repository at www.jacionline.org.

The cohort of *XIAP*-deficient patients was established at the time of the discovery of *XIAP* in our laboratory in 2006. Patients were usually referred to our center because of symptoms evoking XLP-2 syndrome/*XIAP* deficiency, which include HLH, recurrent splenomegaly, and/or recurrent EBV infection. The cohort of patients with early-onset IBD was established between September 2010 and December 2013. In both cohorts colitis was diagnosed according to a combination of clinical (abdominal pain, diarrhea, or both), endoscopic (ulceration, inflammation, or both), and histologic (ulceration, mucosal inflammation, granuloma, and/or cryptic abscesses) findings. Biopsy specimens analyzed were taken before treatment.

When a mutation in *XIAP* is identified in a patient, *XIAP* protein expression is evaluated by using Western blotting, and its function is evaluated by study of activation-induced cell death and NOD2-induced cytokine production in T-cell blasts and PBMCs, respectively. Some patients of this cohort have been reported in previous publications,^{6-9,19} and some patients have been identified since that time.

We screened *XIAP* mutations in 2 pediatric cohorts of 23 and 60 boys with early-onset IBD that were collected at the Necker-Enfants Malades Hospital and Robert Debré Hospital, respectively. In the cohort of 23 boys, 19 were white, 3 originated from north Africa, and 1 originated from Sub-Saharan Africa. Finally, some patients with *XIAP* deficiency and IBD from other centers were included in our study. Among the 17 *XIAP*-deficient patients with IBD analyzed, 13 were white and 4 were Asian (from Japan). For functional assays, *XIAP*-deficient patients with or without IBD have been compared with healthy donors of same age.

Informed consent was obtained from donors, patients, and families of patients. The study and protocols conform to the 1975 Declaration of Helsinki, as well as local legislation and ethical guidelines.

RESULTS

Early-onset IBD is a frequent clinical manifestation in patients with *XIAP* deficiency and is not associated with particular *XIAP* mutations

We undertook a retrospective study of the clinical and histologic features associated with IBD in patients with *XIAP* deficiency. We analyzed a cohort of 54 patients given a diagnosis of *XIAP* deficiency based on the classical clinical features of XLP-2, familial history, or both in which 12 patients had IBD

TABLE I. Clinical parameters of XIAP-deficient patients with IBD

Patient ID*	XIAP mutation	Protein	Age at onset of IBD (y)	IBD localization	Other clinical manifestations	IBD treatments†	Outcome (age [y])
P1.1	E99KfsX129	—	41	Colitis	HLH (18 mo), splenomegaly, cholangitis	NK	Died (42, digestive bleeding)
P1.2	E99KfsX129	—	7	Ileitis, colitis, anoperineal lesions	HLH (7), splenomegaly, cholangitis, skin abscesses	Steroids, azathioprine, anti-TNF- α , colectomy (21)	Alive (26)
P2	I397NfsX405	—	3.5	Colitis	HLH (2)	Steroids, cyclosporine	Alive (6)
P3.1	G466X	+/-	20	Colitis	Skin abscesses	NK	Died (27, IBD)
P3.2	G466X	+/-	33	Colitis, anoperineal lesions	Splenomegaly, arthritis	Anti-TNF- α , antibiotics	Alive (34)
P3.3	G466X	+/-	12	Gastritis, colitis, anoperineal lesions	Splenomegaly, skin abscesses	Steroids, mesalazine, azathioprine, cyclosporine	Alive (14)
P4	R381X	—	4	Colitis	NK	NK	Died (4, IBD)
P5	Del. exons 1-2	—	2	Colitis	HLH (0, 2, 3)	Steroids, cyclosporine, anti-TNF- α	Alive (5)
P6.1	I494N	+/-	12	Colitis	Malignant tumor	Colectomy	Alive (43)
P6.2	I494N	+/-	7	Gastritis, colitis	HLH (12)	Anti-TNF- α , steroids, tacrolimus, colectomy (13)	Alive (14)
P7	H220Y	+/-	7	Duodenitis, ileitis, colitis, anoperineal lesions	HLH (8), splenomegaly	Steroids, mesalazine, azathioprine, anti-TNF- α , thalidomide, colectomy (9)	Alive (14)
P8.1	Q332EfsX347	—	1	Gastritis, ileitis, colitis	Renal failure	Steroids, azathioprine, mycophenolate, mofetil, anti-TNF- α	Alive (4)
P8.2	Q332EfsX347	—	11	Ileitis, colitis	Skin abscesses	Steroids, azathioprine, anti-TNF- α	Alive (27)
P9	Del. exons 1-4	—	3 mo	Colitis		Steroids, mesalazine, azathioprine, anti-TNF- α , thalidomide, ileostomy	Alive (8)
P10	C231Y	+/-	15 mo	Ileitis, colitis, anoperineal lesions		Steroids, anti-TNF- α , G-CSF, tacrolimus, cyclophosphamide, azathioprine, colectomy (4) HSCT (5)	Alive (8)
P11.1	V279TfsX292	—	18	Colitis, anoperineal lesions	Splenomegaly, skin abscesses, arthralgia	Steroids, azathioprine, colectomy (21)	Alive (34)
P11.2	V279TfsX292	—	8	Gastritis, colitis, anoperineal lesions	HLH (4), splenomegaly	Azathioprine, anti-TNF- α , cyclosporine, steroids, HSCT (11)	Alive (13)

+/-, Residual protein expression; *Del.*, deletion of exons; *NK*, not known.

*The list of the treatments for each patient follows the order in which the patient received them.

†Patient identification: the first number corresponds to the family, and the second number corresponds to the individual in the family.

(P1.1, P1.2, P2, P3.1, P3.2, P3.3, P4, P5, P6.1, P6.2, P11.1, and P11.2; Table I). Some of them were previously reported to have colitis.^{6-9,19} The case of a young boy with intractable colitis in whom whole-exome sequencing identified an *XIAP* mutation (reference 8 and P10 in Table I) prompted us to search for *XIAP* mutations in a cohort of 23 boys with early-onset IBD before 1 year and/or severe IBD during infancy refractory to immunosuppressive treatments (range, 1 month to 7 years; median age at onset, 22 months). Three boys were found to be carriers of *XIAP* mutations (P7, P8.1, and P9). One of them (P8.1) has a 27-year-old maternal uncle carrying the same mutation who was also given a diagnosis of a Crohn-like disease at the age of 11 years (P8.2). We also sequenced *XIAP* in a second pediatric cohort of 60 boys with later onset of IBD (range, 2-16 years; median age at onset, 11 years), and we found no mutation. In total, 3 of 83 patients were found to have *XIAP* deficiency in male pediatric cohorts with IBD.

Altogether, we studied 17 male *XIAP*-deficient patients with IBD from 11 families. The occurrence of IBD in the total cohort was 29% (17/59). Clinical data are summarized in Table I. Age at diagnosis of IBD was variable (3 months to 41 years; median age at diagnosis, 7 years). IBD was the main and the most severe clinical trait of the disease in half of the patients. In 10 patients no HLH has occurred thus far, whereas IBD onset preceded HLH

in 2 other patients (P6.2 and P7). Among the same kindred, the clinical phenotypes were variable because some patients had either combined IBD and HLH or isolated manifestations irrespective of age (Fig 1, A). There is no clear correlation between genotype and phenotype because mutations can affect different parts of the protein (Fig 1, B). All mutations excepting *XIAP*^{H220Y}, *XIAP*^{C231Y}, *XIAP*^{G466X}, and *XIAP*^{I494N} led to a complete loss of *XIAP* expression (Table I, Fig 1, and data not shown). Patients with hypomorphic mutations and residual protein, such as P7, had an intestinal pathology as severe as that seen in patients with null mutations, such as patients P8.1 and P9 (Fig 1, C).

IBD in patients with *XIAP* deficiency has the clinical and histologic features of Crohn disease

Endoscopic and histologic data were obtained from 13 patients for whom gut biopsy specimens were available (see Table E1 in this article's Online Repository at www.jacionline.org). As in patients with Crohn disease, different segments of the bowel could be affected, including the stomach, ileum, colon, and anus (Table I). However, the large bowel was involved in all patients. Mucosal inflammatory lesions alternated with healthy mucosa in all 13 patients studied, with focal ulcerations in 12 patients (Fig 2, A). Acute inflammatory lesions included crypt abscesses

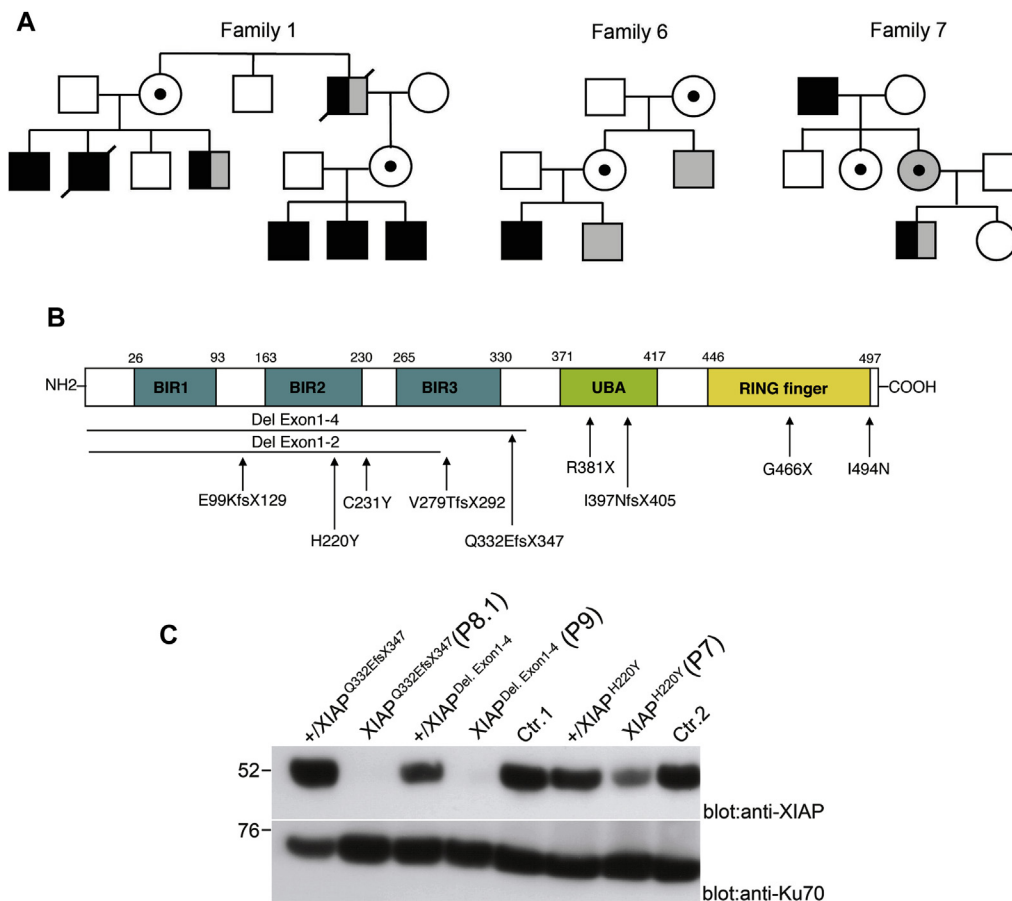


FIG 1. XIAP mutations in patients with IBD. **A**, Pedigrees of 3 families with XIAP deficiency (family 1 [XIAP^{E99KfsX129}], family 6 [XIAP^{I494N}], and family 7 [XIAP^{H220Y}]). Black boxes represent subjects affected by HLH, gray boxes represent those affected by IBD, and mixed boxes represent subjects presenting with both pathologies. Diagonal bars indicate deceased subjects. Circles with black points correspond to female carriers. **B**, Schematic representation of the XIAP protein. Missense and nonsense mutations identified in patients with IBD are indicated by arrows, and deletions are indicated by horizontal lanes. **C**, Expression of XIAP in T-cell blasts from several XIAP-deficient patients (XIAP^{Q332EfsX347} [P8.1], XIAP^{Del. Exon1-4} [P9], and XIAP^{H220Y} [P7]), their mothers (+/XIAP^{Q332EfsX347}, +/XIAP^{Del. Exon1-4}, and +/XIAP^{H220Y}) compared with 2 healthy donors (Ctr. 1 and Ctr. 2). Lysates were immunoblotted with anti-XIAP antibody and anti-Ku70 as a loading control.

in 10 patients (Fig 2, B), polymorphic infiltrations of the lamina propria in all patients (Fig 2, C), and epithelioid granulomas (Fig 2, D) in 6 patients. Signs of chronic inflammation were present in all patients, with transmural (full thickness) inflammation of the bowel wall in some, but not all, patients. In most XIAP-deficient patients, IBD was severe and refractory to medications, including corticosteroids, azathioprine or anti-TNF- α antibodies, requiring colectomy in 5 patients and leading to death in 3 other cases (Table I). Anorectal lesions required surgical treatment in several patients. Two patients (P10 and P11.2) received allogeneic hematopoietic stem cell transplantation (HSCT),⁸ and 2 other patients (P8.1 and P9) are currently undergoing HSCT. HSCT led to a complete and sustained remission of IBD in both patients, as assessed during a follow-up of 38 and 24 months, respectively.

Normal apoptosis in intestinal tissues of XIAP-deficient patients

In a healthy control subject XIAP was expressed in the cytoplasm of mononuclear cells from the lamina propria, as

well as in colonocytes (see Fig E1, A, in this article's Online Repository at www.jacionline.org). By contrast and as expected, in XIAP-deficient patients with a null mutation, results of staining for XIAP were negative (see Fig E1, B). Increased activation-induced cell death of T lymphocytes is one biological hallmark associated with XLP-2 (Fig 3, A).⁶ Yet an excess of apoptosis in a fast-renewing tissue like the intestinal epithelium could be involved in this setting because XIAP was shown to also regulate anoikis of epithelial cells, a form of apoptosis induced by epithelial detachment.²⁰ However, immunostaining of gut biopsy specimens from 8 patients with an anti-cleaved caspase 3 antibody revealed only few apoptotic epithelial cells compared with those seen in patients with Crohn disease without XIAP deficiency (Fig 3, B-E).

NOD2 and NOD1 functions are defective in XIAP-deficient patients

Because XIAP participates in NOD2 signaling, NOD2 function was assessed in PBMCs of XIAP-deficient patients stimulated by muramyl dipeptide (MDP), a NOD2-activating ligand. We tested

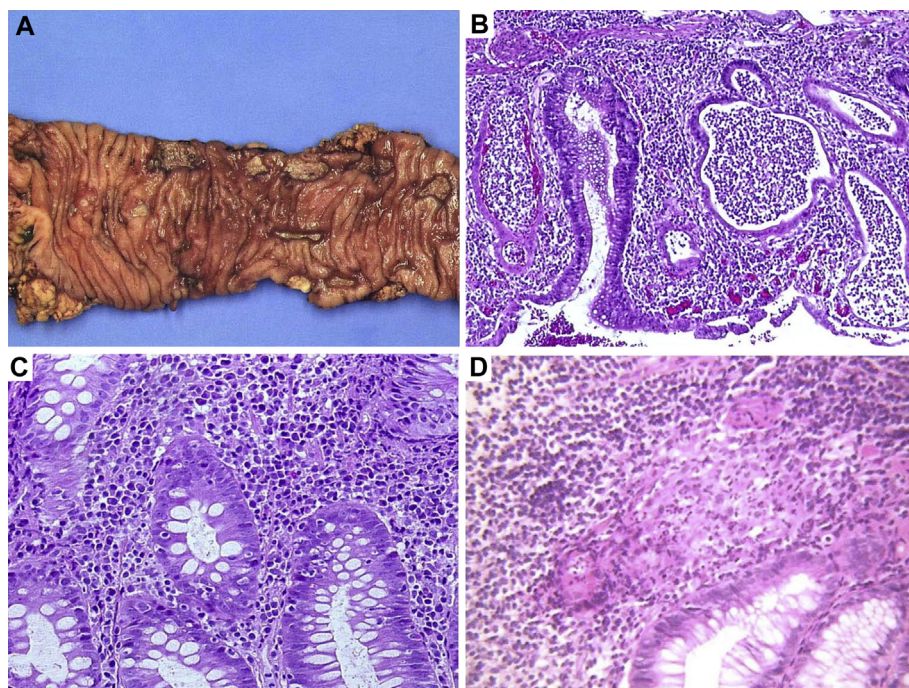


FIG 2. Histologic aspects of IBD associated with XIAP deficiency. **A**, Macroscopic aspects of the colon from a XIAP-deficient patient (P7). Several ulcerations are visible. **B-D**, Histologic examination of colonic tissue shows different lesions in XIAP-deficient patients, such as crypt abscesses (patient P1.2, hematoxylin and eosin stain [HES] $\times 200$; Fig 2, *B*); polymorphic infiltrates containing polynuclear neutrophils, lymphocytes, and plasma cells between the glands (patient P6.2, HES $\times 400$; Fig 2, *C*); and epithelioid granuloma with macrophages and polymorphonuclear neutrophils at the center (patient P7.1, HES $\times 400$; Fig 2, *D*).

PBMCs from XIAP-deficient patients with IBD but also PBMCs from XIAP-deficient patients without IBD (their mutations and ages are shown in Table E2 in this article's Online Repository at www.jacionline.org). In keeping with previous studies showing that MDP stimulation of PBMCs induces NF- κ B-dependent production of cytokines and chemokines by monocytes,^{21,22} PBMCs from healthy control subjects produced large amounts of IL-8 and monocyte chemoattractant protein 1 (MCP-1) in response to MDP stimulation. In contrast, PBMCs from 11 XIAP-deficient patients produced little IL-8 or MCP-1, whereas their response to LPS was comparable with that observed in control subjects (Fig 4, *A* and *B*). There was no difference between XIAP-deficient patients with or without IBD, and both groups exhibited comparable IL-8 and MCP-1 defects (see Fig E2 in this article's Online Repository at www.jacionline.org). No significant production of IL-6, IL-1 β , TNF- α , and RANTES by PBMCs in response to MDP was observed in either XIAP-deficient patients or control subjects (see Fig E3 in this article's Online Repository at www.jacionline.org). Impaired release of IL-8 on NOD2 activation did not result from changes in the frequency of monocyte subsets, which were comparable in patients and healthy control subjects, but rather from their defective response to MDP (see Table E3 in this article's Online Repository at www.jacionline.org). Accordingly, flow cytometry showed reduced intracellular IL-8 staining in MDP-stimulated monocytes from XIAP-deficient patients compared with control subjects, whereas it was comparable in LPS-stimulated cells (Fig 4, *D*). No IL-8 staining was detected in T and B lymphocytes under these conditions. Confirming the NOD2 signaling defect, XIAP-deficient cells from 2 patients exhibited decreased NF- κ B activation on MDP stimulation (see Fig E4 in this article's Online Repository at www.jacionline.org).

However, NOD2 activation alone is not sufficient for IL-10 production, and a synergistic signal through the Toll-like receptor (TLR) 2, TLR3, and TLR4 pathways is required.^{23,24} Thus, MDP-induced production of IL-10 was tested in the presence of specific ligands for TLR2 (Pam3Cys4) and TLR4 (LPS). Although MDP enhanced TLR-dependent production of IL-10 in control PBMCs, this synergistic effect was lost in PBMCs from XIAP-deficient patients (Fig 5, *A*). Because mutations in *NOD2* associated with Crohn disease result in impaired responsiveness to MDP, we checked the most common *NOD2* mutations (rs2066844/*NOD2*^{R702W}), rs2066845/*NOD2*^{G908R}, and rs2066847/*NOD2*^{1007fs}) in 14 XIAP-deficient patients with IBD. Two were heterozygous carriers of one of these polymorphisms (see Table E2). Therefore, this strongly suggests that *NOD2* polymorphisms associated with Crohn disease did not account for the *NOD2* defect in the absence of XIAP in patients.

NOD1 was also shown to require XIAP for signaling.¹⁴ Consequently we analyzed NOD1 function in cells from XIAP-deficient patients. NOD1 in contrast with NOD2 is not expressed in hematopoietic cells²⁵ and was therefore tested in fibroblasts. Although stimulation of control fibroblasts by the NOD1 ligand L-Ala-gamma-D-Glu-meso-diaminopimelic acid (tri-DAP) resulted in increased IL-6 production, no such effect was observed in the XIAP-deficient fibroblasts from 2 XIAP-deficient patients (Fig 5, *B*). A similar defect was observed with IL-8 production analyzed by using intracellular staining (Fig 5, *C*). The DAP-triggered IL-8 response was strongly reduced in XIAP-deficient cells compared with wild-type cells, whereas the IL-8 response to phorbol 12-myristate 13-acetate plus ionomycin stimulation was not altered. Taken together, these results indicate that XIAP deficiency results in an impaired

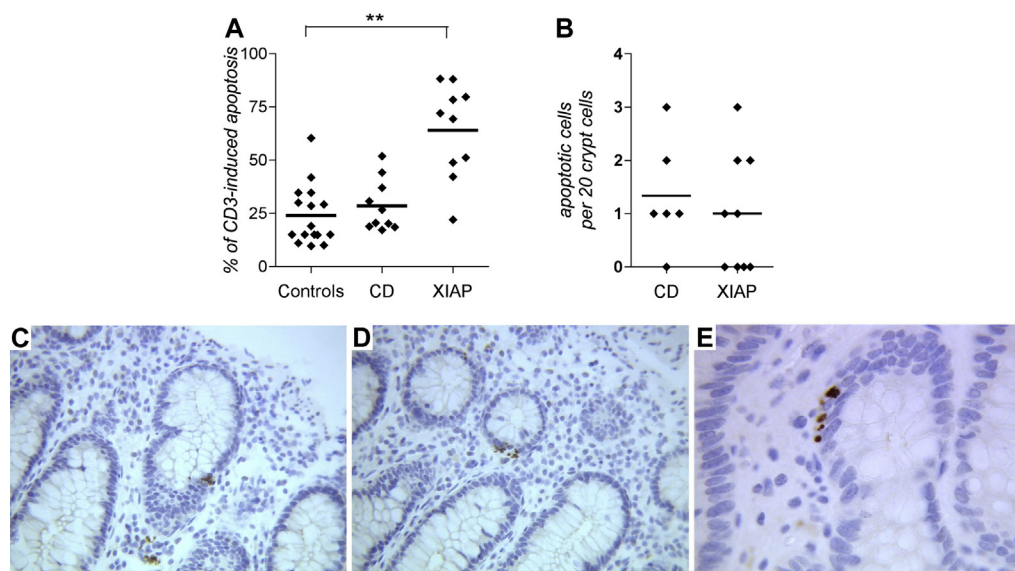


FIG 3. Apoptosis in lymphocytes and intestinal tissues of XIAP-deficient patients. **A**, Activation-induced cell death of T-cell blasts in response to CD3 stimulation from healthy donors (*Controls*; $n = 16$), patients with Crohn disease (*CD*; $n = 10$), and XIAP-deficient patients (*XIAP*; $n = 10$). **B**, Apoptosis quantification in the intestinal tissues assessed by counting caspase 3-positive cells per gland in patients with Crohn disease (*CD*; $n = 7$) and XIAP-deficient patients (*XIAP*; $n = 8$). Results of paired 2-tailed t tests in Fig 3, **A**, and unpaired t tests in Fig 3, **B**, with data means and numbers of patients and control subjects tested in independent experiments are shown. $^{**}P < .01$. **C-E**, Examples of staining with anti-cleaved caspase 3 antibody in intestinal tissues from a healthy control subject (Fig 3, **C**), a patient with Crohn disease (Fig 3, **D**), and an XIAP-deficient patient with IBD (Fig 3, **E**). Few epithelial cells are positive, corresponding to the brown staining. No differences were observed in the 3 samples.

response of both NOD2 and NOD1 independently of the IBD status of the patients.

IBD develops in adult female carriers of *XIAP* mutations with defective NOD2 function

When we reviewed clinical data about relatives of our patients, we found that 2 female carriers of *XIAP* mutations had IBD in adulthood. The mother of patient 7 was given a diagnosis of Crohn disease age 32 years, with ileocecal lesions, which required ileocelectomy (Fig 1, **A**). She is now 43 years old and is well on treatment with azathioprine. The sister of patient 3.3, who is 29 years old, had erythema nodosum and arthralgia at the age of 13 years and skin abscesses in her 20s. At the age of 28 years, she had colitis. These women are heterozygous carriers of the *XIAP*^{H220Y/+} and *XIAP*^{G466X/+} mutations, which are hypomorphic mutations preserving some XIAP expression in T-cell blasts (Fig 1, **C**).²⁶ Thus, we wondered whether they could have had Crohn disease in relation to their heterozygous carrier status. A nonrandom X chromosome inactivation pattern has been observed in leukocytes from heterozygous *XIAP* female carriers,^{6,27} indicating that expression of the wild-type allele provides a selective advantage to leukocytes. This was not the case in the 2 heterozygous carriers of *XIAP*^{H220Y/+} and *XIAP*^{G466X/+} with IBD because the wild-type allele was found to be strongly inactivated in their leukocytes, which is in contrast to 7 other female carriers of *XIAP* mutations, who are healthy (Fig 6, **A**, and see Fig E5 in this article's Online Repository at www.jacionline.org). Accordingly, XIAP expression in the monocytes of the heterozygous carrier of *XIAP*^{H220Y/+} was reduced similarly to her son's monocytes, whereas it was normal in cells from a

healthy *XIAP*^{E118X/+} carrier with a nonrandom X chromosome inactivation (Fig 6, **B**). IL-8 production by PBMCs in response to MDP was reduced to a magnitude almost similar to that of her son (Fig 6, **C**). Thus, it is likely that IBD in the *XIAP*^{H220Y/+} and *XIAP*^{G466X/+} female carriers is the result of a predominant expression of the *XIAP*-mutated allele in their monocytes. These results indicate that heterozygous mutations in *XIAP*, such as hemizygous mutations, represent a genetic factor for Crohn disease.

DISCUSSION

Our data indicate strong similarities between XIAP deficiency-associated IBD and Crohn disease, including focal inflammatory lesions affecting all digestive segments with ulcerations, crypt abscesses, granulomas, and polymorphic cellular infiltrates. However, the ileal inflammation in patients with XIAP deficiency (5/17 had ileitis) seems to be less involved than in patients with Crohn disease (in which >50% of patients had ileal involvement). Importantly, several patients were first given clinical diagnoses of Crohn disease and later given a genetically diagnosis of an XIAP deficiency. This should incite to consider the diagnosis of XIAP deficiency in children with severe IBDs similar to Crohn disease, even in the absence of other XLP-2 clinical phenotypes. We found 3 of 83 patients to have XIAP deficiency by screening 2 pediatric cohorts with IBD; this is an important frequency. A similar frequent occurrence was observed by Zeissig et al,²⁸ who identified 4 *XIAP* variants among 96 male patients with pediatric-onset IBD. Moreover, XIAP deficiency could be also considered in male adult patients with Crohn disease because several XIAP-deficient patients had IBD during adulthood. Although XIAP

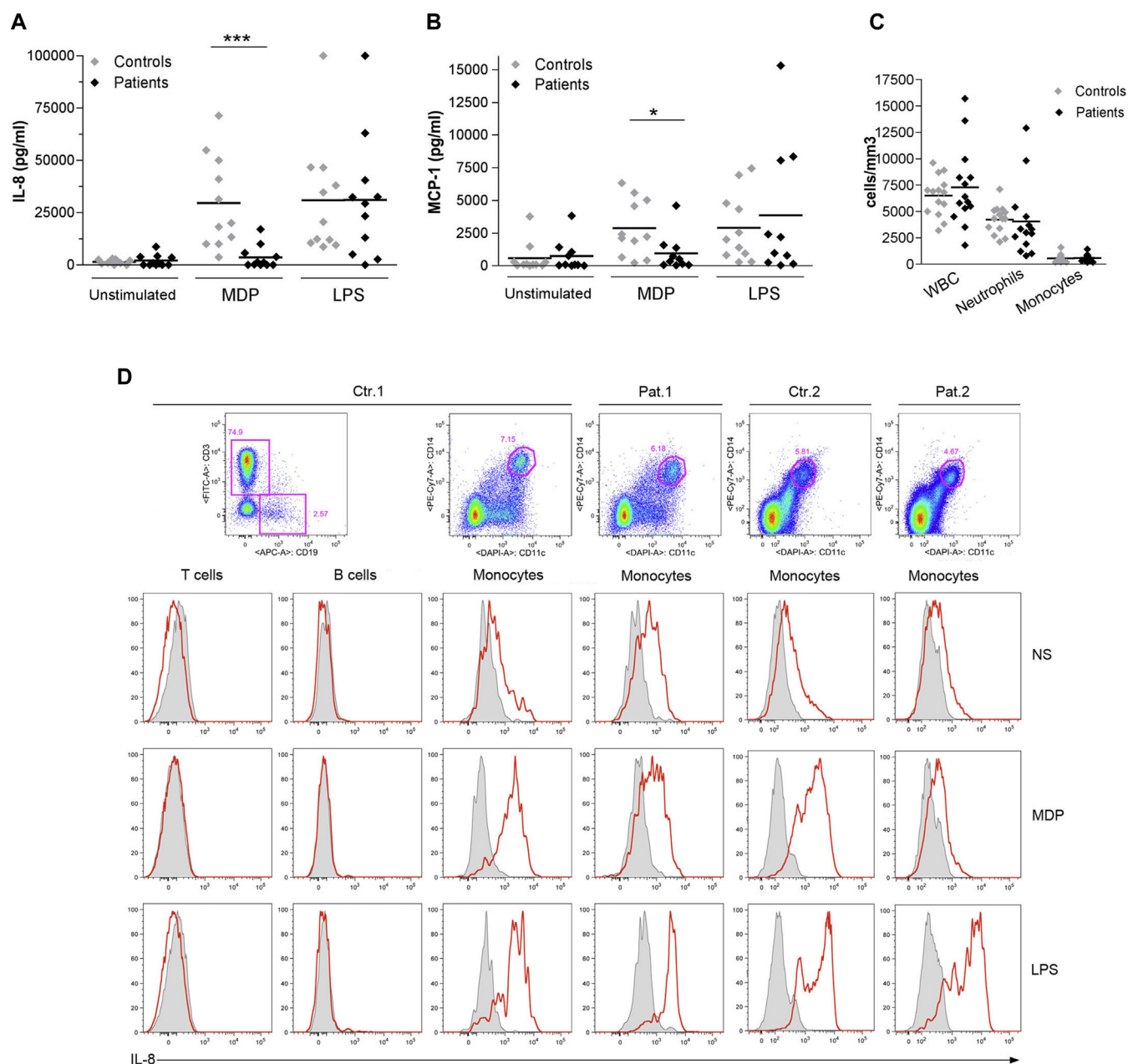


FIG 4. Defective production of myeloid cytokines/chemokines in response to NOD2 stimulation in XIAP-deficient patients. **A**, IL-8 secretion tested after NOD2 or TLR4 stimulation of PBMCs by MDP and LPS. Eleven XIAP-deficient patients were tested compared with 11 healthy control subjects ($n = 11$). **B**, Same as in Fig 4, A, except that MCP-1 secretion was tested. **C**, Counts of white blood cells (WBC), neutrophils, and monocytes in the blood of 14 XIAP-deficient patients and 14 control subjects ($n = 14$). Results of paired 2-tailed t tests with data means and numbers of patients and control subjects tested in independent experiments are shown. * $P < .05$ and *** $P < .001$. **D**, Production of IL-8 in PBMCs, as assessed by means of intracellular staining on gated cells (red circles in dot plots) stained with anti-CD3, anti-CD19, anti-CD14, and anti-CD11c antibodies. Histograms for IL-8 staining are shown for B cells (CD19⁺), T cells (CD3⁺), and monocytes (CD14⁺CD11c⁺) from 1 healthy control subject (Ctr. 1) and only in monocytes from another healthy control subject (Ctr. 2) and 2 XIAP-deficient patients, 1 with IBD (Pat. 1 corresponding to P8.1 in Table I) and 1 without IBD (Pat. 2). Red lines correspond to anti-IL-8 antibody, and gray histograms correspond to control antibody isotype. NS, No stimulation.

deficiency is X-linked, female patients should also be considered because, for the first time, we identified 2 female heterozygous carriers of XIAP mutations who had IBD.

The 2 female heterozygous carriers who were affected had rather random X inactivation or skewed toward the wild-type

allele in their PBMCs when compared with nonaffected female carriers in whom the X inactivation was markedly skewed toward the mutated allele, as we originally reported. The selection of cells preferentially expressing the wild-type allele in female carriers likely results from the antiapoptotic activity of XIAP,

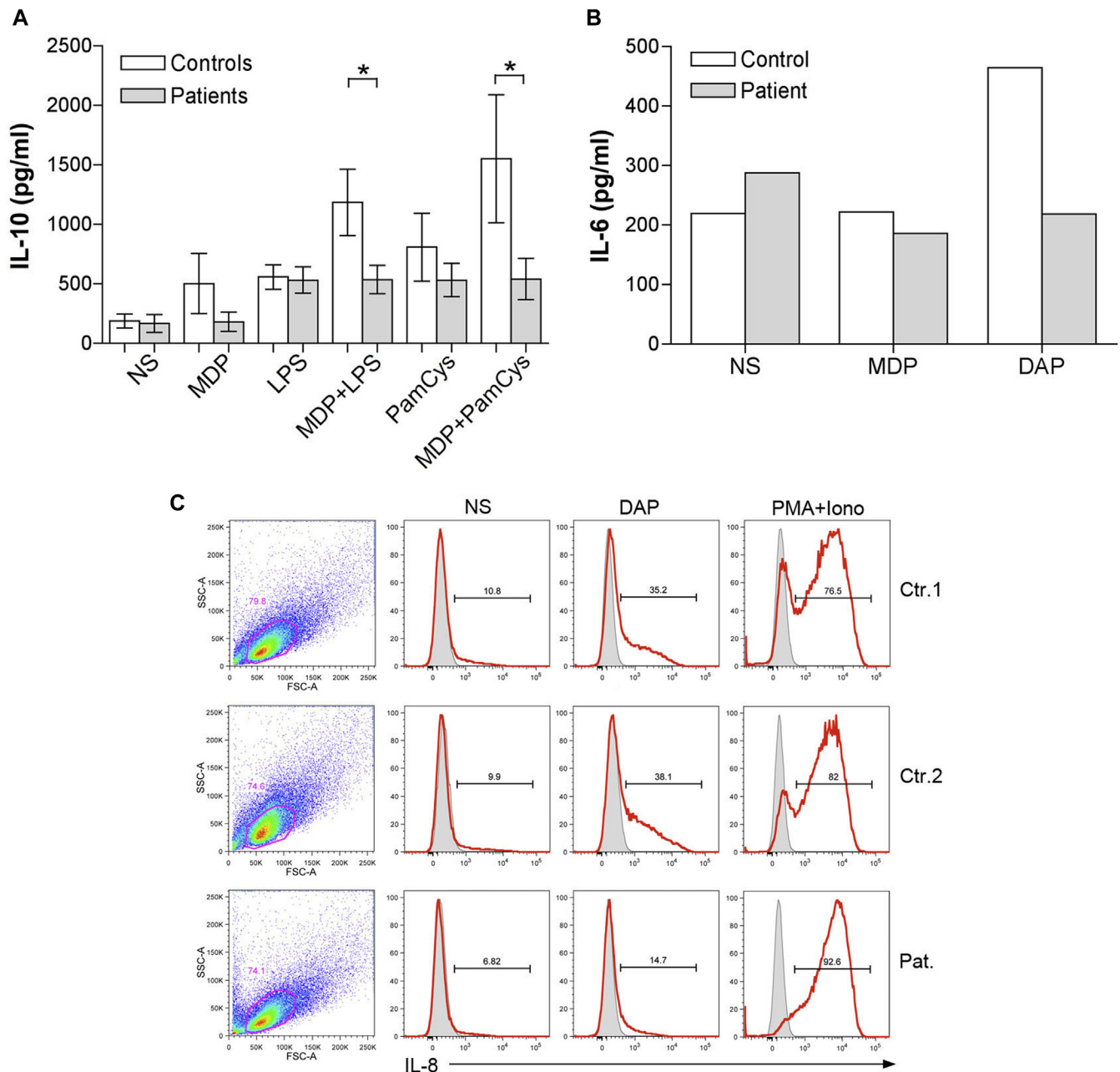


FIG 5. Defects in NOD2 and TLR4/2-mediated IL-10 production and NOD1-mediated IL-6 and IL-8 production. **A**, IL-10 production after NOD2 and TLR4/2 costimulation of PBMCs from 6 healthy control subjects and 6 XIAP-deficient patients by MDP, LPS, MDP plus LPS, Pam3Cys4, or MDP plus Pam3Cys4. Results of paired 2-tailed *t* tests with means $\pm$ SEMs and numbers of patients and control subjects tested in independent experiments are shown. **P* < .05. **B**, IL-6 production by primary fibroblasts from a healthy control subject and an XIAP-deficient patient in response to Tri-DAP (NOD1 ligand). One representative experiment of 2 is shown. **C**, Production of IL-8 in fibroblasts from 2 control subjects (Ctr. 1 and Ctr. 2) and 1 XIAP-deficient patient (Pat.). Red lines correspond to anti-IL-8 antibody, and gray histograms correspond to control antibody isotype. One representative experiment of 2 with 2 patients tested. NS, No stimulation.

which provide a survival advantage compared with cells expressing the mutated allele. In the 1 female subject tested for XIAP expression, a strong decrease in XIAP expression was seen in her monocytes, indicating that the wild-type XIAP allele was strongly inactivated in the monocytes. This correlates with the inactivation skewing toward the wild-type allele observed in PBMCs, more

than 60% of which had a methylated wild-type allele. The mechanism or mechanisms by which the wild-type allele in the 2 affected carriers do not provide a selective advantage as efficient as in nonaffected female carriers is not known. However, one possible explanation could be the hypomorphic nature of the XIAP mutations carried by the 2 women. In cells in which the

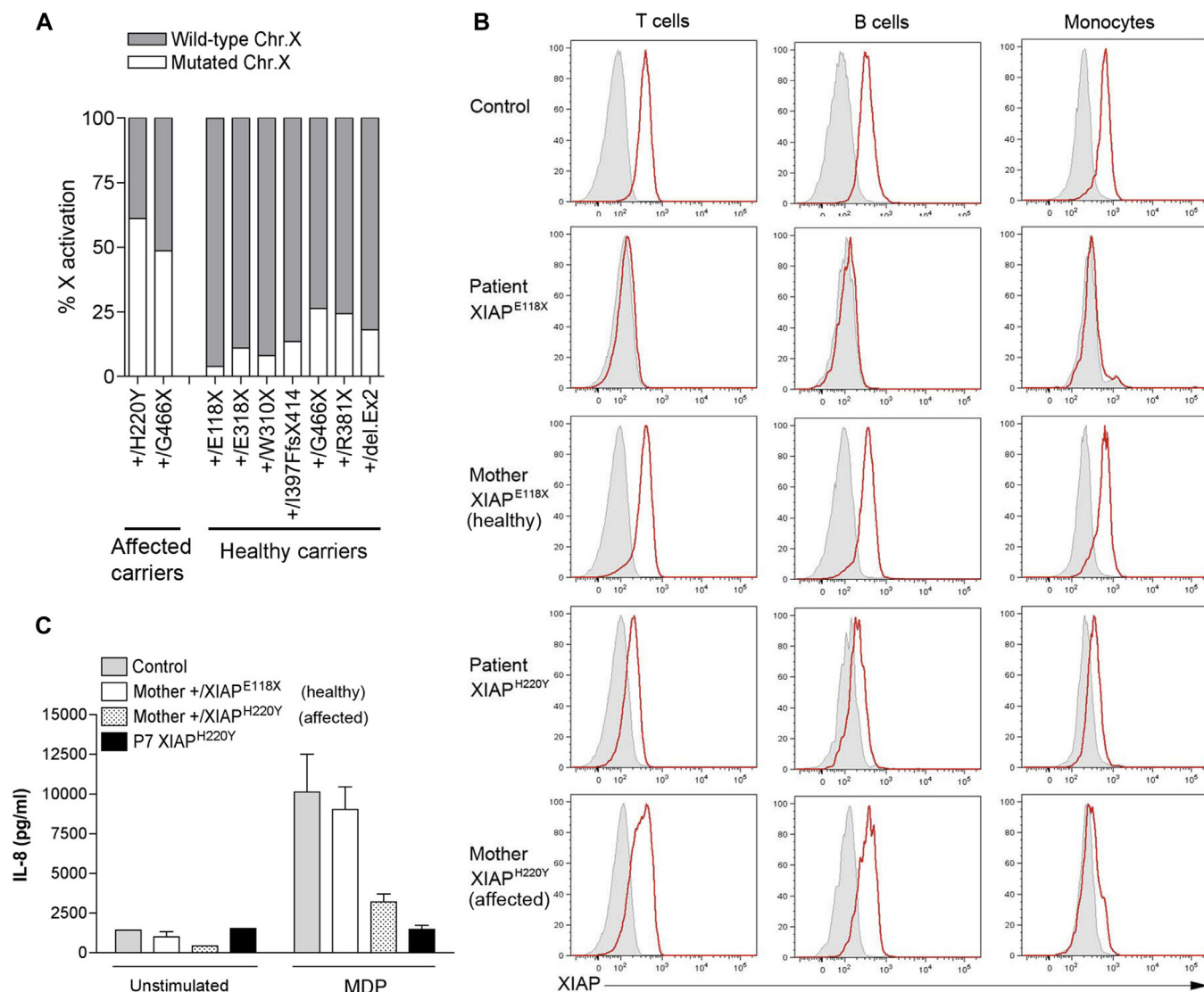


FIG 6. Study of female heterozygous carriers of an *XIAP* mutation with Crohn disease. **A**, Activation of the 2 X chromosomes in PBMCs of the 2 female heterozygous carriers of *XIAP* mutations who had IBD compared with healthy heterozygous carriers of different *XIAP* mutations is indicated. Percentages of activation of the wild-type and mutated alleles are shown. **B**, Flow cytometric histograms of XIAP expression analyzed by means of intracellular staining in T cells, B cells, and monocytes in a healthy mother carrier of a null mutation (+/*XIAP*^{E118X}) and in the mother of P7, a carrier of a hypomorphic mutation (+/*XIAP*^{H220Y}) with Crohn disease, and their respective affected sons. The control subject is a healthy woman matched for age with the 2 female carriers. Histograms of the XIAP staining are shown. **C**, IL-8 production by PBMCs in response to MDP of a healthy mother (+/*XIAP*^{E118X}), the mother carrier who had Crohn disease (+/*XIAP*^{H220Y}), and her affected son.

wild-type allele is inactivated, these mutated alleles (in contrast to null alleles) preserved some protein expression with the capacity to inhibit apoptosis, leading to a less pronounced selective disadvantage and thus allowing these cells to survive similarly as cells expressing the activated wild-type allele. In accordance with this hypothesis, we did not detect an excess of activation-induced cell death in T cells of these 2 affected female subjects (data not shown).

In *XIAP*-deficient patients IBD is often refractory to medications, and thus it requires colectomy, aggressive drugs, or both. The 2 patients who received HSCT had complete remission of digestive symptoms 38 and 24 months after treatment.⁸ Thus,

HSCT seems to represent a therapeutic option for *XIAP*-deficient patients with severe IBD and strengthens the idea, that Crohn disease in the setting of *XIAP* deficiency is the consequence of abnormalities in the hematopoietic compartment rather than in the epithelial compartment. Of interest, no excess in apoptosis in the epithelial compartment of *XIAP*-deficient patients was observed, suggesting compensatory mechanisms by other inhibitor-of-apoptosis proteins. In contrast, T cells of *XIAP*-deficient patients exhibit an increased activation-induced cell death that accounts for the reduction in circulating innate-like T-cell populations and mucosal-associated invariant T cells, which are highly dependent of *XIAP* for their survival.²⁹

The alteration of the NOD2 response in XIAP-deficient patients strongly suggests that the pathophysiologic mechanisms of IBD in patients with XIAP deficiency are quite similar to those proposed for Crohn disease associated with NOD2 variants. Many studies have suggested a pivotal role of NOD2 in immune innate host defense in the gut, although its precise role remains unclear.³⁰ Our data demonstrate that IL-8, MCP-1, and IL-10 production in response to NOD2 stimulation is strongly reduced in XIAP-deficient monocytes. In our assays we did not detect TNF- α secretion in the supernatants of PBMCs in response to MDP. However, a recent report showed that intracellular, membrane, or both type of TNF- α production by PBMCs in response to L18-MDP (which is known to be a stronger NOD2 agonist than the MDP used in our study) could work as a test for the diagnosis of XIAP deficiency.³¹

Because IL-8 and MCP-1 are major chemokines involved in the recruitment of neutrophils, NOD2-impaired signaling in the absence of XIAP might lead to impaired neutrophil influx. Defective neutrophil recruitment is observed in many patients with Crohn disease, who also display impaired cytokine secretion by macrophages derived from blood monocytes.^{32,33} In support of a direct and predominant role of macrophage and neutrophil dysfunction in patients with Crohn disease, colitis indistinguishable from Crohn disease occurs in up to 40% of patients with chronic granulomatous disease, a primary immunodeficiency caused by gene defects affecting the oxidative respiratory burst of phagocytes.^{34,35} Other pathways that involve NOD2 might also participate in the pathogenesis of colitis in XIAP-deficient patients. *NOD2* mutations have been associated with impaired secretion of IL-10,^{24,30} which is also observed in cells from XIAP-deficient patients. IL-10 is a cytokine with a central role in intestinal homeostasis.⁴ Finally, studies have shown that NOD2 is an activator of autophagy, and alterations in autophagy are proposed to play a role in the pathogenesis of Crohn disease.^{36,37} Nevertheless, defects in autophagy in the absence of XIAP are unlikely because recent studies indicate that XIAP acts as an inhibitor of autophagy.³⁸

Our observations point out the importance of the NOD2 pathway in intestinal homeostasis. The preferential expression of NOD2 in myeloid cells is a strong argument that myeloid dysfunctions might play an important role in the initial events leading to Crohn disease. Our data from 1 affected female patient showing that XIAP expression was strongly reduced in monocytes while it was preserved in T and B cells support this notion. However, the penetrance and severity of Crohn disease are higher in patients with XIAP deficiency than in carriers of *NOD2* mutations.¹⁷ This might be explained by additional mechanisms, including impairment of NOD1 functions and increased apoptosis of innate T-cell populations, such as mucosal-associated invariant T cells, that might further weaken local defense and regulation, given their tropism for mucosal tissues and their rapid activation by bacteria.^{14,29,39}

Overall, our study highlights the heterogeneity of Crohn disease and stresses how a primary immune deficiency of Mendelian inheritance can mimic this complex multifactorial disease, as recently discussed.^{10,28} However, additional environmental factors, genetic factors, or both presumably play a role in XIAP deficiency and might explain the variability in penetrance, phenotype, and age at onset of intestinal inflammation. Our observations also strongly support the importance of the NOD2-XIAP pathway in intestinal homeostasis and indicate

that XIAP deficiency represents a privileged model to provide insight into the pathophysiology of Crohn disease.

We thank the patients and their families for cooperation. We thank Drs Rougemont and Henno for providing some histologic data.

Clinical implications: IBD in patients with XIAP deficiency overlaps with Crohn disease. Male and female patients with pediatric-onset IBD, severe IBD, or both should be tested for XIAP, which can be of major importance for therapeutic issues, including HSCT.

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METHODS

Clinical and histologic data

Clinical and histologic data were reviewed independently by at least by 2 persons. Immunostaining was performed on paraffin sections of colonic biopsy specimens to evaluate the expression of XIAP protein with anti-XIAP antibody (clone 28; BD, San Jose, Calif) and apoptosis with a rabbit polyclonal anti-cleaved caspase 3 antibody (Asp175; Cell Signaling, Danvers, Mass). As a control, the staining was performed on paraffin sections from healthy control subjects and patients with Crohn disease.

XIAP sequencing

XIAP gene sequencing was performed by using direct sequencing of exons and intron-exon boundaries, as previously described.^{E1}

Cell counting

Whole blood cells, monocytes, and neutrophils were numbered from the blood of patients and healthy control subjects by using a cell counter (ABX Micros 60; HORIBA Medical, Irvine, Calif).

Cell culture and stimulation

PBMCs were isolated from heparin blood samples by using Ficoll-Paque gradient (Lymphoprep; ProteoGenix, Schiltgheim, France). Cells (2×10^5 per well) in 96-well plates were incubated in RPMI 1640 GlutaMAX medium (Invitrogen, Carlsbad, Calif) with 10% heat-inactivated FCS (Gibco, Life Technologies, Carlsbad, Calif) for stimulation with MDP (10 μ g/mL; Sigma, St Louis, Mo) or LPS (1 ng/mL, Sigma). For experiments with detection of IL-8, intracellular cells have been stimulated similarly in the presence of brefeldin A (GolgiPlug, BD) for 12 hours. For costimulation experiments with NOD2 and TLR pathways, 5×10^5 cells per well were incubated in 96-well plates and stimulated with MDP (10 μ g/mL) and LPS (1 ng/mL) or MDP plus LPS, Pam3CysKKKK (Pam3Cys4; 1 μ g/mL, 1 μ g/mL; EMC Microcollections, Tübingen, Germany), or Pam3Cys4 plus MDP. Supernatants were collected after 18 hours of stimulation and stored at -20°C until the dosage was analyzed for cytokine contents. Primary or SV-40-transformed fibroblasts were cultured in RPMI medium (Invitrogen) with 10% heat-inactivated FCS (Gibco), penicillin (100 U/mL), and streptomycin (100 μ g/mL). Cells (1.25×10^4) have been stimulated in 24-well plates with tri-DAP (20 μ g/mL, InvivoGen, San Diego, Calif) or 10^{-5} mol/L ionomycin (Sigma-Aldrich) plus 10^{-7} mol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich). Supernatants were collected after 18 hours of stimulation and analyzed for their cytokine contents. For intracellular staining with IL-8, cells were stimulated in the presence of brefeldin A (BD GolgiPlug, BD) for 6 hours. I κ B α phosphorylation was analyzed in EBV-immortalized B-cell lines by using Western blotting in response to MDP (10 μ g/mL) stimulation. Cells (2×10^6) were stimulated in 6-well plates.

Cytokine assays

IL-8 and IL-10 were dosed by using ELISAs with commercial kits (DuoSet; R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. IL-6, IL-1 β , TNF- α , MCP-1, and RANTES were dosed by using multiplexed bead-based immunoassays (BD Cytometric Bead Array, BD). Data were collected on a FACSCanto cytometer (BD Biosciences) and analyzed with FCAP Array vb3.0.1 Software (BD).

Flow cytometry

For myeloid phenotyping, PBMCs were stained with anti-CD36-fluorescein isothiocyanate (BD), CD14-PeCy7 (BioLegend, San Diego, Calif), CD11c-VB (BioLegend), and CD16-PerCP (BD) antibodies.

For intracellular staining of cytokines, cells were permeabilized with a permeabilization kit (BD Cytfix/Cytoperm, BD), and cells were incubated with anti-IL-8-phycoerythrin antibody or isotype-matched antibodies (all from BD). For extracellular staining, cells were incubated (before permeabilization) with anti-CD3-allophycocyanin-H7 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD19-FITC (Miltenyi Biotec), anti-CD14-phycoerythrin/Cy7, anti-CD11c-VB, anti-CD66b-allophycocyanin (BD), and anti-CD16-PerCP antibodies.

Western blotting

Cell lysates and immunoblots were performed according to standard protocols. Anti-XIAP antibody (clone 28) was from BD, anti-Ku70 antibody (clone N3H10) was from Neomarkers (Framont, Calif), and anti-phospho-I κ B α (clone 5A5) was from Cell Signaling.

Analysis of NOD2 polymorphisms

The rs2066844 (R702W) and rs2066845 (G908R) polymorphisms were studied by using an allelic discrimination assay with TaqMan predesigned single nucleotide polymorphism genotyping assays (no. C_11717468_20 and no. C_11717466_20; Applied Biosystems, Foster City, Calif), as recommended by the manufacturer. Primers were designed (sequences available on request) for TaqMan genotyping assays of the rs2066847 (1007fs) polymorphism. The assays were carried out with the 7300 HT Fast Real-Time PCR System, and the data were analyzed with SDS Sequence Detector Software (SDSv2.0, Applied Biosystems).

X chromosome inactivation

Inactivation of the X chromosome was determined by analyzing the methylation pattern of the human androgen receptor gene, as previously described.^{E2} Briefly, genomic DNA from cells were first digested with *RsaI* for 3 hours at 37°C with or without *HpaII* for 6 hours at 37°C . Digested DNAs were then used as templates for amplification of the polymorphic CAG repeats contained in the human androgen receptor gene by PCR with specific fluorescent-labeled oligonucleotides. PCR products were evaluated with an ABI 3100 DNA Fragment Analyzer (Applied Biosystems), and data were analyzed with Genescan and Genotyper software (Applied Biosystems). The percentages of activation (or inactivation) of the wild-type and mutated alleles were calculated based on the heights of their respective PCR peaks, with 100% corresponding to the sum of the 2 peaks (Fig E4). As seen in Fig E4, PCR products resolve as several peaks with different intensities corresponding to echoes of the main peak. For the calculation, the height of the highest/main peak was used.

Statistical methods

Paired 2-tailed and unpaired *t* tests were used to analyze the results. They were performed with Prism Version 4.0 software (GraphPad Software, La Jolla, Calif).

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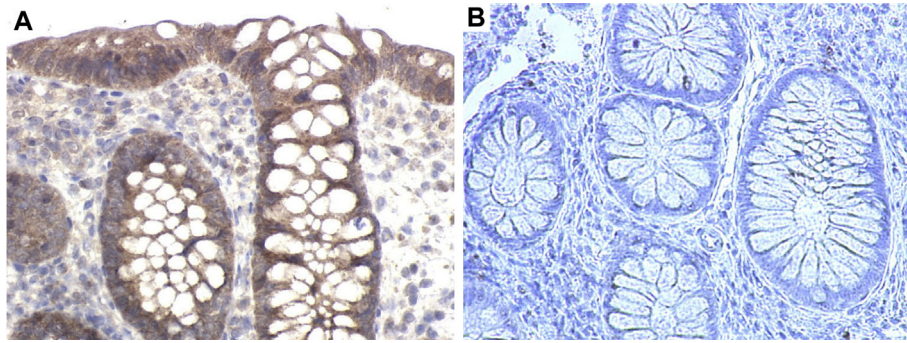


FIG E1. Expression of XIAP in intestinal tissues. **A**, Immunohistochemistry with anti-XIAP antibody on paraffin slides from a colonic biopsy specimen of a healthy control subject shows a strong staining in epithelial cells and less pronounced staining in mononuclear cells (magnification $\times 400$). **B**, The anti-XIAP staining is negative in both mononuclear and epithelial cells from tissues of an XIAP-deficient patient carrier of a null mutation (magnification $\times 400$).

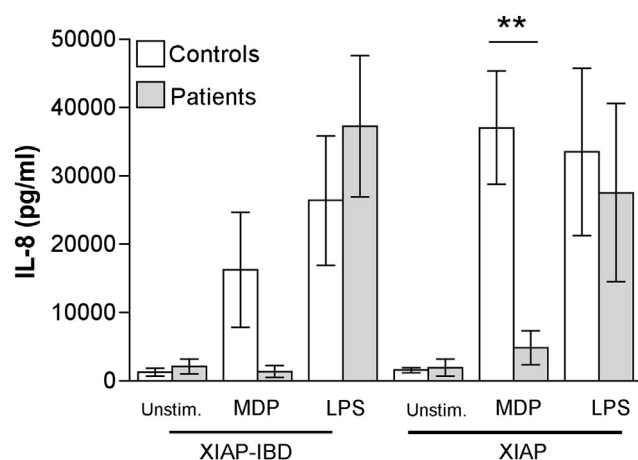


FIG E2. Defective IL-8 production in response to NOD2 stimulation in XIAP-deficient patients with or without IBD. *Left*, IL-8 secretion of PBMCs after stimulation by MDP or LPS or unstimulated (*Unstim.*) in XIAP-deficient patients with IBD ($n = 4$) compared with that seen in healthy control subjects ($n = 4$). *Right*, IL-8 secretion of PBMCs in XIAP-deficient patients without IBD ($n = 7$) compared with that seen in healthy control subjects ($n = 7$). Results of paired 2-tailed t tests with data means and numbers of patients and control subjects tested in independent experiments are shown. $**P < .001$.

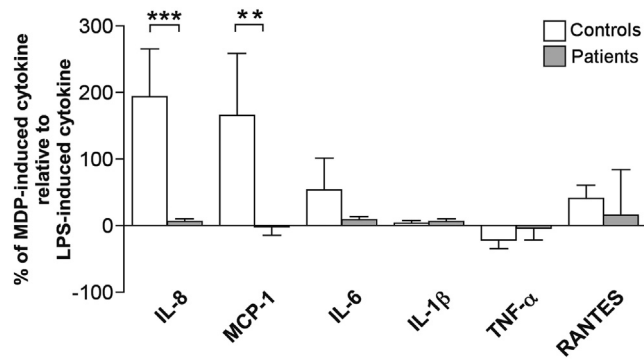


FIG E3. Production of IL-8, MCP-1, IL-6, IL-1 β , TNF- α , and RANTES after NOD2 stimulation with MDP expressed as the percentage of response, with the response induced by LPS stimulation representing 100% of the response. XIAP-deficient patients ($n = 10$) are compared with healthy control subjects ($n = 10$). Results of Mann-Whitney tests and numbers of patients and control subjects tested are shown. ** $P < .01$ and *** $P < .001$.

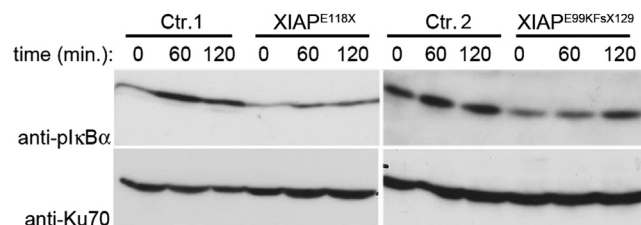


FIG E4. NF- κ B activation is defective in XIAP-deficient cells in response to NOD2 stimulation. B-EBV cell lines from 2 healthy donors (*Ctrl. 1* and *Ctrl. 2*) and 2 XIAP-deficient patients (XIAP^{E118X} [P18] and XIAP^{E99KFsX129} [P1.2]) were stimulated for 60 or 120 minutes with MDP, and cell lysates were analyzed for phospho-I κ B α by using Western blotting. Loading control with anti-Ku-70 antibodies.

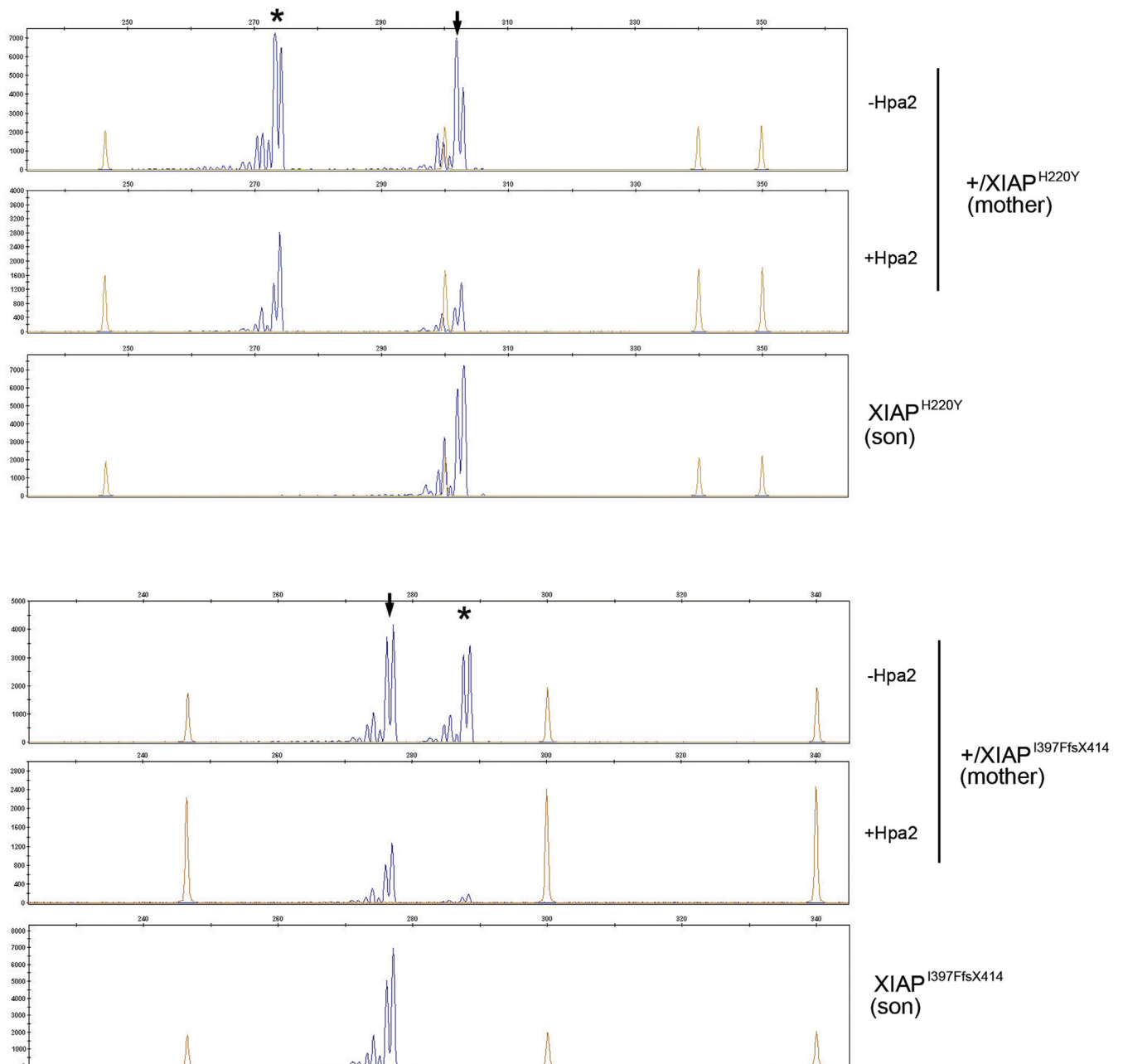


FIG E5. Differential X inactivation in a female patient with Crohn disease who was a carrier of a heterozygous XIAP mutation ($+/\text{XIAP}^{\text{H220Y}}$) and a healthy female carrier ($+/\text{XIAP}^{\text{I397FfsX414}}$). PCR products of polymorphic CAG repeats contained in the human androgen receptor gene from PBMCs of 2 mothers (who were heterozygous carriers) compared with their affected sons are shown after digestion or without digestion by HpaII. For each mother analyzed, the *arrow* indicates the position of the PCR product corresponding to the mutated allele given by the PCR product of their respective sons (who are hemizygous carriers of the mutated allele). The *asterisk* indicates the position of the wild-type allele. PCR products resistant to HpaII digestion correspond to methylated inactive alleles. Size of the PCR products in pair bases are shown on the *horizontal axis*. Relative intensity is shown on the *vertical axis*.

TABLE E1. Histologic findings in digestive samples from XIAP-deficient patients

Patient ID	No. of biopsy specimens analyzed	Ulceration	Granuloma	Infiltrate	Cryptic abscess	Lymphoid hyperplasia
P1.1	11	+	—	+	—	—
P1.2	1	+	+	+	+	+
P2	2	+	—	+	+	—
P3.2	19	+	—	+	+	—
P3.3	15	+	—	+	+	+
P5	1	—	+	+	+	—
P6.2	4	+	+	+	+	+
P7	10	+	+	+	+	+
P8.1	9	+	—	+	—	+
P9	28	+	+	+	+	—
P10	8	+	—	+	+	+
P11.1	3	+	—	+	—	+
P11.2	43	+	+	+	+	+

+, Seen on at least in 1 biopsy specimen; —, not seen in the biopsy specimens analyzed.

TABLE E2. Analysis of the 3 most frequent *NOD2* mutations associated with Crohn disease in XIAP-deficient patients tested for *NOD2* responses

Patient ID	IBD	Mutation	Age at time of experiments (y)	<i>NOD2</i> R702W	<i>NOD2</i> G908R	<i>NOD2</i> 1007fs
P1.1	Yes	E99KFsX129		ND	ND	ND
P1.2	Yes	E99KFsX129		+/+	+/+	+/+
P2	Yes	I397NfsX405		+/+	+/+	+/+
P3.1	Yes	G466X		ND	ND	ND
P3.2	Yes	G466X		+/-	+/+	ND
P3.3	Yes	G466X		+/+	ND	+/+
P4	Yes	R381X		ND	ND	ND
P5	Yes	Del. exons 1-2		+/+	+/+	+/+
P6.1	Yes	I494N		+/+	+/+	+/+
P6.2	Yes	I494N		+/+	+/+	+/+
P7	Yes	H220Y	13	+/+	+/+	+/+
P8.1	Yes	Q332EfsX347	3	+/+	+/+	+/+
P8.2	Yes	Q332EfsX347	26	+/+	+/+	+/-
P9	Yes	Del. exons 1-4	6	+/+	+/+	+/+
P10	Yes	C231Y		+/+	+/+	+/+
P11.1	Yes	V279TfsX292		ND	ND	ND
P11.2	Yes	V279TfsX292		+/+	+/+	+/+
P12.1	No	S361X	16	+/+	+/+	+/+
P12.2	No	S361X	14	ND	ND	ND
P13	No	E318X	3	ND	ND	ND
P14	No	L184X	4	+/-	+/+	+/+
P15	No	W310X	4	ND	ND	ND
P16.1	No	Del. exon2	30	ND	ND	ND
P16.2	No	Del. exon2	17	+/+	+/+	+/+
P17	No	E434AfsX457	19	+/-	+/+	+/+
P18	No	E118X	11	+/+	+/+	+/+

+, Wild-type allele; -, mutant allele; *Del.*, deletion of exons; *ND*, not determined.

TABLE E3. Phenotype of myeloid cells in blood of XIAP-deficient patients and healthy control subjects

Control subjects or patients	CD36 ⁺ (% of PBMCs [myeloid cells])	CD14 ^{high} CD16 ⁻ (% of CD36 ⁺ cells [classical monocytes])	CD14 ^{high} CD16 ⁺ (% of CD36 ⁺ cells [intermediate monocytes])	CD14 ^{low} CD16 ⁺ (% of CD36 ⁺ cells [nonclassical monocytes])	CD14 ⁻ CD11c ⁺ (% of CD36 ⁺ cells [myeloid dendritic cells])	CD14 ⁻ CD11c ⁻ (% of CD36 ⁺ cells)
Ctr. 1	20.1	76.9	3.95	3.18	3.85	12.12
Ctr. 2	14.2	87.4	2.55	0.254	4.14	5.6
Ctr. 3	19	81.7	2.7	0.687	5.47	9.24
Ctr. 4	20	79.9	3.2	6.24	3.53	7.17
Ctr. 5	6.13	71.5	3.3	4.27	7.93	1.98
Ctr. 6	4.97	67.4	4.4	4.01	8.56	1.67
Mean	14.07	77.47	3.3	3.11	5.58	6.3
P2	14.6	83.7	1.8	2.83	4.56	7.11
P7	4.8	44.2	8.5	9.16	1.3	2.85
P9	12.3	80.9	3.2	0.16	1.86	1.86
P14	7.5	77.8	3.74	1.36	7.79	9.31
P16.1	22	85.7	4.38	3.92	2.58	3.42
P16.2	13.6	88.1	1.76	0.627	4.57	4.94
P18	13.9	85.3	3.46	1.68	3.6	5.96
Mean	12.67	77.96	3.84	2.81	5.89	9.49