Excessive deubiquitination of NLRP3-R779C variant contributes to very-early-onset inflammatory bowel disease development

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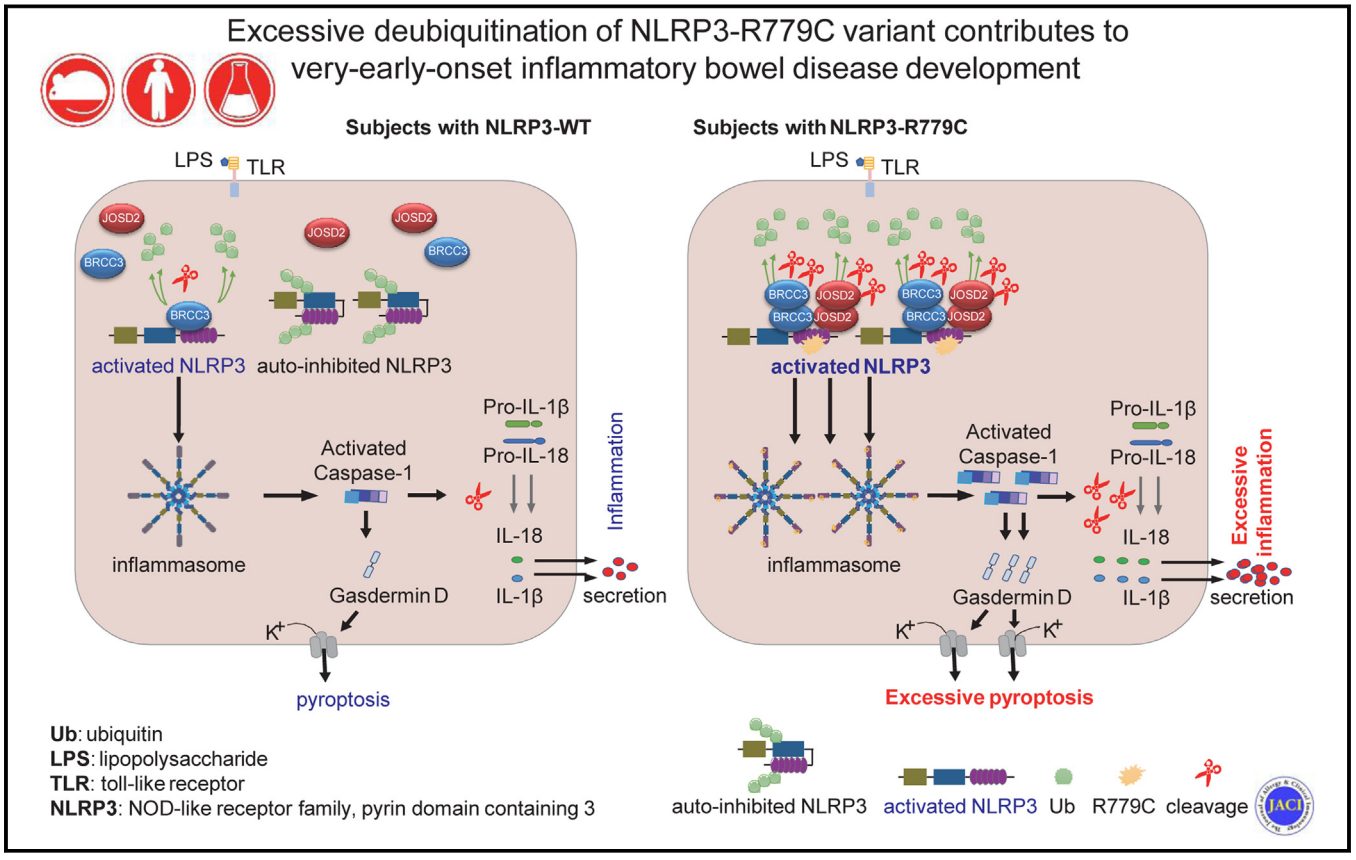
GRAPHICAL ABSTRACT



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Background: Very-early-onset inflammatory bowel disease (VEOIBD) is a chronic inflammatory disease of the gastrointestinal tract occurring during infancy or early childhood. NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome has emerged as a crucial regulator of intestinal homeostasis; however, whether NLRP3 variants may modify VEOIBD risk is unknown.

Objective: We sought to investigate whether and how a rare NLRP3 variant, found in 3 patients with gastrointestinal symptoms, contributes to VEOIBD development.

Methods: Whole-exome sequencing and bioinformatic analysis were performed to screen disease-associated NLRP3 variants from a cohort of children with VEOIBD. Inflammasome activation was determined in reconstituted HEK293T human embryonic kidney cells with NLRP3 inflammasome components, doxycycline-inducible NLRP3 macrophages, as well as PBMCs and biopsies from patients with NLRP3 variants. Pathogenesis of the variants was determined using a dextran sulfate sodium–induced acute colitis model.

Results: We identified a dominant gain-of-function missense variant of *NLRP3*, encoded by rs772009059 (R779C), in 3 patients with gastrointestinal symptoms. Functional analysis revealed that R779C increased NLRP3 inflammasome activation and pyroptosis in macrophages. This was mediated by enhanced deubiquitination of NLRP3 via binding with deubiquitinases BRCC3 and JOSD2, which are highly expressed in myeloid cells. In a dextran sulfate sodium–induced acute colitis model, NLRP3-R779C in hematopoietic cells resulted in more severe colitis, which can be ameliorated via knockdown of BRCC3 or JOSD2.

Conclusions: BRCC3 and JOSD2 mediate NLRP3-R779C deubiquitination, which promotes NLRP3 inflammasome activation and the risk of developing VEOIBD. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: *NLRP3, VEOIBD, deubiquitinase, JOSD2, BRCC3*

Very-early-onset inflammatory bowel disease (VEOIBD) is a more severe form of inflammatory bowel disease (IBD) that occurs in infants and children younger than 6 years. The disease usually manifests as chronic and relapsing inflammation of the gastrointestinal (GI) tract. Clinical remission in some children is difficult to achieve with the commonly prescribed IBD treatment drugs, such as the glucocorticoids or anti-TNF antibodies. In adult patients with IBD, disease development is associated with multiple disease risk genes each having small functional contributions.[1] In infants and children, genetic variants that affect the functions of epithelial, innate, or adaptive immune cells usually have higher disease penetrance and may play dominant roles in IBD development.[2]

The NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome is implicated in a wide variety of systemic and organ-specific inflammatory diseases, including sepsis, hereditary periodic fever syndromes, gout, atherosclerosis, diabetes, Alzheimer disease, and ischemia-reperfusion injuries.[3] Under resting conditions, NLRP3 is regulated through protein ubiquitination. Upon activation, deubiquitinases (DUBs), such as BRCC3, decrease NLRP3 ubiquitination[4] and promote NLRP3 inflammasome activation via association with the adaptor protein apoptosis-associated speck-like protein containing a CARD, and in turn activates caspase-1 to process pro–IL-1β and pro–IL-18 into their active forms. Activated caspase-1 also cleaves gasdermin D, which enables cell membrane pore-formation for the release of IL-1β and IL-18 and triggering of pyroptosis.[5] NLRP3 inflammasome is tightly regulated to balance the need for the immune system to defend pathogen invasion while at the same time allow tissue repair and regeneration of mucosal linings. Gain-of-function NLRP3 variants causing constitutively active inflammasomes and ATP-independent secretion of IL-1β and IL-18 are associated with a clinical spectrum of autoinflammatory disease known as cryopyrin-associated periodic syndromes.[6] So far, no genetic variants of *NLRP3* have been associated with the development of VEOIBD.[7]

By examining a cohort of patients with VEOIBD, we identify and show that tissue-specific deubiquitination of the NLRP3-R779C variant by BRCC3 and JOSD2 can lead to overt gastrointestinal inflammation via heightened NLRP3 inflammasome activation.

Abbreviations used

BM:	Bone marrow
DSS:	Dextran sulfate sodium
DUB:	Deubiquitinase
GI:	Gastrointestinal
IBD:	Inflammatory bowel disease
KO:	Knock out
LRR:	Leucine-rich repeat
NLRP3:	NOD-like receptor family, pyrin domain containing 3
VEOIBD:	Very-early-onset inflammatory bowel disease
WT:	Wild type

METHODS

Patient enrollment

The research was carried out in accordance with the International Ethical Guidelines for Research Involving Human Subjects. Human Ethics Committees of Guangzhou Women and Children's Medical Center approved the study protocol (ID: 2017021504). Legal guardians of all subjects gave written informed consents in accordance with the Declaration of Helsinki. Definition of pediatric-onset colitis or IBD (including Crohn disease and ulcerative colitis) was consistent with our previous publication,[8] and was according to the Porto criteria and the pediatric Paris modification of the Montreal classification.[9,10] The use of bone marrow–derived macrophages and PBMCs was in compliance with institutional ethics guidelines and approved protocols of Sun Yat-sen University.

Plasmids and siRNA transfection

Detailed reagents and methods are described in this article's Online Repository at www.jacionline.org. HA-, Flag- or Myc-tagged NLRP3, BRCC3, or JOSD2 constructs were cloned into the pcDNA3.1 vector. Site-directed mutagenesis was performed with the QuickChange Lightning Kit (210519-5; Agilent Technologies, Santa Clara, Calif) according to the manufacturer's instructions. Chemically synthesized 21-nt siRNA duplexes were obtained from TranSheepBio and transfected using Lipofectamine RNAiMAX (13778150; Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions.

Whole-exome sequencing and bioinformatic analysis

Whole-exome libraries were prepared using the Agilent SureSelect Human All Exon V6 kit, and sequenced on the Illumina platform with 150 base-paired end reads. Sequences were mapped to the human reference genome hg19,

followed by variant calling using the Genome Analysis Toolkit (GATK) HaplotypeCaller. Realignment, base quality score recalibration, and variant quality score recalibration were performed according to the GATK best practice guideline. We hypothesized that homozygous, compound heterozygous, X-linked recessive, and *de novo* were most likely disease relevant and filtered for variants with the following criteria: (1) exclude variants with alternative allele frequency more than 1% in 1000 Genomes Project, dbsnp138, dbsnp141, NHLBI GO Exome Sequencing Project, DiscovEHR, Genome Aggregation Database, and Exome Aggregation Consortium (ExAC r0.3.1); (2) keep variants only with functional effects: missense, startloss, stoploss, stopgain, or splicing; and (3) exclude non–disease-causative variants predicted by logistic regression model.[11]

Evaluation of DUB expression at single-cell resolution

Single-cell RNA sequencing data sets from colon (Genome Sequence Archive, HRA000072)[8] were reanalyzed for DUB expression. In brief, after data preprocessing, cell types were identified by applying dimension reduction and clustering to the gene expression matrix.[12] DUBs were graphed in heat maps to show cell-type–specific expression patterns.

Immunofluorescence of colon biopsies

Paraffin-embedded sections of colonic biopsies were processed as reported previously.[13] For each single colonic mucosa section, 5 areas were randomly selected in 200× magnified images for quantification. The number of positive cells per square millimeter was counted and analyzed using Leica X image analysis software (Leica, Hamburg, Germany) and ImageJ software (National Institutes of Health, Bethesda, Md). Details of the antibodies used are described in the Methods section in this article's Online Repository.

Generation of doxycycline-inducible NLRP3-wild-type and NLRP3-R779C THP-1 cell lines

For NLRP3-inducible expression, lentiviral particles were produced by transfecting HEK293T human embryonic kidney cells with pL-Teton3G-iZ-NLRP3-wild type (WT) or pL-Teton3G-iZ-NLRP3-R779C. NLRP3 knock out (KO) THP-1 cells were infected by incubation with lentivirus-containing supernatant for 48 hours. Cells were treated with doxycycline (100-200 ng/mL) for 24 hours to induce the expression of NLRP3-WT and NLRP3-R779C variant in THP-1–derived macrophages.

Immunoblot and immunoprecipitation analysis

For immunoprecipitation, whole-cell extracts were prepared after transfection or treatment, followed by incubation overnight with anti-FLAG, anti-HA beads (Sigma, Darmstadt, Germany), or Protein A/G beads (Pierce, Rockford, Ill). Beads were washed 5 times with low-salt lysis buffer, and immunoprecipitates were eluted for SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, Calif), and LumiGlo Chemiluminescent Substrate System (KPL, Gaithersburg, Md) was used for protein detection.

Relative cell death assays

Supernatants of treated cells were harvested and analyzed for lactate dehydrogenase activities using a lactate dehydrogenase cytotoxicity assay kit (Pierce), according to the manufacturer's instructions. Relative cell death was determined as (experimental cell death – baseline cell death)/(maximum cell death – baseline cell death) × 100%. Maximum cell death was indicated by a positive control for 100% cell death generated by lysis buffer from the kit, whereas baseline cell death was indicated by a negative control of 0% cell death as a result of no treatment.

Cytokine quantification from culture supernatants

Human IL-1β and IL-18 concentrations were determined by ELISA using BD OptEIA Human IL-1β ELISA Kit II (BD Biosciences, San Jose, Calif) and IL-18 Human Instant ELISA Kit (Invitrogen), respectively. Mouse IL-1β, IL-18, IL-6, and TNF-α were measured by BD OptEIA Mouse IL-1β ELISA Kit (BD Biosciences), IL-18 Mouse ELISA Kit (Invitrogen), BD OptEIA Mouse IL-6 ELISA Kit (BD Biosciences), and BD OptEIA Mouse TNF ELISA Kit (BD Biosciences).

Bone marrow transfer

Bone marrow (BM) cells from 6- to 10-week-old donor male mice (*Nlrp3* KO C57BL/6) were cultured and transduced with NLRP3-WT or NLRP3-R779C variant packaged with the △ 8.9 VSVG lentivirus expression system.[14] Recipient mice (6-10-week-old female *Nlrp3* KO C57BL/6) were irradiated with 900 cGy and randomly separated into 2 groups, which received either NLRP3-WT or NLRP3-R779C–transduced BM cells (5×10^6) intravenously. The reconstituted mice were housed in the specific pathogen-free animal facility of Sun Yat-sen University and analyzed 7 to 9 weeks later. The Institutional Animal Care and Use Committee of Sun Yat-sen University approved all experimental protocols concerning the handling of mice.

Induction of colitis

The BM-reconstituted mice were given 2.5% dextran sulfate sodium (DSS) (molecular weight, 36-50 kD; MP Biomedicals, Solon, Ohio) in drinking water for 7 days. Bleeding scores were determined as follows: 0, no blood by hemoccult test (Beckman Coulter, Brea, Calif); 1, positive hemoccult; 2, blood traces in stool visible; 3, gross rectal bleeding. Mice were euthanized for tissue analyses on day 8. Lengths of the loosely stretched colon were measured from cecum-colon junction to the anus. BM cells were used for detection of NLRP3 WT and R779C expression by immunoblot. Colon tissues were subjected to organ culture and histopathological analyses. Tissue culture supernatants were collected to measure cytokine release by ELISA. Colon tissue was fixed in 4% paraformaldehyde immediately, paraffin-embedded, and stained with hematoxylin and eosin for histological analysis. The lysate of colons from indicated experimental mice (N = 3) were harvested for immunoprecipitation with protein A/G-agarose and anti-NLRP3 antibody.

RESULTS

Whole-exome sequencing identifies a rare NLRP3 variant in patients with gastrointestinal symptoms

We screened a cohort of children with IBD and identified an *NLRP3* rare variant c.C2335T: p.R779C, encoded by rs772009059, in a male patient (patient 1). Whole-exome sequencing of the patient and his biological parents showed that the child inherited this mutation from his mother, who has reported frequent episodes of stress-related severe allergy, diarrhea, and blood in the stool since childhood (**see** Fig E1 in this article's Online Repository at www.jacionline.org). The minor allele frequency for R779C is 0.0009 in gnomAD exome East Asian cohort, approximately 10 times enriched than that from the rest of the gnomAD cohort (6.92×10^{-5}) (see Table E1 in this article's Online Repository at www.jacionline.org).[15] R779C is located at the leucine-rich repeat (LRR) domain of NLRP3 (Fig 1, *A*), and is highly conserved across species (Fig 1, *B*). Blood in the stool was first observed in patient 1 at age 7 months after supplementation of milk powders in the diet. Endoscopy performed at age 2 years uncovered erythema, erosions, and ulcers in the stomach, duodenum, cecum, and colon. Histology revealed mild hyperplasia of stratified squamous epithelium in the esophagus, and marked lymphocyte infiltration, edema, erythema, and bleeding of the colon (Fig 1, *C*, upper panel; see Fig E2, *A*, left panel, in this article's Online Repository at www.jacionline.org). Eosinophil counts were 70/hpf at the descending colon.

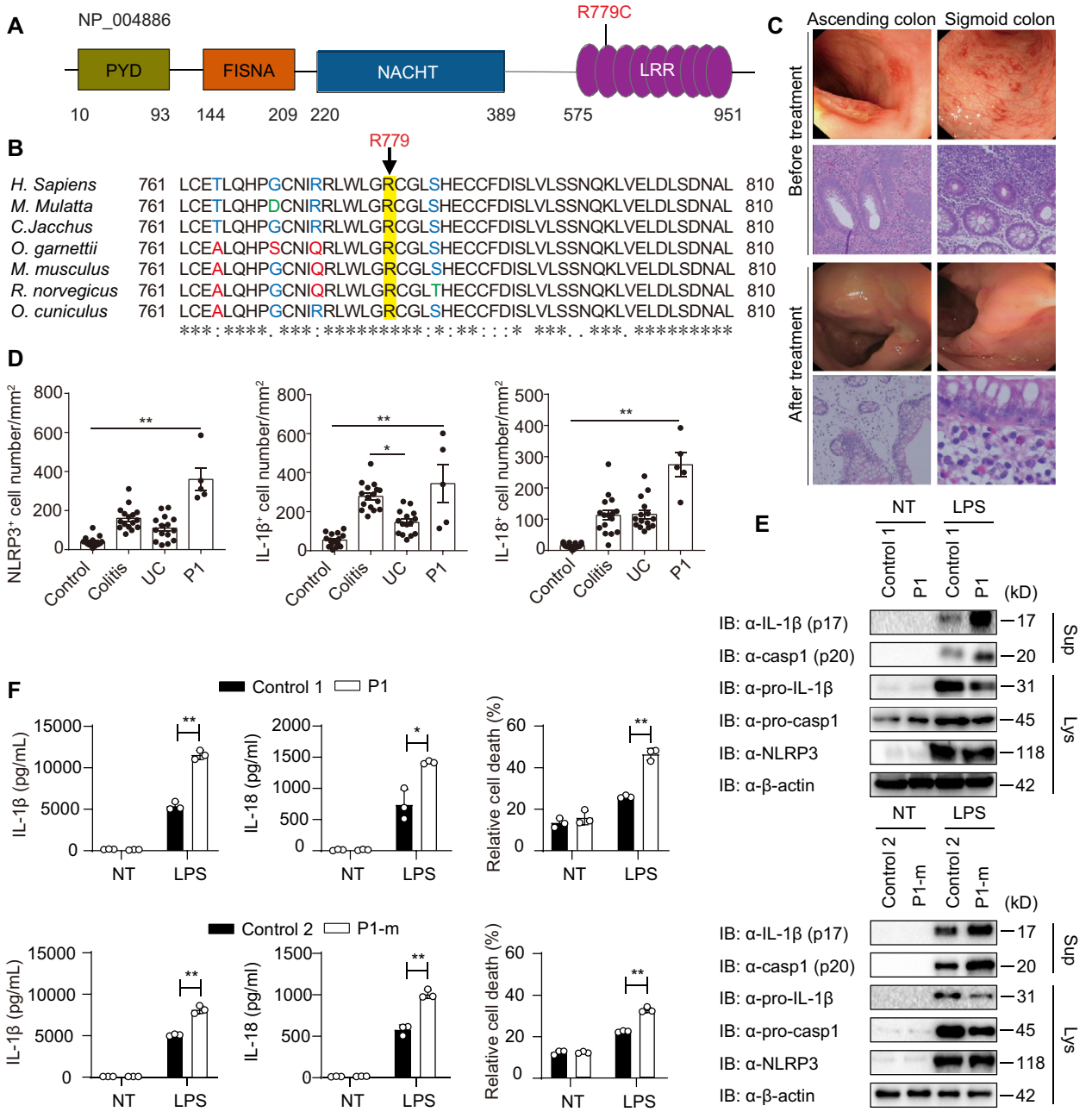

FIG 1. Whole-exome sequencing identifies a rare NLRP3 variant in patients with gastrointestinal symptoms. **A,** Schematic view of NLRP3 and the location of the R779C variant. **B,** Conservation of NLRP3-R779 between species. **C,** Endoscopic and histological appearances of P1 before and after prednisolone treatment. **D,** Summary of immunofiuorescent findings showing increased abundances of NLRP3$^+$, IL-1β$^+$, and IL-18$^+$ cells in colonic mucosae from P1 compared with control (n = 3) or subjects with colitis (n = 3) or UC (n = 3). **E,** IB showing NLRP3 as well as mature and precursor forms of IL-1β and caspase-1 in PBMCs of P1 and P1-m. **F,** IL-1β and IL-18 concentrations in the supernatant and cell death of PBMCs stimulated with LPS (500 ng/mL) for 3 hours. Mixed PBMCs from 6 age- and sex-matched healthy children (control 1) and 6 healthy adult (control 2) subjects were used as controls for P1 and P1-m, respectively. *IB,* Immunoblot; *Lys,* lysates; *NT,* not treated; *P1,* patient 1; *P1-m,* mother of patient 1; *Sup,* supernatant; *UC,* ulcerative colitis. *P* values in Fig 1, *D,* were calculated using the Mann-Whitney *U* test. Data in Fig 1, *F,* are presented as mean ± SEM of 3 independent experiments. **P* < .05 and ***P* < .01.

NLRP3 inflammasome activation and enhanced IL-1β and IL-18 protein levels were observed at the colonic biopsies in patient 1 in comparison to disease-free control subjects (Fig 1, *D*; see Fig E2, *B*). Patient 1 has been on oral prednisone (5 mg every day), omeprazole (10 mg every day), and aminosalicylate (5-ASA, 250 mg twice a day) since diagnosis. Colonoscopy performed 1.5 years after initial diagnosis revealed persistent colonic lesions despite treatment (Fig 1, *C*, lower panel; see Fig E2, *A*, right panel).

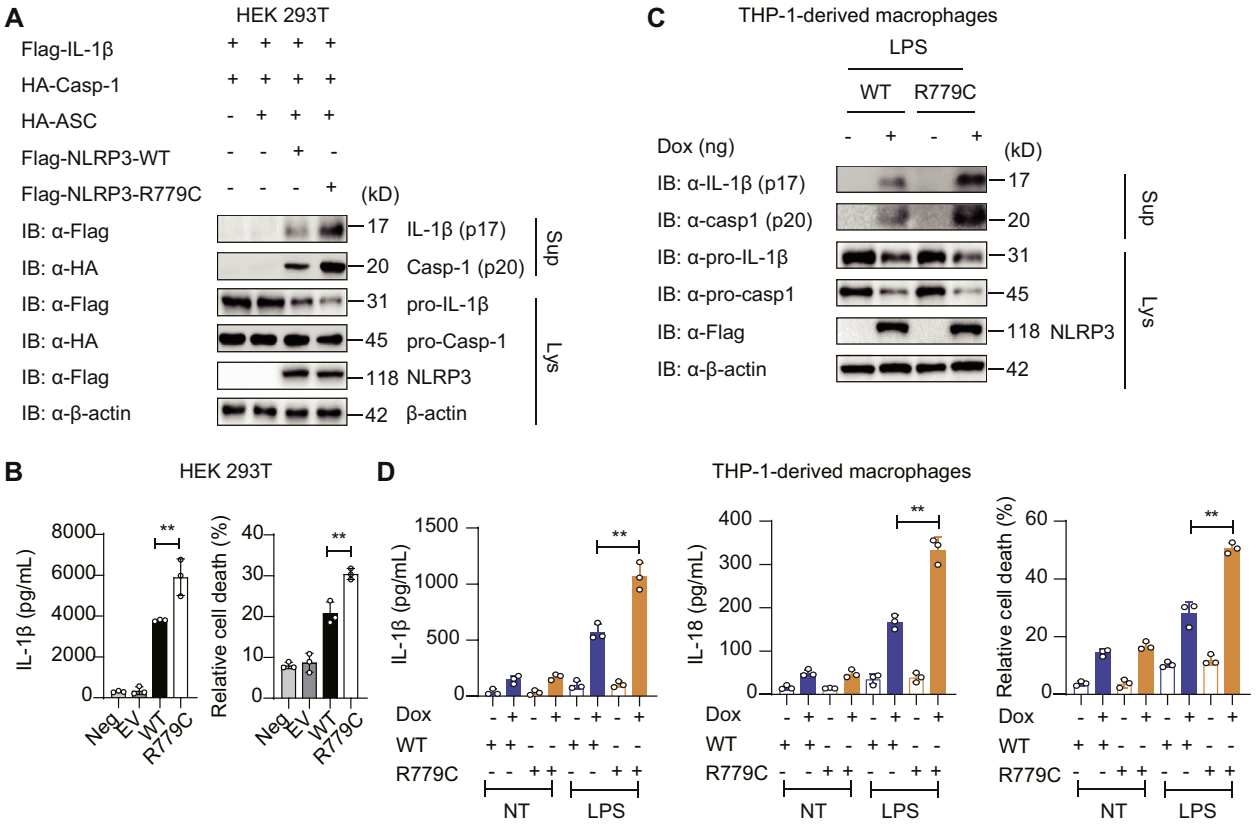

FIG 2. The NLRP3-R779C variant promotes NLRP3 inflammasome activation. HEK293T cells were transiently transfected with expression vectors of ASC, pro–caspase-1, and pro–IL-1β, together with either WT NLRP3 (NLRP3-WT) or NLRP3- R779C variant, respectively. **A,** IB showing NLRP3 as well as mature and precursor forms of IL-1β and caspase-1 in the supernatant and cell lysates. **B,** ELISA showing IL-1β release in the supernatant. Cell death was measured by LDH release. Doxycycline-pretreated NLRP3-WT and NLRP3-R779C THP-1–derived macrophages were primed with LPS (500 ng/mL) for 3 hours. **C,** IB showing NLRP3 as well as mature and precursor forms of IL-1β and caspase-1 in the supernatant (Sup) and cell lysates (Lys) from THP-1–derived macrophages. **D,** ELISA showing IL-1β and IL-18 release in the supernatant (left panel). Cell death was measured by LDH release assay (right panel). *ASC,* Apoptosis-associated speck-like protein containing a CARD; *Dox,* doxycycline; *HEK293T,* HEK293T human embryonic kidney; *IB,* immunoblot; *LDH,* lactate dehydrogenase; *Neg,* negative control; *NT,* not treated. Data in Fig 2, *B* and *D,* are presented as mean ± SEM of 3 independent experiments. $*P < .05$ and $**P < .01$.

To confirm that the R779C variant causes excessive inflammation in patient 1, we stimulated his PBMCs with LPS. Caspase-1 cleavage, release of IL-1β and IL-18, and cell death were markedly increased in patient 1 compared with age- and sex-matched healthy control subjects. Inflammasome activation was also increased in PBMCs of the mother of patient 1 (Fig 1, *E* and *F*), which was consistent with her early-onset disease history. To further validate the pathogenicity of the R779C variant, we searched local and collaborative private patient databases and identified the second patient (patient 2) who carried the same *NLRP3*-R779C variant. Patient 2 was previously diagnosed with activated PI3K delta syndrome and carried a heterozygous mutation, E1021K in PIK3CD. She presented with generalized lymphadenopathy at the age of 7 months, with a clinical course of parotid swelling at 3 years and idiopathic thrombocytopenic purpura at 4 years with evidence of past EBV infection. At age 3 years, endoscopy revealed peptic ulcers and granulomatous lesions in the duodenum and the colon, with polyclonal lymphoproliferation on biopsy (see Table E2 in this Online Repository at www.jacionline.org).

Given that patients with activated PI3K delta syndrome often exhibit VEOIBD-like symptoms early in life,[16] we tested whether the PIK3CD-E1021K variant could contribute to NLRP3 inflammasome activation. We generated doxycycline-inducible PIK3CD-WT and PIK3CD-E1021K THP-1–derived macrophages that coexpressed either NLRP3-WT or NLRP3-R779C variant. When THP-1–derived macrophages were stimulated with LPS, inflammasome activation was comparable between PIK3CD-E1021K and PIK3CD-WT cells. Furthermore, PIK3CD-E1021K mutation did not further increase NLRP3-R779C inflammasome activation (see Fig E3 in the Online Repository at www.jacionline.org). By deduction therefore, we surmised that any increase in inflammasome activation that contributed to patient 2's gastrointestinal symptoms would be due to the NLRP3-R779C mutation.

NLRP3-R779C variant causes increased IL-1β and IL-18 release and pyroptosis

To determine whether the NLRP3-R779C variant causes excessive inflammasome activation, we reconstituted HEK293T

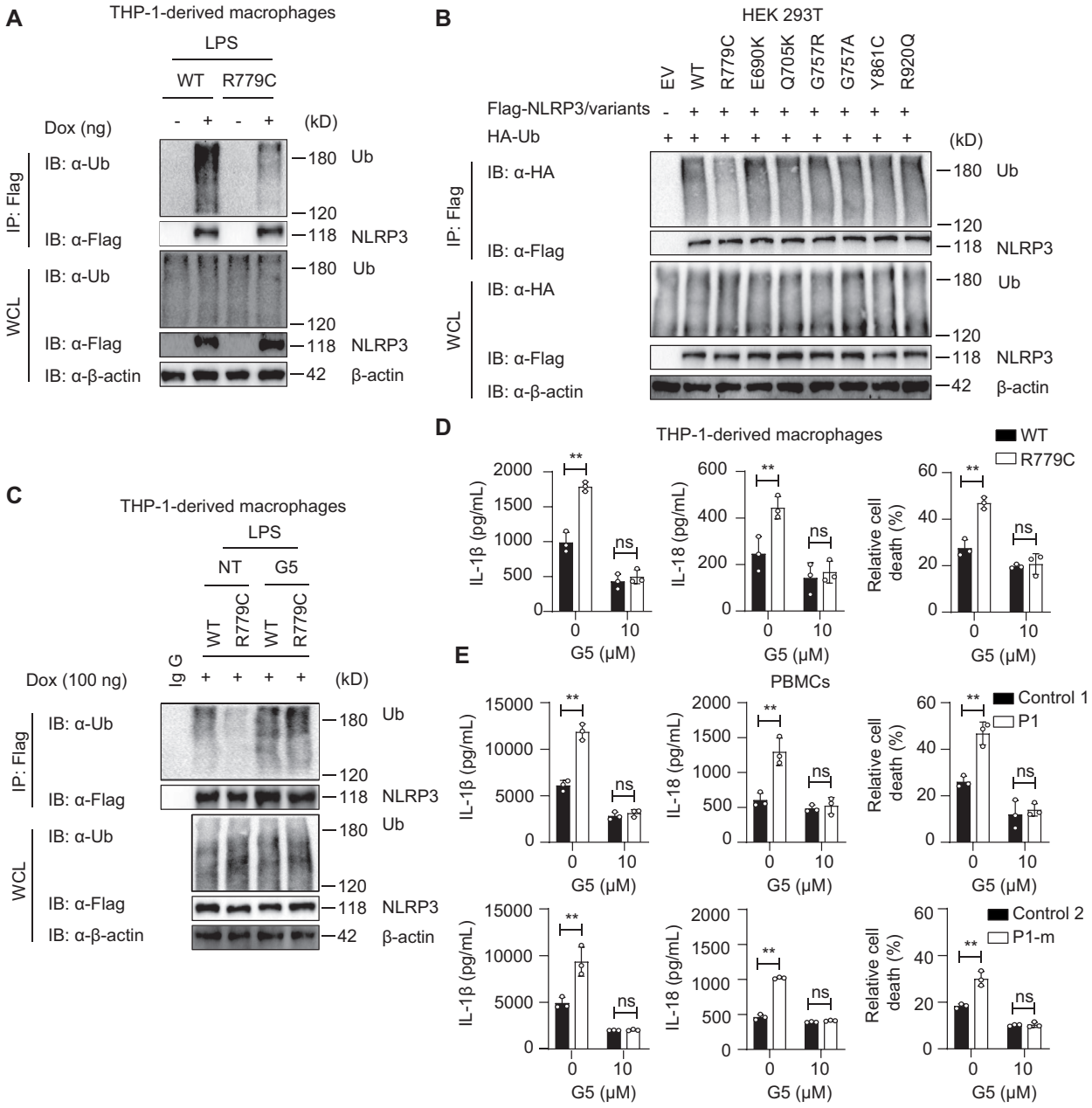

FIG 3. The NLRP3-R779C variant promotes NLRP3 inflammasome activation via deubiquitination. **A,** IB showing Flag-tag IP assay of doxycycline-pretreated inducible NLRP3-WT or NLRP3-R779C THP-1–derived macrophages primed with LPS (500 ng/mL) for 3 hours. **B,** IB showing Flag-tag IP assay of HEK293T cells transiently transfected with Flag-NLRP3-WT or Flag-NLRP3 variants, and HA-Ub. **C,** IB showing Flag-tag IP assay of doxycycline-pretreated inducible NLRP3-WT or NLRP3-R779C THP-1–derived macrophages primed with LPS (500 ng/mL) for 3 hours. G5 (10 μM) was added 15 minutes before LPS stimulation. **D,** ELISA showing IL-1β and IL-18 secretion in the supernatant (left panel). Cell death was measured by LDH release. **E,** ELISA showing IL-1β and IL-18 secretion in the supernatant of PBMCs from P1 and P1-m treated with or without G5 plus LPS performed as in Fig 3, *C* and *D*. PBMCs from 6 age- and sex-matched healthy children were mixed and used as control for P1 (control 1). PBMCs from 6 healthy adult subjects were mixed and used as control for P1-m (control 2). *Dox*, Doxycycline; *HEK293T*, HEK293T human embryonic kidney; *IB*, immunoblot; *IP*, immunoprecipitation; *LDH*, lactate dehydrogenase; *ns*, not significant; *NT*, not treated; *P1*, patient 1; *P1-m*, mother of patient 1; *Ub*, ubiquitin; *WCL*, whole cell lysates. Data in Fig 3, *D* and *E*, are presented as mean ± SEM of 3 independent experiments. **P* < .05 and ***P* < .01.

human embryonic kidney cells with ectopic expression vectors of apoptosis-associated speck-like protein containing a CARD, pro–caspase-1, pro–IL-1β, together with NLRP3-WT or NLRP3-R779C variant. Immunoblot and ELISA analyses demonstrated that the NLRP3-R779C variant markedly increased caspase-1 cleavage, IL-1β secretion, and pyroptosis (Fig 2, *A* and *B*). To further identify the function of the NLRP3-R779C variant in human innate immune cells, we generated

doxycycline-inducible NLRP3-WT and NLRP3-R779C macrophages using a human monocytic THP-1 cell line (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). After doxycycline treatment, expression of NLRP3-WT and NLRP3-R779C variant was comparable to that of the endogenous NLRP3 (see Fig E4, *B*). We then differentiated these THP-1 cells into macrophages and found that NLRP3-R779C cells had significantly increased caspase-1 activation, IL-1β and IL-18 release as well as pyroptosis under LPS stimulation (Fig 2, *C* and *D*). These data confirmed that R779C variant markedly promoted NLRP3 inflammasome activation in human macrophages.

R779C variant promotes NLRP3 deubiquitination

In examining the molecular mechanisms that underlie enhanced NLRP3 inflammasome activation, we found that the NLRP3-R779C variant did not affect NLRP3 expression or stability (see Fig E5, *A* and *B*, in this article's Online Repository at www.jacionline.org). However, NLRP3-R779C showed markedly decreased ubiquitination in comparison to NLRP3-WT (see Fig E5, *C*; Fig 3, *A*). The LRR domain of NLRP3 is generally considered autoinhibitory by folding back onto the NACHT domain, which contains ATPase activity and is vital for NLRP3 self-association and function.[17] We asked whether other reported gain-of-function LRR domain variants (E690K, Q705K, G757R, G757A, Y861C, and R920Q)[18-23] may also promote NLRP3 deubiquitination. When overexpressed in 293T cells, all tested variants promoted IL-1β secretion and pyroptosis (see Fig E6 in this article's Online Repository at www.jacionline.org). However, none of these variants altered NLRP3 ubiquitination when compared with that of the NLRP3-WT (Fig 3, *B*).

Given that deubiquitination is a prerequisite for its activation, we reasoned that the R779C variant might reduce the threshold of NLRP3 activation by maintaining a low-ubiquitination state. Indeed, the low ubiquitination state of NLRP3-R779C variant was reversed by G5, a pan DUB inhibitor (Fig 3, *C*). At the same time, G5 also inhibited excessive secretion of IL-1β and IL-18 and cell death from NLRP3-R779C THP-1–derived macrophages (Fig 3, *D*) and from PBMCs of patient 1 and his mother (Fig 3, *E*). These results indicated that the R779C variant enhanced NLRP3 inflammasome activation by promoting NLRP3 deubiquitination.

NLRP3-R779C variant promotes interactions with BRCC3 and JOSD2, DUBs highly expressed in the inflamed colon

Given that all patients manifested GI symptoms, we hypothesized that DUBs highly expressed in the myeloid cells of the GI tract may activate the R779C variant and increase the risk of developing VEOIBD. We thus reanalyzed a previously generated single-cell RNA transcriptome data set[8] to identify colon-enriched DUBs (Fig 4, *A*). By performing a deubiquitination assay, we found that JOSD2 and BRCC3 both promoted NLRP3-R779C deubiquitination (Fig 4, *B*; see Fig E7 in this article's Online Repository at www.jacionline.org). We confirmed that JOSD2 and BRCC3 expression levels were highest in colonic biopsies from patient 1 with the NLRP3-R779C variant. However, JOSD2 and BRCC3 were also expressed in more sterile organs such as liver (see Fig E8, *A-D*, in this article's Online Repository at www.jacionline.org). We next analyzed single-cell RNA sequencing data of different myeloid cells from heart, liver, kidney, and lung,[24-27] and found that BRCC3 and JOSD2 are ubiquitously expressed in myeloid cells (see Fig E8, *E-H*). Further analysis using single-cell RNA sequencing data showed that the expression of BRCC3 was relatively high in macrophages, neutrophil-like cells, DCs, and pDCs, and the expression of JOSD2 was relatively high in macrophages and DCs (see Fig E9, *A*, in this article's Online Repository at www.jacionline.org). Immunofluorescent staining of JOSD2 and BRCC3 together with either macrophage (CD68), monocyte (CD11b or CD14), DC (CD11c), or neutrophil (MPO) markers indicated that JOSD2 and BRCC3 were both expressed in macrophages and monocytes, whereas JOSD2 was also expressed in $CD11c^+$ DCs (see Fig E9, *B-D*). BRCC3 has previously been shown as a DUB that deubiquitinates and activates NLRP3.[4] We found that the NLRP3-R779C variant not only had enhanced interaction with BRCC3 but also strongly interacted with JOSD2, a DUB that did not interact with NLRP3-WT (Fig 4, *C*). Thus, BRCC3 mediated the deubiquitination of both NLRP3-WT and NLRP3-R779C variant (see Fig E10, *A*, in this article's Online Repository at www.jacionline.org), whereas JOSD2 mediated the deubiquitination of only NLRP3-R779C (Fig 4, *D*). Concordantly, knockdown of *BRCC3* significantly increased the ubiquitination of both NLRP3-WT and NLRP3-R779C (see Fig E10, *B* and *C*), whereas knockdown of *JOSD2* only markedly increased the ubiquitination of NLRP3-R779C (see Fig E10, *B*; Fig 5, *A*). We demonstrated that knockdown of either *BRCC3* or *JOSD2* significantly decreased IL-1β and IL-18 release and pyroptosis in NLRP3-R779C macrophages (see Fig E10, *D* and *E*), and that knockdown of both DUBs completely eliminated excessive NLRP3 inflammasome activation (Fig 5, *B*).

We confirmed the above findings in the colonic biopsies from patient 1 and found that *BRCC3* and *JOSD2* were highly expressed (see Fig E11, *A* and *B*, in this article's Online Repository at www.jacionline.org) in myeloid-derived cells and colocalized strongly with NLRP3 (Fig 5, *C-F*). In addition, expressions of BRCC3 and JOSD2 were significantly increased in PBMCs from patient 1 compared with those from his mother (see Fig E11, *C*). Together, these data suggested that BRCC3 and JOSD2, DUBs enriched in the inflamed GI track, promote NLRP3-R779C inflammasome activation and pyroptosis.

NLRP3-R779C variant promotes more severe intestinal inflammation in an *in vivo* colitis model

To further confirm that the NLRP3-R779C variant causes excessive inflammation *in vivo*, we reconstituted irradiated *Nlrp3* KO mice[14] with *Nlrp3* KO bone marrow transduced with human either *NLRP3*-WT or *NLRP3*-R779C variant, respectively (hereafter called NLRP3-WT mice or NLRP3-R779C mice, respectively). Colitis was induced with DSS (see Fig E12, *A* and *B*, in this article Online Repository at www.jacionline.org). Compared with NLRP3-WT mice, NLRP3-R779C mice were highly susceptible to DSS-induced colitis and showed significantly increased clinical severity (Fig 6, *A*), more dramatic weight loss (Fig 6, *B*), reduced survival rate (Fig 6, *C*), and shortened colon length (Fig 6, *D* and *E*). In hematoxylin and eosin–stained colonic sections, *NLRP3*-R779C mice had increased histological scores as measured by inflammatory cell infiltration and basal lamina thickness (Fig 6, *F* and *G*).

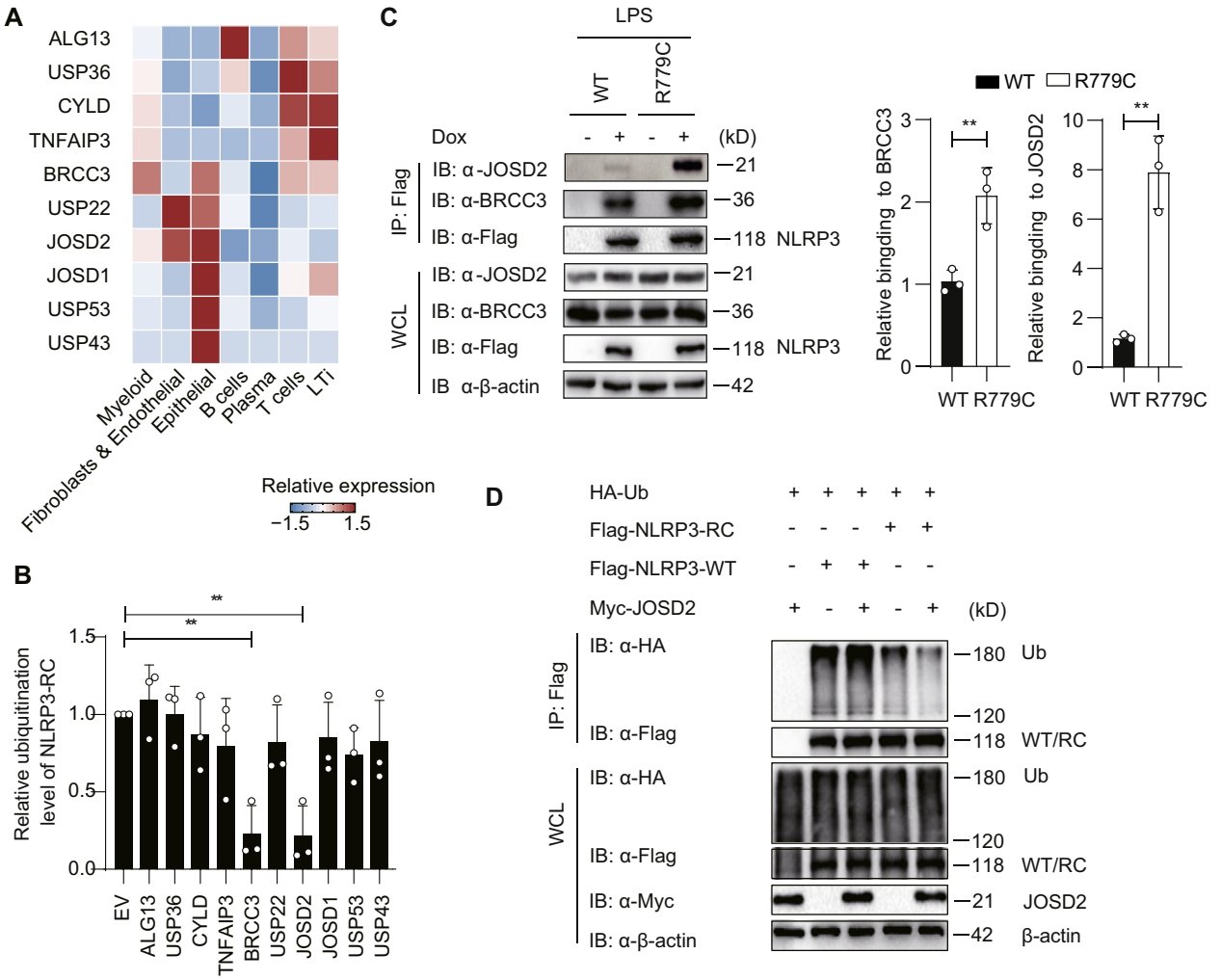

FIG 4. The NLRP3-R779C variant interacts with BRCC3 and JOSD2. **A,** Heat map showing mRNA of DUBs highly and differentially expressed in the colonic myeloid, epithelial, and stromal cell subsets. **B,** Quantitative statistics showing relative ubiquitination level of NLRP3-R779C from HEK293T cells transfected with Flag-NLRP3-R779C, HA-Ub, and the indicated DUB plasmids. Cell lysates were IPed with anti-Flag beads and IBed with anti-HA antibody. NLRP3-R779C ubiquitination was quantified relative to EV. **C,** IB showing binding of NLRP3-WT or NLRP3-R779C with DUBs in doxycycline-pretreated THP-1–derived macrophages that were treated with LPS (500 ng/mL) for 3 hours. Densitometry analysis of BRCC3 and JOSD2 binding with NLRP3-WT and NLRP3-R779C proteins was shown. DUBs binding to NLRP3-WT were used as reference. **D,** IB showing JOSD2-mediated deubiquitination of NLRP3-R779C but not NLRP3-WT protein. *Dox,* Doxycycline; *EV,* empty vector; HEK293T, HEK293T human embryonic kidney; *IB,* immunoblot; *Ub,* ubiquitin; *WCL*, whole cell lysates. Data in Fig 4, *B* and *C*, are presented as mean ± SEM of 3 independent experiments. ***P* < .01.

Furthermore, the concentrations of the proinflammatory cytokines IL-1β, IL-18, IL-6, and TNF-α in the NLRP3-R779C mice colon were significantly higher than those in the NLRP3-WT mice (Fig 6, *H*; see Fig E12, *C*). We also found that NLRP3-R779C bound to mouse Brcc3 and Josd2 (Fig 6, *I*), and exhibited decreased ubiquitination in colonic tissues (Fig 6, *J*). We also examined colitis severity in mice with human NLRP3-R920Q variant, which has been reported to cause tissue-specific autosomal-dominant sensorineural hearing loss. In a DSS-induced colitis model, colonic inflammation of NLRP3-R920Q mice was comparable to that in NLRP3-WT mice (see Fig E13 in this article's Online Repository at www.jacionline.org). In addition, PIK3CD-E1021K did not increase NLRP3 inflammasome activation compared with PIK3CD-WT mice in the DSS-induced colitis model (see Fig E14 in this article's Online Repository at www.jacionline.org).

Consistent with requirement for BRCC3 and JOSD2, we showed that knockdown of either DUBs (Fig 6, *K*) or administration of G5 (Fig 6, *L*) in colonic single-cell suspensions from the reconstituted mice largely eliminated excessive inflammasome activation associated with the NLRP3-R779C variant. These results were further confirmed *in vivo*. We showed that knockdown of either *Brcc3* or *Josd2* significantly decreased colitis severity of NLRP3-R779C mice to levels that were comparable with those of NLRP3-WT mice (Fig 7, *A-E*). Hematoxylin and eosin–stained colonic sections of DSS-fed NLRP3-R779C mice displayed similar histological scores to those of DSS-fed NLRP3-WT mice (Fig 7, *F* and *G*). IL-1β and IL-18 concentrations in the

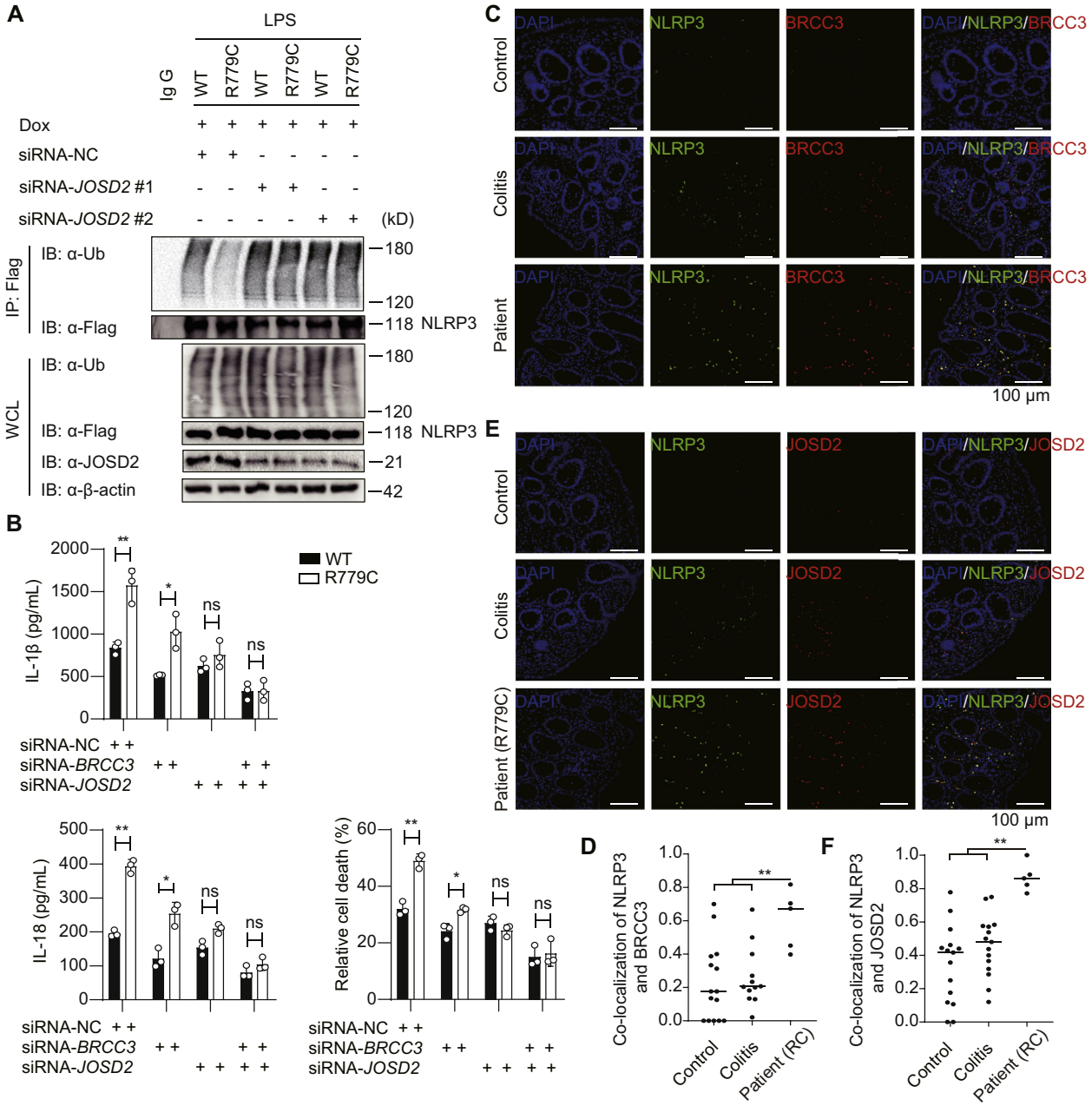

FIG 5. The NLRP3-R779C variant promotes inflammation by colon-specific BRCC3 and JOSD2. **A,** IB showing JOSD2-mediated NLRP3-R779C deubiquitination. Inducible NLRP3-WT or NLRP3-R779C THP-1–derived macrophages were transfected with control siRNA (siRNA-NC) or *JOSD2* siRNAs for 24 hours, followed with doxycycline for 10 hours and LPS (500 ng/mL) for 3 hours. **B,** ELISA showing IL-1β and IL-18 secretion in the supernatant of inducible NLRP3-WT or NLRP3 R779C THP-1–derived macrophages. Cells were transfected with *BRCC3* siRNA #2 and/or *JOSD2* siRNA #2 for 24 hours, followed by doxycycline and LPS treatment performed as in Fig 5, *A* (left panel). Cell death was measured by LDH release (right panel). **C-F,** Immunofluorescent staining of NLRP3 and BRCC3 (Fig 5, *C*) or JOSD2 (Fig 5, *E*) in cells at the colonic mucosae from control (n = 3), patient with colitis (n = 3), and patient 1. Bar graph showing colocalization of NLRP3 with BRCC3 (Fig 5, *D*) and JOSD2 (Fig 5, *F*). Data in Fig 5, *B*, are presented as mean ± SEM of 3 independent experiments. *P* values of Fig 5, *D* and *F*, were calculated using the Mann-Whitney *U* test. *Dox*, Doxycycline; *IB*, immunoblot; *LDH*, lactate dehydrogenase; *ns*, not significant; *WCL*, whole cell lysates. **P* < .05 and ***P* < .01.

colon of NLRP3-R779C mice were decreased after knockdown of either *Brcc3* or *Josd2* (Fig 7, *H*).

Together, these data demonstrate that Brcc3 and Josd2, enriched in inflamed colonic myeloid-derived cells, promote overt NLRP3-R779C inflammasome activation *in vivo*.

DISCUSSION

Mutations in NLRP3 have been shown to cause a spectrum of hereditary systemic autoinflammatory diseases known as cryopyrin-associated periodic syndromes.[28] These activating mutations normally induce spontaneous inflammasome formation

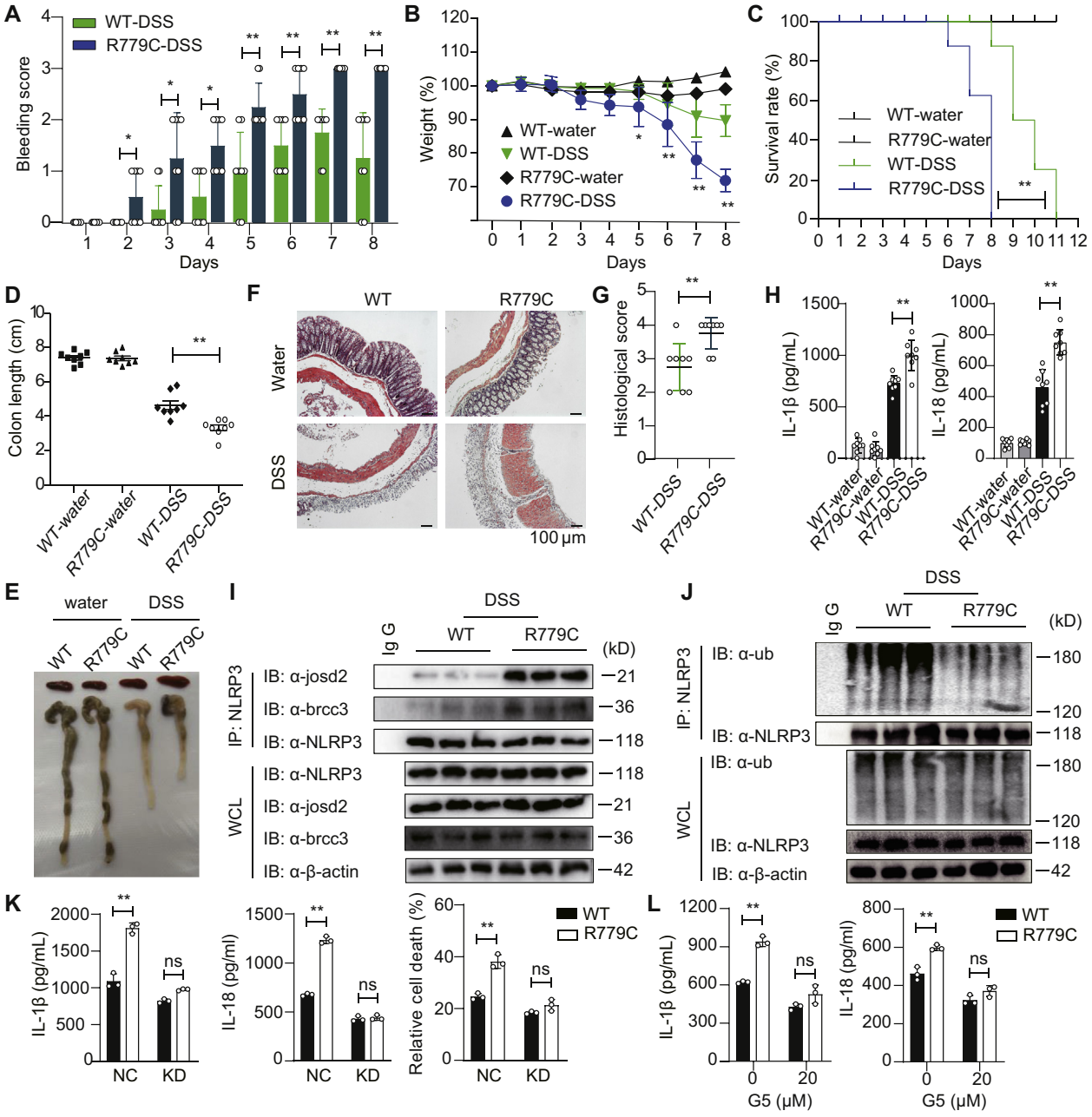

FIG 6. The NLRP3-R779C variant promotes intestinal inflammation in mice. (**A-D**) showing bleeding scores (Fig 6, *A*), change in body weights (Fig 6, *B*), survival rates (Fig 6, *C*), and lengths of the colon (Fig 6, *D*) of the NLRP3-WT and NLRP3-R779C mice. (**E** and **F**) Representative appearances (Fig 6, *E*) and H&E-stained sections (Fig 6, *F*) of the colon. The scale bar is 100 μm. **G,** Histological scores of colonic H&E examination. **H,** ELISA showing IL-1β and IL-18 released into colonic culture supernatants. **I,** IB showing endogenous interaction of NLRP3-WT or NLRP3-R779C with Josd2 and Brcc3. **J,** IB showing ubiquitination of NLRP3-WT or NLRP3-R779C. **K,** ELISA showing IL-1β and IL-18 released from small intestine cells transfected with *BRCC3* siRNA #2 and *JOSD2* siRNA #2 (KD group) or control siRNA (siRNA-NC). **L,** ELISA showing IL-1β and IL-18 released by colonic macrophages treated with or without G5 (20 μM). Data in Fig 6, *A-D*, *G-H*, and *K-L*, are presented as mean ± SD with 8 mice per group. Survival rate (Fig 6, *C*) difference is tested by log-rank (Mantel-Cox) test. *H&E*, Hematoxylin and eosin; *IB*, immunoblot; *ns*, not significant. *$P < .05$ and **$P < .01$.

and ATP-independent secretion of IL-1β, IL-18, and pyroptosis.[29] In this study, we showed that a gain-of-function variant located in the LRR domain of NLRP3, R779C, was associated with the development of VEOIBD-like symptoms in 3 patients.

NLRP3 consists of an N-terminal pyrin (PYD) domain, a central nucleotide-binding oligomerization (NACHT) domain, and an LRR domain at the C terminus. In the resting state, the LRR domain folds back onto the NACHT domain and maintains NLRP3 in an autoinhibitory state.[30] TRIM31, FBXL2, and

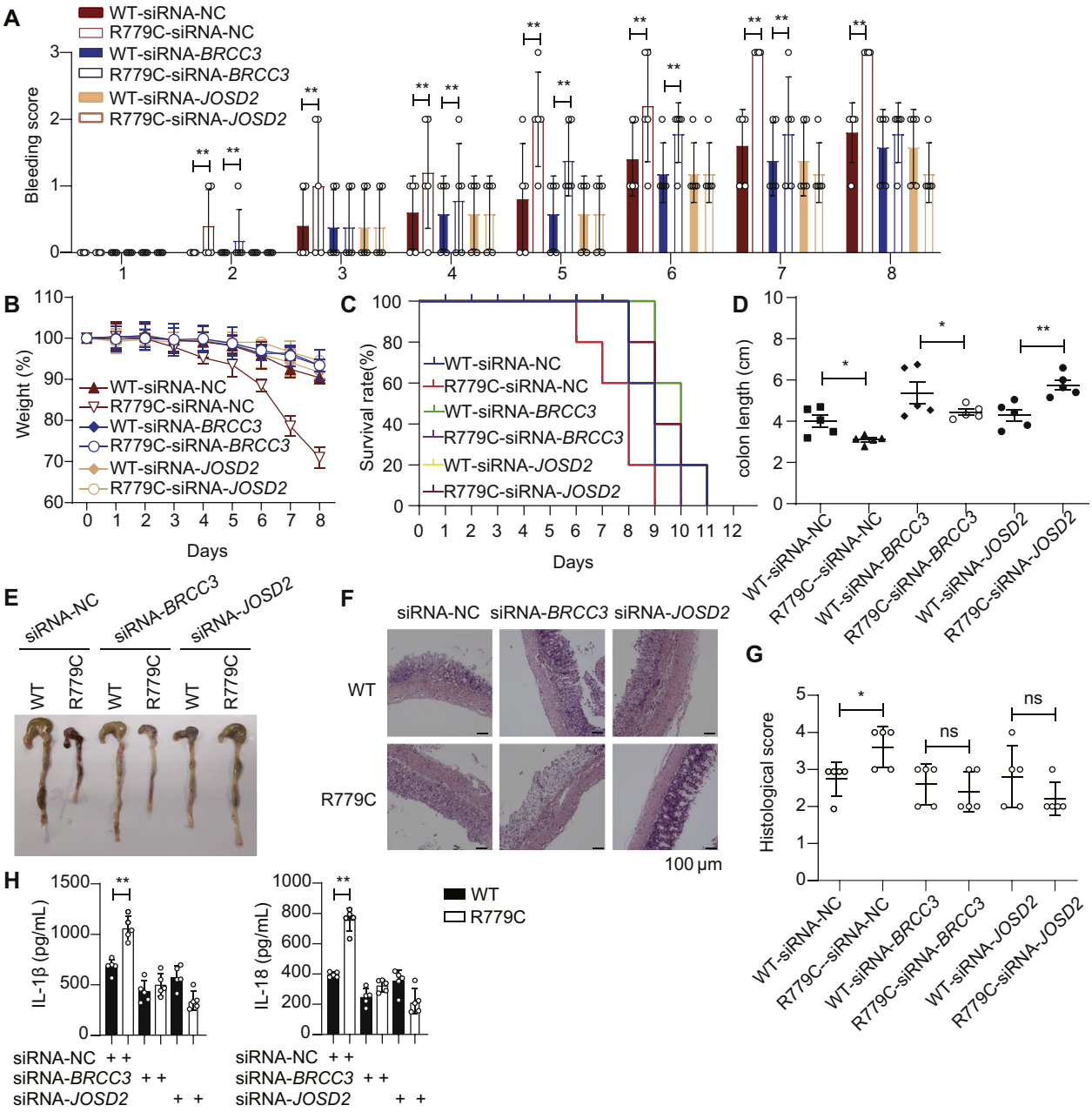

FIG 7. BRCC3 and JOSD2 promote NLRP3-R779C inflammasome activation. (**A-C**) Graphs showing bleeding scores (Fig 7, *A*), changes in body weights (Fig 7, *B*), survival rates (Fig 7, *C*), and lengths of the loosely stretched colon (Fig 7, *D*) of the NLRP3-WT and NLRP3-R779C mice. (**E** and **F**) Representative appearances (Fig 7, *E*) and H&E-stained sections (Fig 7, *F*) of the colon. The scale bar is 100 μm. **G,** Histological score for H&E examination. **H,** Single-cell suspensions from colons of indicated mice were cultured for 36 hours. ELISA showing IL-1colons of indicated mice were cultured Fig 7, *A-D* and G-*H*, are presented as mean olons of indicated mice were culture Fig 7, *F*, are presented for 3 independent biological experiments with similar results. Survival rate (Fig 7, *C*) difference is tested by log-rank (Mantel-Cox) test. *H&E*, Hematoxylin and eosin. **P* < .05 and ***P* < .01.

MARCH7 further induce NLRP3 K48-linked ubiquitination for proteasomal and autophagic degradation.[31-33] IL-10 has been shown to promote K48-linked ubiquitination of NLRP3 for proteasome degradation.[34] Thus, excessive NLRP3 inflammasome activation due to reduced K48-linked ubiquitination may contribute to the severe IBD-like symptoms in the first months of life in patients with IL-10 and IL10R mutations.[35]

Upon inflammatory stimulation, deubiquitination of NLRP3 promotes oligomerization and activation of NLRP3.[4] By siRNA-mediated screening of the DUB family, Py et al[4] found that BRCC3 was required for NLRP3 deubiquitination and activation. We showed that the NLRP3-R779C variant bound strongly with BRCC3. Unexpectedly, R779C gained a new ability to interact with JOSD2, a DUB that does not deubiquitinate NLRP3-WT.

Considering that BRCC3 and JOSD2 are enriched in myeloid cells in the inflamed GI tract, it is highly likely that patients with the NLRP3-R779C variant may develop early-onset GI symptoms although mechanisms regulating BRCC3 and JOSD2 expression remain to be determined. It is also worth mentioning that although patients with PIK3CD gain-of-function variants may have enteropathy,[16,36] our study suggests that PIK3CD does not directly affect NLRP3 inflammasome activation in stimulated THP-1–derived macrophages and mice with DSS-induced colitis model. Thus, the NLRP3-R779C variant may have contributed to the formation of peptic ulcers and gastrointestinal granulomata in patient 2 with the digenic PIK3CD-E1021K and NLRP3-R779C variants.

It should be mentioned that the structural basis underlying NLRP3-DUB interactions are currently unknown. However, preliminary analysis based on the reported structure of the NLRP3-NEK7 complex[37] suggested that R779 of NLRP3 was likely exposed to the solvent (cysteines are found frequently buried within the cores of proteins). Furthermore, whether C779 might form a disulfide link with C780 that lead to structural rearrangements of NLRP3 will require further investigation. Nevertheless, considering that controlled activation of NLRP3, which is likely involving BRCC3-mediated NLRP3 deubiquitination, is pivotal to maintain intestinal homeostasis,[38] inhibition of JOSD2-mediated NLRP3 deubiquitination may offer a precision treatment for patients carrying the R779C variant.

Conclusions

We show that the NLRP3-R779C variant is associated with excessive inflammasome activation by greater binding to 2 DUBs (BRCC3 and JOSD2) and may predispose infants to increased risk of VEOIBD development. This study highlights that posttranslational mechanisms are critical in shaping clinical spectrums of NLRP3-associated diseases.

We thank the patients and their guardians for participating in this work.

Key messages

- **BRCC3 and JOSD2 promote deubiquitination and activation of the NLRP3-R779C variant. Patients with the NLRP3-R779C variant may experience very-early-onset gastrointestinal symptoms.**

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