Supplemental Materials

Patients' case histories

Proband:

The Proband is a 16-year-old male residing in Cleveland, OH who was admitted to UH Medical Center on 8/2014 with abdominal pain and bloody diarrhea with greater than 2 liters of liquid stool output/day of 2 months duration. EGD/colonoscopy disclosed macroscopic pancolitis and microscopic esophagitis, gastritis, duodenitis, and terminal ileitis. The patient exhibited a partial response to treatment with high dose steroids and 5-ASA with mild decrease in liquid stool output, but relapsed upon weaning of the steroids despite addition of imuran. Subsequent treatment with anti-TNF-α (Remicade) was also unable to bring about remission and the patient was transferred to Children's Hospital of Philadelphia for further management. At this site, the patient's abdominal pain and bloody diarrhea persisted despite an increase in the Remicade dose to 10 mg/kg every 4 weeks concomitant to high doses of IV steroids. In addition, patient had significant protein losing enteropathy as evident of Albumin levels of 1.5-2.5 requiring albumin infusions and TPN supplementation. Repeat EGD/colonoscopy at this point revealed markedly increased intestinal inflammation with evidence of pancolitis as well as macroscopic duodenitis and ileitis. Macroscopic examination of the colon exhibited scattered areas of superficial erythema and ulceration having a lenticular pattern especially evident in the rectosigmoid region; this was associated with colonic and ileal friability (Figure 1B). Biopsies from the terminal ileum (TI) and colon showed the presence of a severe inflammatory mononuclear infiltrate associated with loss of villous pattern and apoptosis of epithelial cells; this was thought to be similar to intestinal inflammation sometimes associated with GvHD (Figure 1C a-e). In view of continued refractory disease, additional opinion as to appropriate therapy was sought from NIH clinicians. Because of the presence of certain histologic features sugestive of GvHD, NIH clinicians advised treatment with anakinra, an IL-1β receptor antagonist and such treatment was susbsequently instituted(1). However, prior to such therapy, the patient had been started on Vedolizumab and had received 2 induction doses without significant improvement. Anakinra therapy resulted in rapid

clinical improvement marked by decreased in stool output to < 500 cc/day and improved albumin level (no longer requiring albumin infusions). This led to shortly thereafter transfer back to UH Medical center where repeat histology examination revealed decrease in inflammatory process without evidence of GvH disease but that of routine Crohn's disease (Figure 1 Cd-e). He was maintained on Anakinra and Vedolizumab with further improvement as correlated by improvement in albumin levels and change in stool outout to semi-formed stools. Subsequent to discharge from hospital the patient was followed at NIH where he was transitioned from Anikinra to Canakinumab (anti-IL-1β monoclonal antibody). On this therapy stools remained formed and free of blood/mucous; in addition, abdominal symptoms of pain/cramping markedly improved. Patient remained on Canakinumab q 4 weeks and Vedolizumab q 7-week therapy. Of note the patient was recently diagnosed with avascular necrosis (AVN) of the hip after developing a limp upon ambulation. The latter was thought to be due to prior high dose steroid treatment. In follow-up a repeat colonoscopy in May, 2017, disclosed a left and transverse colon area of persistent microscopic inflammatory changes requiring an increase in Canakinumab dose from 3 to 4 mg/Kg/dose q 4 weeks and decrease interval dosing of Vedolizumab to q 4 weeks. At present the patient is predominately free of GI symptoms and enjoys normal growth. Laboratory correlates reveal a normalization of albumin levels (3.5-4.1) and as shown in Figure 1D fecal calprotectin and serum IL-1β levels have marked improved on corresponding therapy.

The Proband's Mother:

The mother of the Proband is a 55-year-old female residing in Cleveland, OH. In 2014, she presented with a one month history of abdominal pain, weight loss and diarrhea. These symptoms prompted an abdominal CT scan which revealed marked circumferential wall thickening of small bowel corresponding to loop of ileum. However, she did not undergo colonoscopic evaluation due to concern that this might cause bowel perforation. She was then administered oral prednisone and on this treatment obtained cessation of abdominal pain and diarrhea symptoms over 2-3 month time period. Patient was then transitioned to budesonide therapy for one year duration with follow-up daily mesalamine treatment. Subsequent MRE studies demonstrate no acute GI illness.

The Proband's Maternal Aunt:

The maternal aunt of the proband is a 48 y.o female who presented at age 21 with abdominal pain, vomiting, and mild diarrhea. She was found to have both proximal and distal ileal strictures suggesting the presence of CD, but had no colonic disease. She was treated with, but did not improve on, 5-ASA or prednisone therapy and subsequently underwent a distal ileal resection (resulting in removal of ≈30 cm of small bowel) and a stricturoplasty of the proximal stricture. The histology of the resected tissue revealed small intestinal mucosa with acute inflammation, mucosal erosions and occasional granuloma, all consistent with CD (Figure 1C f-g).

In 1993, she again developed recurrent obstructive GI symptoms and at this point was treated with 6-MP. However, no improvement was observed and underwent a second resection (resulting in loss of an additional ≈30 cm of bowel due to the occurance of an ileal stricture. She remained on 6-MP post-operatively and concomitantly received IV iron infusions for severe iron deficiency anemia. She was not considered a candidate for anti-TNF-α therapy because of a history of a positive PPD in childhood that was treated with INH. Obstructive symptoms recurred in 2006 that once again led to a bowel resection secondary to stricture formation. Colonoscopy at that point revealed anastomotic recurrence but no colonic disease. Approximately 6 cm from the ileocecal valve there appeared to be a stricture 3-4 mm in diameter with ulcerations on the proximal side of the stricture. Ileal mucosa was extremely friable.

In between surgical procedures she has been relatively pain-free and maintained a stable weight; however, she has 4-5 loose watery stools/day. 6-MP was discontinued in 2008 while she was pregnant and breast feeding. She has not been on any medications since then but has been treated with acupuncture and probiotics.

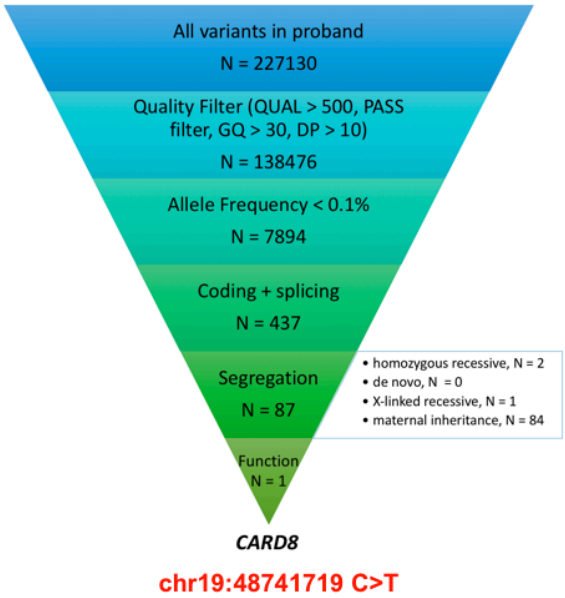
In June 2016, she again experienced nausea and abdominal pain without emesis, or weight loss. Currently, she continues with 4-5 loose BMs/day (her baseline since first surgery) associated with occasional bloating. These symptoms improve with probiotics. Her weight remains stable and she has no extra-intestinal symptoms. Recent

colonoscopy revealed acute inflammation at the anastomosis site associated with histiocytic aggregates and mucosal erosions. No stricture formation noted.

Supplemental references:

1. Antin JH, Weinstein HJ, Guinan EC, McCarthy P, Bierer BE, Gilliland DG, Parsons SK, Ballen KK, Rimm IJ, Falzarano G, et al. Recombinant human interleukin-1 receptor antagonist in the treatment of steroid-resistant graft-versus-host disease. *Blood.* 1994;84(4):1342-8.
2. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, and Sunyaev SR. A method and server for predicting damaging missense mutations. *Nat Methods.* 2010;7(4):248-9.
3. Kumar P, Henikoff S, and Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc.* 2009;4(7):1073-81.
4. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, and Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet.* 2014;46(3):310-5.
5. Cheung MS, Theodoropoulou K, Lugrin J, Martinon F, Busso N, and Hofer M. Periodic Fever with Aphthous Stomatitis, Pharyngitis, and Cervical Adenitis Syndrome Is Associated with a CARD8 Variant Unable To Bind the NLRP3 Inflammasome. *J Immunol.* 2017;198(5):2063-9.
6. Ito S, Hara Y, and Kubota T. CARD8 is a negative regulator for NLRP3 inflammasome, but mutant NLRP3 in cryopyrin-associated periodic syndromes escapes the restriction. *Arthritis Res Ther.* 2014;16(1):R52.

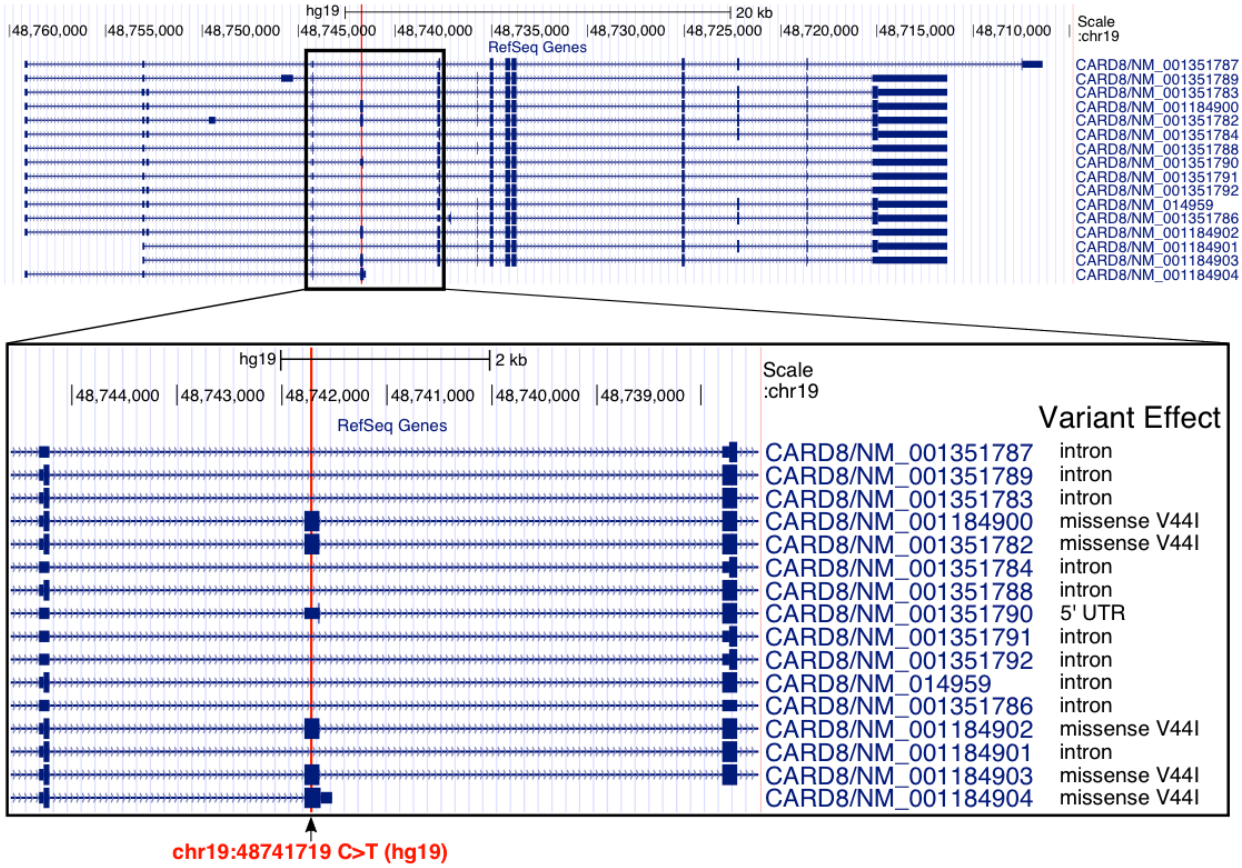
Supplemental Figures:



Supplemental Figure 1. Whole Exome Sequencing of Proband DNA Identified CARD8 To Be a Potential Damaging Gene in The Proband with CD

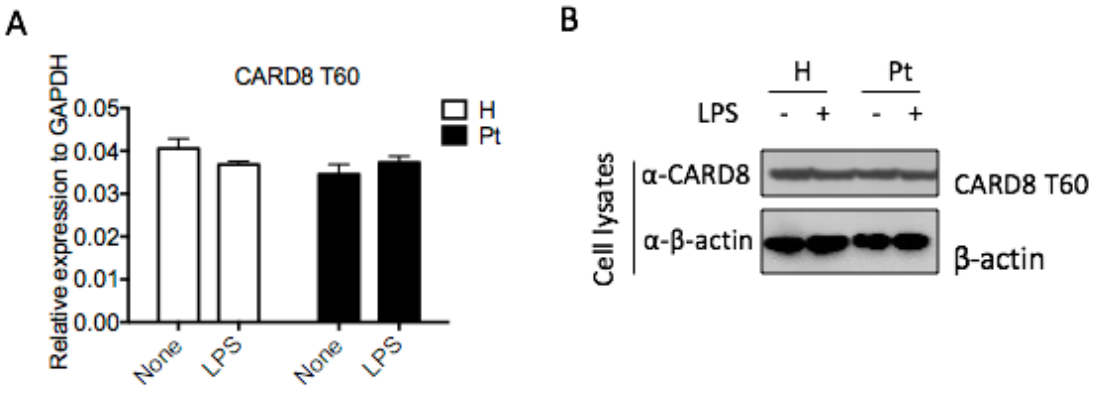
Sequencing of proband DNA was initially performed at the Children's Hospital of Philadelphia (CHOP) and revealed a single allele (heterozygote) CARD8 c.130G>A (V44I) mutation that was not predicted to be located in a functional domain. Subsequently, DNA from the blood of the patient and the patient's mother and father were subjected to whole exome sequencing at NIH. 10x sequencing coverage for 89.9%, 90.2%, and 90.7% of coding regions in proband, mother and father was obtained, respectively. After applying various filters for variant quality, population allele frequency, variant impact and inheritance models, 87 variants in the proband were obtained. Of these, there was a single variant following the X-linked recessive model, 2 variants within the homozygous recessive model, and zero *de novo* variants passing filters. The X-linked recessive variant was a missense variant in *DCAF12L1*, predicted to be benign/tolerated by PolyPhen (2) and SIFT (3), with a low CADD-phred score (4) of 1.5. The two homozygous recessive variants were missense and inframe insertion in *TROVE2*, and *LNP1* genes, respectively.

None of these three candidates were interesting based on predicted deleteriousness scores or gene function. Since the patient's maternal aunt also carried a diagnosis of CD, 84 heterozygous variants common to the patient and the aunt were identified. Among these, the variant chr19:48741719 C>T (hg19) in *CARD8* stood out because of its role in the inflammasome(5, 6). It was predicted to be possibly damaging/deleterious for CARD8.



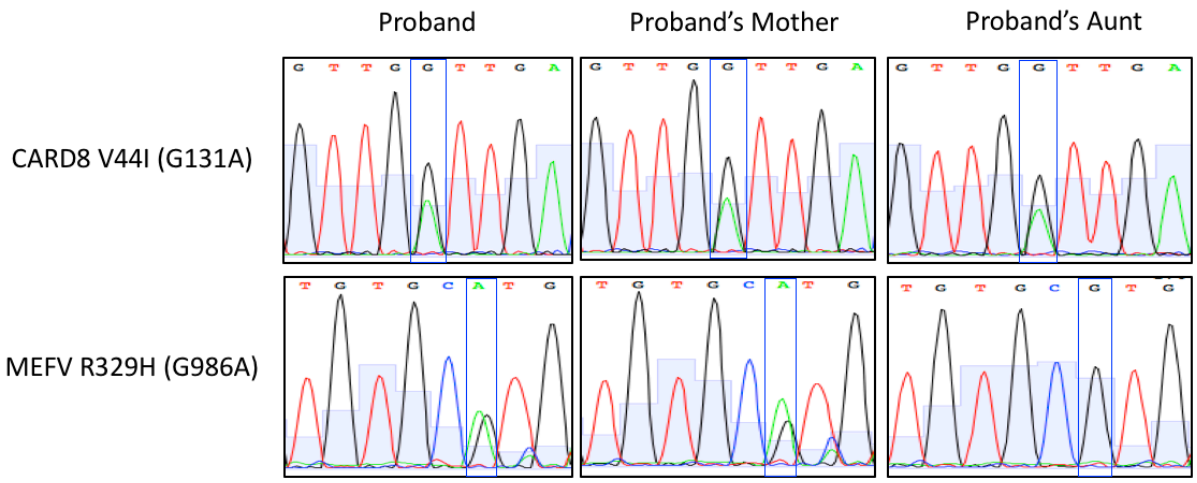
Supplemental Figure 2. The CARD8 Variant chr19:48741719 C>T (hg19) Overlaps Multiple Transcripts of CARD8

Multiple transcripts overlapped by the CARD8 variant chr19:48741719 C>T (hg19) identified in whole exome sequencing data were shown. The possible outcome of the variant could be in the intron, in the 5' UTR, or else a V44I missense mutation.



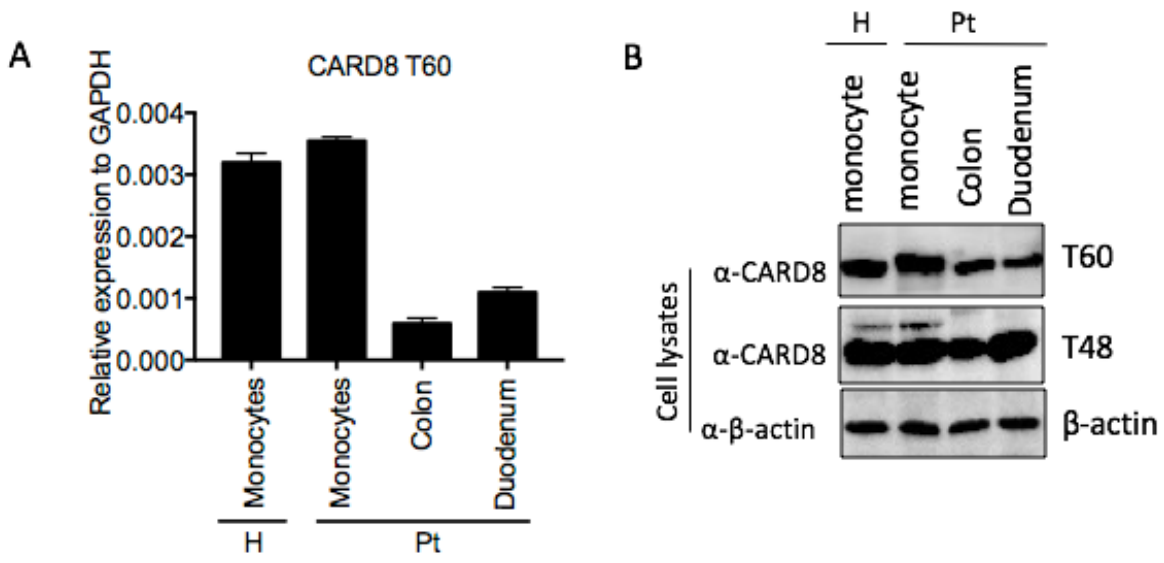
Supplemental Figure 3. CARD8 Variant chr19:48741719 C>T Does Not Affect CARD8 Transcription and Protein Expression

Primary mDCs from Proband carrying the variant chr19:48741719 C>T (Pt) and healthy control (H) were treated with or without LPS (100ng/ml) for 6 hours and then were harvested for RNA or protein extraction. (A) qRT-PCR was performed to examine the expression of CARD8 T60 at the mRNA level. Data are shown as means ± SEM. (B) Western blot was performed to examine CARD8 T60 protein. Data in A are representative of three independent experiments, data in B are representative of two independent experiments.



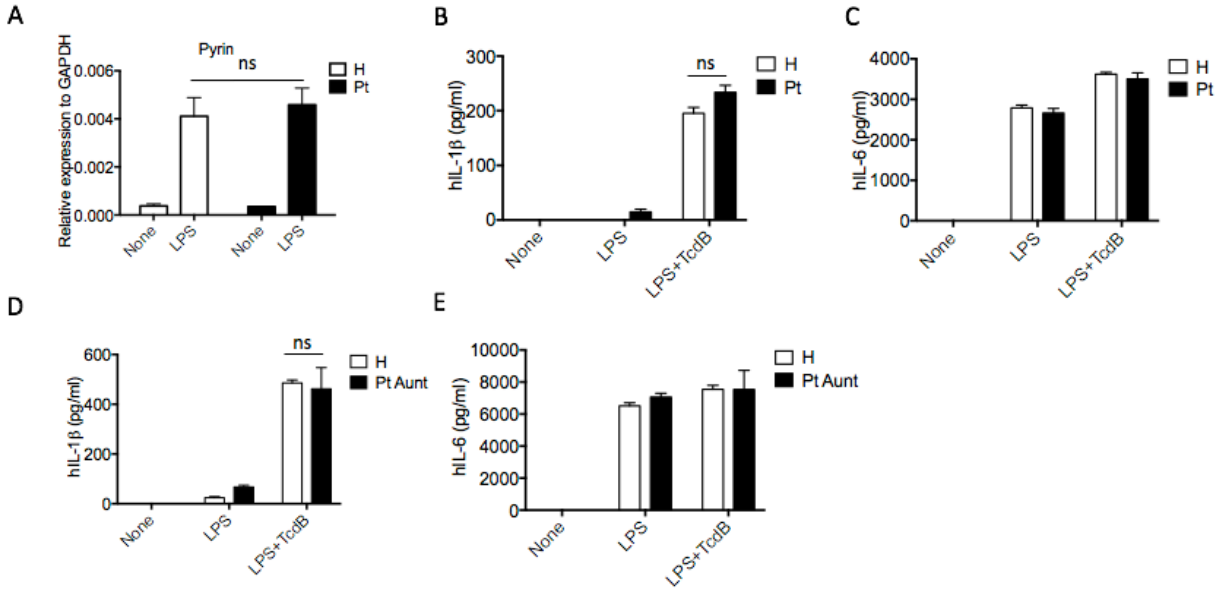
Supplemental Figure 4. PCR Product Sequencing Confirmed CARD8 and MEFV Mutations in the Proband, His Mother and Aunt

mDCs from the Proband, his mother and aunt were treated with LPS (100ng/ml) for 6 hours and subjected to mRNA extraction. RT-PCR was performed to amplify CARD8 or MEFV. The PCR products were then subjected to DNA sequencing. The mutation sites in CARD8 T60 (upper panel) and MEFV (lower panel) were highlighted. Data are representative of three independent experiments.



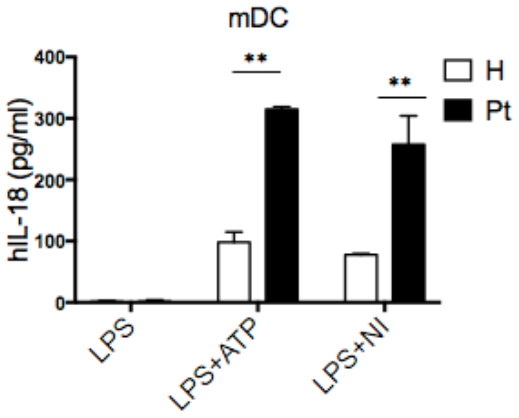
Supplemental Figure 5. CARD8 Bearing the V44I Mutation was Expressed at a Substantial Level in the Proband's Small Intestine and Colonic Tissues

Primary monocytes from Proband (Pt) and healthy control (H) were treated with or without LPS (100ng/ml) for 6 hours and then were harvested for mRNA or protein extraction. (A) Colon and duodenum biopsies from the patient was subjected to mRNA or protein extraction. qRT-PCR was performed to examine the expression of CARD8 T60. Data are shown as means ± SEM. (B) Western blot was performed to examine the protein expression of CARD8 T60 and T48. Data in A are representative of three independent experiments, data in B are representative of two independent experiments.



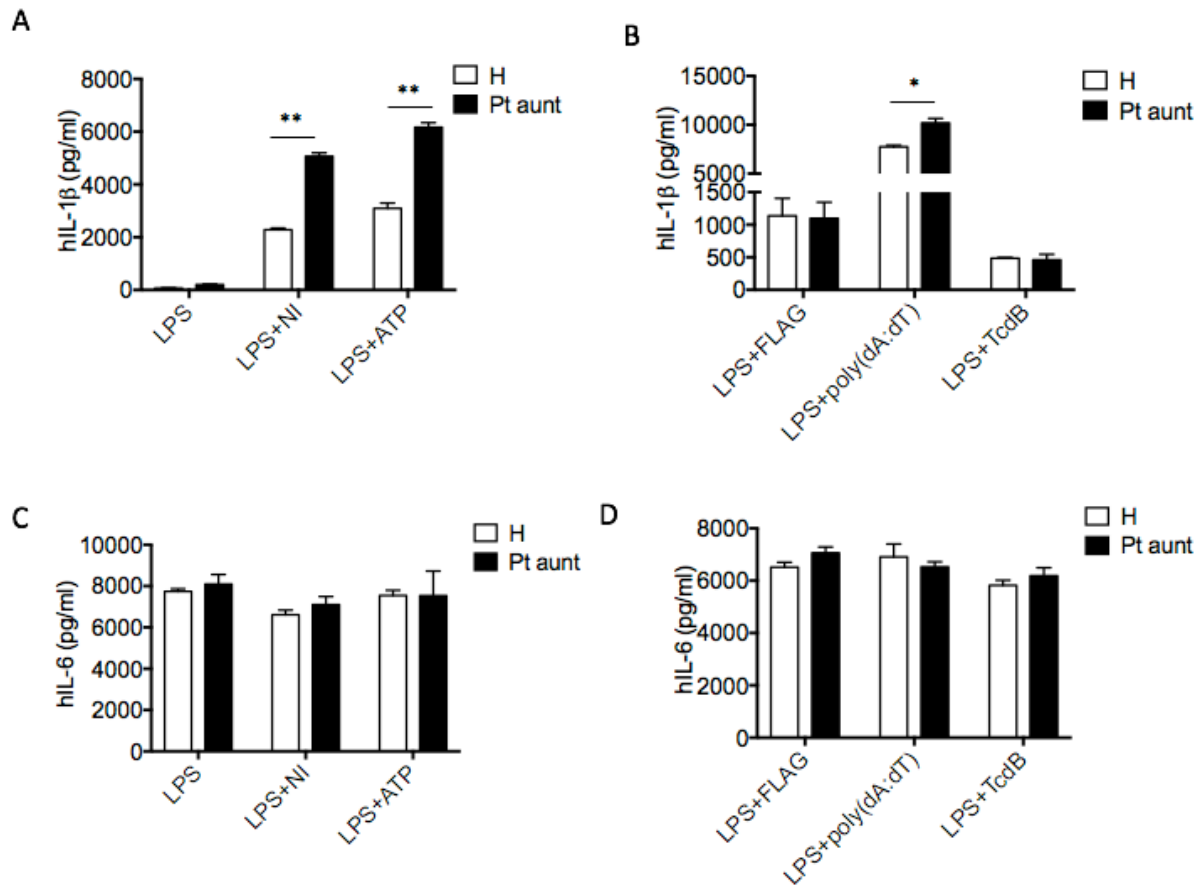
Supplemental Figure 6. The mDCs from the Proband Display Normal Pyrin Inflammasome Activation

(A) Primary mDCs from the Proband (Pt), the Proband's maternal aunt (Pt Aunt), both carrying CARD8 V44I mutation, and a healthy control individual (H) were treated with or without LPS (100ng/ml) for 6 hours and then were harvested for mRNA extraction; mRNA was then subjected to qRT-PCR to determine the transcription of pyrin. (B-E) LPS (100ng/ml, 6 hours) primed mDCs from the Proband, the Proband's aunt and H were stimulated with TcdB (1μg/ml) for 2 hours to activate pyrin inflammasome. The cultural supernatants were collected and subjected to assays of IL-1β (B, D) and IL-6 (C, E) by ELISA. Data are shown as means ± SEM. ns: not significant. Two-tailed student's *t*-test. All the data are representative of three independent experiments.



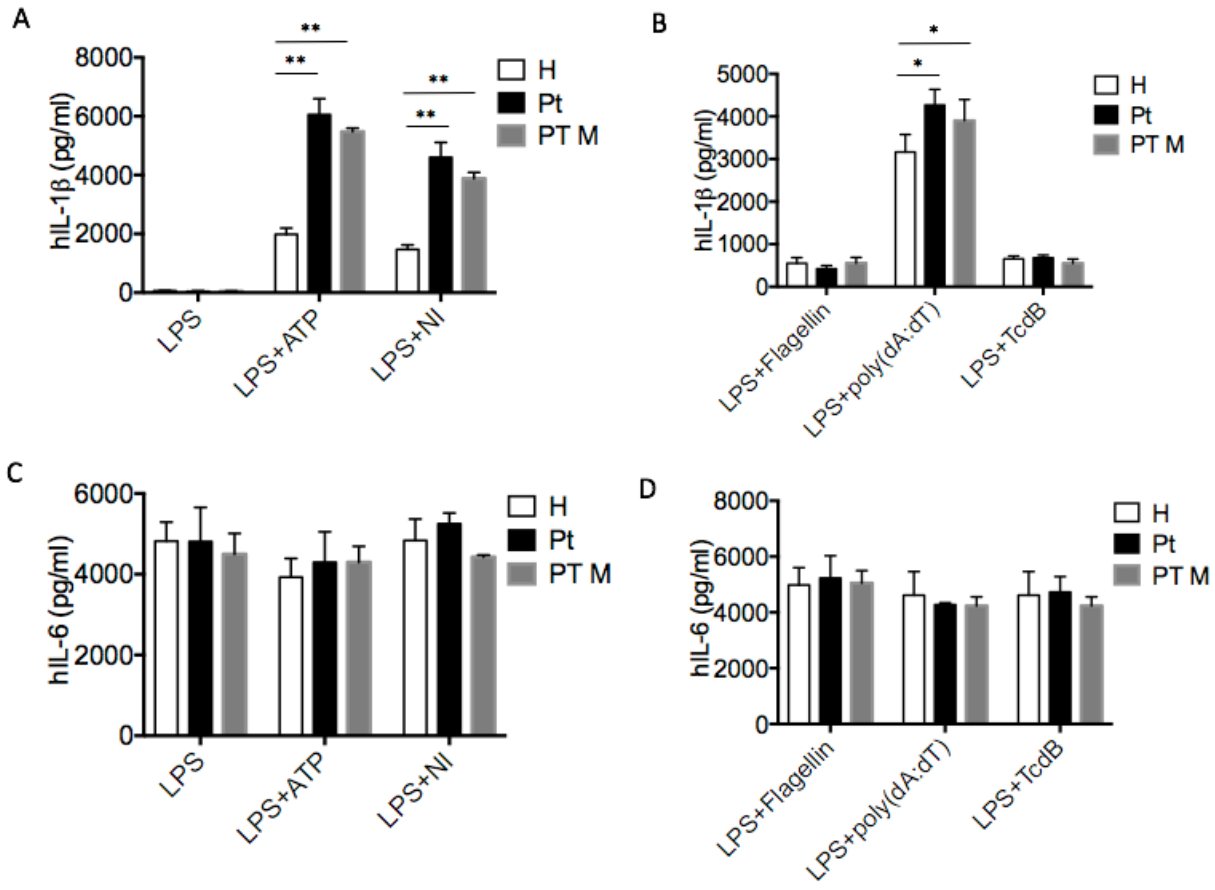
Supplemental Figure 7. Over production of IL-18 by peripheral monocytes from Proband patient with CARD8 mutation

mDCs from the Proband's patient (Pt) and a healthy control individual (H) were treated with LPS (100ng/ml) for 6 hours and then stimulated with ATP (5mM, 30 minutes) or nigericin (1.2μM, 30 minutes, NI). The cultural supernatants were collected and then subjected to assays for IL-1β by ELISA. Data are shown as means ± SEM. ***p*<0.01. Two-tailed student's *t*-test. All the data are representative of three independent experiments.



Supplemental Figure 8. Over-Activation of the NLRP3 Inflammasome in mDCs from the Proband's Aunt

mDCs from the Proband's aunt (Pt aunt) carrying the CARD8 V44I mutation and a healthy control individual (H) were treated with LPS (100ng/ml) for 6 hours and then stimulated with ATP (5mM, 30 minutes) or nigericin (1.2μM, 30 minutes, NI), poly(dA:dT) (1μg/ml, 2 hours), flagellin (1μg/ml, 2 hours) or TcdB (1μg/ml, 2 hours) to activate the NLRP3, AIM2, NLRC4 or pyrin inflammasomes, respectively. The cultural supernatants were collected and then subjected to assays for IL-1β (A, B) and IL-6 (C, D) by ELISA. Data are shown as means ± SEM. *p<0.05; **p<0.01. Two-tailed student's t-test. All the data are representative of three independent experiments.



Supplemental Figure 9. Over-Activation of the NLRP3 Inflammasome in mDCs from the Proband's Mother

mDCs from the Proband (Pt), his mother (Pt M) and a healthy control individual (H) were treated with LPS (100ng/ml) for 6 hours and then stimulated with ATP (5mM, 30 minutes) or nigericin (1.2μM, 30 minutes, NI), poly(dA:dT) (1μg/ml, 2 hours), flagellin (1μg/ml, 2 hours) or TcdB (1μg/ml, 2 hours) to activate the NLRP3, AIM2, NLRC4 or pyrin inflammasomes, respectively. The cultural supernatants were collected and then subject to assays of IL-1β (A, B) and IL-6 (C, D) by ELISA. Data are shown as means ± SEM. *p<0.05; **p<0.01. One-way ANOVA with Tukey's post-hoc test. All the data are representative of three independent experiments.