

SUPPLEMENTARY INFORMATION

Supplementary Methods:

Human subjects

XLA patient 1 is a 39-year-old male who had a history of recurrent sinusitis, otitis media and pneumonia along with diarrhea and failure to thrive during his first 6 months of life. At that time, examination revealing lack of tonsillar tissue and low IgG, IgM, and IgA levels led to a diagnosis of XLA. The latter was confirmed by *Btk* sequencing which revealed c.1684 C>T, p.562R>W). The patient was treated with IMiG therapy initially and subsequently with IViG therapy. Since age 19 the patient has had recalcitrant osteomyelitis infection of lower extremities due to *Flexispira Rappini* infection

XLA patient 2 is a 9-year-old male with a family history of XLA. Immunological analysis at birth indicated a reduced CD19+ B cell number and monocytes negative for BTK expression. At one month of age he was noted to have reduced IgG, IgM and IgA levels and was started on SC immunoglobulin treatment. At age 5 years he was observed to have recurrent *C. difficile* infection and concomitant inflammatory bowel disease. Sequencing data showed that the patient carries a *Btk* mutation consisting of 1753G loss causing a frameshift and an early stop codon at amino acid 584.

XLA patient 3 is an 18-year-old male who developed recurrent sinus and otitis media infections at 6 weeks of age and had pneumococcal pneumonia at 13 months of age. Studies of immunoglobulin levels at the latter time revealed decreased IgG, IgM and IgA levels as well as decreases in mature B cell numbers (CD19+ and CD20+ cells); as a result he was started on IViG replacement. At 21 months of age exome sequencing demonstrated a *de novo* *Btk* mutation (c.46C>T, p.Gln16Ter). Since age 15 the patient as had skin cellulitis and osteomyelitis infection of the lower extremities secondary to *Helicobacter Cinaedi*.

XLA patient 4 carrying a *Btk* mutation (c.953 C>T, p.318S>F), is a 24-year-old male with a history of recurrent sinopulmonary infections first occurring at 3 years of age.

Concomitant skin infections led to evaluation of immunoglobulin levels which revealed decreased IgG, IgM and IgA levels and he was placed on IVIG replacement therapy. At age 16 he developed intestinal inflammation thought to be consistent with Crohn's disease. This has been unresponsive to conventional biologic therapies such as infliximab, adalimumab, certolizumab and vedolizumab and has resulted in ileocolonic resections at ages 18 and 24 years.

CLL Patient 1 is a 74-year-old male with CLL who was on ibrutinib therapy for four years at the time of NLRP3 inflammasome study of his peripheral blood cells. He had responded to therapy but at the time of study still had a high lymphocyte count due to increased numbers of B cells. Nevertheless, he was free of acute or chronic infection. His course on ibrutinib was marked by diarrhea beginning after 9 months of therapy, but this subsided after about one year. He also had episodes of atrial fibrillation during the treatment period and this necessitated lowering of the ibrutinib dose to 140mg/day (as compared to initial dose of 420mg/day). Other medications at the time of study included, alprazolam, finasteride, flomax, lipitor and doxycycline.

CLL Patient 2 is an 81-year-old female with CLL who was on ibrutinib therapy for four years and three months at the time of NLRP3 inflammasome study of her peripheral blood cells. Ibrutinib therapy had been initiated after failure of treatment with rituximab and bendamustine. She was in complete remission at the time of study with absence of palpable nodes and normal peripheral blood parameters. Her course during treatment with ibrutinib was marked by constipation. She was on a standard dose of ibrutinib of 420mg/day. Other medications at the time of study included amlodipine, chrothalidone, loratadine and trazodone.

Mice

BTK-KO mice(1) in C57BL/6 background were kindly provided by Dr. Wasif N. Khan in University of Miami and Michail S. Lionakis in NIAID. The female homozygous BTK deficient mice were first crossed with male wild type mice purchased from Jackson lab to obtain female heterozygous BTK-KO mice, which were then crossed with male

homozygous BTK-KO mice to obtain male homozygous BTK-KO mice and male wild type littermates. Male mice were used in this study. In Figure 4 and Figure 6E-F, BTK-KO mice and their wild type littermates were used. In Figure 5 and Figure 6A-D, 6H-L, wild type control mice were ordered from Jackson lab because of difficulties in littermate breeding. All procedures of animal experiments were approved by the ethics committees of the National Institutes of Health.

Reagents and Constructs

Lipopolysaccharide (LPS, Ultrapure, L3012), ATP (tlrl-atpl), Nigericin (tlrl-nig), Poly (dA:dT) (tlrl-patn), flagellin (tlrl-epstfla-5), Alum crystals (tlrl-alk), were purchased from InvivoGen. DSS crosslinker (Disuccinimidyl suberate, S1885) was purchased from Sigma-aldrich. Ibrutinib (S2680) and LFM-A13 (S7734) were purchased from Selleckchem. Myc-DDK-tagged pCMV6 BTK (MR209829) and Myc-DDK-tagged pCMV6 PP2A (RC201334) were purchased from Origene. pcDNA3-N-HA-NEK7 (75142) and Flag-Gsdmd (80950) were purchased from Addgene. NLRP3 truncations, BTK truncations or kinase dead point mutation R430E were introduced by using Q5 Site-Directed Mutagenesis Kit (E0554S, New England Biolabs). The primers used are shown in Table 1. Myc or Flag tag was deleted from pCMV6 constructs using Q5 Site-Directed Mutagenesis Kit, the primers used are shown in Table1. pEF6 NLRP3-N-flag and pEF6 NLRP3-N-HA are gifts from Dr. Luke A J O'Neill (Trinity College Dublin). pcDNA3.1 flag-ASC, pcDNA3.1 flag-Caspase-1 and pcDNA3.1 flag-IL-1 β is provided by Dr. Guangxun Meng from Pasteur institute of Shanghai.

Table 1 Primers used in generating mutations or truncations of NLRP3 and BTK

Mutation or truncation	Forward Primer	Reverse Primer
NLRP3 Pypin	TAGCCAGAGTGGAATGACACG	GTCCTTCTTAGCTTTTTCCCA
NLRP3 Δ LRR	TAGCAGGCCCGGGAGGCCTTCA	CTCGTTGGAGAAATACTTAAAG
NLRP3 Δ Pypin	CAGCCAGAGTGGAATGACACG	CAGCTTGCAACGGACTCTGT
NLRP3 LRR	CAGGCCCGGGAGGCCTTCAAG	CAGCTTGCAACGGACTCTGT
BTK PH	ACGCGTACGCGCCGCTCGAG	TTGGCAGCCCATAGCATTCTT
BTK PTK	CAAAACAAAACGCGCCTTCT	CATGGCGATCGCGCGGCAGAG
BTK Δ SH	CAAAACAAAACGCGCCTTCT	TTGGCAGCCCATAGCATTCTT

BTK Δ PTK	ACGCGTACGCGGCCGCTCGAG	AGACACAGGATATTTTCAGCCT
BTK K430E	GAGATGATCAGAGAAGGTTCC	GATGGCCACATCATATTGGCC
Δ Myc tag	ATCCTGGATTACAAGGATGACGACGATAAGG	CTCGAGCGGCCGCGTACG
Δ Flag tag	GTTTAAACGGCCGGCCGC	TGCCAGATCCTCTTCTGAGATGAG

Induction of experimental colitis

To induce TNBS (2,4,6-Trinitrobenzenesulfonic acid) colitis, 3 mg of TNBS (P2297, Sigma-Aldrich) was administered per rectum in 45% ethanol on day 0 and day 2. During the procedure, the mice were first anesthetized using isoflurane (NDC 10019-360-40, Taxter), then TNBS solution was injected into the rectum using a vinyl catheter positioned 4 cm proximal to the anus. After the injection, the mice were kept vertical for 30 seconds. Mice survival, body weight, stool consistency and rectal bleeding were monitored daily. The mice were euthanized on day 4 in a CO₂ cage, colons were subjected to histology, protein or RNA expression analysis. Colitis disease score was evaluated according to the method described previously(2).

To evaluate the effect of anakinra or LFM-A13 on TNBS-induced colitis, the mice were I.P. pre-injected with anakinra (0.5mg/mouse/day) or LFM-A13 (10 μ g/mouse/day) in 100 μ l PBS 4 hours prior to TNBS administration. Anakinra or LFM-A13 was injected daily and TNBS was injected per rectum on day 0 and day 2.

To evaluate the roles for IL-1 α or IL-1 β in TNBS-induced colitis, BTK-KO mice were I.P. injected with anti-IL-1 α antibody (1mg/mouse, BE0243, Bio X cell), anti-IL-1 β antibody (1mg/mouse, BE0246, Bio X cell) or isotype control (1mg/mouse, BE0091, Bio X cell) diluted in 150 μ l PBS one day prior to TNBS administration. TNBS was injected per rectum on day 0 and day 2.

To induce DSS-colitis, mice were administered drinking water containing 4% DSS for 5 days. The DSS-containing water was subsequently replaced by regular water for 2 days. Mice were monitored daily for weight loss and then euthanized on day 7 for histologic assessment of colitis. MLN cells were harvested for assay of inflammatory cytokine production, the stimulating methods were shown in figure legends.

Induction of peritonitis

Peritonitis model was induced as described previously with modifications(3). Wild type or BTK-KO mice were i.p. injected with 700 µg alum crystals (tlrl-alk, Invivogen) dissolved in PBS. After 12 hours, the mice were euthanatized, peritoneal cavities were washed by i.p. injection of 0.5 ml PBS, after a brief massage, PBS was harvested for ELISA analysis of cytokines, IL-1 β and IL-6. Another 8 ml of PBS was injected to peritoneal cavities and harvested for collecting the peritoneal cells. These peritoneal exudates cells (PECs) were analyzed by FACS.

Human cell Isolation

The PBMCs from both patients and healthy individuals were freshly isolated using LSM Lymphocytes separation medium (50494, MP Biomedicals) following the manufacturer's instruction. Peripheral monocytes were isolated using human pan monocyte isolation kit (130-096-537, Miltenyi Biotec). The monocytes obtained were maintained in RPMI 1640 medium with 10% FBS and 100U/ml penicillin-streptomycin. All human cell isolation was approved by the Review Board of National Institutes of Health for human subject research.

Generation of mouse BMDMs and BMDCs

Mouse BMDMs were generated using a protocol described previously with modifications(4). Briefly, femurs were dissected from 6-12-week-old wild type and BTK-KO mice using scissors. The bones were flushed with a syringe filled with IMDM medium to extrude bone marrow, which was then homogenized to obtain bone marrow cell suspension using a 1 ml pipettor by pipetting up and down. The bone marrow cells were maintained in a mixture of IMDM medium with 10% FBS and M-CSF (20ng/ml, Peprotech) to allow the cells differentiate to BMDMs. The bone marrow cells were also maintained in RPMI medium with 10% L929 cell conditional medium for BMDM differentiation. Medium was replenished on day 3. The differentiated BMDMs were harvested on day 7 to do inflammasome activation experiments. To generate mouse BMDCs, the bone marrow cells were allowed to differentiate in this RPMI 1640 medium mixture with recombinant granulocyte-macrophage colony-stimulating factor (20 ng/ml; Peprotech). Medium was replenished on day 3. The differentiated BMDCs were harvested on day 6 to do

inflammasome activation experiments.

Generation of immortalized mouse macrophages

Generation of Immortalized mouse macrophages were conducted following a protocol described previously with modifications(5). Briefly, J2 murine recombinant retrovirus virus carrying v-raf and v-myc oncogenes was generated using AMJ2-C11 cells (ATCC CRL-2456). The culture supernatants containing the viruses were harvested and were filtered using a 0.22µm tube top filter. J2 virus was pelleted by centrifugation at 100,000 g for 3 hours at 4 °C and were then resuspended using 5 ml fresh DMEM. The resuspended virus was added to a 10cm dish plated with mouse primary bone marrow progenitor cells containing M-CSF (final concentration 20ng/ml). The medium was replaced by fresh culture supernatant with J2 virus after 5 days. The medium was then replaced by fresh culture medium without J2 virus every 5 days. Immortalized cell colonies were seen 3 weeks after the first J2 virus infection.

HEK293T cell transfection and reconstitution of the NLRP3 inflammasome

HEK293T cell line was a gift from Dr. Harry Malech. The cells (2.5×10^5 /well) were plated in a 12-well plate in 1 ml complete DMEM cell culture medium. After 12 hours, the cells were transfected with constructs expressing NLRP3, BTK, ASC, pro-caspase-1, pro-IL-1 β , NEK7, PP2A or GSDMD as indicated particularly. The total amount of DNA was adjusted to same concentration per well through the use of empty vector. The plasmid DNA was first diluted in 50 µl opti-MEM and then mixed with 3.5 µl lipofectamine LTX (15338100, Thermo Fisher Scientific) diluted in 50 µl opti-MEM, the mixture was incubated in room temperature for 10 minutes and were then added to HEK293T cells with 1 ml complete DMEM culture medium. For experiments detecting protein interactions or PP2A activity, 100ng plasmid DNA was transfected for each construct. Twenty-four hours later, the cells were lysed without stimulation and the cell lysates were subjected to subsequent test. The reconstitution of NLRP3 inflammasome in HEK293T cells has been reported previously(6, 7) with minor changes. Briefly, the cells were co-transfected with constructs expressing NLRP3 (100ng/well), ASC (10ng/well), pro-caspase-1(10ng/well), pro-IL-1 β (100ng/well), BTK (100ng/well) or PP2A (100ng/well). The cells

were washed with culture medium 24 hours later and were stimulated with nigericin (1 μ M) for 30 minutes, then the culture supernatants were collected for ELISA assay.

Cell viability assay

Cell viability was evaluated by examining LDH activity using Pierce LDH cytotoxicity assay kit (Thermo scientific, 88953) following the manufacturer's instructions. Briefly, BMDMs were primed with LPS (200ng/ml) for 3 hours, and then stimulated with ATP (5mM) or nigericin (1uM) for 30 minutes. HEK293T cells with reconstitution of NLRP3 inflammasome and transfection of BTK were treated with nigericin for 30 minutes. The supernatants from both cell types were transferred to a new plate and mixed with reaction mixture. After incubation at room temperature for 30 minutes, the reactions were stopped by adding stop solution. The absorbance at 490nm and 680nm was measured using a spectrophotometer to determine the LDH activity. Water-treated cells were used as spontaneous LDH activity control. Lysis buffer treated cells were used as maximum LDH activity control.

Real time PCR

Total RNA was extracted from BMDMs or colon tissues of the wild type and BTK-KO by using RNeasy mini kit (Qiagen, 74104) following manufacturer's instruction. Reverse transcription of mRNA and synthesis of cDNA was performed using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4374966). Real-time PCR was performed using the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, A25742) and the 7900HT Fast real-time PCR system (Applied Biosystems). Relative quantification of genes was achieved via normalization against GAPDH. The primers used were shown in Table 2.

Table 2. qPCR primer sequences.

Genes	Forward Primer	Reverse Primer
<i>Nlrp3</i>	GCTAAGAAGGACCAGCCAGA	CAGCAAACCCATCCACTCTT
<i>IL-1b</i>	TGTGAAATGCCACCTTTTGA	CAGGTCAAAGGTTTGGGAAGC
<i>Pycard</i>	CCAGTGTCCCTGCTCAGAGT	AGCACTCCGTCCACTTCTGT
<i>Casp1</i>	TCAGCTCCATCAGCTGAAAC	AGTCCTGGAAATGTGCCATC

<i>Il18</i>	GCTGTGACCCTCTCTGTGAA	TTTCAGGTGGATCCATTTCC
<i>Btk</i>	ACGTGGGAGAAGAGGCAGTA	GTCTTTCTGGTGGGGGATTT
<i>Il6</i>	GTTCTCTGGGAAATCGTGGA	TTCTGCAAGTGCATCATCGT
<i>Tnf</i>	CCCCAAAGGGATGAGAAGTT	TCCTCCACTTGGTGGTTTGT
<i>Ifng</i>	GCGTCATTGAATCACACCTG	ACCTGTGGGTTGTTGACCTC
<i>Cln2</i>	CAACTGGTGGGCTACATCCT	GCTGGCACCAACATAGGAAC
<i>Il17a</i>	TCCAGAAGGCCCTCAGACTA	TGAGCTTCCCAGATCACAGA
<i>Aim2</i>	ACCCGCAGTGACAATGACTT	CTTCCTGTTCTGCCACCATC
<i>Nlr4</i>	GGCCTGCAACCTCTTTCTTA	ATTCTGGGCCAAAACATTCA
<i>Il10</i>	CAAAGGACCAGCTGGACAAC	TCATTTCCGATAAGGCTTGG
<i>Cxcl1</i>	CTTGAAGGTGTTGCCCTCAG	AAGGGAGCTTCAGGGTCAAG
<i>Ccl2</i>	AAGAGGATCACCAGCAGCAG	TCTGGACCCATTCTTCTTG
<i>Elane</i>	CACTGTGTGAACGGCCTAAA	TGCACAGAGAAGGTCTGTCTG
<i>Gapdh</i>	GTTTCCTCGTCCCGTAGACA	AATCTCCACTTTGCCACTGC
<i>Ly6g</i>	TTGCAAAGTCCTGTGTGCTC	AGGTGGGACCCCAATACAAT

Western blot and immunoprecipitation

Mouse BMDMs or HEK293T cells after transfection or appropriate stimulation were lysed by applying lysis buffer (250 μ l/well of 6-well plate; 125 μ l/well of 12-well plate;) containing 50 mM Tris (pH 7.5), 0.5% NP-40, 50 mM NaCl, 1mM NaVO₄, 1mM NaF, and protease and phosphatase inhibitor cocktail. The cell lysates were allowed to stay on ice for 15 minutes and pelleted by centrifugation at 13,000 \times g for 15 min. The supernatants were collected and the protein concentration was determined using BCA protein assay kit (ThermoFisher Scientific, 23225). Appropriate amount of protein was mixed with LDS loading buffer, heated at 70°C for 10 minutes and subjected to LDS electrophoresis. The proteins were transferred onto nitrocellulose membranes and blocked with 5% fat-free milk in 1xTBS containing 0.05% Tween 20 and probed with the corresponding primary antibodies to detect BTK (Cell signaling Technology, 8547), pro-IL-1 β (sc-7884; clone H-153; Santa Cruz Biotechnology Inc.), NLRP3 (ALX- 804-881; clone cryo2; Enzo Life Sciences), ASC (sc-22514-R; clone N-15; Santa Cruz Biotechnology Inc.), pro-caspase-1 (sc-514; clone C-20; Santa Cruz Biotechnology Inc.), Myc tag (sc-40, clone 9E10; Santa Cruz Biotechnology Inc.), Flag tag (F1804, clone M2; Sigma-Aldrich), HA-probe (sc-7392,

clone F-7, Santa Cruz Biotechnology Inc.) and β -actin (Cell signaling Technology, 8457), rabbit IgG isotype control (Cell signaling Technology, 2729). For detecting mature caspase-1 and IL-1 β , the cell culture supernatants were collected and concentrated using methanol/chloroform, the harvested protein pellets were mixed with LDS loading buffer for the detection of caspase-1 p10 and IL-1 β p17. For detecting NLRP3 general phosphorylation, anti-tyrosine antibody (9411s, Cell signaling) and anti-serine/threonine antibody (9631s, Cell signaling) were used. For detecting NLRP3 pyrin domain serine phosphorylation, anti-phosphoserine antibody (ab9332, Abcam) was used. After incubation over night at 4°C, the primary antibodies were removed. The membranes were washed with 1xTBS containing 0.05% Tween-20 for 3x5 minutes and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. The membranes were then washed for 5x15 minutes with TBS. The immunoreactive bands were visualized using enhanced chemiluminescence reagent (K-12043-D10, Advansta).

Immunoprecipitation was performed using Dynabeads™ protein G immunoprecipitation kit (ThermoFisher Scientific, 10007D). Dynabeads (10ul per IP sample) were washed twice in PBS, resuspended in 250ul Ab binding and washing buffer, and mixed with appropriate antibodies (1 μ g per IP sample). Beads-antibody mixture were incubated at room temperature for 30 minutes. After incubation, the buffer was removed, beads (with antibody binding) were resuspended in cell lysates harvested after centrifugation. The beads-antibody-cell lysate mixtures were incubated on a rotator overnight at 4°C, then the beads were washed with the lysis buffer 3x5 minutes and subjected to Western blot.

ASC, NLRP3 and NEK7 oligomerization

ASC oligomerization detection was carried out according to methods described previously(8). Briefly, BMDMs from wild type or BTK-KO mice were seeded in 6-well plates (2×10^6 cells per well) and treated with LPS (200 ng/ml) for 3 h and nigericin (NI, 1uM) for 30 minutes. The cells were lysed with 250 μ l lysis buffer containing 50 mM Tris (pH 7.5), 0.5% NP-40, 50 mM NaCl, 1mM NaVO₄, 1mM NaF, and protease and phosphatase inhibitor cocktail. The cell lysates were put through a 21-gauge needle 10

times and incubated on ice for 15 minutes. The cell lysates were then centrifuged at 13,000 × g for 15 min at 4°C. The supernatants were removed, the insoluble cell debris was resuspended in 0.5 ml of PBS and was crosslinked with freshly prepared disuccinimidyl suberate (DSS; S1885, Sigma-Aldrich) in working concentration of 0.8 mM for 30 minutes at 37°C. The crosslinked cell debris was then pelleted by centrifugation at 6,000 × g for 10 min and resuspended in 30 µl LDS loading buffer for Western blot detection of ASC. To examine NLRP3 oligomerization, the mouse BMDMs were stimulated and lysed using the above methods. The cell lysates were mixed with DSS crosslinker (0.8 mM, final concentration) and were incubated at 37°C for 30 minutes. Then the crosslinked cell lysates were mixed with LDS loading buffer for Western blot. To examine NEK7 oligomerization, the mouse BMDMs after appropriate stimulation or HEK293T cells transfected with appropriate plasmids were lysed, the cell lysates were mixed with LDS loading buffer in nonreducing condition and subjected to Western blot.

Immunocytochemistry for ASC speck

BMDMs were seeded in 4-chamber slide (3×10^4 cells per chamber) and treated with LPS (200 ng/ml) for 3 h and nigericin (NI, 1 µM) for 30 minutes. The cells were then fixed with 4% paraformaldehyde for 15 minutes at room temperature and washed with PBS for 2x5 minutes. The cells were treated with 0.1% Triton X100 for 20 minutes at room temperature followed by 2x5 minutes wash with PBS. After blocking with 2% serum (sheep plus donkey serum in 1:1 ratio) at room temperature for 30 minutes, the cells were then incubated with anti-ASC antibody (1:500) overnight at 4°C. The cells were washed 3x15 minutes with PBS and incubated with secondary antibody at room temperature for 1 hour. After washing 3x10 minutes with PBS, the cells were incubated with DAPI for 10 minutes at room temperature. The cells were then washed with PBS for 2x5 minutes, then the chamber was removed and the cover slide was mounted. The resultant slides were examined under microscope.

Histology

Colon tissues for histological analysis were dissected from indicated mice and immediately fixed with 10% formaldehyde. The tissues were then embedded with paraffin,

then sections were cut and subjected to H&E staining. The resultant slides were examined under a light microscope. Colon histological score was determined by two experienced pathologists in a single-blind test according to the method described previously(9).

PP2A phosphatase assay

PP2A phosphatase activity was examined using PP2A immunoprecipitation phosphatase assay kit following manufacturer's instructions. Briefly, HEK293T cells were transfected with plasmids expressing mouse flag-tagged PP2A, NLRP3, BTK, kinase-dead BTK or PTK domain of BTK with different combinations in a 12-well plate. 24 hours later, the cells were lysed with phosphatase extraction buffer containing 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, with 10 µg/mL each of aprotinin, leupeptin, antipain, soybean trypsin inhibitor, 1 mM benzamidine, and 1 mM PMSF. The cell lysates were sonicated for ten seconds and subjected to centrifugation at 2000 x g for 5 minutes. Supernatants were mixed with anti-flag antibody and protein G Dynabeads (ThermoFisher Scientific, 10007D). The mixture was incubated 2 hours at 4°C with constant rocking. The beads were then washed 3 times with TBS and one time with Ser/Thr assay buffer. The wash buffer was then removed and phosphopeptide was added. This mixture was incubated at 30°C for 10 minutes in a shaking incubator. After a brief centrifugation, the supernatants were transferred into a microtiter plate and were mixed with malachite green phosphate detection solution. The mixture was incubated at room temperature for 15 minutes. The absorbance was read at 650 nm to obtain the relative PP2A phosphatase activity of each sample.

BTK over-expression in BMDMs

A lentiviral construct expressing wild type BTK (pReceiver-Lv181-BTK, N-flag tag, EX-Mm34445-Lv181) was purchased from GeneCopoeia. A kinase-dead mutation (K430E) was introduced by Site-Directed Mutagenesis using the primers shown in Table 1. Viral particles were generated using Lenti-Pac Lentiviral packaging kit (LT001, GeneCopoeia) according to manufacturer's instructions. Briefly, Lenti-Pac 293Ta cells (1.5×10^6) were seeded into a 10-cm dish with culture medium (DMEM, 10 ml) with 10% FBS; then, 48

hours later, plasmids expressing wild type or kinase-dead BTK (2.5 µg) were transfected into the above cells. Forty-eight hours post-transfection, the cell culture supernatants containing viral particles were harvested. To concentrate the virus particles, 50 ml original culture supernatant containing virus was mixed with 10 ml Lenti-Pac concentration solution (LT007, GeneGopoeia). The mixture was incubated overnight at 4°C and then were subjected to centrifugation at 3500g for 25 minutes at 4°C. The virus pellet was resuspend using 10 ml fresh culture medium. To perform transduction, BMDMs (10^7 cells) in differentiation (day 3) were transferred to 10-cm petri dish, then the culture medium containing virus particles and polybrene (8 µg/ml) were added into the dish. The dish containing BMDMs and virus particles were incubated at 4°C for 2 hours and then were transferred to an incubator at 37°C for 12 hours, after which the virus containing medium was replaced by fresh complete medium without polybrene; after incubating at 37°C for 48 hours, the cells were stimulated (as indicated) and harvested to examine BTK expression using Western blotting.

ELISA

Human serum or culture supernatants of human PBMCs, mDCs, elutriated monocytes, THP-1 cells, HEK293T cells, mouse BMDMs or BMDCs were harvested, and IL-1 β as well as IL-6 secretion were analyzed in an enzyme-linked immunosorbent assay (ELISA; BD Biosciences). From mouse BMDCs, the supernatants were harvested for IL-1 β and IL-6 ELISA assay (eBioscience). All the procedures were performed according to the manufacturers' instructions.

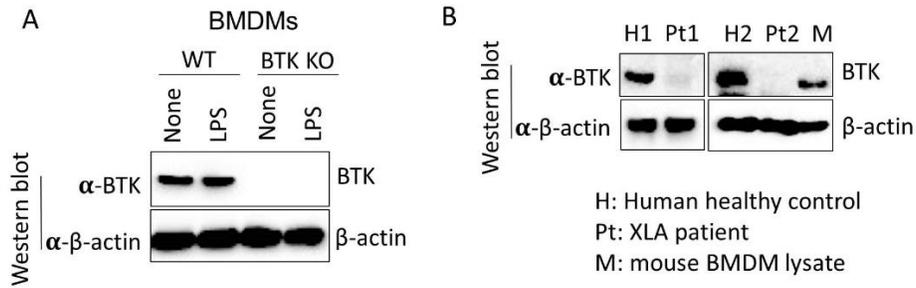
Statistical analysis

Data with two groups of samples were analyzed using a two-tailed Students' t test. Experiments with more than two groups of samples were analyzed using a one-way ANOVA followed by Dunnett's or Tukey's post-hoc multiple comparisons. All the data are presented as mean \pm SD except where indicated otherwise. A *p* value of ≤ 0.05 was considered statistically significant.

References:

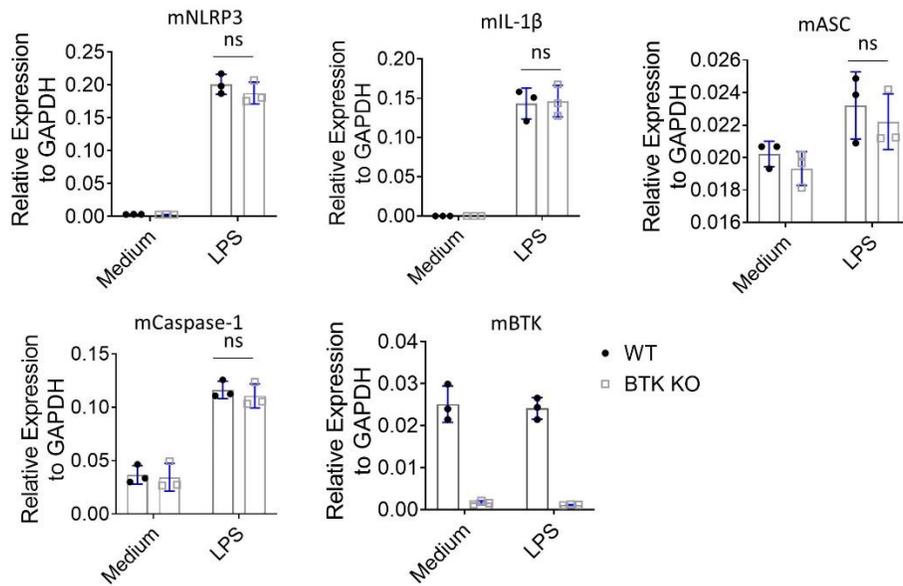
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SUPPLEMENTAL FIGURES



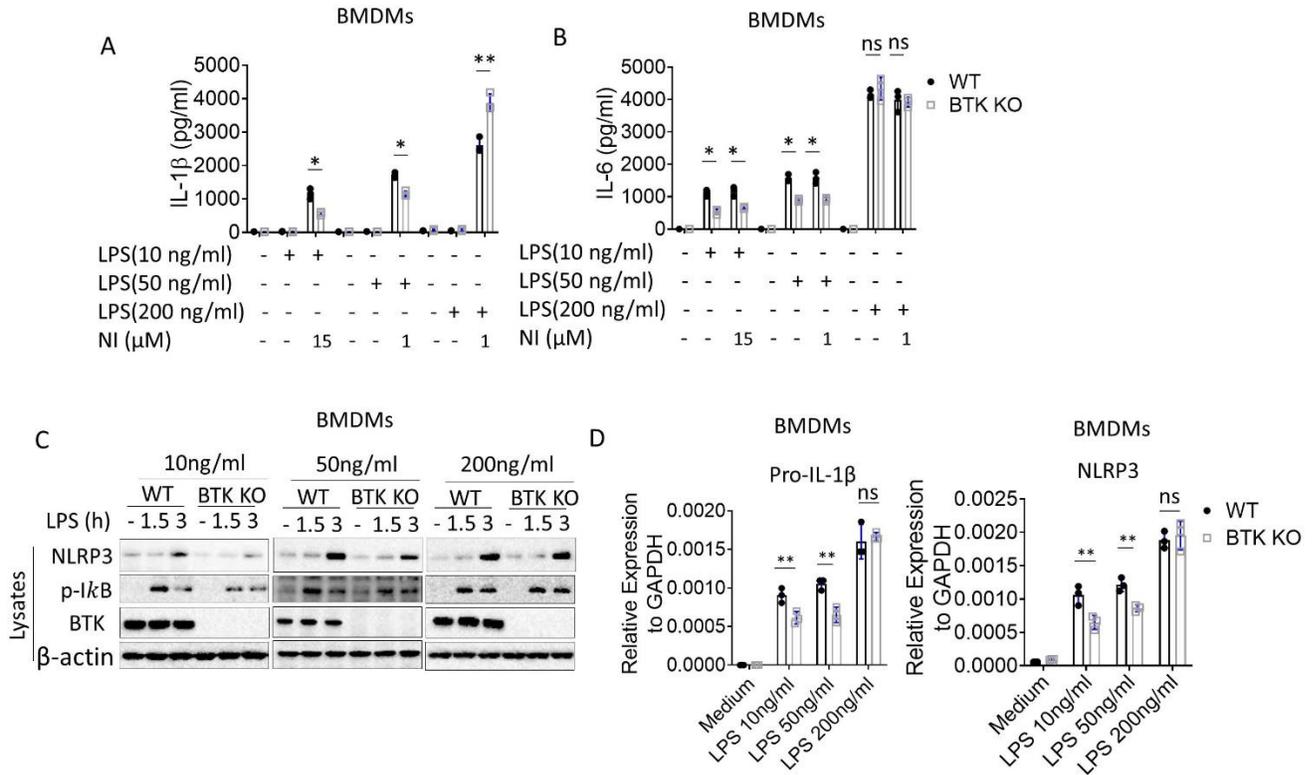
Supplemental Figure 1. Absence of BTK Protein Expression in XLA (BTK-KO) Mice and XLA Patients with BTK Mutations.

(A) BMDMs from wild type and BTK-KO mice were stimulated with or without LPS (200ng/ml, 3 hours); the cells were then lysed and subjected to Western blotting; (B) Human monocytes from two XLA patients (Pt1 and Pt2) and two healthy individuals (H1 and H2) were stimulated with LPS, the cells were lysed and the cell lysates were subjected to Western blotting. Mouse wild type BMDMs (M) were used as controls. Data displayed are representative of two independent experiments.



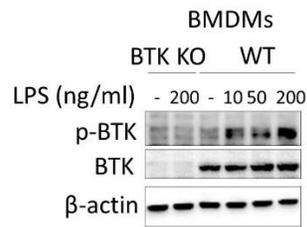
Supplemental Figure 2. Transcription of NLRP3 Inflammasome Components in BTK-KO cells.

BMDMs from WT and BTK-KO mice were primed with LPS (200ng/ml) for 3 hours after which they were lysed; total RNA was then extracted and subjected to qPCR assays for expression of NLRP3, IL-1 β , ASC, pro-caspase-1 and BTK. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ns: not significant. All the data are representative of two independent experiments.



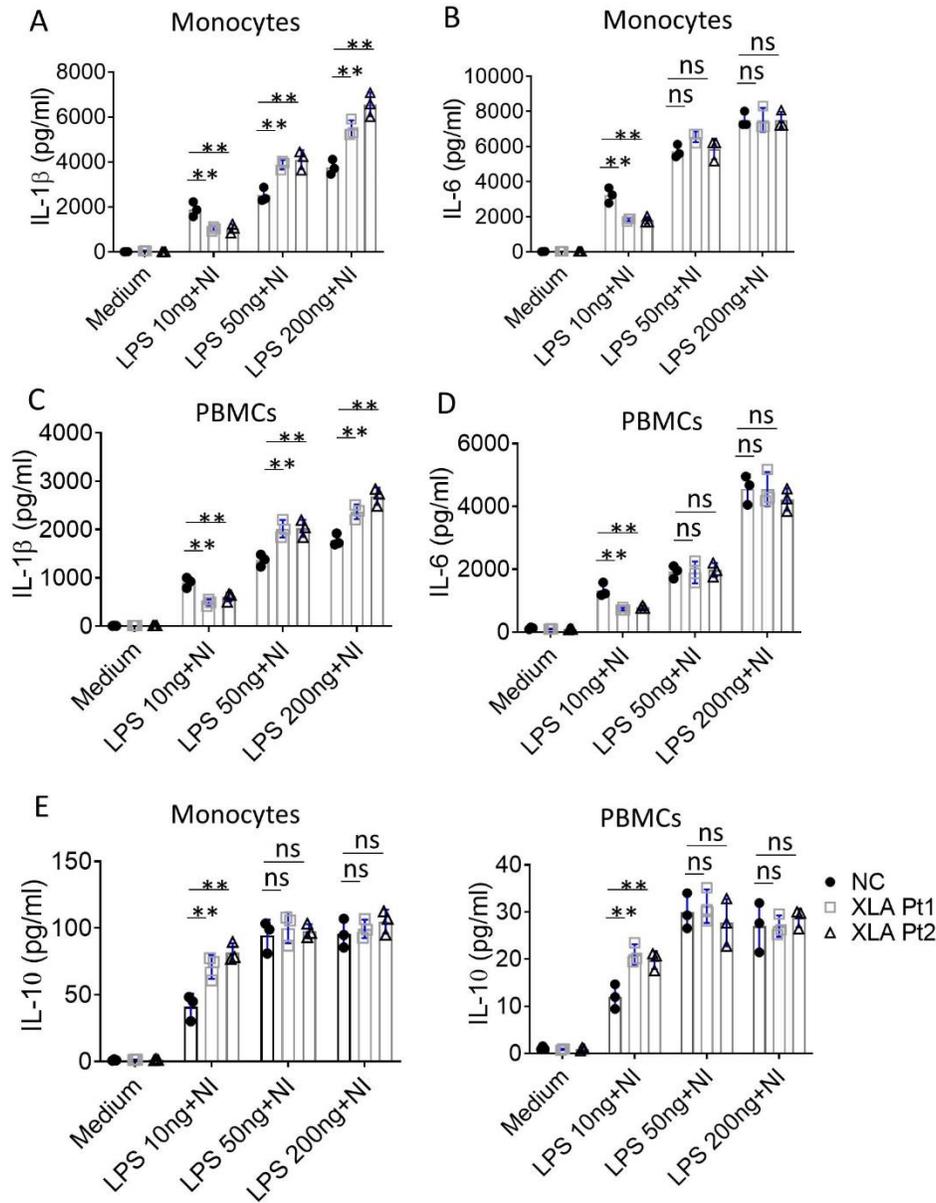
Supplemental Figure 3. BTK-KO BMDMs Exhibit Decreased Production of IL-1 β when Stimulated with Low Concentrations of LPS.

(A, B) Mouse BMDMs were primed with LPS (10ng/ml, 50ng/ml or 200ng/ml) and then stimulated with nigericin; the culture supernatants obtained were then subjected to IL-1 β (A) and IL-6 (B) ELISA assays. (C) BMDMs were stimulated with LPS (10ng/ml, 50ng/ml or 200ng/ml) as indicated; the cells were then lysed and the cell lysates were subjected to Western blotting for detection of NLRP3 and I κ B phosphorylation. (D) BMDMs from wild type mice and BTK-KO mice were primed with LPS (10ng/ml, 50ng/ml or 200ng/ml) for 3 hours after which the cells were lysed for RNA extraction; the RNA thus obtained was then reverse transcribed to cDNA which was then used as template for qPCR detection of IL-1 β and NLRP3. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, * p <0.05; ** p <0.01; ns: not significant. All the data are representative of two independent experiments.



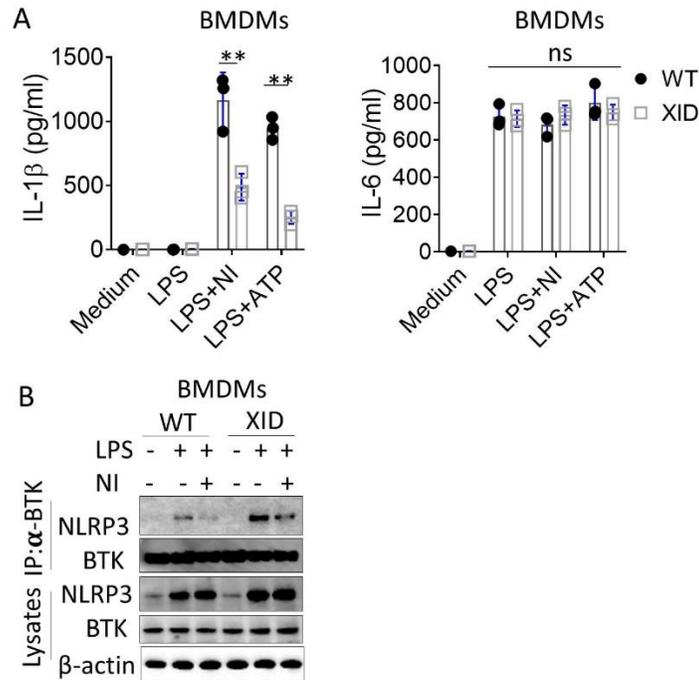
Supplemental Figure 4. BTK Phosphorylation at Y223 is Reduced in Cells Stimulated by 10 or 50mg/ml LPS as Compared to BTK Phosphorylation on Cells Stimulated by 200ng/ml LPS.

BMDMs from WT mice or BTK KO mice were stimulated with various concentrations of LPS as indicated; the cells were then lysed and the cell lysates were subjected to Western blotting for detection of BTK and BTK tyrosine phosphorylation. Data are representative of two independent experiments.



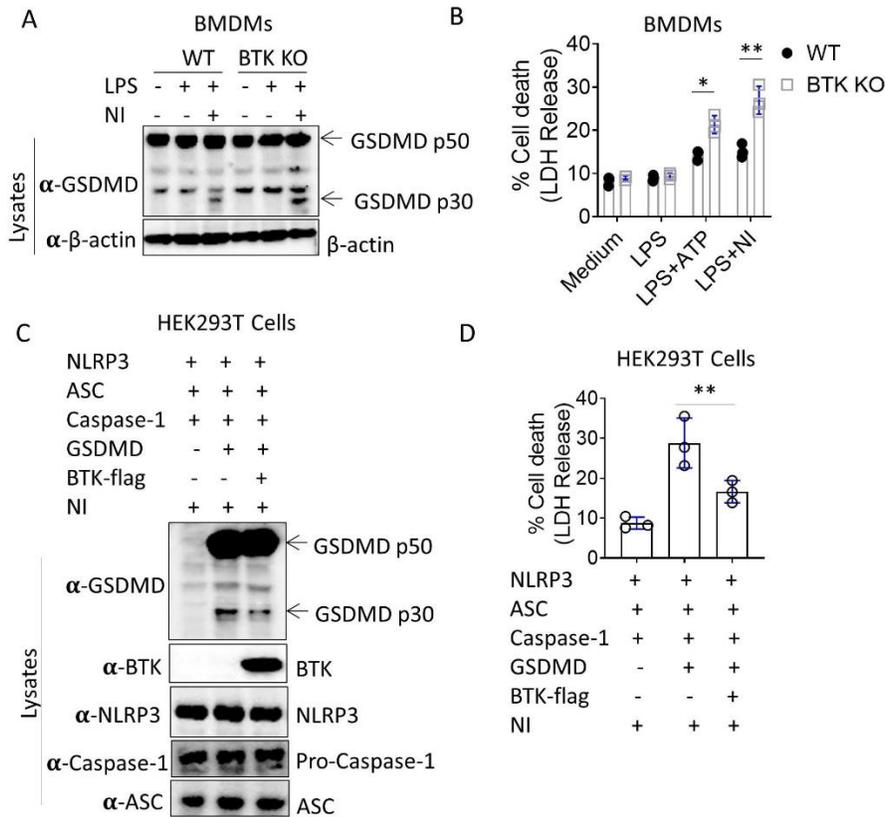
Supplemental Figure 5. Cells from XLA Patients Produce Decreased Amounts of IL-1β and Increased Amounts of IL-10 When Stimulated with Low Concentrations of LPS vs. High Concentrations of LPS.

(A, B, E) Human monocytes from two XLA patients (XLA Pt1, Pt2) and a healthy control individual (NC) were primed with LPS (10ng/ml, 50ng/ml or 200ng/ml) for 3 hours after which the cells were treated with nigericin (NI, 1uM) for 30 minutes; the culture supernatants obtained were then subjected to IL-1β (A), IL-6 (B) and IL-10 (E) ELISA assays. (C, D, F) PBMCs from two XLA patients (XLA Pt1, Pt2) and a healthy control individual (NC) were stimulated as in (A); the culture supernatants obtained were then subjected to IL-1β (C), IL-6 (D) and IL-10 (F) ELISA assays. Data were analyzed using a one-way ANOVA with multiple comparisons and were displayed as mean ± SD, ** $p < 0.01$; ns: not significant. All data are representative of three independent experiments.



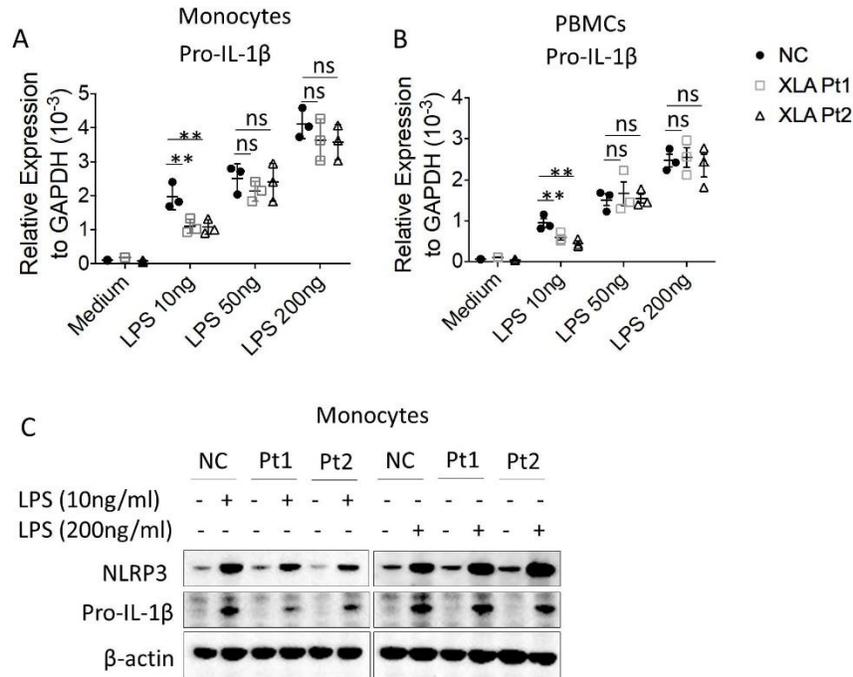
Supplemental Figure 6. BTK Bearing the XID Mutation Exhibits Enhanced Binding to NLRP3.

(A) BMDMs from wild type mice and XID mice were primed with LPS (50ng/ml) for 3 hours and then stimulated with nigericin (NI, 1 μ M) for 30 minutes; the culture supernatants obtained were then subjected to IL-1 β (left panel) and IL-6 (right panel) ELISA assays. (B) BMDMs were treated as in (A) after which the cells were lysed and the cell lysates subjected to IP with anti-BTK antibody and Western blotting for NLRP3 and BTK detection. Data were analyzed using a one-way ANOVA with multiple comparisons and were displayed as mean \pm SD, ** p <0.01; ns: not significant. Data are representative of two independent experiments.



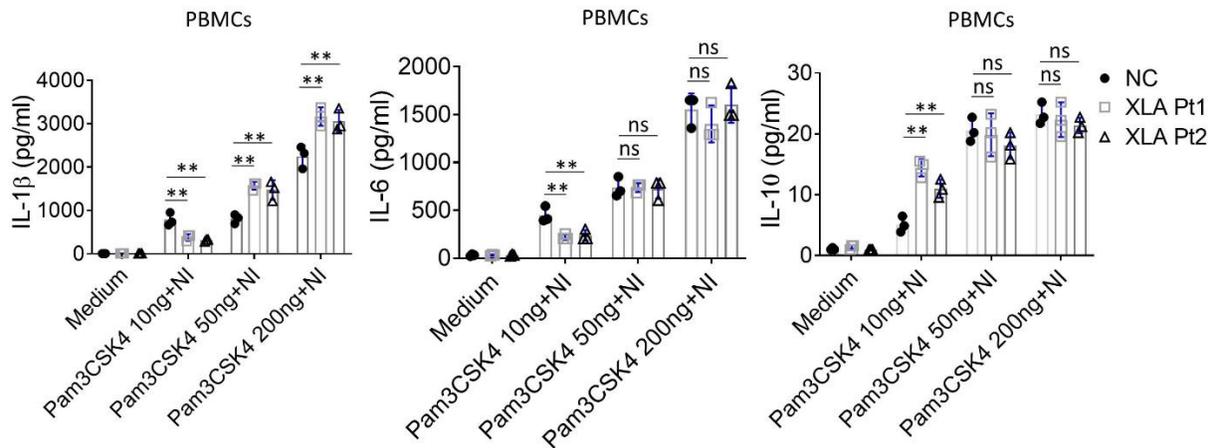
Supplemental Figure 7. BTK-KO Cells Exhibit Enhanced GSDMD Cleavage and Pyroptosis Following NLRP3 Inflammasome Activation.

(A-B) BMDMs from WT mice and BTK-KO mice were primed with LPS (200ng/ml) for 3 hours and then stimulated with nigericin (NI, 1 μ M) for 30 minutes; the cells were then lysed and cell lysates were subjected to Western blotting for GSDMD detection(A). (B) BMDMs were primed with LPS (200ng/ml) for 3 hours and then stimulated with nigericin (NI, 1 μ M) or ATP (5mM) for 30 minutes; the culture supernatants obtained were then subjected to LDH assay (B). (C-D) HEK293T cells were transfected with plasmids expressing NLRP3, ASC, pro-caspase-1 and GSDMD and co-transfected with a plasmid expressing BTK or a control plasmid; 24 hours later, the cells were stimulated with NI for 30 minutes and then lysed; cell lysates were then subjected to Western blotting (C) and the culture supernatants obtained were subjected to LDH assay (D). Data were analyzed using a one-way ANOVA with multiple comparisons and were displayed as mean \pm SD, * p <0.05; ** p <0.01. Data are representative of three independent experiments.



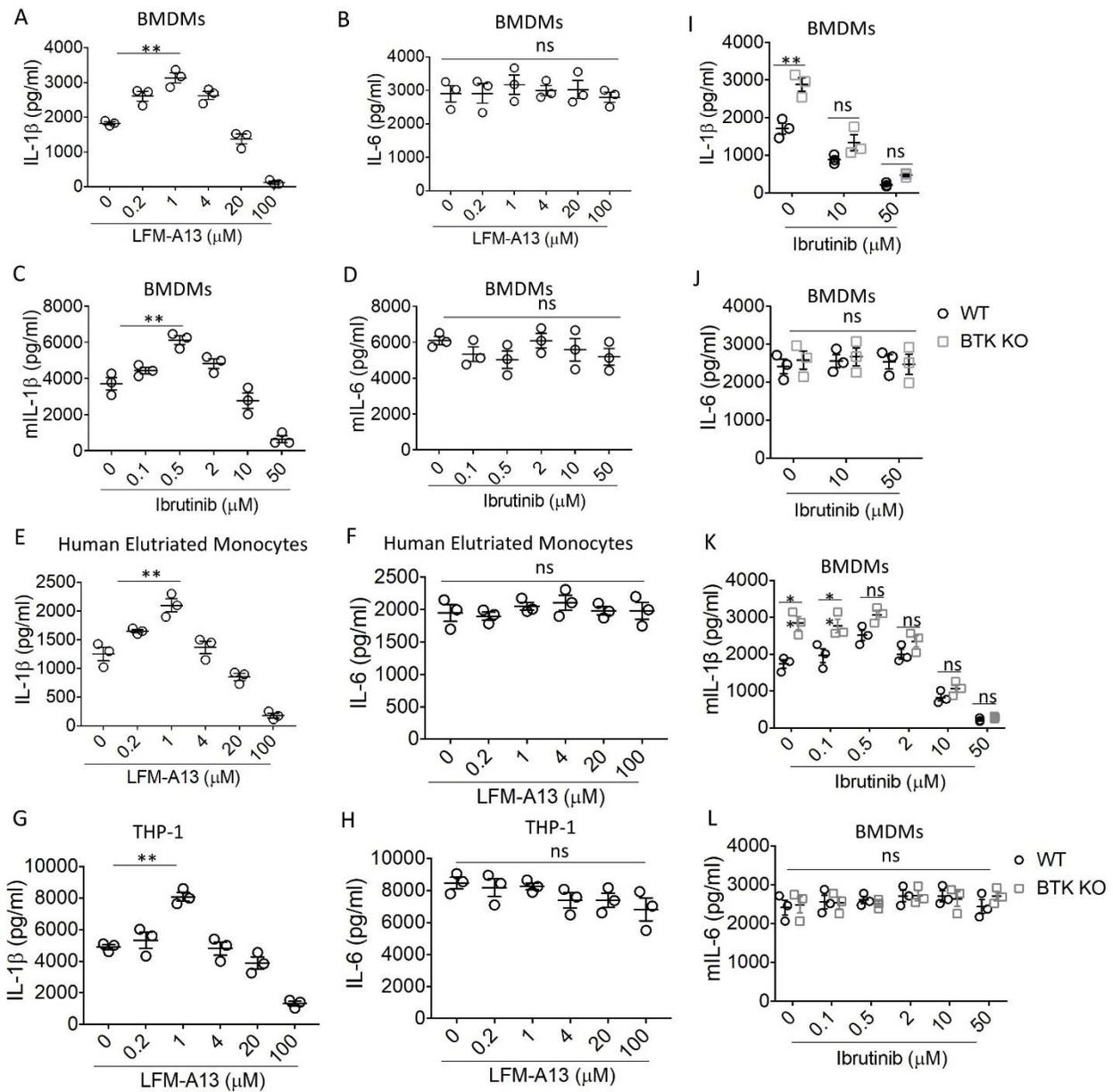
Supplemental Figure 8. XLA Patient Cells Exhibit Decreased NLRP3 and Pro-IL-1β Expression When Stimulated with Low Concentrations of LPS vs High Concentrations of LPS.

(A-B) Human monocytes (A) or PBMCs (B) from two XLA patients (Pt1, Pt2) and a healthy control individual (NC) were primed with LPS (10ng/ml, 50 ng/ml or 200ng/ml) for 3 hours; the cells were then harvested for RNA extraction and reverse transcription, the resultant cDNA was subjected qPCR analysis for pro-IL-1β detection. (C) Monocytes from above individuals were stimulated as indicated and were then lysed, cell lysates were subjected to Western blotting for detection of NLRP3 and pro-IL-1β. Data were analyzed using a one-way ANOVA with multiple comparisons and were displayed as mean ± SD, ** $p < 0.01$; ns: not significant. Data are representative of three independent experiments.



Supplemental Figure 9. PBMCs from XLA Patients Produce Decreased Amounts of IL-1 β and Increased Amounts of IL-10 When Stimulated with Low Concentrations of Pam₃CSK₄ vs. High Concentrations of Pam₃CSK₄.

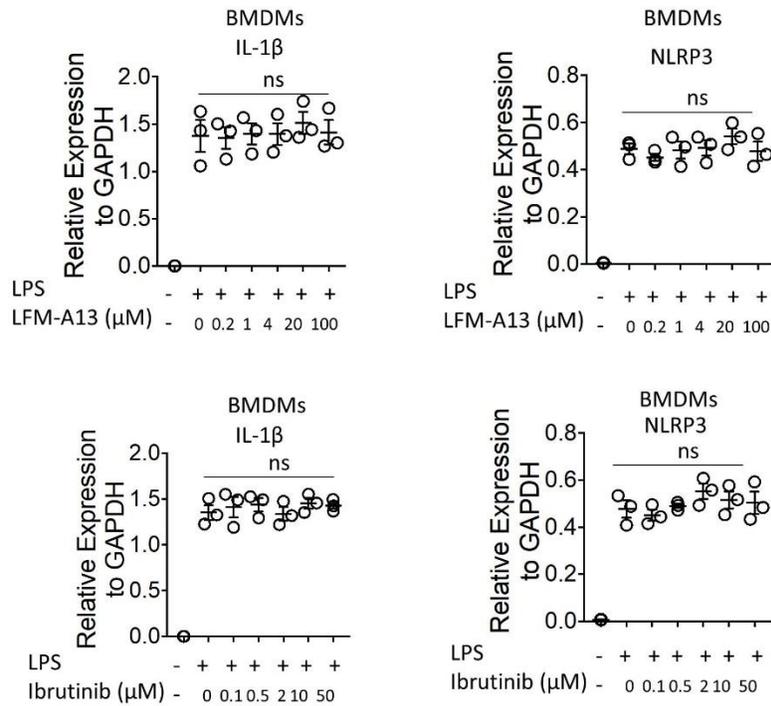
Human PBMCs from two XLA patients (Pt1, Pt2) and a healthy control individual were primed with LPS (200ng/ml) for 3 hours; the cells were then stimulated with nigericin (NI, 1uM) or ATP (5mM) for 30 minutes and the culture supernatants obtained were subjected to IL-1 β , IL-6 and IL-10 ELISA assays. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ** p <0.01; ns: not significant. All the data are representative of three independent experiments.



Supplemental Figure 10. BTK Inhibitors Augment NLRP3 Inflammasome IL-1 β Production at Low Concentrations and Inhibit Inflammasome IL-1 β Production at High Concentration.

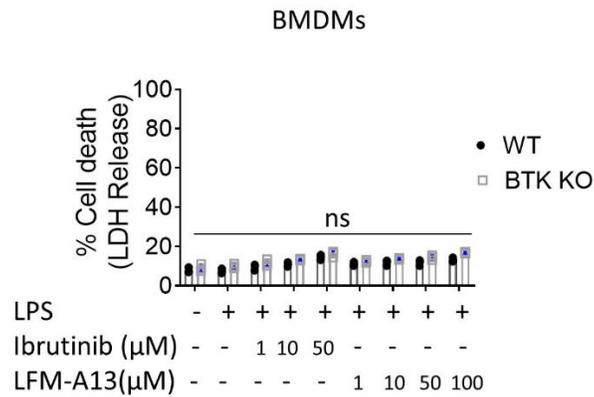
(A-D) BMDMs were stimulated with LPS (200ng/ml) for 3 hours and treated with various concentrations of LFM-A13 (A, B) or ibrutinib (C, D) for 30 minutes; the cells were then stimulated with nigericin (NI, 1 μ M) for 30 minutes and the culture supernatants obtained were subjected to ELISA for IL-1 β (A, C) and IL-6 (B, D). Human elutriated monocytes (E, F) or human THP-1 cells (G, H) were stimulated with LPS (200ng/ml) for 3 hours and treated with various concentrations of LFM-A13 for 30 minutes; the cells were then stimulated with nigericin (NI, 1 μ M) for 30 minutes and the culture supernatants obtained were subjected to ELISA assay for IL-1 β (E,G) and IL-6 (F, H); (I-L) BMDMs from WT mice and BTK-KO mice were primed with LPS (200ng/ml) for 3 hours and then treated

with various concentrations of ibrutinib for 30 minutes, after which the cells were stimulated with nigericin (NI, 1uM) for 30 minutes and the culture supernatants obtained were subjected to IL-1 β (I, K) and IL-6 (J, L) ELISA assays. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ** p <0.01; ns: not significant. All the data are representative of three independent experiments.

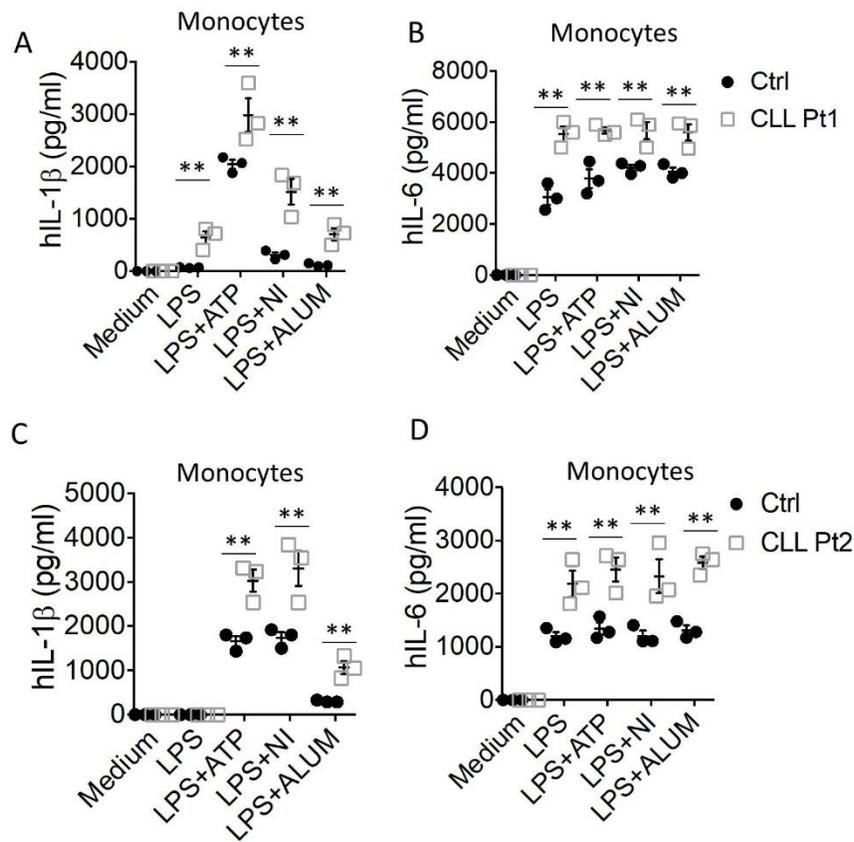


Supplemental Figure 11. BTK Inhibitors Do not Affect IL-1 β Transcription during Priming of the NLRP3 Inflammasome.

Mouse BMDMs were primed with LPS (200ng/ml) for 3 hours; the cells were then stimulated with various concentrations of LFM-A13 (A, B) or Ibrutinib (C, D) for 30 minutes after which the cells were lysed for RNA extraction and qPCR for IL-1 β (A, C) and NLRP3 (B, D) detection. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ns: not significant. All the data are representative of two independent experiments.

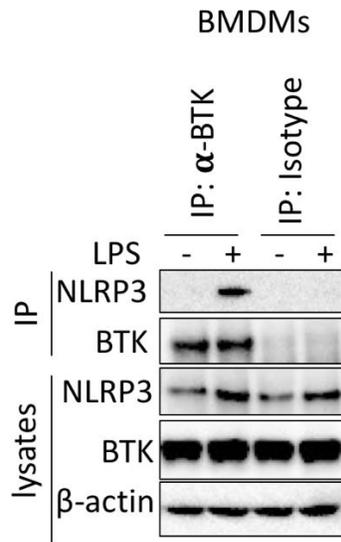


Supplemental Figure 12. BTK Inhibitors Do Not Cause Cytotoxic Loss of BMDMs. Mouse BMDMs were primed with LPS (200ng/ml) for 3 hours; the cells were then stimulated with various concentrations of Ibrutinib or LFM-A13 for 30 minutes after which the culture supernatants were subjected to LDH assay. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ns: not significant. Data are representative of two independent experiments.



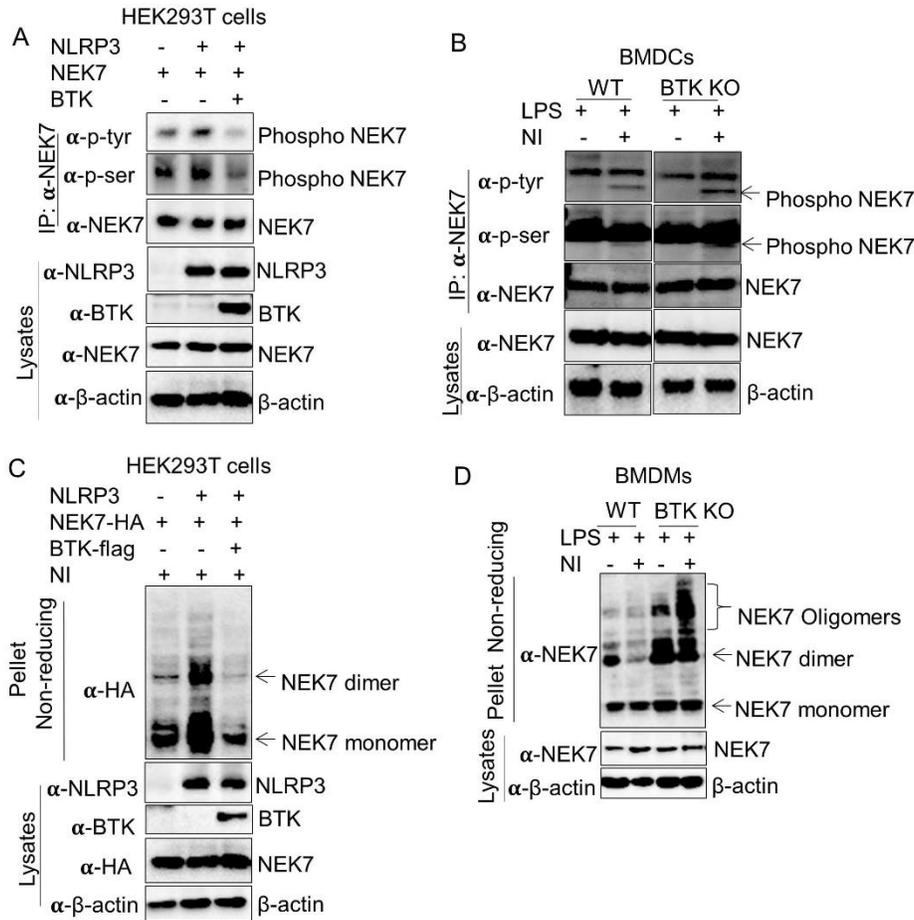
Supplemental Figure 13. Cells from CLL Patients with Low Blood Concentrations of Ibrutinib Exhibit Increased NLRP3 Inflammasome Production of IL-1 β Following Inflammasome Activation.

Peripheral monocytes from two CLL patients (Pt1 and Pt2) and two different healthy control individuals were stimulated with LPS (200ng/ml) for 3 hours and then stimulated with nigericin (NI, 1 μ M) for 30 minutes; the culture supernatants obtained were then subjected to ELISA assays for IL-1 β (A, C) and IL-6 (B, D). Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ** p <0.01. Data are representative of three independent experiments.



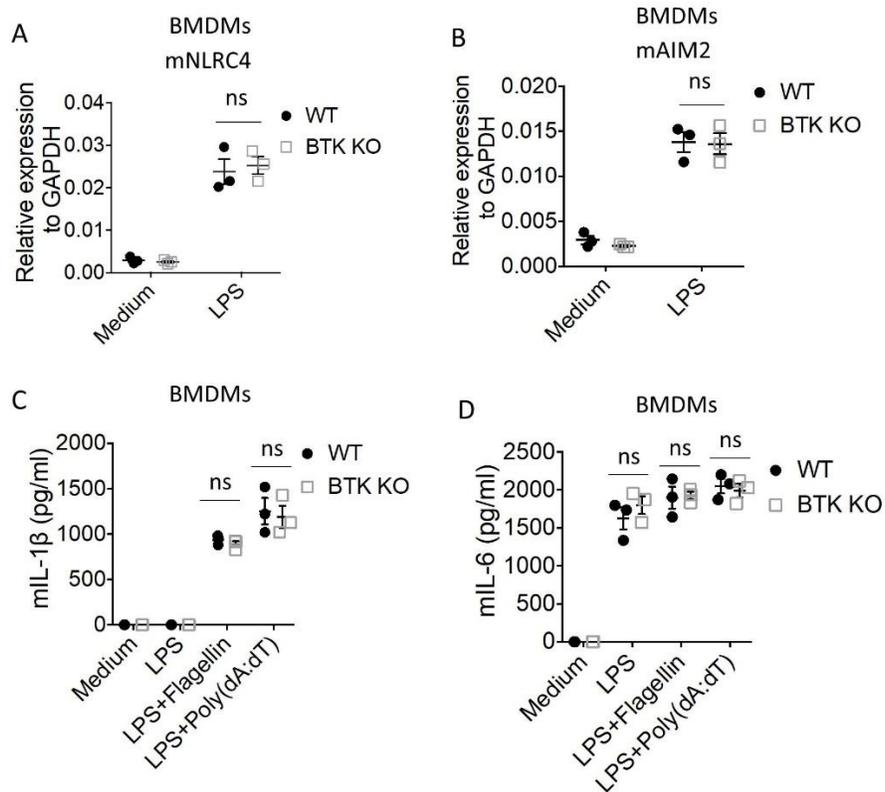
Supplemental Figure 14. NLRP3 Inflammasome Priming by LPS Stimulation of BMDMs Induces BTK-NLRP3 Interaction.

BMDMs from wild type mice were stimulated with or without LPS (200ng/ml) for 3 hours after which the cells were lysed and the cell lysates were subjected to IP with anti-BTK antibody or the isotype control antibody and Western blotting for NLRP3 and BTK detection. Data are representative of two independent experiments.



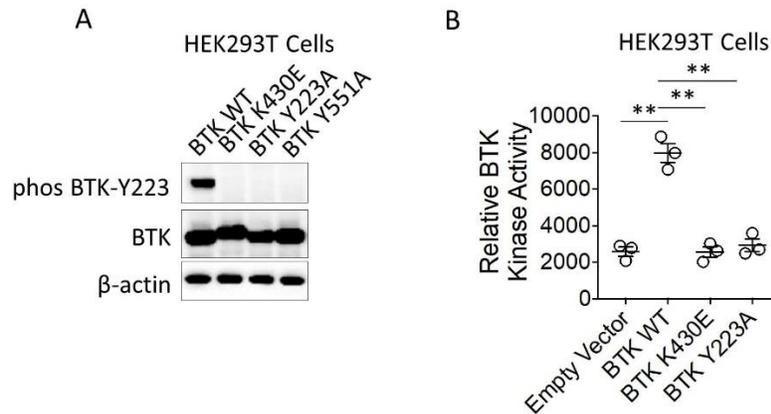
Supplemental Figure 15. BTK Inhibits NEK7 Phosphorylation and Oligomerization.

(A) HEK293T cells were transfected with plasmids expressing NLRP3 and NEK7 with or without co-transfection of a plasmid expressing BTK; 24 hours later, the cells were lysed and cell lysates obtained were subjected to IP and Western blotting. (B) BMDCs from WT and BTK KO mice were primed with LPS (200ng/ml) for 3 hours; the cells were then stimulated with nigericin (NI, 1 μ M) for 30 minutes after which cell lysates were obtained and subjected to IP and Western blotting; (C) HEK293T cells were transfected with plasmids expressing NLRP3 or NEK7 with or without co-transfection of a plasmid expressing BTK; 24 hours later the cells were stimulated with NI (1 μ M) for 30 minutes, after which the cells were lysed and the cell lysates obtained were treated using the procedures described in “Methods” for NEK7 oligomerization or subjected to Western blotting. (D) BMDMs were primed with LPS (200ng/ml) for 3 hours and then stimulated with NI (1 μ M) for 30 minutes and then treated using the procedures described in “Methods” for NEK7 oligomerization or subjected to Western blotting. All of the data are representative of three independent experiments.



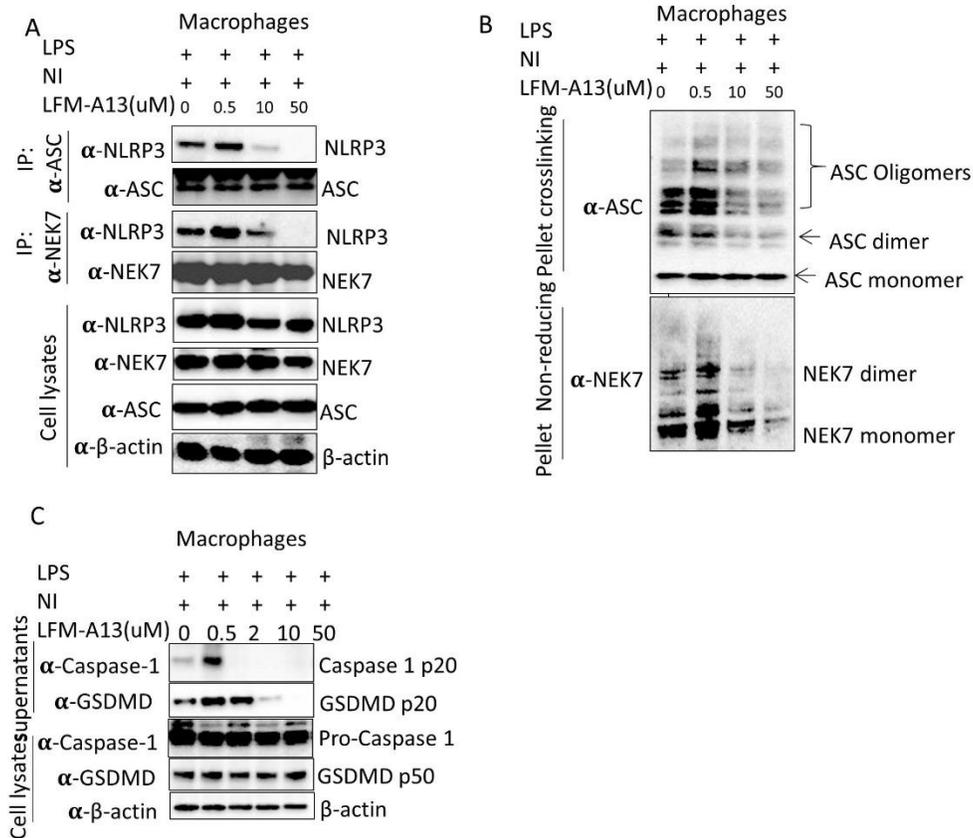
Supplemental Figure 16. BTK Does Not Regulate the NLRC4 or AIM2 Inflammasomes.

(A, B) BMDMs from WT and BTK KO mice were primed with LPS (200ng/ml) for 3 hours and then were subjected to total RNA extraction; the RNA was then subjected to qPCR for detection of mouse NLRC4 (A) and AIM2 (B); (C, D) The above cells were primed with LPS (200ng/ml) for 3 hours and then transfected with flagellin (1ug/ml) or poly(dA:dT) (1ug/ml) for 2 hours after which the culture supernatants obtained were subjected to ELISA assays for IL-1 β (C) and IL-6 (D). Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ns: not significant. All the data are representative of three independent experiments.



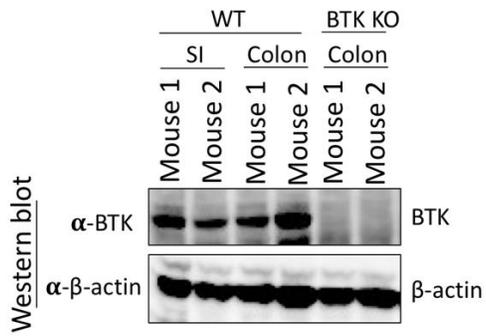
Supplemental Figure 17. BTK Mutations Causing Decreased Y223 Phosphorylation Result in Decreased BTK Kinase Activity.

(A) HEK293T cells were transfected with plasmids expressing WT BTK or BTK with various mutations; 24 hours later the cells were lysed and the cell lysates were subjected to Western blotting for BTK and BTK phosphorylation detection. (B) HEK293T cells were transfected as above; 24 hours later the cells were lysed and the cell lysates were subjected to IP with anti-flag antibody and then BTK kinase assay. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ** $p < 0.01$. All the data are representative of two independent experiments.



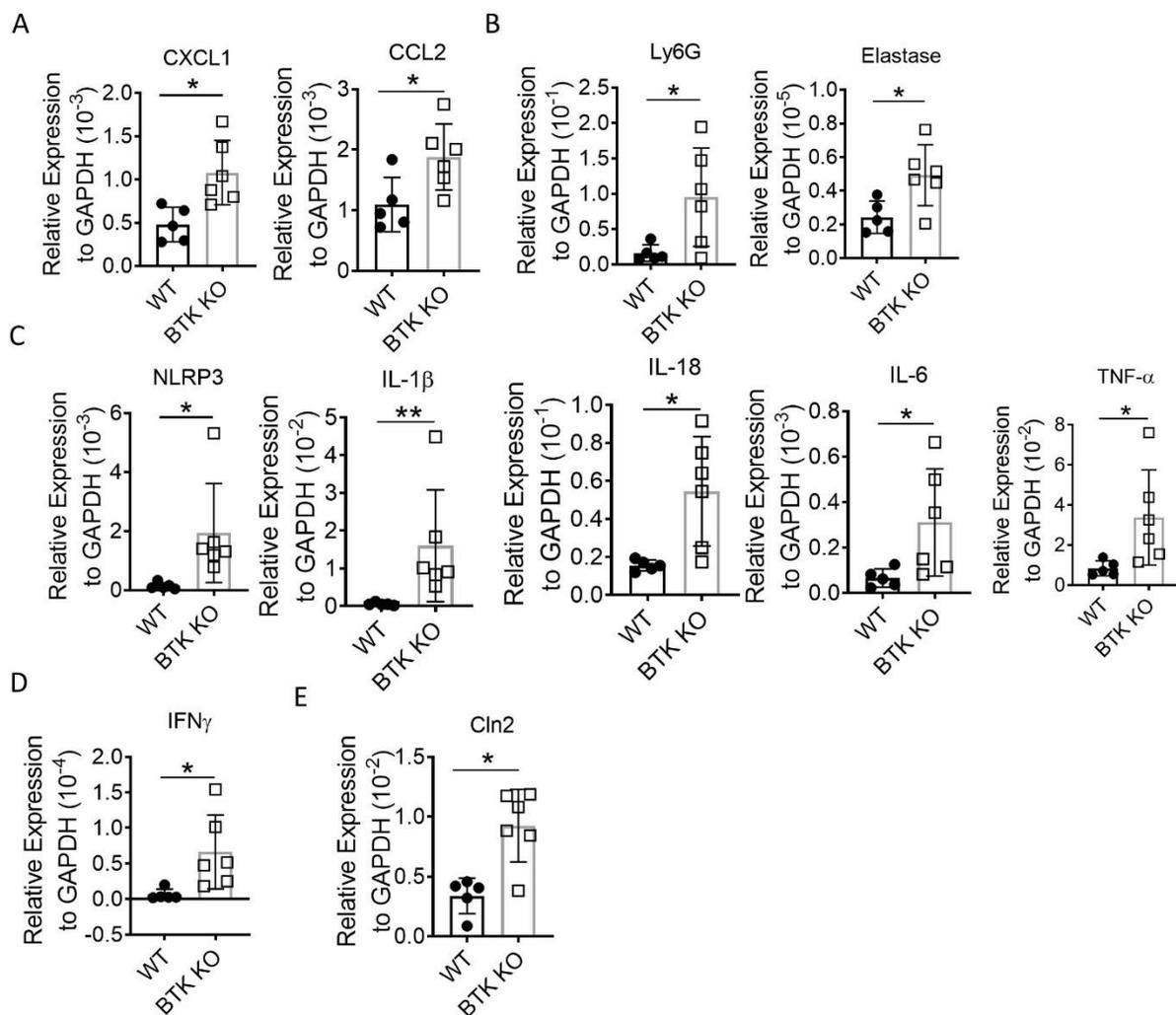
Supplemental Figure 18. High Concentrations of A BTK Inhibitor (LFM-130) Disrupts NLRP3 Inflammasome Activation.

(A) Immortalized mouse macrophages were primed with LPS (200ng/ml) for 3 hours and treated with various concentrations of LFM-A13 for 30 minutes; the cells were then stimulated with nigericin (NI, 1 uM) for 30 minutes after which the cells were lysed and cell lysates obtained were subjected to IP and Western blotting as indicated; (B) Immortalized mouse macrophages stimulated as above were treated using the procedures described in “Methods” for ASC oligomerization and subjected to Western blotting for ASC detection (B, upper panel); the same cells were treated using the procedures described in “Methods” for NEK7 oligomerization and subjected to Western blot for NEK7 detection under non-reducing condition (B, lower panel). (C) Immortalized mouse macrophages were treated as above after which the culture supernatants were precipitated and subjected to Western blot for detection of mature caspase-1 and GSDMD p20. All the data are representative of two independent experiments.



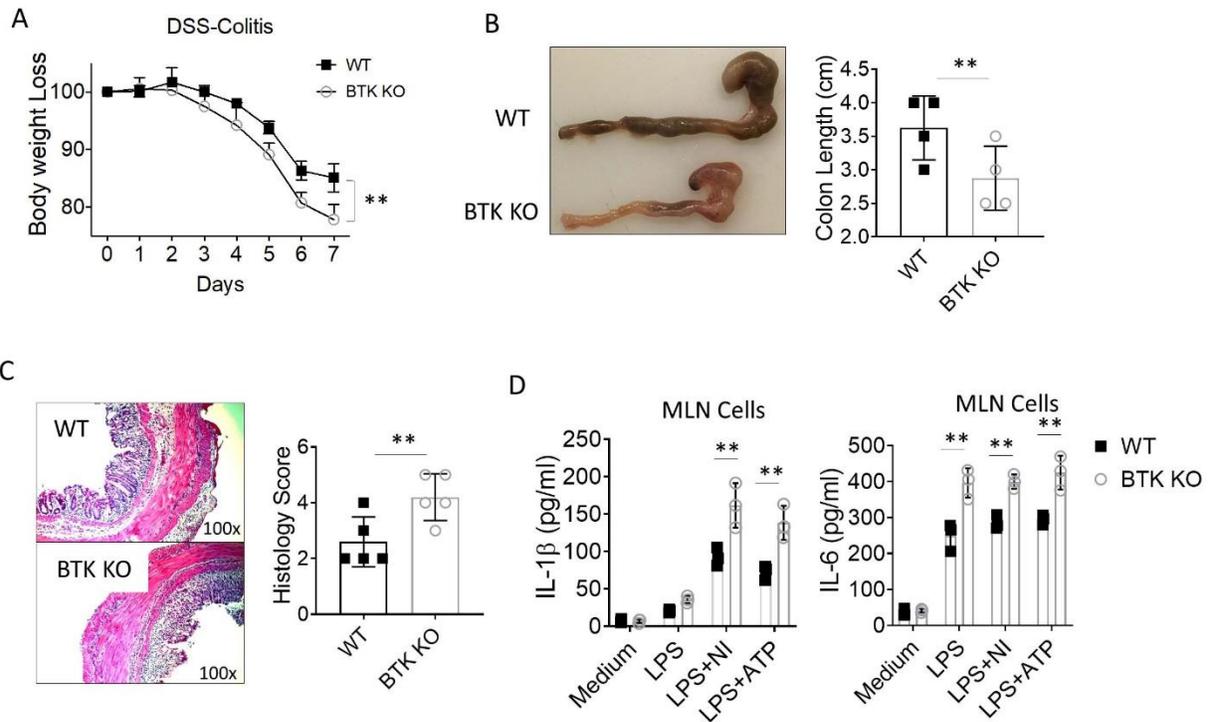
Supplemental Figure 19. BTK Expression in Mouse Small Intestine and Colon.

Tissue obtained from the small intestine (SI) and colon of wild type or BTK KO mice were homogenized and subjected to centrifugation. The supernatants obtained were subjected to Western blotting for detection of BTK expression. Data are representative of two independent experiments.



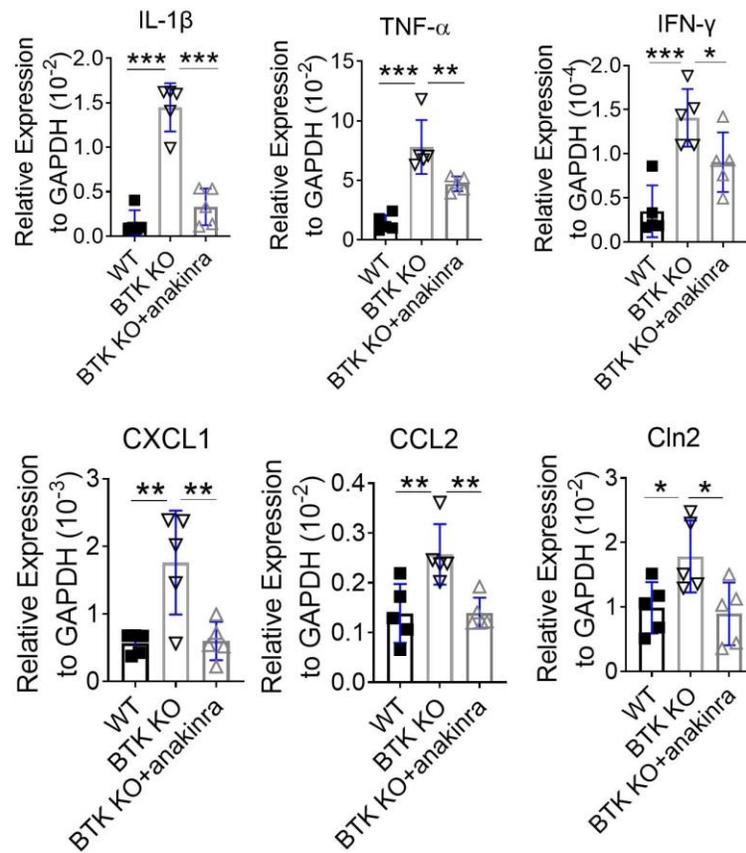
Supplemental Figure 20. Pro-inflammatory Gene Transcription in BTK-KO and WT Mice with TNBS-Colitis.

Total RNA was extracted from colon tissues and then subjected to qPCR for assay of expression of CXCL1, CCL2 (A), Ly6G, Elastase (B), NLRP3, IL-1 β , IL-18, IL-6, TNF- α (C), IFN- γ (D) and claudin-2 (E); Data were analyzed by two tailed Student's *t*-test and are displayed as mean \pm SD, **p*<0.05; ***p*<0.01. All the data are representative of three independent experiments.



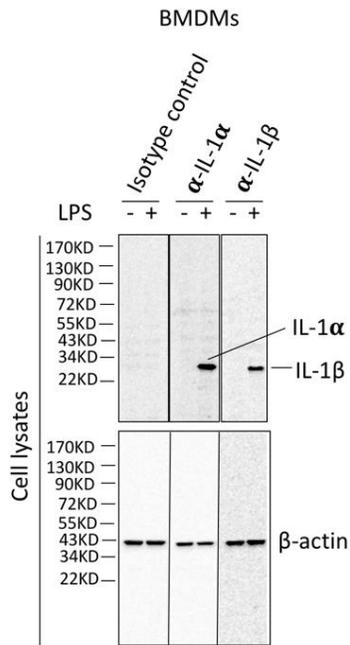
Supplementary Figure 21. BTK KO Mice Exhibit More Severe DSS-Colitis than WT Mice.

Male BTK-KO mice (n=4) and their wildtype littermates (n=4) were administered 4% DSS in drinking water for 5 days (day 0-5) to induce DSS-colitis. On day 6-7, the drinking water was changed to fresh water without DSS. Mouse body weight loss on day 7 (A), colon length (B), and histological damage (C, Original magnification, x100) due to colitis were measured. (D) Mononuclear cells from mesenteric lymph nodes (MLN) were primed with LPS (1ug/ml) for 12 hours and then stimulated with ATP (5mM) or NI (1 μM) for 30 minutes; the culture supernatants obtained were then subjected to ELISA assays of IL-1β (left panel) and IL-6 (right panel). Data were analyzed by two tailed Student's t test (A, B, C) or one-way ANOVA with multiple comparisons (D) and are displayed as mean ± SEM, **p<0.01. All the data are representative of three independent experiments.



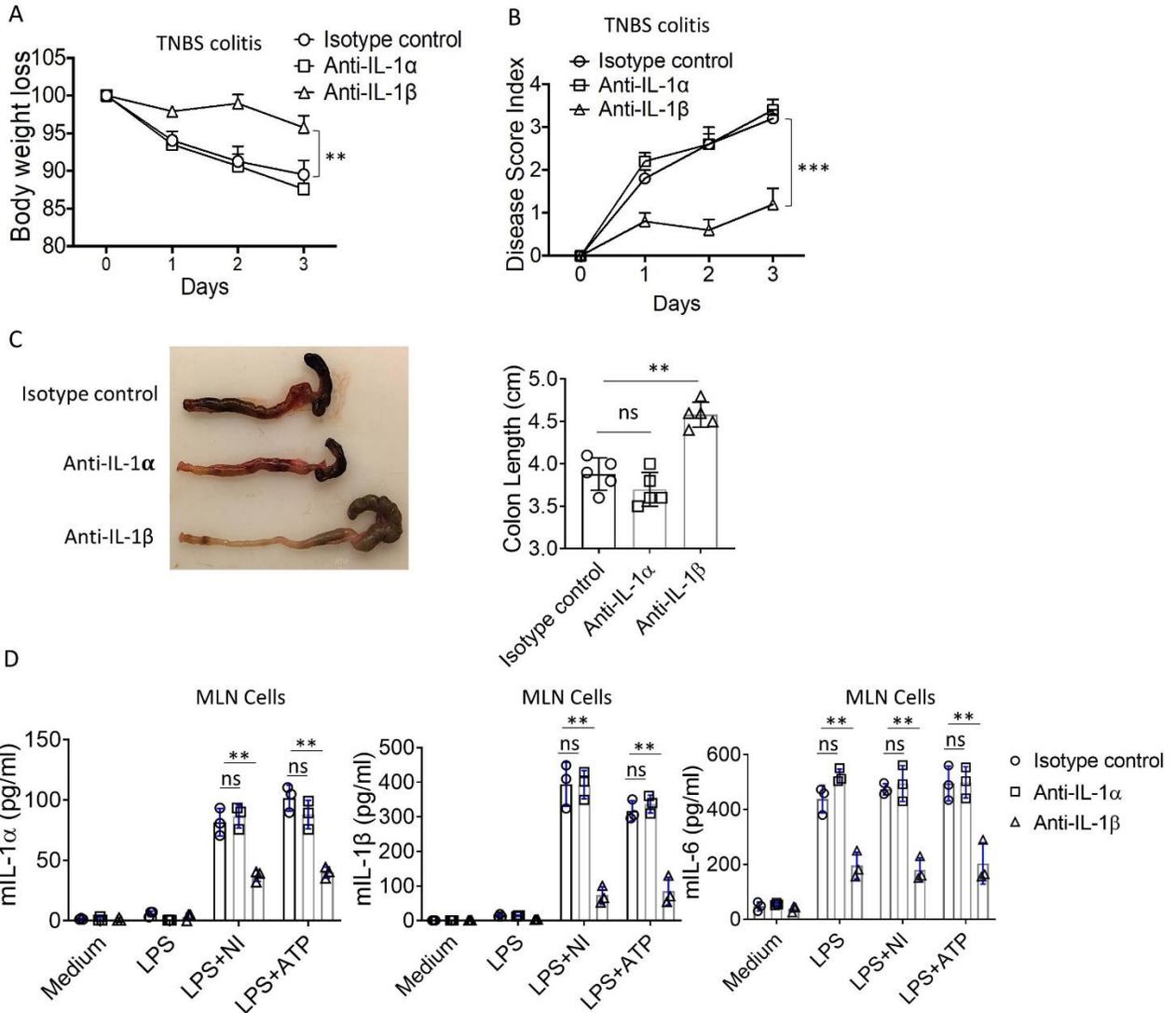
Supplemental Figure 22. Blockade of IL-1β Signaling by Anakinra Inhibits Colon Transcription of Pro-inflammatory Cytokines, Chemokines and Claudin 2 in BTK-KO Mice with TNBS-Colitis.

Total RNA was extracted from colon tissues and subjected to qPCR for detection of the expression of IL-1β, TNF-α, IFN-γ, CXCL1, CCL2 and Cln2. Data were analyzed by a one-way ANOVA with multiple comparisons and are displayed as mean ± SD, * $p < 0.05$; ** $p < 0.01$. All the data are representative of three independent experiments.



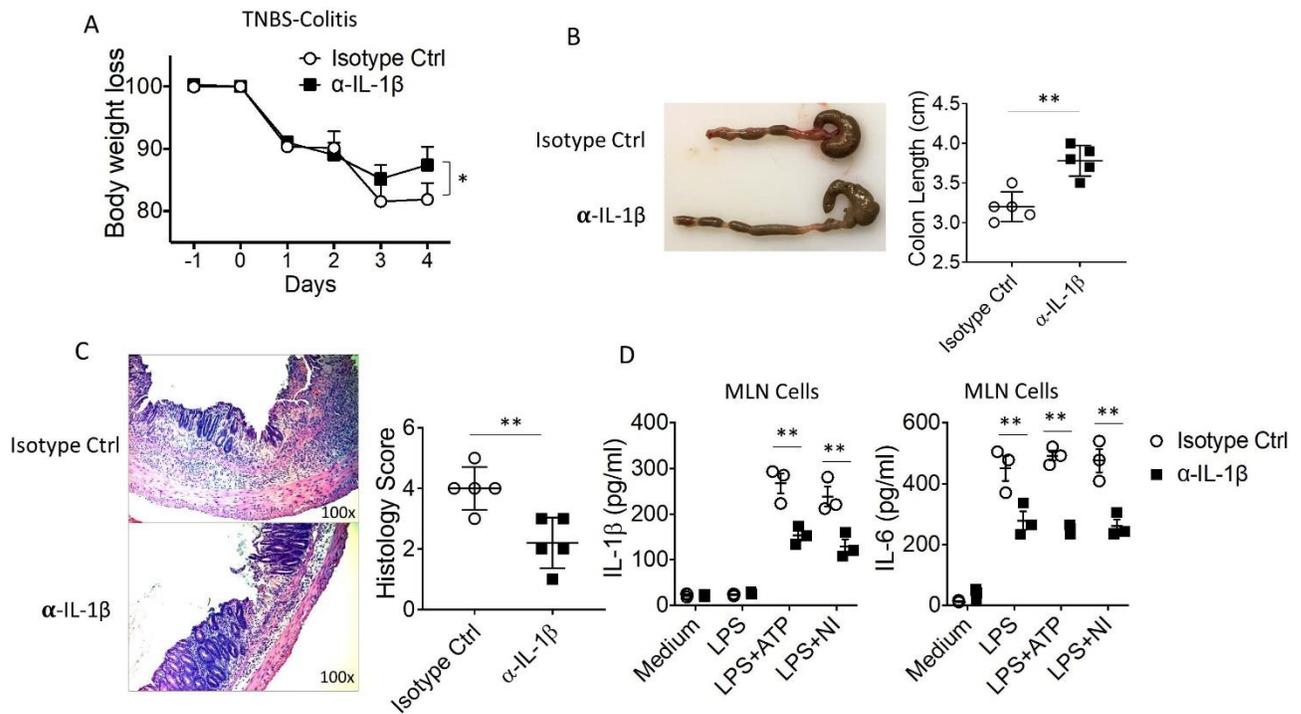
Supplemental Figure 23. Specificity of IL-1 α and IL-1 β Antibodies Used in Studies of TNBS-Colitis.

Wild type BMDMs were stimulated with LPS (200ng/ml) for 6 hours and were then lysed; the cell lysates were then subjected to Western blotting for detection of IL-1 α or IL-1 β . Data are representative of three independent experiments.



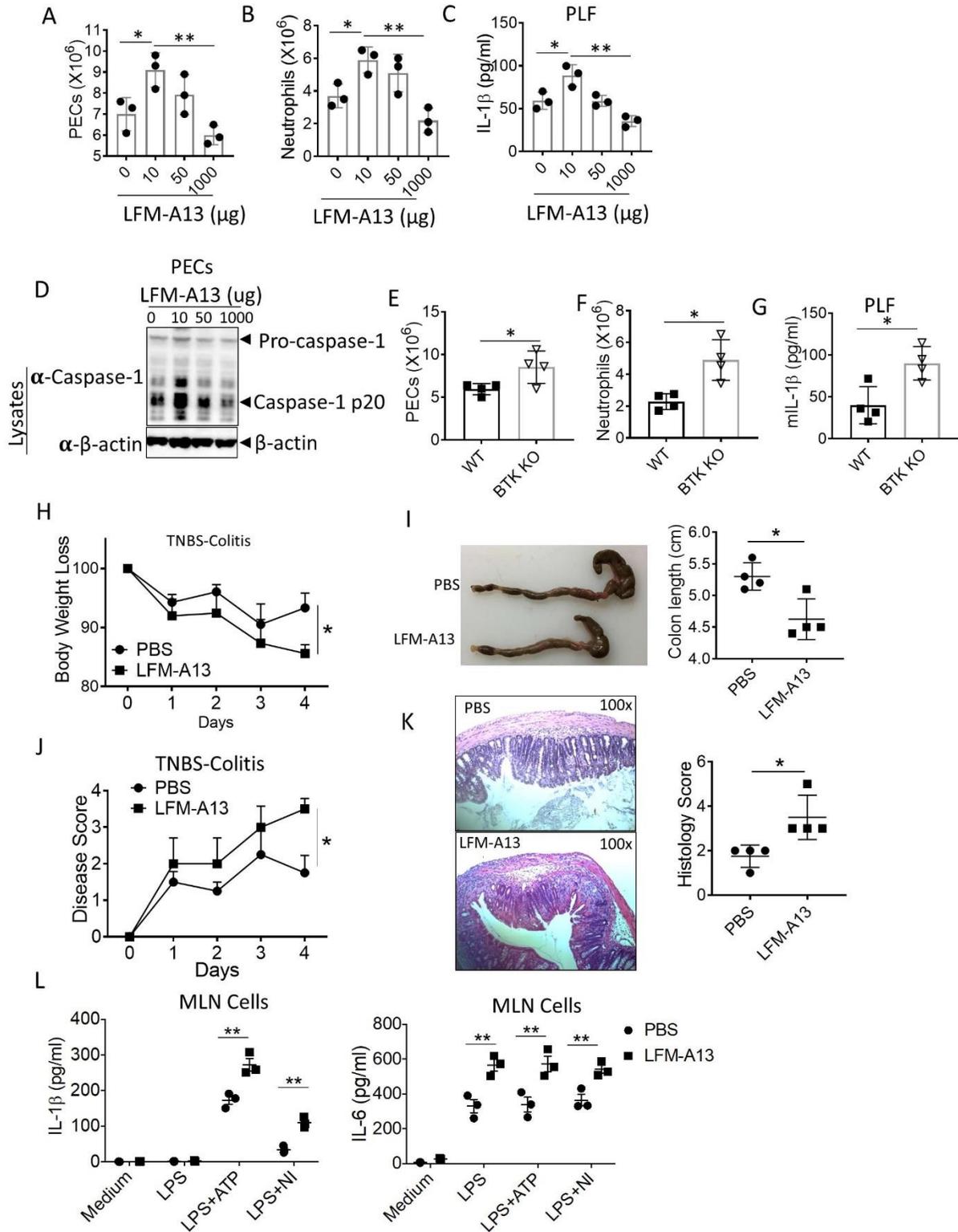
Supplemental Figure 24. Blockade of IL-1 β , but not IL-1 α Signaling Ameliorates TNBS-Colitis in BTK-KO Mice.

(A) Male BTK-KO mice were administered anti-IL-1 α (n=5) anti-IL-1 β (n=5) or the isotype control antibody (n=5) on day -1 (1mg/mouse, IP) and then were administered 3mg TNBS per rectum on days 0 and 2. Mouse body weight loss (A), disease score (B), colon length (C) were measured. (D) Mononuclear cells from MLN were primed with LPS (1ug/ml) for 12 hours and then stimulated with ATP (5mM, 30 minutes) or NI (1 μ M, 30 minutes), the culture supernatants were subjected to ELISA assay of IL-1 α (left panel), IL-1 β (middle panel) and IL-6 (right panel). All of the data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, ** p <0.01; *** p <0.001; ns: not significant. Data are representative of two independent experiments.



Supplemental Figure 25. IL-1 β Neutralizing Antibody Protected Wildtype Mice from TNBS Colitis.

Wild type mice were injected with anti-IL-1 β antibody (1mg/mouse, n=5) or appropriate isotype control antibody (1mg/mouse, n=5) (IP) on day -1; the mice were then administered 3 mg TNBS per rectum on day 0 and day 2 to induce TNBS-colitis. Mouse body weight loss after 4 days (A), colon length (B) and histological damage (C, Original magnification, $\times 100$) due to colitis were measured. (D) Mononuclear cells from mesenteric lymph nodes (MLN) were primed with LPS (1ug/ml) for 12 hours and then stimulated with ATP (5mM, 30 minutes) or NI (1 μ M, 30 minutes); the culture supernatants obtained were then subjected to ELISA assays of IL-1 β (upper panel) and IL-6 (lower panel). Data were analyzed by two tailed Student's t test (A, B, C) or one-way ANOVA with multiple comparisons (D) and are displayed as mean \pm SD, * p <0.05; ** p <0.01. Data are representative of two independent experiments.



Supplemental Figure 26. Administration of BTK Inhibitors Affects NLRP3 inflammasome Activation in a Dose Dependent Manner.

(A) Wild type mice were pre-injected (IP) with various doses of LFM-A13 (n=3 per group) as indicated and 2 hours later administered alum crystals (700 μ g/mouse, IP); 12 hours later the mice were euthanized after which peritoneal exudate cells (PECs) were collected and counted. (B) Gr1⁺ neutrophils in PECs were determined from cell counts and the neutrophil fraction obtained by FACS. (C) Peritoneal lavage fluids (PLF) were collected and subjected to ELISA assay of IL-1 β . (D) PECs were lysed and the cell lysates obtained were subjected to Western blotting. (E) BTK KO mice (n=4) and their wild type littermates (n=4) were injected with alum crystals (700 μ g/mouse, IP) and euthanized 12 hours later for collection and counting of PECs. (F) Gr1⁺ neutrophils in PECs were determined from cell counts and the neutrophil fraction obtained by FACS. (G) The peritoneal lavage fluids were collected and subjected to ELISA assay of IL-1 β . (H-L) Male wild type mice were pre-injected with LFM-A13 (10 μ g/mouse/day, n=4) or PBS (n=4) and were then administered 3 mg TNBS per rectum on day 0 and day 2. On the day TNBS was administered, LFM-A13 or PBS were injected 4 hours prior to TNBS administration. Mouse body weight loss (H), colon length (I), disease score (J) and histological damage (K, Original magnification, \times 100) at day 4 were measured. (L) Mononuclear cells from MLN were primed with LPS (1 μ g/ml) for 12 hours and then stimulated with ATP (5mM, 30 minutes) or NI (1 μ M, 30 minutes) after which the culture supernatants were subjected to ELISA assays of IL-1 β (left panel) and IL-6 (right panel). Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean \pm SD, * p <0.05; ** p <0.01. All the data are representative of three independent experiments.