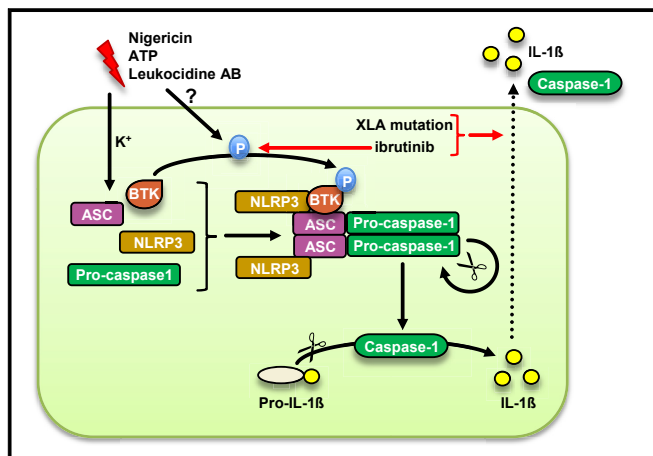


Human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) inflammasome activity is regulated by and potentially targetable through Bruton tyrosine kinase



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GRAPHICAL ABSTRACT



Background: The Nod-like receptor NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) and Bruton tyrosine kinase (BTK) are protagonists in innate and adaptive immunity, respectively. NLRP3 senses exogenous and endogenous insults, leading to inflammasome activation, which occurs spontaneously in patients with Muckle-Wells syndrome; BTK mutations cause the genetic immunodeficiency X-linked agammaglobulinemia (XLA). However, to date, few proteins that regulate NLRP3 inflammasome activity in human primary immune cells have been identified, and clinically promising pharmacologic targeting strategies remain elusive. **Objective:** We sought to identify novel regulators of the NLRP3 inflammasome in human cells with a view to exploring interference with inflammasome activity at the level of such regulators.

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Methods: After proteome-wide phosphoproteomics, the identified novel regulator BTK was studied in human and murine cells by using pharmacologic and genetic BTK ablation. **Results:** Here we show that BTK is a critical regulator of NLRP3 inflammasome activation: pharmacologic (using the US Food and Drug Administration–approved inhibitor ibrutinib) and genetic (in patients with XLA and *Btk* knockout mice) BTK ablation in primary immune cells led to reduced IL-1 β processing and secretion in response to nigericin and the *Staphylococcus aureus* toxin leukocidin AB (LukAB). BTK affected apoptosis-associated speck-like protein containing a CARD (ASC) speck formation and caspase-1 cleavage and interacted with NLRP3 and ASC. *S aureus* infection control *in vivo* and IL-1 β release from cells of patients with Muckle-Wells syndrome were impaired by ibrutinib. Notably, IL-1 β processing and release from immune cells isolated from patients with cancer receiving ibrutinib therapy were reduced. **Conclusion:** Our data suggest that XLA might result in part from genetic inflammasome deficiency and that NLRP3 inflammasome–linked inflammation could potentially be targeted pharmacologically through BTK. (J Allergy Clin Immunol 2017;140:1054-67.)

Key words: IL-1, ibrutinib, Bruton tyrosine kinase, NLRP3, inflammasome, macrophage, X-linked agammaglobulinemia, *Staphylococcus aureus*, Muckle-Wells syndrome, inflammation

The human immune system relies on both innate and adaptive mechanisms to defend the host against infections (eg, from pathogenic bacteria, such as *Staphylococcus aureus*). Initial pathogen sensing by the innate immune system uses so-called pattern recognition receptors, such as Toll-like receptors (TLR) and Nod-like receptors (NLRs). Their activation by microbe-associated molecular patterns or invader-induced cellular insults leads to production of proinflammatory cytokines, which establish an inflammatory state and are critical in activating adaptive immunity.¹ Conversely to other cytokines, the important proinflammatory cytokine IL-1 β is induced (“primed”) at the mRNA level but requires processing and secretion initiated by NLR activation.² NACHT, LRR, and PYD domain-containing protein 3 (NLRP3), the most prominent NLR member, is activated by various pathogenic, environmental, and endogenous stress-related insults and thus plays a role in both microbe-elicited and sterile inflammation.^{3,4} On activation, NLRP3 assembles a so-called inflammasome complex, with the adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 leading to caspase autoactivation and consequent proteolytic cleavage of pro-IL-1 β into bioactive IL-1 β for subsequent secretion.² The latter is a vital step in host defense against infectious agents, such as *S aureus*.⁵ Interestingly, the IL-1 axis also has pathophysiologic significance, as exemplified by autoinflammatory periodic fever syndromes, such as Muckle-Wells syndrome (MWS), in which rare gain-of-function polymorphisms in NLRP3 lead to spontaneous inflammasome activation.^{4,6} Furthermore, NLRP3 inflammasome activity has been implicated in a diverse range of complex human diseases, including gout, rheumatoid arthritis, type 2 diabetes, atherosclerosis, and neurodegeneration.⁴ Thus the NLRP3 inflammasome can be considered an attractive therapeutic target, but efforts to develop inflammasome inhibitors have been hampered by incomplete knowledge regarding the identity and role of the molecular

Abbreviations used

APC:	Allophycocyanin
ASC:	Apoptosis-associated speck-like protein containing a CARD
BMDM:	Bone marrow–derived macrophage
BTK:	Bruton tyrosine kinase
CFU:	Colony-forming unit
DMSO:	Dimethyl sulfoxide
DSS:	Disuccinimidylyl suberate
FDA:	US Food and Drug Administration
FITC:	Fluorescein isothiocyanate
GFP:	Green fluorescent protein
HEK:	Human embryonic kidney
IVIG:	Intravenous immunoglobulin
KO:	Knockout
LukAB:	Leukocidin AB
M-CSF:	Macrophage colony-stimulating factor
MoMac:	Monocyte-derived macrophage
MWS:	Muckle-Wells syndrome
Ni-NTA:	Nitrilotriacetic acid
NLR:	Nod-like receptor
NLRP3:	NACHT, LRR, and PYD domain–containing protein 3
PE:	Phycoerythrin
PIP ₃ :	Phosphatidylinositol (3,4,5)-trisphosphate
PMA:	Phorbol 12-myristate 13-acetate
PVL:	Panton Valentine Leukocidin
SCX:	Strong cation exchange
SH:	Src homology
shRNA:	Short hairpin RNA
TBP:	TATAA-box binding protein
TH:	Tec homology
TLR:	Toll-like receptor
Xid:	X-linked immunodeficiency
XLA:	X-linked agammaglobulinemia

steps involved in activating and regulating the inflammasome. Additionally, how the thus far proposed experimental inhibitors work is unclear. Thus identification of well-defined and therapeutically tractable regulatory proteins would be highly desirable.

Bruton tyrosine kinase (BTK) has long been regarded as a protagonist in adaptive antimicrobial defense because in the 1990s *BTK* mutations were discovered to be the cause for X-linked agammaglobulinemia (XLA),⁷ the first described primary immunodeficiency.⁸ XLA, also termed Bruton disease, is characterized by the almost complete absence of B cells and, consequently, antibodies, leading to severe immunodeficiency. More recently, BTK has also become appreciated as an important therapeutic target, such as in patients with B-cell malignancies, which are characterized by continuous B-cell receptor signaling through BTK.⁹ Promising results have been obtained by using US Food and Drug Administration (FDA)–approved ibrutinib (PCI-32765), an orally administered, selective, and covalent BTK inhibitor, in patients with mantle cell lymphoma and chronic lymphocytic leukemia trials. Ibrutinib was both efficacious and well tolerated.^{10,11}

BTK encodes a cytoplasmic protein tyrosine kinase expressed at high levels in B cells, controlling development, survival, differentiation, and activity from very early stages of development.^{12,13} Additionally, *BTK* is also expressed in cells of the myeloid lineage, including macrophages, neutrophils, mast cells, and dendritic cells,^{1,2} so that a contribution of the myeloid compartment to the overall phenotype of XLA cannot

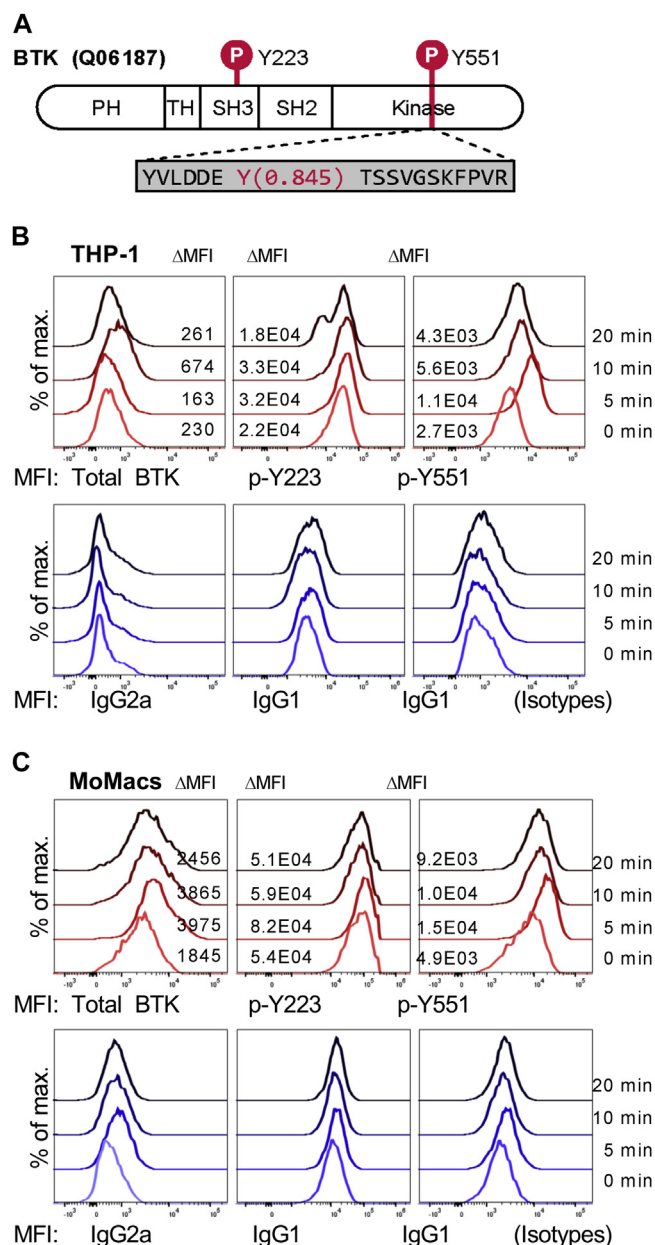


FIG 1. BTK is rapidly phosphorylated in myeloid cells on NLRP3 inflammasome triggering. **A**, Domains, phosphosites (red), and the regulated phosphopeptide (gray) in BTK. **B** and **C**, PMA-primed THP-1 cells (Fig 1, B) or LPS-primed human primary MoMacs (Fig 1, C) were stimulated with nigericin, stained, and analyzed by means of flow cytometry. Δ MFI differences for each antibody-isotype pair are given. One representative of 3 experiments each is shown.

be excluded. BTK contains several functional domains (Fig 1, A): an N-terminal pleckstrin homology domain that binds phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), toward which BTK can translocate from the cytoplasm to PIP₃-containing membranes; this property is abrogated in X-linked immunodeficiency (*Xid*) mutant mice (R28C mutation), which exhibit, like *Btk*-deficient (*Btk*^{-/-}) mice, characteristics similar to the human XLA phenotype, although the phenotype is less severe.¹⁴ Furthermore, BTK features central Tec homology (TH) and Src homology (SH) 3 and SH2 domains involved in protein-protein interactions and a C-terminal catalytically active kinase domain.

Two critical tyrosine phosphorylation sites, Y223 and Y551, play a pivotal role in BTK activation. Y551 is first transphosphorylated by upstream Syk or Lyn kinases, which promotes the catalytic activity of BTK with subsequent autophosphorylation at position Y223. A known downstream target of BTK is phospholipase C, but additional signaling pathways regulating cell proliferation, differentiation, and apoptosis have been shown to at least partially depend on BTK function.^{12,15} Because XLA can be adequately managed clinically by means of regular administration of intravenous immunoglobulin (IVIG) and antimicrobial therapy,¹⁴ a BTK-related defect in the humoral arm of the adaptive immune system has been taken as the primary immunologic explanation for the observed severe susceptibility of patients with XLA for pyogenic bacteria, including *S aureus*. However, it is known that immunity against these bacteria also strongly depends on innate immunity exerted by macrophages and neutrophils,^{16,17} posing the question of whether XLA might also encompass BTK-related defects in the innate immune system.

We show here that BTK is a critical NLRP3 inflammasome regulator in both human subjects and mice and thus functions in a key innate immune process. Inflammasome activity was found to be impaired in *Btk*-deficient mice and patients with XLA, suggesting that the XLA phenotype might indeed encompass the first known primary genetic inflammasome deficiency. Furthermore, we demonstrated that pharmacologic BTK inhibition is able to block IL-1 β release in a murine *in vivo* model, as well as human primary cells from healthy donors, patients with MWS, and ibrutinib-treated patients.

METHODS

Reagents

Nigericin, LPS, phorbol 12-myristate 13-acetate (PMA), and ionomycin were purchased from InvivoGen (San Diego, Calif), ATP from Sigma (St Louis, Mo), ibrutinib and CG11746 were purchased from Selleckchem (Houston, Tex), recombinant GM-CSF or macrophage colony-stimulating factor (M-CSF) were purchased from PeproTech (Rocky Hill, NJ), and Ficoll was purchased from Merck Millipore (Temecula, Calif). Antibodies are listed in the Methods section in this article's Online Repository at www.jacionline.org.

Study subjects and sample acquisition

All human subjects provided written informed consent in accordance with the Declaration of Helsinki, and the study was approved by local ethics committees. Detailed information regarding buffy coats and blood samples from healthy donors, patients with XLA, patients with MWS, and ibrutinib-treated patients is provided in the Methods section in this article's Online Repository.

Isolation and stimulation of primary immune cells

PBMCs from healthy donors and patients with XLA were isolated from whole blood by using Ficoll density gradient purification, primed with 10 ng/mL LPS for 3 hours, and stimulated with 15 μ M/L nigericin for 1 hour or instead with 50 ng/mL PMA and 1 μ M/L ionomycin for 4 hours. PBMCs from patients with MWS were treated with 10 ng/mL LPS and 1 mmol/L ATP concomitantly with the indicated doses of ibrutinib or a dimethyl sulfoxide (DMSO) control for 4 hours. For macrophage differentiation, monocytes were purified by means of positive selection from PBMCs (from buffy coats using Ficoll purification) by using anti-CD14 magnetic beads (>90% purity; Miltenyi Biotec, Bergisch Gladbach, Germany) differentiated into macrophages (GM-CSF for 5 days). The resulting monocyte-derived macrophages (MoMacs) were primed with 300 ng/mL LPS for 3 hours and pretreated with

ibrutinib at 20 or 60 $\mu\text{mol/L}$ for 10 minutes before stimulation with 15 $\mu\text{mol/L}$ nigericin or the indicated amounts of leukocidin AB (LukAB) or Pantone Valentine Leukocidin (PVL) for 1 hour. Further details are provided in the [Methods](#) section in this article's Online Repository.

Plasmid constructs

ASC, NLRP3, and BTK coding sequences in pENTR clones were generated, as described by Wang et al¹⁸ and in the [Methods](#) section in this article's Online Repository.

Cell culture

All cells were cultured at 37°C and 5% CO₂ in Dulbecco modified Eagle medium or RPMI supplemented with 10% FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g/mL}$); all from Life Technologies, Grand Island, NY), unless described otherwise in the [Methods](#) section in this article's Online Repository. THP-1-null cells (InvivoGen) stably express a nontargeting short hairpin RNA (shRNA) and are referred to throughout as THP-1 cells, unless otherwise stated. NLRP3-deficient THP-1 cells express an NLRP3-targeting shRNA (InvivoGen). iGluc THP-1¹⁹ and THP-1 cells with stable BTK knockdown and corresponding mock cells²⁰ were kind gifts of V. Hornung, Gene Center, Munich, Germany, and R. Morita, Keio University School of Medicine, Tokyo, Japan, respectively. ASC-mCerulean expressing immortalized macrophages or THP-1 cells were described previously.^{21,22}

Mice and generation of bone marrow-derived macrophages

Bone marrow cells were isolated from femurs and tibiae of 8- to 12-week-old *Btk* knockout (KO)²³ mice and wild-type littermates (all on a C57BL/6 background) grown and differentiated with GM-CSF (M1 polarization) or M-CSF (M2 polarization). Cells were always counted and reseeded before *in vitro* assays to ensure equal cell numbers. For *in vivo* infections, 8-week-old C57BL/6 female mice (Jackson Laboratory, Bar Harbor, Me) were used. All mouse colonies were maintained in specific pathogen-free conditions. All animal experiments were approved by local authorities and done in accordance with local institutional guidelines and animal protection laws, as detailed in the [Methods](#) section in this article's Online Repository.

Mass spectrometry

THP-1-null cells (InvivoGen) labeled to 97% with "light," "medium-heavy," and "heavy" SILAC medium were primed with 300 ng/mL PMA for 3 hours and left to rest overnight. The next day, the cells were detached and either left unstimulated (light) or stimulated with 15 $\mu\text{mol/L}$ nigericin for 5 (medium) or 10 (heavy) minutes. After washing with ice-cold PBS (containing phosphatase and protease inhibitors; Roche, Mannheim, Germany), cell pellets were snap-frozen and stored at -80°C before analysis. Further details on phosphopeptide enrichment, liquid chromatography-tandem mass spectrometry, and peptide identification are described in the [Methods](#) section in this article's Online Repository.

ELISA

IL-1 β , IL-2, TNF, and IFN- γ levels in supernatants were determined by using half-area plates by ELISA (BioLegend, San Diego, Calif) with triplicate points on a standard plate reader.

Quantitative RT-PCR

mRNA was isolated with the RNeasy Mini Kit on a Qiacube robot (both from Qiagen, Hilden, Germany) and transcribed to cDNA (High Capacity RNA-to-cDNA Kit; Life Technologies), and *Il1b*, *Nlrp3*, *IL1B*, and *NLRP3* mRNA expression was quantified in triplicates relative to TATAA-box binding protein (*Tbp/TBP*) by using TaqMan primers (Life Technologies) on a real-time cyclor (7500 fast; Applied Biosystems, Foster City, Calif), as described

by Wang et al.¹⁸ Comparable cycle threshold values for *TBP* (data not shown) in all treatment groups confirmed equal cell numbers.

ASC speck formation assay and confocal microscopy

Human embryonic kidney (HEK) 293T cells (4×10^4) were plated, transiently transfected, fixed with 100% methanol, and stained for nucleic acids (To-pro-3; Thermo Fisher, Waltham, Mass) and BTK-HA (anti-HA-Alexa Fluor 594). Immortalized *Nlrp3* KO macrophages overexpressing NLRP3-FLAG and ASC-mCerulean were pretreated with ibrutinib or CGI1745 (and a solvent control) for 10 or 60 minutes, respectively, before stimulation with either 5 $\mu\text{mol/L}$ nigericin (Life Technologies) for 60 minutes or 1 mmol/L Leu-Leu-OMe•HCl (Chem-Impex, Wood Dale, Ill) for 90 minutes and then fixed (4% formaldehyde), and nucleic acids were stained (DRAQ5; eBioscience, San Diego, Calif). Details regarding analysis with a Zeiss confocal microscope (Zeiss, Oberkochen, Germany) and image quantification with ImageJ (National Institutes of Health, Bethesda, Md) or CellProfiler software are described in the [Methods](#) section in this article's Online Repository.

Pro-IL-1 β and caspase-1 cleavage

Equal amounts of cells were primed and then stimulated in Opti-MEM medium (Gibco, Grand Island, NY). Proteins in supernatants were precipitated with methanol (VWR International, Radnor, Pa) and chloroform (Sigma, St Louis, Mo). Cells were lysed in RIPA buffer with protease inhibitors (Sigma). Where applicable, recombinant Protein A was added before precipitation to control for equal precipitation. Fifteen percent and 12% SDS-PAGE gels were used for protein from supernatants and whole-cell lysates, respectively, and probed with the indicated antibodies.

Co-immunoprecipitation

HEK293T cells were transfected with CaPO₄ and lysed 48 hours later in buffer (50 mmol/L Tris [pH 8], 280 mmol/L NaCl, 0.5% NP-40, 0.2 mmol/L EDTA, 2 mmol/L EGTA, 10% glycerol, and 1 mmol/L dithiothreitol) with protease/phosphatase inhibitors (Roche). Cleared lysates were subjected to immunoprecipitation of the BTK-Protein A fusion protein with Dynabeads (M-280 Sheep Anti-Mouse IgG, Thermo Fisher Scientific). Washed beads were boiled in loading buffer and applied to SDS-PAGE and immunoblots (see the [Methods](#) section in this article's Online Repository).

Crosslinking of ASC oligomers

THP-1 ASC-mCerulean cells (2×10^6) were primed with 300 ng/mL LPS for 2 hours and then treated with 60 $\mu\text{mol/L}$ ibrutinib for 1 hour and then 15 $\mu\text{mol/L}$ nigericin for 1 hour. After washing with PBS, cells were lysed, and lysates and pellets were cross-linked with disuccinimyl suberate (DSS; Thermo Scientific) and analyzed as described by de Almeida et al²⁴ and in the [Methods](#) section in this article's Online Repository.

Flow cytometry and phospho-flow

Whole blood from healthy donors and patients with XLA was subjected to standard cell-surface staining and flow cytometry by using anti-CD3-fluorescein isothiocyanate (FITC), anti-CD19-Pacific Blue, anti-CD14-phycoerythrin (PE), and anti-CD11b-allophycocyanin (APC) by using a standardized protocol and identical flow cytometer settings for all donors. For phospho-flow analysis, the indicated primed cells were treated, fixed (Lyse/Fix Buffer; BD Biosciences, San Jose, Calif), and permeabilized with 1 mL of cold methanol; Fc receptors were blocked (Human AB serum); and cells were stained with anti-Btk (pY551) PE, anti-Btk (pY223) BV421, and anti-total BTK Alexa Fluor 647 antibodies (all from BD Biosciences). Corresponding isotype controls were from ImmunoTools (Friesoythe, Germany). For further details, see the [Methods](#) section in this article's Online Repository.

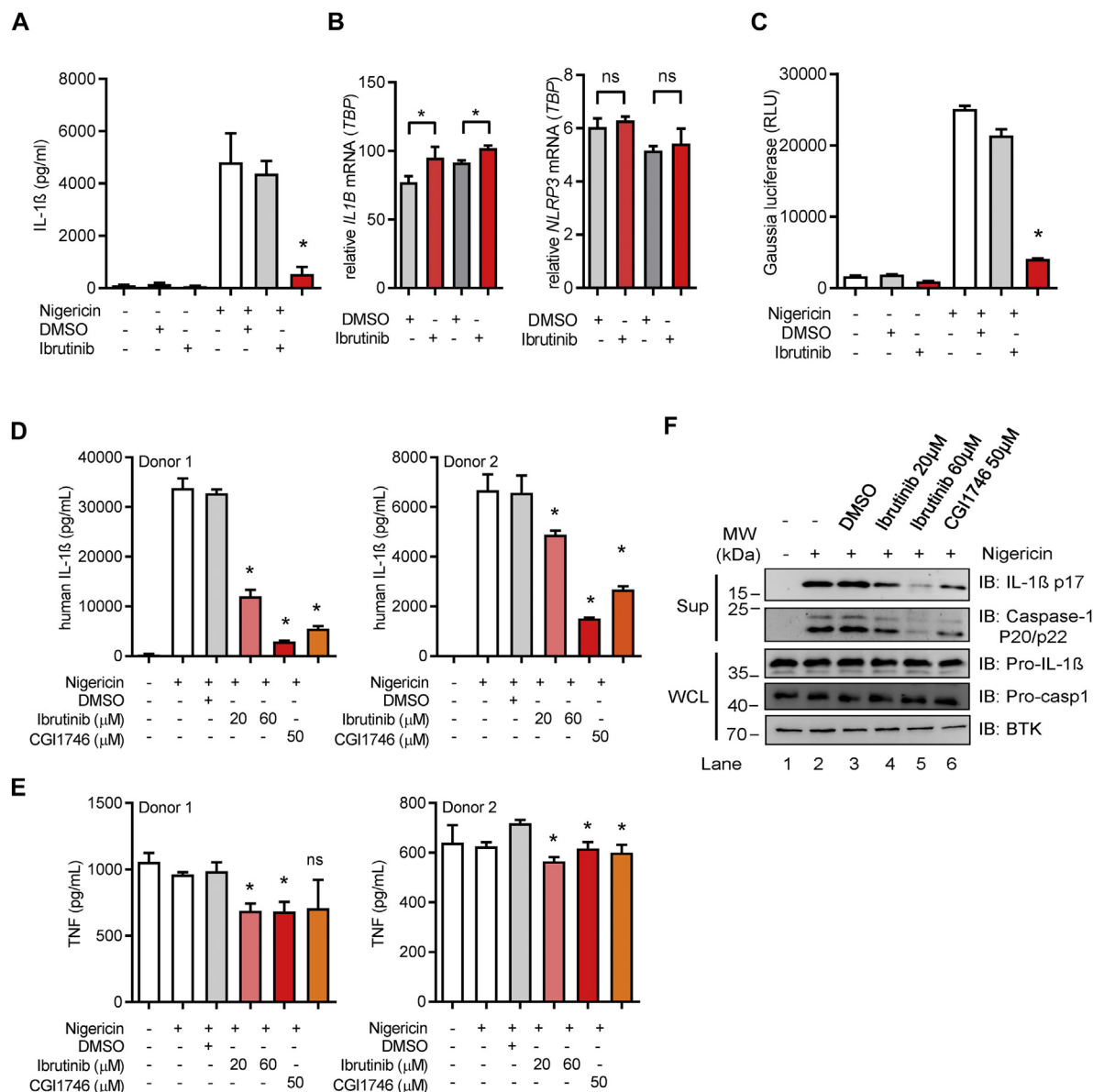


FIG 2. Ablation of BTK impairs NLRP3 inflammasome activity. **A**, IL-1β release from PMA-differentiated and nigericin-treated THP-1 cells preincubated with ibrutinib (60 μmol/L). **B**, mRNA levels before nigericin addition. **C**, IL-1β–Gaussia levels assessed as in Bartok et al.¹⁹ **D–F**, Cleaved IL-1β (Fig 2, **D** and **F**), caspase-1 (Fig 2, **F**), and TNF (Fig 2, **E**) from primed and nigericin-treated human primary MoMacs pretreated with DMSO (mock), ibrutinib, or CGI-1746 for 10 minutes. Numbers of experiments shown are as follows: 1 of 2 in Fig 2, **A**; 1 of 3 in Fig 2, **B**; and 1 of 3 in Fig 2, **C**. In Fig 2, **D** and **E**, 2 of 5 and in Fig 2, **F**, 1 of 3 donors are shown. Means + SDs are shown, and 2-sided Student *t* tests were used. Comparison with the DMSO control is shown in the gray bar. **P* < .05. ns, Not significant.

S aureus strains

The community-acquired methicillin-resistant *S aureus* strain USA300 bioluminescent derivative LAC::Lux was grown according to standard microbiological practice and used as indicated (see the [Methods](#) section in this article's Online Repository).

In vivo infection model

Ibrutinib (6 mg/kg) in 3% DMSO and 5% corn oil in PBS or vehicle control was injected intravenously through the retro-orbital vein in anesthetized mice on days -1, 0, and 1 and topically on day 2 (6 mg/kg ibrutinib in 10 μL of DMSO or vehicle control). On day 0, the dorsal backs of anesthetized mice (2% isoflurane) were shaved and injected intradermally with 3×10^7 colony-forming units (CFU) of *S aureus* LAC::Lux; digitally imaged

on 1, 3, 7, 10, and 14, and total lesion size (in square centimeters) was analyzed by using ImageJ software with a millimeter ruler as a reference.

In vivo *S aureus* bioluminescent imaging

Mice were anesthetized (2% isoflurane), *in vivo* bioluminescent imaging was performed (Lumina III IVIS; PerkinElmer, Waltham, Mass), and total flux (photons per seconds) within a circular region of interest measuring 1×10^3 pixels was measured with Living Image software (PerkinElmer; limit of detection, 2×10^4 photons/s).

Purification of LukAB and PVL

The pQE30 vector (Qiagen) was used to produce recombinant His-tagged LukS-PV, LukF-PV, LukA, and LukB, as described in the [Methods](#) section in

this article's Online Repository, by using nitrilotriacetic acid (Ni-NTA) affinity chromatography. Protein stock solutions were dialyzed against PBS/50% glycerol, checked for endotoxin contamination (<0.25 EU/mL; Lonza, Basel, Switzerland), and stored at -20°C until use. Single components were mixed in an equal molar ratio (IL-1 β secretion was not detectable in stimulations using single components, data not shown).

Data analysis and statistics

Data were analyzed with GraphPad Prism software (version 5.0; GraphPad Software, La Jolla, Calif) by using Student *t* tests and nonparametric Mann-Whitney *U* or Wilcoxon matched-pairs signed-rank tests, as indicated. All tests were 2-tailed unless stated otherwise. A *P* value of less than .05 was generally considered statistically significant and, even if considerably lower, is marked with an asterisk throughout the figures.

RESULTS

BTK is rapidly phosphorylated on NLRP3 inflammasome activation

To identify novel NLRP3 regulators of the NLRP3 inflammasome, we used unbiased triple SILAC phosphoproteomics²⁵ in primed THP-1 macrophages activated by the microbial potassium ionophore and NLRP3 agonist nigericin (see the [Methods](#) section). Differences in the phosphoproteome of unstimulated cells versus cells stimulated for 5 or 10 minutes to capture early NLRP3-proximal events included a phosphopeptide harboring the well-known BTK regulatory site tyrosine 551 (Y551; [Fig 1, A](#)), which was significantly upregulated 2.6-fold within 5 minutes of stimulation. Phospho-flow cytometric analysis in primed THP-1 cells ([Fig 1, B](#)) and primary human MoMacs ([Fig 1, C](#)) confirmed that although Y223 showed only subtle phosphorylation, Y551 was rapidly and robustly phosphorylated, peaking at 5 minutes after nigericin addition, indicating a rapid activation of BTK by an NLRP3 inflammasome trigger.

Pharmacologic inhibition of BTK impairs inflammasome activation

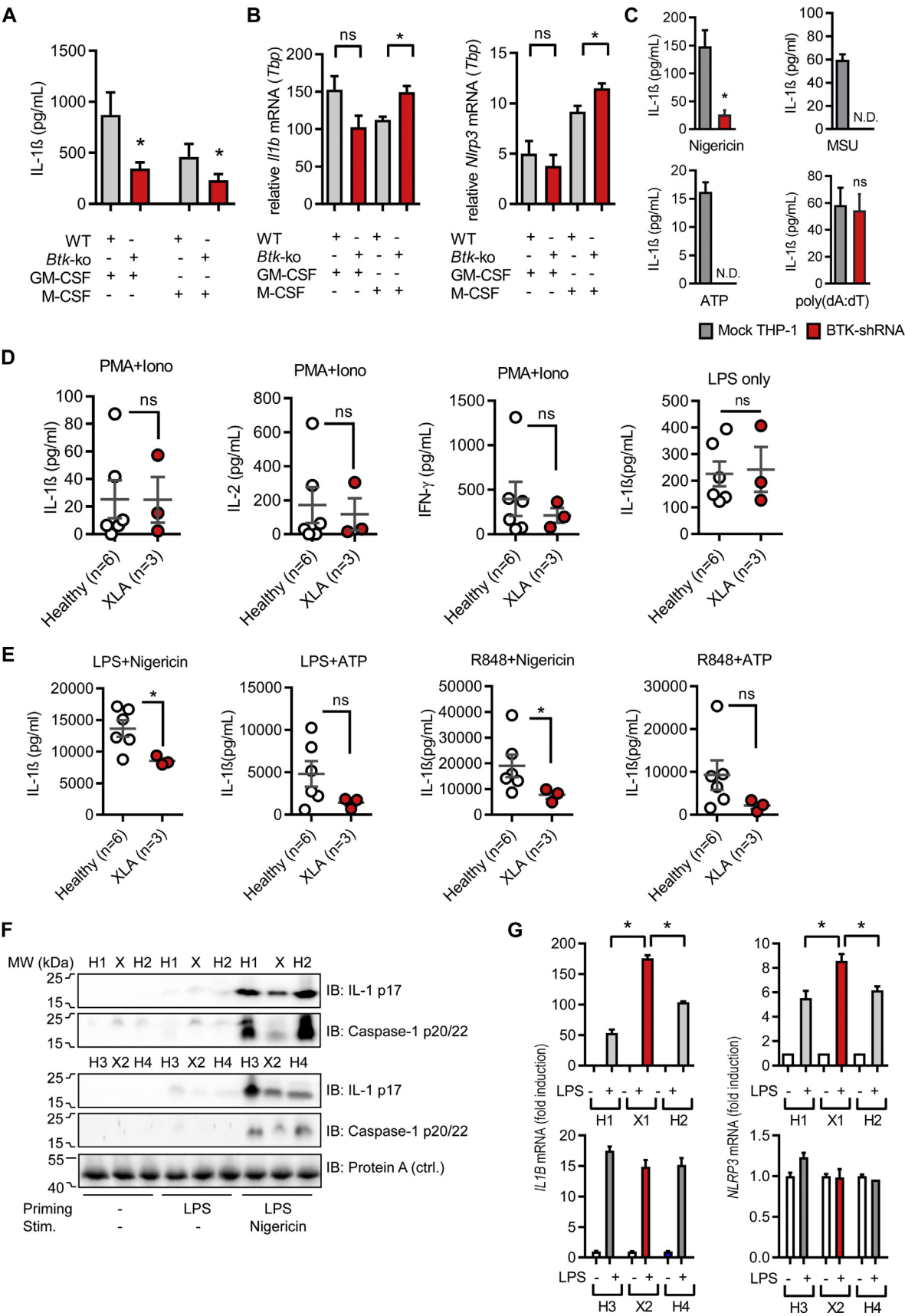
To assess whether such BTK activation translated to an effect on inflammasome function, we investigated NLRP3-dependent IL-1 β release. THP-1 cells pretreated with the FDA-approved inhibitor ibrutinib (PCI-32765) responded with reduced IL-1 β release ([Fig 2, A](#)),¹⁹ despite comparable *IL1B* and *NLRP3* mRNA levels ([Fig 2, B](#)). This was confirmed in so-called iGluc THP-1 cells, in which IL-1 β release can be quantified in the form of a Gaussia Luciferase assay ([Fig 2, C](#)).¹⁹ Importantly, in primary MoMacs pharmacologic BTK inhibition strongly decreased IL-1 β levels ([Fig 2, D](#)), whereas the effect on TNF release was minor ([Fig 2, E](#)). Similar results were obtained with an additional specific BTK inhibitor, CGI1746 ([Fig 2, D and E](#)).²⁶ Generally, ibrutinib and CGI1746 did not show cytotoxicity at the concentrations used here, as assessed by means of simultaneous CCK8 viability testing (data not shown). Minor effects of BTK inhibition at the level of *IL1B* and *NLRP3* mRNA ([Fig 2, B](#)) or secreted TNF ([Fig 2, E](#)) suggest that the observed effect related directly to IL-1 β processing rather than priming. Indeed, pro-IL-1 β and caspase-1 processing in primary MoMacs was strongly reduced by both ibrutinib and CGI1746 ([Fig 2, F](#)). Collectively, this pharmacologic approach implicated BTK in NLRP3 inflammasome function.

Genetic ablation of BTK confirms a role in inflammasome activation

To rule out off-target effects and further confirm a role of BTK in NLRP3 inflammasome function, we used genetic ablation of BTK and first analyzed IL-1 β release from bone marrow-derived macrophages (BMDMs) from *Btk* KO mice. Evidently, IL-1 β release ([Fig 3, A](#)), but not *Il1b* or *Nlrp3* mRNA synthesis ([Fig 3, B](#)), was strongly reduced in BMDMs from GM-CSF- and M-CSF-differentiated *Btk* KO versus WT mice. THP-1 cells, in which BTK was constitutively downregulated by shRNA, showed reduced IL-1 release compared with the corresponding mock THP-1 cells in response to the NLRP3-dependent inflammasome stimuli nigericin, monosodium urate, and ATP but not the AIM2 inflammasome stimulus poly(dA:dT), see [Fig 3, C](#). More importantly, we were able to compare PBMCs from healthy donors and matched patients with XLA with genetically and cytometrically confirmed BTK deficiency (see the [Methods](#) section and [Fig E1](#) in this article's Online Repository at www.jacionline.org). PMA plus ionomycin, a non-NLRP3-dependent²⁷ and poorly IL-1 β -inducing stimulus, prompted IL-2 and IFN- γ release from T cells, in which BTK is not expressed and thus is functionally irrelevant ([Fig 3, D](#)),²⁸ thus indicating similar overall cellular viability in PBMC preparations from healthy donors and those with XLA. However, IL-1 β release in response to NLRP3 activation by nigericin was substantially lower in PBMCs of patients with XLA compared with those of healthy donors ([Fig 3, E](#)), irrespective of whether priming conducted with R848 or ATP was used as an alternative NLRP3 trigger ([Fig 3, E](#)). Although differences in IL-1 β levels in response to ATP were not statistically significant because of donor-to-donor variation, a clear trend toward lower IL-1 β levels was clearly discernible. As expected, secreted mature caspase-1 and IL-1 β levels were also reduced in PBMCs from patients with XLA compared with those from healthy donors ([Fig 3, F](#)), even though LPS-primed *IL1B* and *NLRP3* mRNA levels at the time of NLRP3 agonist addition were higher ([Fig 3, G](#)). Thus genetic ablation of BTK activity in both mice and human subjects impairs NLRP3 inflammasome activity and confirms a role for BTK in the NLRP3 inflammasome.

BTK interacts directly with ASC and NLRP3 and promotes inflammasome formation

Given the early phosphorylation of BTK and its effect on downstream caspase-1 cleavage, we speculated whether BTK might directly interact with the core inflammasome components ASC and NLRP3. Indeed, when expressed in HEK293T cells, BTK interacted with both ASC ([Fig 4, A](#)) and NLRP3 ([Fig 4, B](#)) in co-immunoprecipitations. Additionally, overexpression-induced ASC speck formation, a surrogate visual readout for inflammasome formation,²² in HEK293T cells transfected with ASC-green fluorescent protein (GFP) was significantly enhanced by BTK coexpression ([Fig 4, C](#); quantified in [Fig 4, D](#)). Interestingly, a spheroid ASC signal typical for ASC specks was surrounded by a spherical localization of BTK (see [Fig E2, A](#), in this article's Online Repository at www.jacionline.org). In agreement with earlier experiments, ibrutinib ([Fig 4, E and F](#)) and CGI1746 (see [Fig E2, B](#)) reduced ASC speck formation in stably ASC-mCerulean-expressing murine macrophages²² when treated with nigericin or the NLRP3 trigger Leu-Leu-OMe, a lysosomal destabilizing agent.²⁹ Furthermore, the potential for ASC



cross-linking/oligomerization²⁴ in THP-1 cells stably expressing ASC-mCerulean was enhanced by nigericin stimulation but sensitive to ibrutinib (Fig 4, G). Collectively, BTK appears to directly influence inflammasome activation at the level of NLRP3 and ASC.

BTK functionality is required for full *S aureus* toxin-elicited inflammasome activity

BTK-deficient patients with XLA experience recurrent bacterial infection with pathogens, including *S aureus*. Based on the observation that nigericin- and ATP-triggered IL-1 β processing and secretion were impaired in patients with XLA (Fig 3), we wondered whether IL-1 β release induced by the *S aureus* toxins, PVL, and LukAB, which both activate NLRP3 (Fig 5, A, and see Fig E3 in this article's Online Repository at www.jacionline.org),^{30,31} also required BTK. Indeed, BTK inhibition (Fig 5, B) and stable shRNA-mediated *BTK* knockdown (Fig 5, C) in THP-1 cells and ibrutinib treatment in primary MoMacs (Fig 5, D) strongly reduced IL-1 β release in response to PVL and LukAB. Additionally, in LPS-primed PBMCs from patients with XLA, LukAB led to reduced IL-1 β cleavage compared with that seen in healthy donors (Fig 5, E). Because LPS does not occur in gram-positive bacteria and *S aureus* instead activates TLR2,³² the TLR2 agonist Pam₃CSK₄ was also used for priming instead of LPS. Again, TLR2-primed PBMCs from patients with XLA showed reduced levels of cleaved IL-1 β compared with those from healthy donors in response to LukAB (Fig 5, E). These genetic and pharmacologic *in vitro* results suggest that *in vivo* BTK might play a role in NLRP3 inflammasome/IL-1 β -dependent host defense, as well as pathophysiological autoinflammation.

BTK inhibition negatively affects IL-1 β -dependent *S aureus* clearance *in vivo* and blocks IL-1 β release in patients with MWS and ibrutinib-treated patients with cancer *ex vivo*

We next explored the possibility of whether BTK inhibition would affect the outcome of IL-1 β -dependent infection in an *in vivo* setting. Murine experimental models of *S aureus* skin infection dependent on Nlrp3/ASC-inflammasome-dependent IL-1 β for clearance.^{5,33} Therefore we applied ibrutinib treatment to C57BL/6 mice intradermally infected with a bioluminescent community-acquired methicillin-resistant *S aureus* strain (USA300 LAC::lux).³³ Ibrutinib-treated mice showed increased bacterial burden, as measured based on *in vivo* bioluminescent signals (Fig 6, A and B) compared with vehicle-treated control mice. They also had larger skin lesions (Fig 6, C, and see Fig E4 in this article's Online Repository at www.jacionline.org). Although additional *in vivo* effects of ibrutinib cannot be

ruled out, our data suggest a relevance for BTK in inflammasome-related host defense *in vivo*.

Our results thus far posed the question of whether BTK might be a plausible point of therapeutic intervention to target the many human inflammasome/IL-1 β -related inflammatory processes or disorders.⁴ MWS is an autoinflammatory disease caused by gain-of-function mutations in the NLRP3 gene *CIAS* and is characterized by excessive IL-1 β release compared with that seen in healthy donors (see Fig E6 in this article's Online Repository at www.jacionline.org).

To explore whether this phenotype could be ameliorated by BTK inhibition, we triggered IL-1 β release by LPS (no second stimulus required because of NLRP3 autoactivation, see Prota et al³⁴) in PBMCs from 4 patients with MWS in the absence or presence of ibrutinib. As shown in Fig 6, D, LPS-dependent IL-1 β and caspase-1 release was strongly reduced for all assessed patients, and the level of inhibition corresponded to the dose of ibrutinib (Fig 6, E). General toxicity or anti-inflammatory effects were ruled out by using cytokine release and viability tests (data not shown). Thus pharmacologic BTK inhibition blocked excessive IL-1 β release that characterizes the autoinflammatory MWS. To gain a first insight into whether application of ibrutinib in human patients would affect inflammasome activity *in vivo*, we stimulated PBMCs from male patients with cancer receiving ibrutinib daily *ex vivo* and compared their ability to process or release IL-1 β or caspase-1 in response to the NLRP3 triggers nigericin, ATP, or both by means of immunoblotting or ELISA, respectively (Fig 6, G and H), whereas TNF release was comparable (Fig 6, H). Evidently, *in vivo* application of ibrutinib specifically correlated with reduced *ex vivo* IL-1 β and caspase-1 processing and lower IL-1 β release in these patients compared with subjects not receiving ibrutinib. Collectively, our data suggest that BTK inhibitors might block the NLRP3 inflammasome *in vivo* in patients.

DISCUSSION

In the present work we show that BTK is a critical regulator of NLRP3 inflammasome activation in both human subjects and mice. This is illustrated by significant NLRP3 inflammasome loss-of-function phenotypes observed in functionally *BTK*-deficient cells from patients and mice and effects of pharmacologic inhibition of BTK in an *in vivo* *S aureus* infection model, which is congruent with inefficient bacterial control evidenced in human patients with XLA and *Xid* or *Nlrp3*^{-/-} mice.^{12,33} Additionally, our molecular analysis suggests that BTK regulates (and that consequently BTK inhibitors might act on) the inflammasome proximally to NLRP3 and ASC, as evidenced by interaction and colocalization of BTK with these inflammasome components. Furthermore, we

FIG 3. Genetic ablation of BTK in primary immune cells impairs NLRP3-mediated IL-1 β release. **A**, IL-1 β release from equal numbers of GM-CSF- or M-CSF-differentiated, LPS-primed, and nigericin-treated WT or *Btk* KO BMDMs (means + SEMs). **B**, quantitative RT-PCR (mean + SEM) before nigericin addition. **C**, IL-1 β release from primed THP-1 cells expressing either a nontargeting (mock) or BTK shRNA. **D** and **E**, IL-1 β , IL-2, and IFN- γ release from LPS-primed PBMCs from male patients with XLA and age-matched male healthy donors (mean $\pm$ SEM of biological replicates. Each symbol represents 1 donor. **F** and **G**, Supernatant immunoblot (Fig 3, F) or quantitative RT-PCR relative to *TBP* (mean + SD; Fig 3, G) of the stimulated PBMCs. Pooled data from 3 mice (biological replicates) per group (1 of 2 identical experiments) in Fig 3, A and B, and from 6 versus 3 donors (biological replicates; mean + SEM in gray) in Fig 3, D and E, are shown, respectively. In Fig 3, C, 1 representative of 2 experiments and in Fig 3, F and G, 2 versus 4 donors are shown, respectively. In Fig 3, A-C, G, and H, the Student *t* test and in Fig 3, D and E, a Mann-Whitney *U* test was used. **P* < .05. N.D., Not detected; ns, not significant.

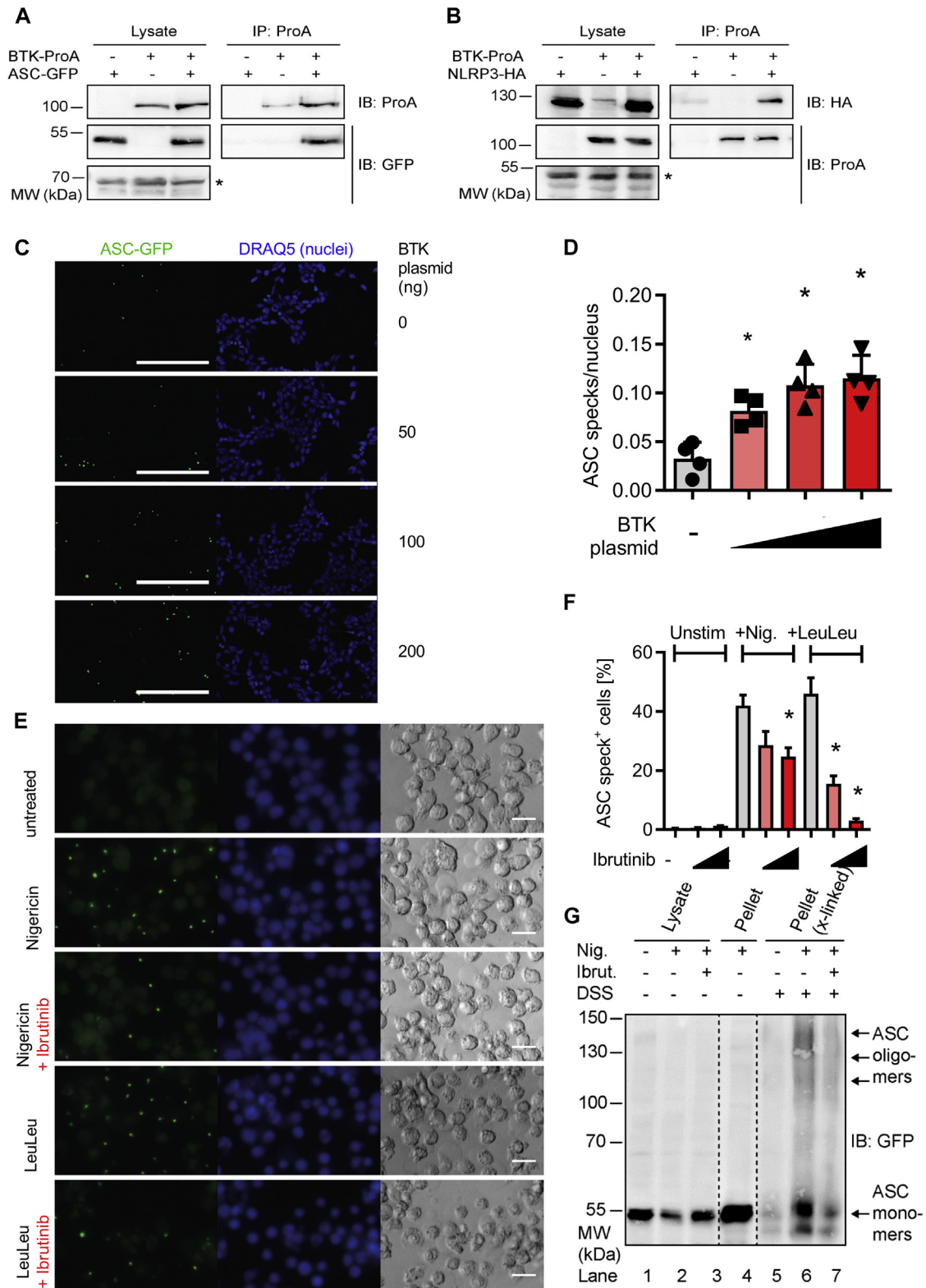


FIG 4. BTK directly interacts with ASC and NLRP3 and promotes inflammasome formation. **A** and **B**, Immunoblot (IB) of transfected HEK293T cells. *Nonspecific loading control. **C** and **D**, Confocal microscopy of fixed and stained transfected HEK293T cells (scale bar = 200 μ m; Fig 4, **C**) and quantification thereof (Fig 4, **D**). **E** and **F**, Representative fluorescence microscopy images of ASC specks (Fig 4, **E**) and quantification thereof (Fig 4, **F**) from immortalized *Nlrp3* KO macrophages overexpressing NLRP3-FLAG ASC-mCerulean. Scale bar = 20 μ m. **G**, DSS cross-linking of ASCs from primed and nigericin-treated THP-1-ASC-mCerulean cells. In Fig 4, **A-D**, 1 of 2 and in Fig 4, **E** and **G**, 1 of 3 identical experiments is shown. Fig 4, **F**, shows the combined analysis of 3 experiments. In Fig 4, **D** and **F**, the Student *t* test was used. **P* < .05.

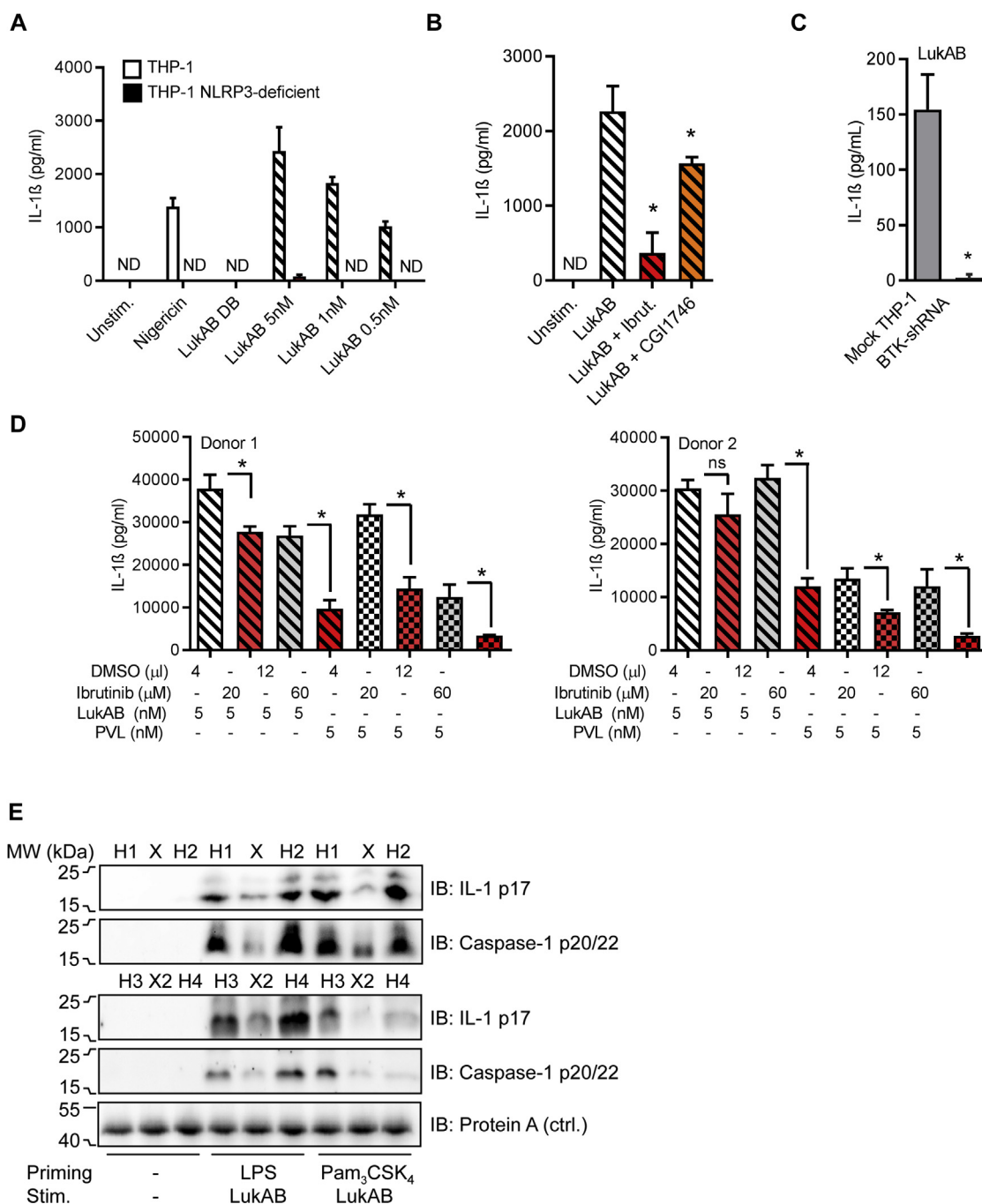
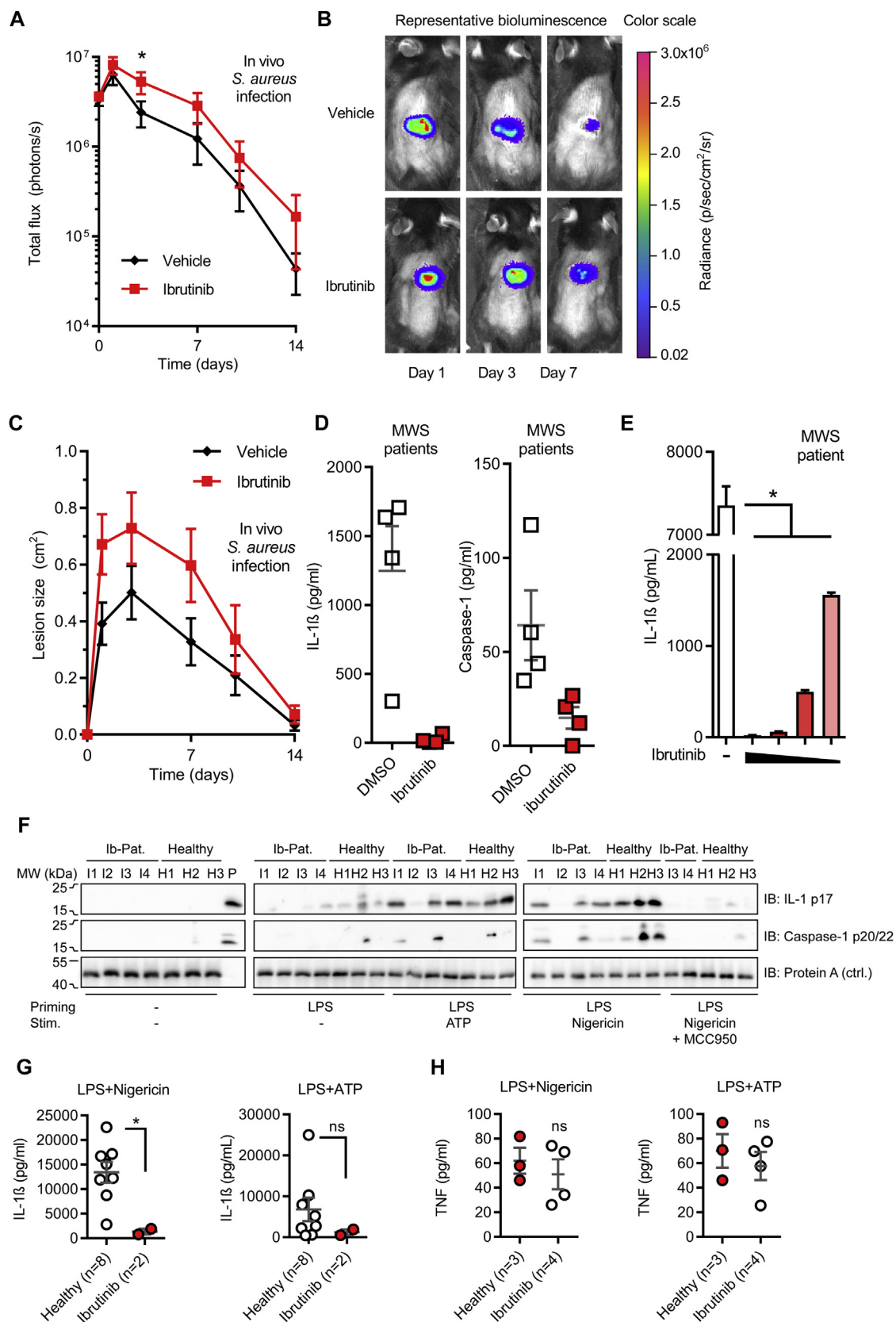


FIG 5. BTK functionality is required for full *S aureus* toxin-elicited inflammasome activity. **A** and **B**, IL-1 β release from PMA-differentiated null (control; Fig 5, **A** and **B**) or NLRP3-deficient (Fig 5, **A**) THP-1 inhibitor-pretreated (Fig 5, **B**, only), with stimulation as indicated. **DB**, LukAB dialysis buffer. **C**, Primed THP-1 cells expressing either a nontargeting (mock) or BTK shRNA were stimulated as indicated. **D**, Primed human primary MoMacs analyzed as in Fig 2, **E** and **F**, but treated with LukAB or PVL. **E**, Supernatant immunoblot of LPS- or Pam₃CSK₄-primed PBMCs from male patients with XLA and age-matched male healthy donors. In Fig 5, **A-C**, 1 of 2 and in Fig 5, **D** and **E**, 2 of 2 identical experiments are shown. In all graphs means + SDs are shown, and the Student *t* test was used. **P* < .05. ND, Not detectable; ns, not significant.

demonstrate that in human primary macrophages (as well as their murine counterparts), BTK inhibition impaired IL-1 β processing and release. In blood samples from patients with MWS, BTK inhibition strongly reduced this pathologically relevant inflammatory event. Notably, reduced IL-1 β secretion was also observed in PBMCs from patients receiving ibrutinib *in vivo*.

These findings represent considerable advances regarding our understanding of the NLRP3 inflammasome, its therapeutic tractability, and BTK-related immunodeficiency, which warrant further discussion.

First, our data significantly expand on the thus far known roles of BTK and highlight that BTK not only critically contributes to



adaptive immunity but is in fact also a key player in innate immunity. Although few reports described a role for BTK in TLR^{35,36} and Fc receptor signaling,²⁶ recruitment,³⁷ and development¹³ in myeloid cells, the abovementioned insights were almost exclusively gained for the murine system or human cell lines. Whether BTK played a prominent role in human innate immunity thus remained somewhat unaddressed. It is also for the murine system that Ito et al²⁰ recently provided a first glimpse into a possible role for BTK in inflammasome activation. In this study BTK loss or inhibition in murine cells reduced IL-1 β release. Our study in primary cells from healthy volunteers, patients with XLA, patients with MWS, and ibrutinib-treated patients now provides evidence that BTK is indeed a key regulator of the NLRP3 inflammasome in human subjects. How the observed residual amounts of IL-1 β released from PBMCs from patients with XLA (Fig 3, D and E) can be reduced further (Fig 6, F, and see Fig E5 in this article's Online Repository at www.jacionline.org) by an NLRP3 inhibitor with unknown target, MCC950,³⁸ warrants further exploration. It seems plausible that the BTK mutations identified in the patients studied here (see the Methods section in this article's Online Repository) display low residual activity³⁹ or expression of BTK that is sufficient for low inflammasome activity but too low to support the development of B cells, thus leading to clinical XLA; alternatively, BTK might only be required for one of potentially several NLRP3 inflammasome pathways.⁴⁰

Nevertheless, our study supports the notion that BTK is a master regulator of myeloid cell functions spanning the entire functional spectrum from development through initial pathogen recognition by TLRs to initiation of inflammation through the NLRP3 inflammasome to postadaptive functions involving the response to antibodies bound through Fc receptors. The versatility of BTK to participate in these diverse processes is staggering and clearly warrants further exploration, not least to investigate cross-talk between the different signaling pathways, as well as to closely define how pharmacologic inhibition of BTK affects these different BTK-dependent immune functions.

Indeed, this is necessary to explore potential therapeutic opportunities that could be deduced from the observation that BTK inhibitors reduce NLRP3-dependent IL-1 β release. Human macrophages, which can be effectively blocked in their ability to release IL-1 β , as shown here, have been seen as primary mediators in many inflammatory disorders. For example, in experimental mouse models for atherosclerosis, cholesterol crystals contribute to disease progression in a macrophage/IL-1-dependent manner.⁴¹ Similarly, in Alzheimer models amyloid- β -triggered neuroinflammation depends on NLRP3.⁴² Targeting the IL-1 axis through BTK can have

considerable advantages over previously proposed strategies: for example, although proposed inhibitors, such as MCC950,³⁸ are highly effective, the precise mechanism of action and the actual molecular target seem unclear at current. Biological-based anti-IL-1 therapies, though proved and FDA approved, target inflammasome-distal events. Targeting the inflammasome through BTK might eventually overcome some of these disadvantages by focusing on a well-studied and well-defined molecular target, BTK, and an NLRP3 inflammasome (and thus caspase/IL-1 β -processing) proximal event. Use of ibrutinib in treating human patients with MWS (Fig 6), acute stroke (as hinted to by Ito et al²⁰), or atherosclerosis should be considered and is encouraged by the observed good tolerability and efficacy of BTK inhibitors in the cancer setting,^{10,11} primary human immune cells (Figs 2-6), and even human platelets, as suggested by our most recent study.⁴³ The data shown in Fig 6, F and G, suggest that BTK inhibition can be considered for blocking inflammasome activity *in vivo*. Even if administration would have to be temporary (eg, to avoid adverse effects from infection, see below), a temporary blockade of the IL-1 axis might help break a vicious cycle of chronic inflammation.

For effective therapeutic exploitation, additional details need to be clarified. First, as with any pharmacologic inhibitor, off-target effects (ie, the possibility of other kinases apart from BTK being affected by ibrutinib or other "BTK" inhibitors) cannot be entirely ruled out and require further investigation. Second, although a direct interaction with NLRP3 and ASC has been demonstrated by us (Fig 4), it remains to be explored how exactly BTK participates in the inflammasome activation process. Ito et al²⁰ rule out an effect of Ca²⁺ signaling, and by measuring additional cytokine or transcript levels, we can rule out that BTK ablation primarily affects caspase-1 or IL-1 β cleavage or release through TLR-dependent priming, although this would have been conceivable because of the role of BTK in TLR signaling.³⁵ The SH2 and SH3 domains found in BTK are well-known motifs for protein-protein interactions, so that BTK might act as a scaffold protein for inflammasome nucleation or extension. However, because ibrutinib targets BTK kinase activity, it seems unlikely that BTK only acts as a molecular scaffold but rather participates as a kinase, possibly at the level of ASC phosphorylation.²⁰ The verification of ASC as a BTK interactor and substrate should be the subject of future studies.

Another important question to be addressed is what links the exposure of cells to upstream NLRP3 agonists with BTK activation. Studying the role of the pleckstrin homology domain, a common motif for receptor/membrane engagement, or different reported gain- or loss-of-function mutants, such as the *Xid* loss-of-function mutation (R28C), in the assays described here

FIG 6. BTK inhibition negatively affects IL-1 β -dependent *S aureus* clearance *in vivo* and blocks excessive IL-1 β release in patients with MWS *ex vivo*. **A** and **C**, Mean $\pm$ SEM total flux (photons/s; Fig 6, A) and mean $\pm$ SEM total lesion size (in square centimeters; Fig 6, C) from C57BL/6 mice ($n = 10$ per group) treated with vehicle or ibrutinib and inoculated intradermally with bioluminescent methicillin-resistant *S aureus* USA300 LAC::lux. **B**, Representative *in vivo* bioluminescent signals. **D**, LPS-induced IL-1 β or caspase-1 release from LPS-stimulated PBMCs³⁴ from a patient with MWS. **E**, Ibrutinib was titrated from 60 to 30 to 15 to 7.5 μ mol/L for 2 patients with MWS; 1 patient is shown (mean $\pm$ SD). **F-H**, IL-1 β , caspase-1, TNF, and/or IL-6 cleavage and/or release (mean $\pm$ SEM of biological replicates, each symbol represents 1 donor) from PBMCs from male patients with cancer receiving daily ibrutinib and male healthy donors. **P**, Blotting control. Pooled data from 4 versus 3 donors in Fig 6, F, and from 8 versus 2 donors in Fig 6, G, or 3 versus 4 donors in Fig 6, H, are shown (means $\pm$ SEMs of biological replicates, each symbol represents 1 donor). In Fig 6, A, C, G, and H, the Mann-Whitney *U* test; in Fig 6, D, the Wilcoxon matched-pairs signed-rank test; and in Fig 6, E, the Student *t* test was used. **P* < .05. ns, Not significant.

might shed light on these important mechanistic questions in the future.

BTK acting as a novel inflammasome component would make any genetic BTK insufficiency also a genetically determined functional NLRP3 inflammasome deficiency. Thus our work supports the notion that the BTK-mediated human XLA phenotype might represent the first reported genetic functional NLRP3 inflammasome deficiency in human subjects. Given that *S aureus* toxin-mediated release is BTK dependent (Fig 5) and that BTK-dependent IL-1 β production is required for bacterial clearance in skin *S aureus* infection in mice (Fig 6, A-C),³³ human inflammasome-deficient patients would be expected to show a susceptibility to pyogenic bacteria, such as *S aureus*. This is indeed a clinical feature in patients with XLA.^{12,14} It can be speculated whether high-dose IVIG and antibiotic therapy applied routinely to patients with XLA might mask a simultaneous NLRP3 inflammasome deficiency contributing to bacterial susceptibility in patients with XLA. Of note, a certain fraction of patients in ongoing clinical trials with ibrutinib show moderate-to-severe infections with the same pathogens frequently encountered in patients with XLA despite unaltered antibody levels (ie, a remaining adaptive/humoral defense component).^{10,11} Potentially, this temporary bacterial susceptibility conferred by BTK inhibition might be significantly attributable to temporary inflammasome inhibition. Further studies should address this possibility in ongoing clinical trials with ibrutinib and explore the notion of a potential inflammasome deficiency to contribute to immunodeficiency in patients with XLA.

Collectively, unexpectedly, the data presented here imply that in human subjects and mice 2 protagonists of innate and adaptive immunity, NLRP3 and BTK, cooperate in activation of the inflammasome, an innate defense mechanism that is critical for activation of adaptive immunity. Our data in human primary cells provide a rationale for further investigations at the molecular level and for exploring therapeutic implications. Targeting the NLRP3 inflammasome at the level of BTK might bring inflammasome targeting unexpectedly within reach if larger clinical studies confirm the observed effects and FDA approval was extended beyond B-cell malignancies. Thus the growing number of BTK inhibitors developed for the treatment of hematologic malignancies might be interesting for clinicians working on the many reported IL-1 β -driven inflammatory diseases to follow to glean insights into how to possibly apply BTK inhibition to their clinical settings.

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Clinical implications: Based on our results, it could be speculated that the NLRP3 inflammasome, which contributes to inflammatory pathologies in human subjects, could be targeted with BTK inhibitors.

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METHODS

Reagents

Nigericin, LPS, PMA, and ionomycin were from InvivoGen; ATP was from Sigma; ibrutinib and CGI1746 were from Selleckchem; recombinant GM-CSF for monocyte differentiation was from PeproTech; Ficoll was from Merck Millipore; and anti-CD14-PE was from BD. Antibodies for immunoblots were as follows: caspase-1 (D7F10) antibodies were from Cell Signaling Technology (Danvers, Mass), ASC (F-9) was from Santa Cruz Biotechnology (Dallas, Tex), IL-1 β was from R&D Systems (Minneapolis, Minn), and BTK was from BD Biosciences. For flow cytometry, anti-CD11b-APC and anti-CD14-PE were from ImmunoTools, anti-CD19-Pacific Blue was from BioLegend, and anti-CD3-FITC, anti-Btk (pY551)-PE, and anti-Btk (pY223)-Alexa Fluor 647 were from BD Biosciences. For co-immunoprecipitation experiments, all primary antibodies were purchased from Sigma-Aldrich: anti-HA (H9658) and anti-GFP (G1544) both used 1:5000 and anti-Protein A (P3775, dilution 1:62,500). Secondary horseradish peroxidase-coupled antibodies were anti-mouse (PA1-86015; Thermo Fisher Scientific) and anti-rabbit (5127; Cell Signaling Technology). Anti-ASC (Santa Cruz Biotechnology sc-271054) was used for DSS-crosslinking. Recombinant protein was from Thermo Fisher Scientific.

Study subjects and sample acquisition

All human subjects provided written informed consent in accordance with the Declaration of Helsinki, and the study was approved by the local ethics committees. Buffy coats from healthy donors were provided by the Tübingen University Hospital Transfusion Medicine Department. Male patients with XLA with confirmed genetic and clinical BTK deficiency were recruited at the Center for Immunodeficiency at Freiburg University Hospital. Male healthy donors in a similar age range were recruited at the Department of Immunology, Tübingen, and blood was taken on the same day as that from patients with XLA. Samples were processed in the same way and measured together. Patients with MWS were recruited at the Pediatrics Department of the University Hospital Tübingen, as previously described.^{E1} Patients are described in detail below.

Isolation and stimulation of primary immune cells

PBMCs were isolated from whole blood or buffy coats by using Ficoll density gradient purification and washed 3 times with RPMI to remove residual platelets and then seeded in RPMI-1640 (Sigma), 10% FBS (GE Healthcare, Fairfield, Conn), 2 mmol/L L-glutamine (Life Technologies), and 1% Pen-Strep (Life Technologies). PBMCs were isolated from peripheral blood of 3 patients with XLA and 6 healthy donors. Cells were then treated with 10 ng/mL LPS for 3 hours and 15 μ mol/L nigericin for 1 hour or instead with 50 ng/mL PMA and 1 μ mol/L ionomycin for 4 hours. PBMCs from patients with MWS were seeded at a concentration of 1×10^6 cells/mL in 24-well tissue culture plates. Cells were then treated with 10 ng/mL LPS, 1 mmol/L ATP concomitantly with 60 μ mol/L ibrutinib, or a DMSO control for 4 hours, and supernatants were collected for ELISA. For macrophage differentiation, monocytes were purified by means of positive selection from PBMCs with anti-CD14 magnetic beads (>90% purity assessed by using the anti-CD14-PE flow cytometry; Miltenyi Biotec) and seeded at a concentration of 1×10^6 cells/mL in 96-well tissue-culture plates. Cells were differentiated into macrophages in the presence of 25 ng/mL recombinant human GM-CSF for 5 days.^{E2} MoMacs were then primed with 300 ng/mL LPS for 3 hours and pretreated with ibrutinib at 20 or 60 μ mol/L for 10 minutes before stimulation with 15 μ mol/L nigericin or indicated amounts of LukAB or PVL for 1 hour. In all cases supernatants were then collected for ELISA.

Plasmid constructs

ASC, NLRP3, and BTK coding sequences in pENTR clones were from the genomics core facility at DKFZ Heidelberg (Heidelberg, Germany). The inserts were transferred into pDEST plasmids containing N-terminal streptavidin-hemagglutinin (NLRP3; T. Bürckstümmer, CeMM, Vienna,

Austria, and M. Gstaiger, ETH Zurich, Zurich, Switzerland) and C-terminal Protein A (M. Kögl, DKFZ Heidelberg) or GFP tags (Stefan Pusch, Neuropathology, Heidelberg University) by LR Gateway cloning (Invitrogen, Carlsbad, Calif). Correct transfer was checked by means of restriction digest and DNA sequencing.

Cell culture

HEK293T cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μ g/mL; all from Life Technologies). THP-1 (ATCC) cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μ g/mL; all from Life Technologies). THP-1-null (stably expressing a nontargeting shRNA) and NLRP3-deficient (stably expressing an NLRP3-targeting shRNA, both from InvivoGen) were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), streptomycin (100 μ g/mL; all from Life Technologies), sodium pyruvate (1 mmol/L; from Invitrogen), HEPES buffer (10 mmol/L; from Sigma), Normocin (100 μ g/mL; from InvivoGen), and Hygromycin B (100 μ g/mL; from Invitrogen). iGluc THP-1 cells were a kind gift of V. Hornung, Institute of Molecular Medicine, Munich, Germany, and cultured as previously described.^{E3} BTK-shRNA- and mock control-THP-1 cells were a kind gift of R. Morita, Keio University School of Medicine, Tokyo, Japan, and were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μ g/mL; all from Life Technologies) in the presence of 1.5 and 2 mg/mL G418, respectively. ASC-mCerulean-expressing immortalized macrophages or THP-1 cells were described previously.^{E4,E5} All cell lines and primary cells were cultured at 37°C and 5% CO₂.

Mice and generation of BMDM

Btk KO mice have been described previously.^{E6} Btk KO and wild-type littermates (all C57BL/6 background) were used at an age of 8 to 12 weeks. In brief, bone marrow cells were isolated from femurs and tibiae by using standard procedures (details available on request) and 3×10^6 cells/mL plated in 10-cm non-tissue culture-coated dishes in 10 mL of complete RPMI media containing 10% GM-CSF (M1 polarization)- or M-CSF (M2 polarization)-conditioned medium for 5 to 7 days. Cells were always counted and reseeded before *in vitro* assays to ensure equal cell numbers. *Ex vivo* animal experiments (Fig 3, A and B) were in accordance with institutional guidelines and German animal protection laws. *In vivo* infection experiments (Fig 6) were approved by the Johns Hopkins University Animal Care and Use Committee (ACUC protocol no. MO15M421). C57BL/6 female mice at 8 weeks of age were obtained from Jackson Laboratories (Bar Harbor, Me). All mouse colonies were maintained in specific pathogen-free conditions.

Mass spectrometry

THP-1-null cells (Invitrogen) were grown in "light" (L-lysine/Lys0, L-arginine/Arg0), "medium-heavy" (D4-L-lysine/Lys4, ¹³C6-L-arginine/Arg6), and "heavy" (¹³C₆¹⁵N₂-L-lysine/Lys8, ¹³C₆¹⁵N₄-arginine/Arg10) SILAC medium for 3 passages. Incorporation of the labeled amino acids was in each case confirmed to be greater than 97%. For the experiment, cells were primed with 300 ng/mL PMA for 3 hours and left to rest overnight. The next day, the cells were detached and either left unstimulated (light) or stimulated with 15 μ mol/L nigericin for 5 (medium) or 10 (heavy) minutes. After washing with ice-cold PBS (containing phosphatase and protease inhibitors, Roche), cell pellets were snap-frozen and stored at -80°C before analysis. For phosphopeptide enrichment, cells were lysed in denaturation buffer (6 mol/L urea and 2 mol/L thiourea in 10 mmol/L Tris buffer, pH 8.0), and DNA was removed by means of addition of benzonase.

After determining the amount of protein in the lysates by using a Bradford assay, 3 mg of protein of each condition were pooled, and the mixture was digested in solution with trypsin. The resulting peptide mixture was subjected to phosphopeptide enrichment, as described previously,^{E7} with minor

modifications: peptides were separated by using strong cation exchange (SCX) chromatography with a gradient of 0% to 35% SCX solvent B, resulting in 8 fractions that were subjected to phosphopeptide enrichment by titanium dioxide beads. Elution from the beads was performed 3 times with 100 μ L of 40% ammonia hydroxide solution in 60% acetonitrile (pH > 10.5). Peptide-rich fractions were subjected to titanium dioxide enrichment multiple times. Enrichment of phosphopeptides from the SCX flowthrough was done in 5 cycles. liquid chromatography–tandem mass spectrometry analyses were performed on an EasyLC nano-HPLC coupled to an LTQ Orbitrap XL (Thermo Scientific), as described previously.^{E8} The peptide mixtures were injected onto the column in HPLC solvent A (0.5% acetic acid) at a flow rate of 500 nL/min and subsequently eluted with a 127-minutes (phosphoproteome) segmented gradient of 5–33–90% HPLC solvent B (80% ACN in 0.5% acetic acid). During peptide elution the flow rate was kept constant at 200 nL/min. The 5 most intense precursor ions were fragmented by means of multistage activation of neutral loss ions at -98 , -49 , and -32.6 Th relative to the precursor ion.^{E9} Sequenced precursor masses were excluded from further selection for 90 seconds. Full scans were acquired at resolution of 60,000 (Orbitrap XL, Thermo Fisher Scientific). The target values were set to 5000 charges for the LTQ (MS/MS) and 10^6 charges for the Orbitrap (MS), respectively; the maximum allowed fill times were 150 ms (LTQ) and 1000 ms (Orbitrap). The lock mass option was used for real-time recalibration of MS spectra.^{E7}

MS data were processed by using default parameters of MaxQuant software (v1.2.2.9).^{E10} Extracted peak lists were submitted to database search by using the Andromeda search engine^{E11} to query a target-decoy^{E12} database of *Homo sapiens* proteome (downloaded from Uniprot on December 25, 2012), containing also 248 commonly observed contaminants. In a database search full tryptic specificity was required, and up to 2 missed cleavages were allowed. Carbamidomethylation of cysteine was set as fixed modification; protein N-terminal acetylation, oxidation of methionine, and phosphorylation of serine, threonine, and tyrosine were set as variable modifications. Initial precursor mass tolerance was set to 6 ppm at the precursor ion and 0.5 Da at the fragment ion level. False discovery rates were set to 1% at the peptide, phosphorylation site, and protein group levels. Details regarding other phosphopeptides with nigericin-dependent are available on request.

ELISA

IL-1 β , IL-2, TNF, and IFN- γ levels in supernatants were determined with half-area plates by ELISA (BioLegend) with triplicate points on a standard plate reader.

Quantitative RT-PCR

mRNA was isolated with the RNeasy Mini Kit on a Qiacube robot (both from Qiagen) transcribed to cDNA (High Capacity RNA-to-cDNA Kit; Life Technologies), and *IL1b*, *Nlrp3*, *IL1*, and *NLRP3* mRNA expression was quantified relative to that of *TBP* by using TaqMan primers (Life Technologies) on a real-time cycler (7500 fast; Applied Biosystems), as described by Wang et al.^{E13} Comparable cycle threshold values for *TBP* (data not shown) in all treatment groups confirmed equal cell numbers.

ASC speck formation assay and confocal microscopy

HEK293T cells (4×10^4) were plated (24-well format; Greiner Bio-One, Monroe, NC) and transiently transfected as indicated. Forty-eight hours later, cells were fixed with 100% methanol, nucleic acids were stained with TO-PRO-3 (Thermo Fisher), and BTK-HA was stained with anti-HA–Alexa 594–conjugated antibodies. Samples were analyzed with a Zeiss LSM 510 confocal microscope (Zeiss, Oberkochen, Germany) under the $\times 20$ objective. Excitation and emission wavelengths are available on request. Nuclei and ASC-GFP particle counting and analysis were performed with ImageJ software (National Institutes of Health, Bethesda, Md), with further details on request. Immortalized *Nlrp3* KO macrophages overexpressing NLRP3-FLAG and ASC-mCerulean were pretreated with ibuprofen or solvent

control for 10 minutes before stimulation (or with CG1746 or solvent control) for 60 minutes before stimulation with either 5 μ mol/L nigericin (Life Technologies) or 1 mmol/L Leu-Leu-OMe-HCl (Chem-Impex) for 90 minutes. After stimulation, cells were fixed with 4% formaldehyde and nucleic acids were stained with DRAQ5 (eBioscience). Cells were imaged with a Zeiss Observer.Z1 epifluorescence microscope by using a $\times 20$ objective, as previously described.^{E5} Numbers of cells and specks were counted for 10 images per condition by using CellProfiler.^{E14}

Pro-IL-1 β and caspase-1 cleavage

Equal numbers of cells were primed with PMA (100 ng/mL, InvivoGen) overnight or LPS (300 ng/mL, InvivoGen) for 3 hours and then stimulated with indicated stimuli in Opti-MEM (Gibco) to monitor caspase-1 and pro-IL-1 β processing. Protein in supernatants was precipitated by using methanol (VWR International) and chloroform (Sigma). Where applicable, recombinant Protein A was added before precipitation as a control. Where applicable, cell fractions were lysed in RIPA buffer with protease inhibitors (Sigma). Fifteen percent and 12% SDS-PAGE gels were used for protein from supernatants and whole-cell lysates, respectively, and probed with the indicated antibodies.

Co-immunoprecipitation

For co-immunoprecipitations, 1.5×10^6 HEK293T cells were plated in 10-cm dishes (Greiner Bio-One) and 2 to 6 hours later were transfected with a calcium phosphate precipitation method with 5 μ g of appropriate plasmids. Cells were lysed 48 hours later in a buffer (50 mmol/L Tris [pH 8], 280 mmol/L NaCl, 0.5% NP-40, 0.2 mmol/L EDTA, 2 mmol/L EGTA, 10% glycerol, and 1 mmol/L dithiothreitol) with protease/phosphatase inhibitors (Roche). Cleared lysates were stored for immunoblot analysis. The remainder were subjected to immunoprecipitation of the BTK–Protein A fusion protein, 30 μ L of lysis buffer–equilibrated Dynabeads (M-280 Sheep Anti-Mouse IgG, Thermo Fisher Scientific) were incubated with lysates at 4°C for 3 hours. After washing of precipitated protein complexes 4 times, samples were boiled in Invitrogen's NuPAGE 4 $\times$ LDS loading dye supplemented with 10 $\times$ Sample Reducing Agent and applied for SDS-PAGE and immunoblotting.

Crosslinking of ASC oligomers

THP-1 null or ASC-mCerulean cells (2×10^6 ; Veit Hornung, Gene Center, Munich, Germany) were plated in a 6-well format (Greiner Bio-One), primed with 100 ng/mL PMA overnight or with 300 ng/mL LPS for 2 hours, respectively, and then treated with 60 μ mol/L ibuprofen for 1 hour and 15 μ mol/L nigericin for 1 hour, unless otherwise stated. After washing with PBS, cells were lysed at 4°C in 100 μ L of buffer^{E15} containing 20 mmol/L HEPES (pH 7.4), 100 mmol/L NaCl, 1% NP-40, and 1 mmol/L sodium orthovanadate and supplemented with protease inhibitors (Roche) and Benzonase (Sigma-Aldrich). Lysates were cleared (16,000g at 4°C for 15 minutes) and used for immunoblot analysis. Pellets were resuspended in 250 μ L of PBS and subjected to 2 mmol/L DSS (Thermo Scientific) for 1 hour at room temperature. Crosslinked samples were spun (16,000g, at 4°C for 15 minutes), and the pelleted fraction was resuspended and boiled in Invitrogen's NuPAGE LDS loading dye supplemented with Sample Reducing Agent and applied for immunoblotting.

Flow cytometry and phospho-flow

For whole-blood analysis of healthy donors and patients with XLA, 200 μ L of whole blood from patients and healthy control subjects was stained with a mix of antibodies detecting cell surface antigens (anti-CD3–FITC, anti-CD19–Pacific Blue, anti-CD14–PE, and anti-CD11b–APC) for 30 minutes at room temperature in the dark. Samples were then fixed and permeabilized (Lyse/Fix Buffer, BD Biosciences) for 20 minutes, washed, and resuspended in 200 μ L of PBS 0.5% BSA for analysis (BD Fortessa). A standardized protocol and identical flow cytometer settings were used for all donors. Further settings are available on request. For phospho-flow analysis, the indicated primed cells were treated and then fixed (Lyse/Fix Buffer, BD

Biosciences). For phosflow analysis, the indicated primed cells were treated and then fixed (Lyse/Fix Buffer, BD Biosciences). LIVE/DEAD Fixable Aqua was used to stain dead cells (Life Technologies). Cells were permeabilized with 1 mL of cold methanol, Fc receptors were blocked (Human AB serum), and cells were stained with antibodies against anti-Btk (pY551) PE, anti-Btk (pY223) BV421, and anti-total BTK Alexa Fluor 647 (all from BD Biosciences). Corresponding isotype controls were from ImmunoTools.

S aureus strains

For experiments in mice, a modified USA300 strain, LAC::lux,^{E16} which possesses a modified *luxABCDE* operon from *Photobacterium luminescens* stably integrated into the bacterial chromosome that was transduced from bioluminescent strain Xen29 (PerkinElmer) was used. Live and metabolically active USA300 LAC::lux bacteria constitutively emit a blue-green light, which is present in all progeny. USA300 strain LAC::lux was streaked onto a tryptic soy agar plate (tryptic soy broth plus 1.5% bacto agar, BD Biosciences) and grown overnight at 37°C in a bacterial incubator. Single colonies were picked and placed into tryptic soy broth and grown overnight in shaking culture in a 37°C shaker at 240 rpm. After overnight culture (18 hours), a 1:50 subculture in tryptic soy broth was prepared for 2 hours at 37°C to achieve midlogarithmic phase bacteria. Bacteria were pelleted, resuspended in sterile PBS, and washed 3 times. Absorbance (A_{600}) was measured to estimate the number of CFU, which was verified after overnight culture on tryptic soy agar plates.

In vivo infection model

Ibrutinib (6 mg/kg, Selleckchem) in 3% DMSO (Sigma-Aldrich) and 5% corn oil (Sigma-Aldrich) in PBS or vehicle control was injected intravenously through the retro-orbital vein in anesthetized mice on days -1, 0, and 1. In addition, mice received 1 application of ibrutinib (6 mg/kg) in 10 μ L of DMSO or vehicle control on day 2. For infection, the dorsal backs of anesthetized mice (2% isoflurane) were shaved and injected intradermally with 3×10^7 CFU of *S aureus* LAC::Lux: in 100 μ L of PBS by using a 29-gauge insulin syringe on day 0. Digital photographs were taken on days 1, 3, 7, 10, and 14, and total lesion size (in square centimeters) measurements were analyzed with Image J software (<http://imagej.nih.gov/ij/>) and a millimeter ruler as a reference.

Quantification of *in vivo* *S aureus* bioluminescent imaging

Mice were anesthetized (2% isoflurane), *in vivo* bioluminescent imaging was performed (Lumina III IVIS, PerkinElmer), and total flux (photons/s) within a circular region of interest measuring 1×10^3 pixels was measured by using Living Image software (PerkinElmer; limit of detection, 2×10^4 photons/s).

Purification of LukAB and PVL

The pQE30 vector (Qiagen) was used to produce recombinant His-tagged LukS-PV, LukF-PV, LukA and LukB (the 2 single components of PVL and LukAB, respectively) by using the following oligonucleotides: LukF (Bam-lukF forward: GGGGGGATCCTCCAATACACTTGATGCAGCT and PstlukF reverse: GCGCCTGCAGTCTATCTGTTAGCTCATAGGATT); LukS (BamHluk-S1: GGGGGGATCCAAAGCTGATAACAATATTGAGAA and Pstluk-S2: GGGGCTGCAGTCAATTATGCTCTTTCACCT); LukB (PstblhA forward: BCGCGCTGCAGGGCAACTTTTATTACTTATTCTT and BamblhA reverse: CCCCGGATCCCCAGCTACTTTCATTTGCAAA GATT); and LukA (Pstblh-S1: CCCCTGCAGCGCCCTTTCAATATTA TCCT and BamHblh-S2: CCCCGGATCCAATTGAGCTCATAAGACTCT CAA). Primers were chosen to omit the region predicted to encode the signal peptide. PCR fragments were cloned into the *BamHI*-*PstI* cloning site of pQE30. Plasmids were transformed in *Escherichia coli* BL21 and verified by means of sequencing. LukS-PV, LukF-PV, and LukA proteins were purified by means of affinity chromatography on Ni-NTA columns (Qiagen) under native conditions according to the instructions of the manufacturer (Qiagen)

by using 50 mmol/L NaH_2PO_4 , 300 mmol/L NaCl, 250 mmol/L imidazole, and 20% glycerol for elution. LukB was purified under denaturing conditions from inclusion bodies.

Briefly, inclusion bodies were resuspended in 6 mol/L guanidine HCl, 20 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, and 20% glycerol and centrifuged, and the supernatant was packed with Ni-NTA on columns. Columns were washed (6 mol/L guanidine HCl, 20 mmol/L Tris [pH 8.0], 500 mmol/L NaCl, 20% glycerol, and 50 mmol/L imidazole), and LukB was eluted with 6 mol/L guanidine HCl, 20 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, 20% glycerol, and 300 mmol/L imidazole. The fusion proteins were dialyzed against PBS/50% glycerol and stored at -20°C until use. Endotoxin contamination of the toxin stock solutions was excluded by using a limulus assay with a detection limit of 0.25 EU/mL (Lonza) and found to be negative. Single components were mixed in an equal molar ratio for use. IL-1 β secretion was not detectable in stimulations using the single components (data not shown).

Data analysis and statistics

Data were imported into and analyzed in GraphPad Prism version 5.0 by using 2-tailed Student *t* tests and nonparametric Mann-Whitney *U* or Wilcoxon matched-pairs signed-rank test unless stated otherwise. A *P* value of less than .05 was generally considered statistically significant and marked by an asterisk throughout the figure legends, even if considerably lower.

Characteristics of patients with XLA

All patients were adult males and provided written informed consent for participation in the study.

Patient 1 (Fig 4, D and E) was given a diagnosis of XLA caused by agammaglobulinemia and absence of B cells after recurring bronchopulmonary infections. A *BTK* mutation mapping to a splicing site was confirmed by means of sequencing (g.IVS17+5G>A, c.1750+5G>A).

Patient 2 (Fig 4, D and E) was given a diagnosis of XLA because of the absence of all immunoglobulin subtypes and absence of B cells after recurring bronchopulmonary infections. A coding *BTK* mutation was confirmed by means of sequencing (c1361A>T, p.454H>L). The patient has been receiving IVIG since 1984.

Patient 3 (Fig 4, D and E) was given a diagnosis of XLA caused by absence of all immunoglobulin subtypes and absence of B cells after recurring bronchopulmonary infections, episodes of pneumonia, otitis media and externa, and mild bronchiectasis. A *BTK* mutation mapping to a splicing site was confirmed by means of sequencing (Intron 7: 721+1 G-C). Drastically reduced but detectable *BTK* expression was seen.

Patient 4 (Figs 4, F and G, and 5, E) was given a diagnosis of Bruton agammaglobulinemia caused by IgG, IgA, and IgM levels of less than the detection limit and absence of peripheral B cells. The patient had recurring respiratory tract infections, including *Haemophilus influenzae*, and bronchiectasis. The patient also had an autoimmune enteropathy and ulcerating duodenitis treated with ileal resection.

Characteristics of patients with MWS

All patients provided written informed consent for participation in the study. For further information, see to Rieber et al.^{E1}

Patient 1 (Fig 6, D) was a 30-year-old woman with a confirmed p.E311K mutation. Complete response to anti-IL-1 therapy was seen.

Patient 2 (Fig 6, D) was a 54-year-old man with a confirmed p.E311K mutation. Complete response to anti-IL-1 therapy was seen.

Patient 3 (Fig 6, D) was a 54-year-old man with a confirmed p.E311K mutation. Complete response to anti-IL-1 therapy was seen.

Patient 4 (Fig 6, D) was a 5-year-old girl with a confirmed p.Q703K mutation who was currently not receiving anti-IL-1 therapy.

Patient 5 (Fig 6, E) was a 46-year-old man with a confirmed p.R260W mutation. Complete response to anti-IL-1 therapy was seen.

Patient 6 (Fig 6, E) was a 60-year-old man with a confirmed p.Val198Met mutation. Complete response to anti-IL-1 therapy was seen.

Ibrutinib-treated patients

All patients provided written informed consent for participation in the study.

Patient 1 (Fig 6, G) was an 81-year-old man. Primary diagnosis/indication for ibrutinib treatment was mantle cell lymphoma (first diagnosed September 2006). Other secondary disorders were prostate cancer, hypertension, hyperthyroidism, and chronic kidney disease. Ibrutinib (Imbruvica) at a dosage of 560 mg/d was administered orally. Time of last administration before blood sampling (10 AM) was 8 AM.

Patient 2 (Fig 6, G) was an 80-year-old man. Primary diagnosis/indication for ibrutinib treatment was chronic lymphocytic leukemia (first diagnosed June 2008). Other secondary disorders were hypertension and hypogammaglobulinemia. Ibrutinib (Imbruvica) at a dosage of 420 mg/d was administered orally. Time of last administration before blood sampling (10 AM) was 8 AM.

Patient 3 (Fig 6, F) was an 86-year-old woman. Primary diagnosis/indication for ibrutinib treatment was chronic lymphocytic leukemia (first diagnosed May 2007). The other secondary disorders was atrial fibrillation. Ibrutinib (Imbruvica) at a dosage of 280 mg/d was administered orally. Time of last administration before blood sampling (10 AM) was 8 AM.

Patient 4 (Fig 6, F) was a 64-year-old man. Primary diagnosis/indication for ibrutinib treatment was mantle cell lymphoma (first diagnosed August 2008). Other secondary disorders were hypogammaglobulinemia, diabetes mellitus, and polymyalgia rheumatica. Ibrutinib (Imbruvica) at a dosage of 560 mg/d was administered orally. Time of last administration before blood sampling (10 AM) was 8 AM.

Patient 5 (Fig 6, F) was a 60-year-old man. Primary diagnosis/indication for ibrutinib treatment was chronic lymphatic leukemia (first diagnosed December 1999). Other secondary disorders were secondary antibody deficiency syndrome, history of autoimmune hemolysis with incomplete warm autoantibody, polyneuropathy in both feet, history of gram-negative sepsis at the time of colitis with thickening of cecum and colon ascendens, *E coli* detection (BK), history of septic pneumonia with detection of *E coli* (BK), and antibiotics with Tazobac Oesophageal varices of grade III fundus varices grade I. Ibrutinib (Imbruvica) at a dosage of 420 mg/d was administered orally. Time of last administration before blood sampling (10 AM) was between 6 and 9 AM.

Patient 6 (Fig 6, F) was an 87-year-old man. Primary diagnosis/indication for ibrutinib treatment was chronic lymphatic leukemia (first diagnosed February 1994). Other secondary disorders were pacemaker implantation, sick sinus syndrome, bradytachycardia, permanent atrial fibrillation, currently taking Apixaban, history of duodenal ulcer, Forrest III hemorrhage, choledocholithiasis History of stent implantation, and postinterventional cholangiosepsis (*E coli* in pBK). Ibrutinib (Imbruvica) dosage of 420 mg/d was administered orally. Time of last administration before blood sampling (10 AM) was between 6 and 9 AM.

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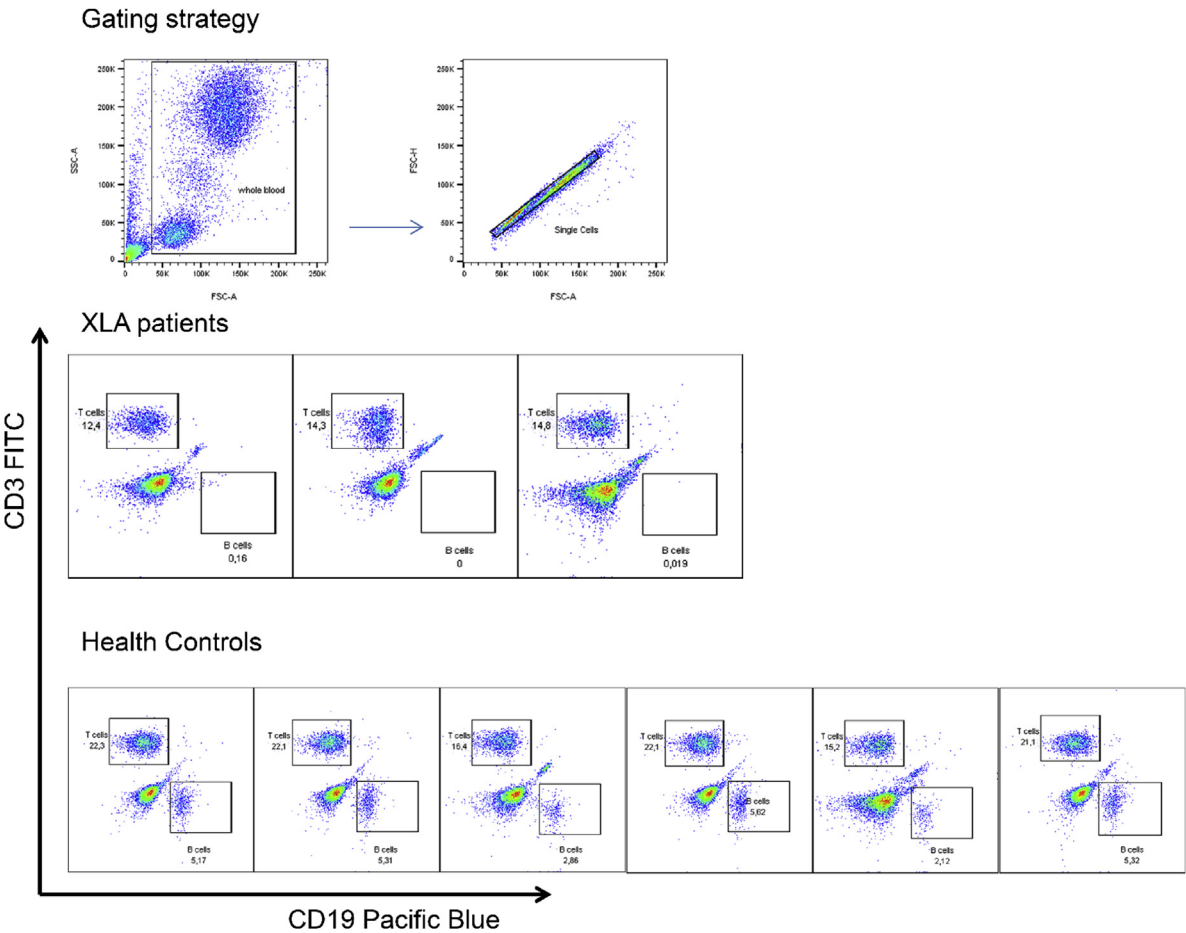


FIG E1. Whole-blood analysis of donors with XLA and healthy donors. Before PBMC purification, whole blood from the 3 donors with XLA and 6 healthy donors shown in Fig 3, C and D, was stained with anti-CD3-FITC and anti-CD19-Pacific blue mAbs and analyzed by using flow cytometry, as indicated.

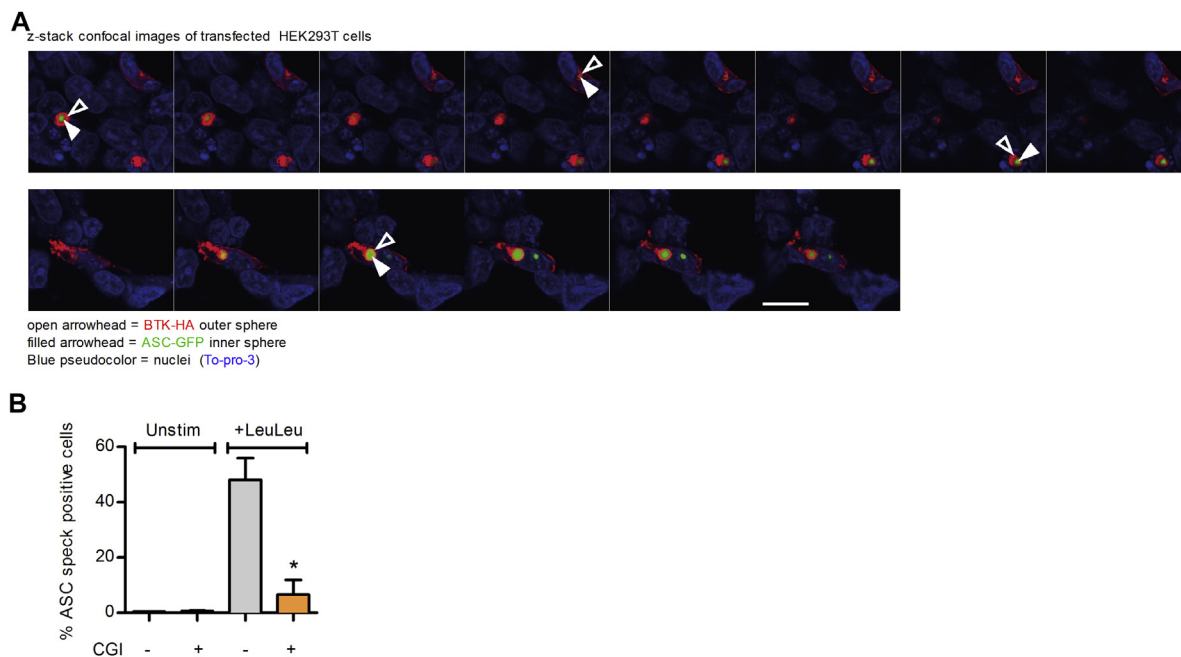


FIG E2. BTK localizes around ASC specks, and CGI1746 blocks ASC speck formation. **A**, HEK293T cells were transfected with BTK-HA and ASC-GFP plasmids and fixed with methanol 48 hours later, nucleic acids were stained with TO-PRO-3, and samples were analyzed by using confocal microscopy. Z-stacks from 2 fields of view from at least 3 experiments are shown. *Scale bar* = 20 μ m. **B**, Quantification of ASC specks of immortalized *Nlrp3* KO macrophages overexpressing NLRP3-FLAG and ASC-mCerulean treated with CGI1746 at 50 μ mol/L or solvent control (–) for 60 minutes before stimulation with 1 mmol/L Leu-Leu-OMe•HCl for 90 minutes. Cells were fixed after stimulation, and nucleic acids were stained with DRAQ5. Quantification was performed on 10 fields per condition by using CellProfiler software, and mean + SEM percentages of cells with ASC specks from 2 experiments + SEM were shown. The Student *t* test was used. **P* < .05.

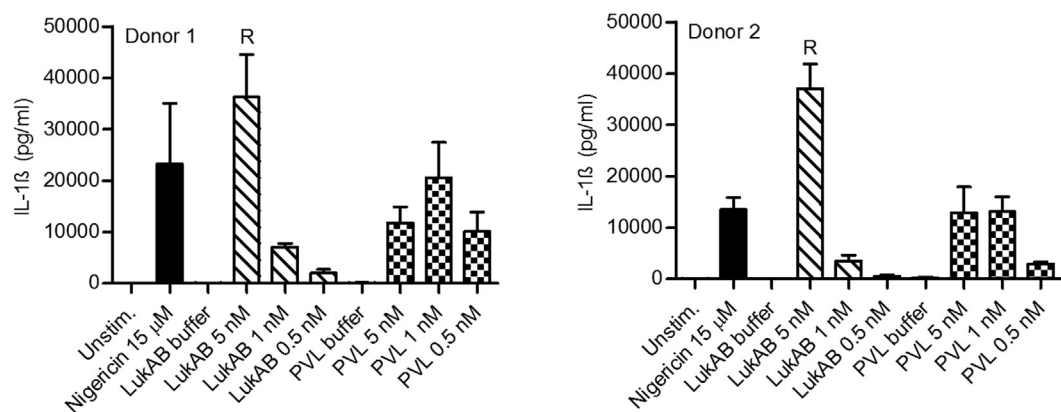


FIG E3. LukAB and PVL dose-dependently activate NLRP3-dependent IL-1 β release in THP-1 cells and primary MoMacs. Human primary MoMacs were primed with 300 ng/mL LPS for 3 hours and then stimulated with the indicated amounts of LukAB, PVL, nigericin, or dialysis buffer for 60 minutes. IL-1 β levels in the supernatant were quantified by using triplicate ELISA (mean + SD). R, Out of range. One representative of 2 identical experiments is shown.

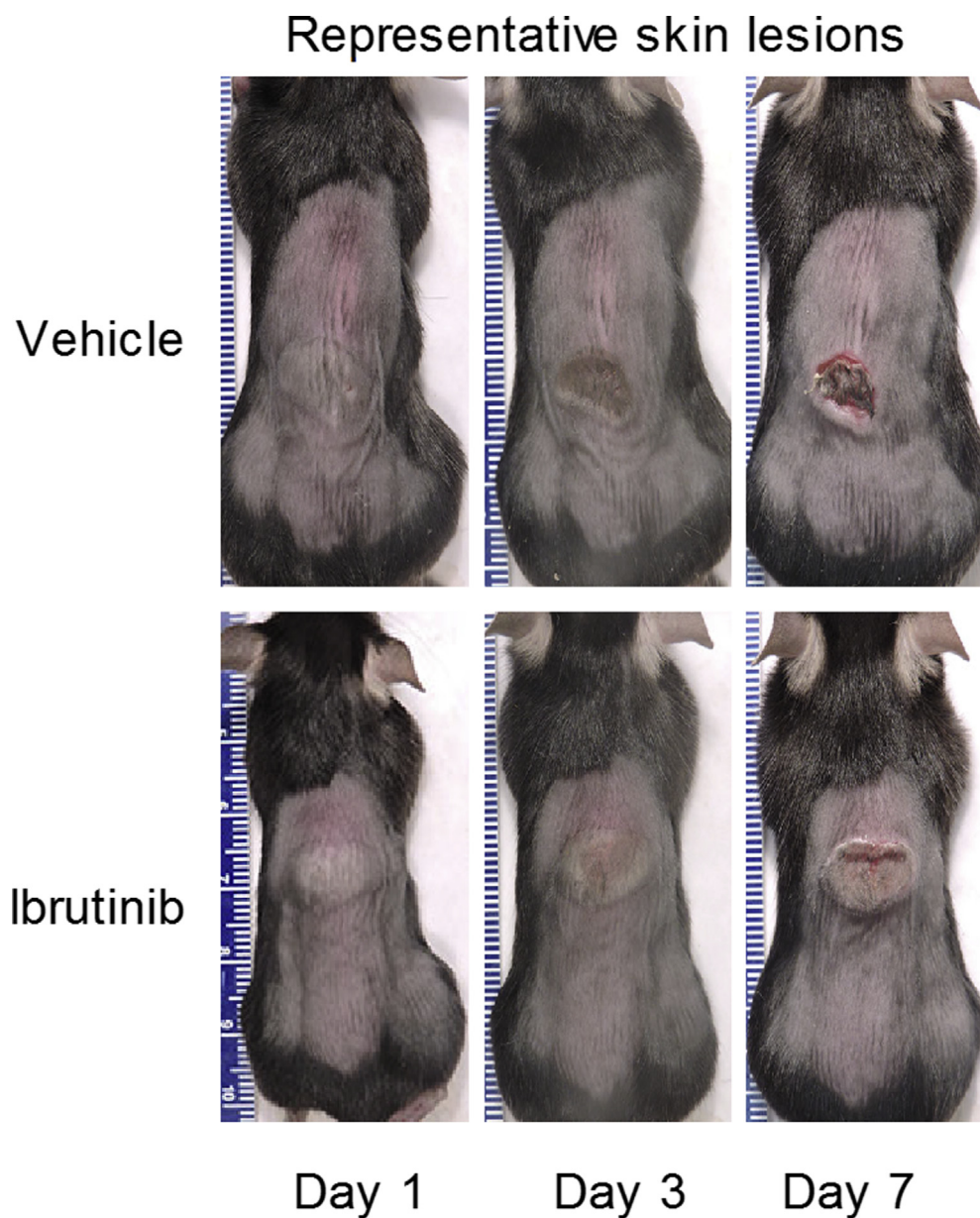


FIG E4. BTK inhibition negatively affects IL-1 β -dependent *S aureus* clearance *in vivo*. C57BL/6 mice (n = 10 per group) received 3 consecutive intravenous injections of vehicle or ibrutinib on days -1, 0, and 1, followed by 2 topical ibrutinib application on day 2. Inhibitor- and vehicle-treated mice were inoculated intradermally with a bioluminescent community-acquired methicillin-resistant *S aureus* strain (USA300 LAC::lux). At the indicated days, mean total lesion size (in square centimeters) was measured, and representative skin lesions are shown.

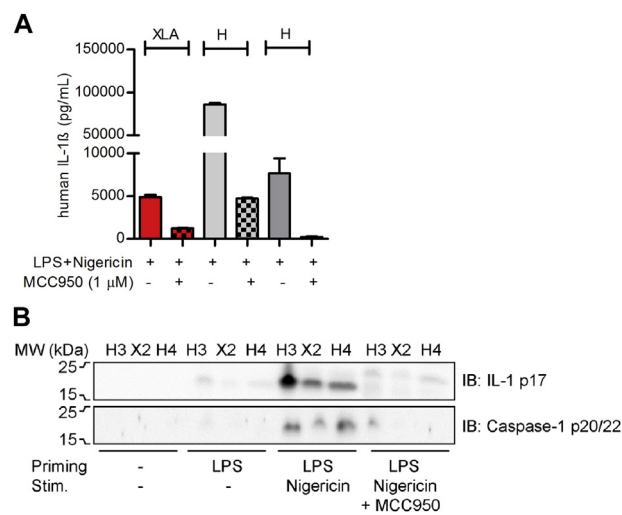


FIG E5. The NLRP3 inhibitor MCC950 reduces IL-1 and caspase-1 processing further in patients with XLA. **A**, LPS-primed PBMCs from a male patient with XLA and age-matched male healthy donors ($n = 2$) were treated with $15 \mu\text{mol/L}$ nigericin for 1 hours in the presence of absence of $1 \mu\text{mol/L}$ MCC950. IL-1 β was quantified by means of ELISA (mean $\pm$ SD of technical). **B**, Stimulated PBMCs were also analyzed by mean of immunoblotting with anti-IL-1 β p17 or anti-caspase-1 p20/p22-specific antibodies.

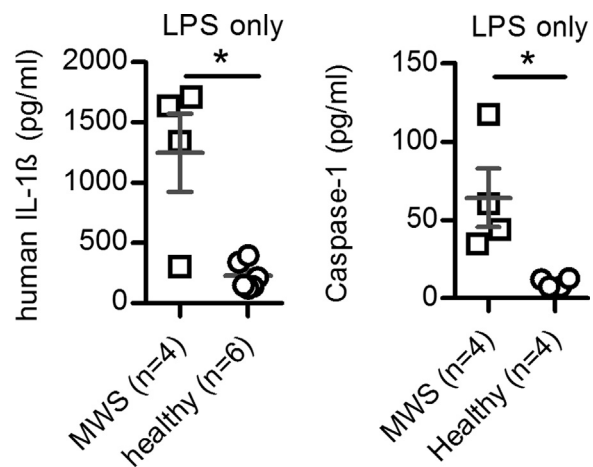


FIG E6. Excessive IL-1 β and caspase-1 release in patients with MWS in response to only LPS. PBMCs from patients with MWS ($n = 4$) and healthy donors ($n = 6$) were treated with LPS only for 4 hours. IL-1 β and caspase-1 release was quantified by means of ELISA (mean $\pm$ SEM of biological replicates). Each *symbol* represents 1 donor. * $P < .05$.