Bruton tyrosine kinase deficiency augments NLRP3 inflammasome activation and causes IL-1β–mediated colitis

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Bruton tyrosine kinase (BTK) is present in a wide variety of cells and may thus have important non–B cell functions. Here, we explored the function of this kinase in macrophages with studies of its regulation of the NLR family, pyrin domain–containing 3 (NLRP3) inflammasome. We found that bone marrow–derived macrophages (BMDMs) from BTK-deficient mice or monocytes from patients with X-linked agammaglobulinemia (XLA) exhibited increased NLRP3 inflammasome activity; this was also the case for BMDMs exposed to low doses of BTK inhibitors such as ibrutinib and for monocytes from patients with chronic lymphocytic leukemia being treated with ibrutinib. In mechanistic studies, we found that BTK bound to NLRP3 during the priming phase of inflammasome activation and, in doing so, inhibited LPS- and nigericin-induced assembly of the NLRP3 inflammasome during the activation phase of inflammasome activation. This inhibitory effect was caused by BTK inhibition of protein phosphatase 2A–mediated (PP2A-mediated) dephosphorylation of Ser5 in the pyrin domain of NLRP3. Finally, we show that BTK-deficient mice were subject to severe experimental colitis and that such colitis was normalized by administration of anti–IL-β or anakinra, an inhibitor of IL-1β signaling. Together, these studies strongly suggest that BTK functions as a physiologic inhibitor of NLRP3 inflammasome activation and explain why patients with XLA are prone to develop Crohn's disease.

Introduction

The NLR family, pyrin domain–containing 3 (NLRP3) inflammasome, when appropriately activated and enabled to release mature IL-1β and IL-18, serves as a powerful mechanism for the induction of the proinflammatory cytokines participating in host defense (1–3). However, this potentiality requires strict regulation in order to suppress responses that would inadvertently lead to inflammatory disease. Lack of regulation and excessive responses are, in fact, the origin of inflammation in cryopyrin-associated periodic syndromes (CAPS), characterized by mutations in *Nlrp3* that allow its activation by TLR ligands (or other primary stimulants) in the absence of cofactors such as ATP or nigericin (4, 5).

One mechanism of regulation of NLRP3 inflammasome activation involves the phosphorylation of NLRP3 that is manifest in the complex array of phosphorylation and/or dephosphorylation events that serve as known checkpoints of inflammasome activation (6). Another mechanism of regulation involves the binding of molecules, such as NIMA related kinase 7 (NEK7) (7, 8) or caspase recruitment domain family member 8 (CARD8) (9), which have the capacity to modify NLRP3 function. In the present study, we investigated the regulation of the NLRP3 inflammasome by Bruton tyrosine kinase (BTK), a molecule that falls into both categories of NLRP3 inflammasome activation modifiers in that it binds NLRP3 and affects NLRP3 phosphorylation.

Previous studies of NLRP3 inflammasome regulation by BTK, most notably those conducted by Liu et al. (10) and Ito et al. (11) using BTK-deficient cells, have led to the conclusion that BTK enhances NLRP3 inflammasome activity. However, these studies were based in part on comparisons of inflammasome responses of WT and BTK-deficient cells after stimulation with low doses of LPS and therefore overlooked the fact that deficient NLRP3 inflammasome responses in BTK-KO cells, presumably indicative of deficient BTK-enhancing activity, could be due to defects in TLR4 activation (12). In the present studies in which WT and BTK-KO cells were stimulated with LPS concentrations that elicited comparable TLR4 responses in WT and BTK-KO cells, we overcame this problem and show that absent BTK, NLRP3 inflammasome activity is augmented. These studies were therefore in full accord with reciprocal (BTK overexpression) studies involving HEK293T cells transfected with plasmids allowing construction of the NLRP3 inflammasome, in which we showed that inflammasome activity by the transfected cells was suppressed by cotransfection of BTK-express-

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ing plasmids. Importantly, the negative regulation of the NLRP3 inflammasome by BTK in these in vitro studies was verified by in vivo studies showing that BTK-KO mice had increased severity of trinitrobenzene sulfonic acid (TNBS) colitis and that the colitis was driven by IL-1β, since it was attenuated by the administration of an IL-1β inhibitor. Finally, in mechanistic studies, we showed that BTK-suppressive activity depended on its capacity to block NLRP3 (Ser5) dephosphorylation and thus prevented NLRP3 oligomerization.

Overall, our studies suggest that the activation of BTK in macrophages and DCs by TLR ligands or other primary activation factors serves as a physiologic mechanism of NLRP3 inflammasome regulation, in that BTK activation blocks the NLRP3 activation of the NLRP3 inflammasome in the absence of a costimulator. In addition, our findings provide an explanation for the fact that patients with XLA lacking functional BTK are prone to develop intestinal abnormalities (13).

Results

BTK regulation of murine NLRP3 inflammasome activity. To investigate the role of BTK in activating the NLRP3 inflammasome, we evaluated inflammasome activation in bone marrow–derived macrophages (BMDMs) obtained from mice and humans with verified BTK deficiency (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI128322DS1). In initial studies focused on murine cells, we evaluated the priming phase of NLRP3 inflammasome stimulation in cells from BTK-KO mice by measuring the transcription of NLRP3 and IL-1β by quantitative PCR (qPCR) in these cells following stimulation with LPS only and found that transcription of these components was similar to that of similarly stimulated cells from WT mice (Supplemental Figure 2). We next investigated the activation phase of NLRP3 inflammasome stimulation in BTK-KO mice by stimulating BMDMs from these mice with LPS and ATP or nigericin. We found that BTK-KO cells secreted significantly higher levels of IL-1β than did WT cells (Figure 1A), whereas the secretion of IL-6 by these cells was equivalent (Figure 1B). Moreover, this enhanced IL-1 secretion was accompanied by increased cleavage of IL-1β and caspase-1 (Figure 1C). As expected, we also observed enhanced production of IL-1β by BMDMs from BTK-KO mice (Figure 1, D and E).

The above findings indicating that murine cells lacking BTK activity had increased NLRP3 inflammasome activity are at odds with data from studies by Liu et al. (10) and Ito et al. (11), which showed that such cells exhibit decreased inflammasome activity. One possible explanation of this discrepancy with respect to the Liu et al. study arises from the fact that cells in this study were stimulated with a low dose of LPS that failed to fully activate BTK-KO cells, which have previously been shown to have impaired TLR4-mediated responses (12). To examine this possibility, we stimulated WT and BTK-KO mouse BMDMs with 10 ng/mL LPS (the dose used by Liu et al.) and 200 ng/mL (the dose used in the present study) plus nigericin or ATP (at doses used in the respective studies) and found that, indeed, IL-1β production was deceased in BTK-KO cells stimulated with the lower dose and was increased in cells stimulated at the higher dose as compared with WT cells (Supplemental Figure 3, A and B). In related studies, to explain this difference, we found that the transcription of pro–IL-1β and NLRP3 as well as Iκβ phosphorylation and NLRP3 protein expression were decreased in BTK-KO cells as compared with WT cells stimulated with the lower dose of LPS, whereas these parameters were equivalent in the 2 cell populations stimulated with the higher dose (Supplemental Figure 3, C and D). Our findings strongly suggest that the previous data showing that the decreased inflammasome activity of BTK-KO cells compared with WT cells — presumably because of a lack of BTK enhancement of such activity — was actually due to reduced TLR4-mediated activation of BTK-KO cells relative to WT cells when these cells were stimulated with low doses of LPS and thus generated reduced amounts of NLRP3 when compared with WT cells.

It should be noted that the problem introduced by decreased activation of BTK-KO cells by TLR ligands such as LPS also applies on the basis of previous studies using a TLR7/-8 stimulant, R848, which was also used in the study by Liu et al. (14, 15). In addition, whereas Liu et al. found that stimulation of cells with a low dose of LPS induced nearly equal levels of pro–IL-1 in PBMCs, in more extensive studies of both mouse and human cells, we found that low doses of LPS induced less pro–IL-1β transcription, which was in turn reflected in the production of less IL-1β protein.

Yet another potential problem arising from the use of the low dose of LPS in the Liu et al. study to evaluate NLRP3 activation in WT versus BTK-KO cells concerns whether this dose was sufficient to activate BTK. To evaluate this, we examined LPS dose effects on BTK (Tyr223) phosphorylation, shown previously to be a necessary initial step of BTK activity (16). Our results showed that cell activation with the concentration of LPS used by Liu et al. (10 ng/mL) induced a lower level of BTK (Tyr223) phosphorylation (as evaluated by Western blotting [WB]) than did the higher dose (200 ng/mL) used in the present studies (Supplemental Figure 4). Given that BTK was not fully activated at the lower dose and that BTK kinase activity is necessary for BTK regulation (see below), it is reasonable to assume that BTK was not fully able to inhibit the NLRP3 inflammasome under these conditions and therefore leads to a higher level of NLRP3 activity in suboptimally activated WT cells than would be present in fully activated WT cells.

The discrepancy between the results in the Ito et al. study (11) and the data provided here could also be due, at least in part, to the LPS concentration used in priming, despite the fact that in this study a somewhat higher LPS concentration was used (50 ng/mL). This follows from the fact that stimulation of mouse BTK-KO cells with this concentration of LPS again resulted in lower phosphorylated IκB (p-IκB) and NLRP3 expression than was seen with a similar stimulation of WT cells and thus an inappropriate comparison of NLRP3 activation in the 2 cell populations as discussed above (Supplemental Figure 3, A and B). It should be noted, however, that human XLA cells, in contrast to mouse BTK-KO cells, exhibit enhanced NLRP3 activity when stimulated with 50 ng/mL LPS, and therefore this concentration may be adequate for the evaluation of BTK activity in human cells but not in mouse cells (Supplemental Figure 5, A–D).

Another and more fundamental problem with the results reported by Ito et al. is that the authors used cells from CBA/N (XID) mice with which to define the effect of BTK deficiency. Like Ito et al., we found that BTK-deficient cells from XID mice

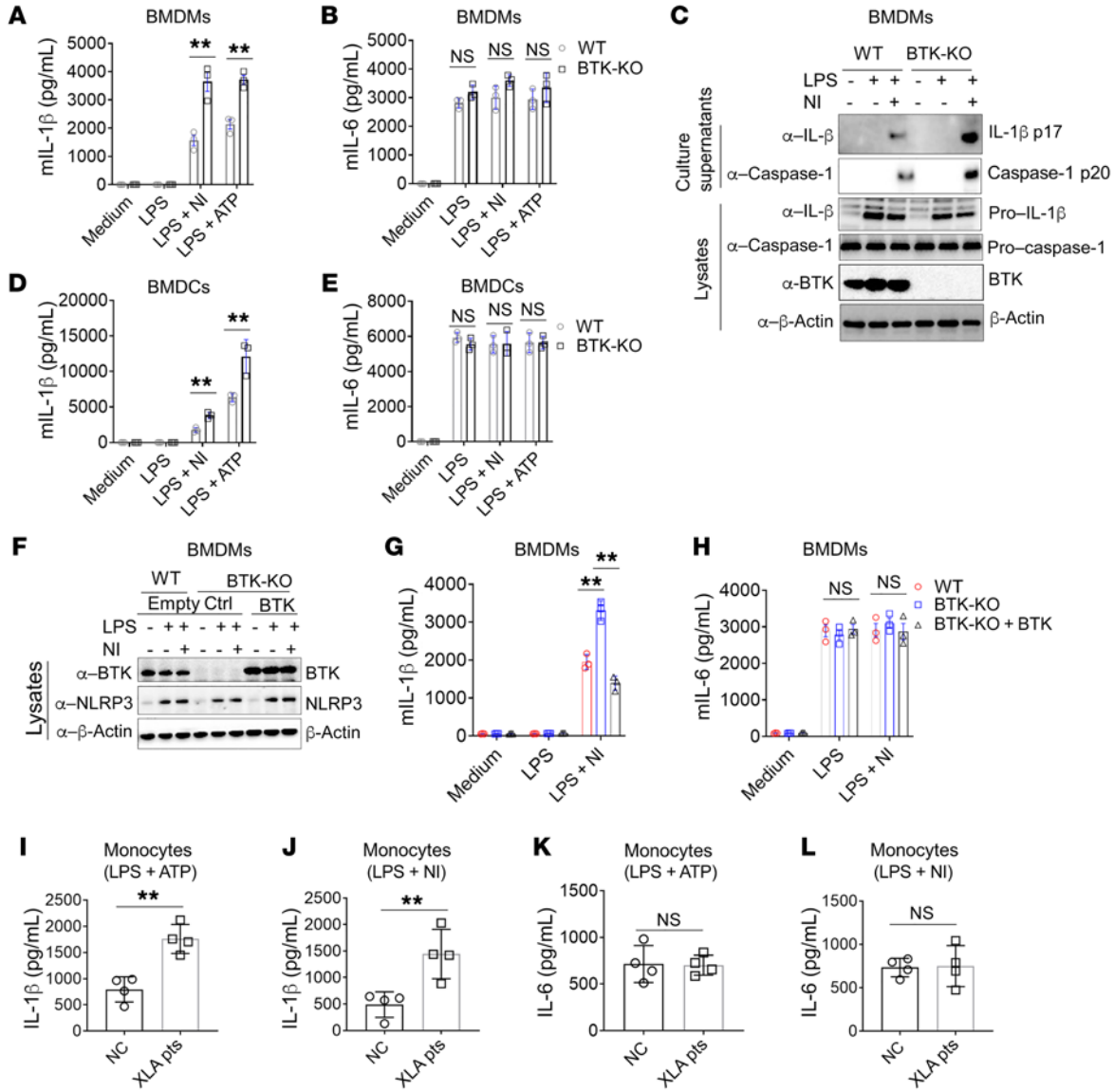

Figure 1. BTK deficiency causes increased NLRP3 inflammasome activation in murine and human cells. (**A**–**C**) Murine BMDMs were primed with LPS (200 ng/mL) for 3 hours and then stimulated with nigericin (NI) (1 μM) or ATP (5 mM) for 30 minutes. The culture supernatants were then subjected to ELISA to detect mature IL-1β (mIL-1β) (**A**) and mature IL-6 (mIL-6) (**B**) and WB to detect mature IL-1β and caspase-1 (**C**). (**D** and **E**) Mouse BMDCs were treated as above, and the culture supernatants were subjected to IL-1β (**D**) and IL-6 (**E**) ELISAs. (**F**–**H**) BMDMs from WT or BTK-KO mice were transduced with a lentiviral vector expressing BTK or an empty vector control for 48 hours and then primed with LPS (200 ng/mL) for 3 hours, followed by stimulation with nigericin (1 μM) for 30 minutes. Next, the cells were lysed and the lysates subjected to WB for detection of BTK and NLRP3 (**F**). Culture supernatants were collected for IL-1β (**G**) and IL-6 (**H**) ELISAs. (**I** and **J**) Human monocytes from patients with XLA (XLA pts) ($n = 4$) and healthy individuals (NCs) ($n = 4$) were primed with LPS (200 ng/mL) for 3 hours and then stimulated with ATP (5 mM) or nigericin (1 μM) for 30 minutes. The culture supernatants were then subjected to IL-1β (**I**) and IL-6 (**J**) ELISAs. $^{**}P < 0.01$, by 1-way ANOVA with multiple comparisons test (**A**, **B**, **D**, **E**, **G**, and **H**) and 2-tailed Student t test (**I** and **J**). Data are presented as the mean ± SD and are representative of 3 independent experiments. α, anti.

indeed had lower inflammasome activity than did WT cells (Supplemental Figure 6A). However, this result is problematic, because the R28C BTK mutation responsible for BTK deficiency in these mice results in B cells that express normal amounts of BTK with normal in vitro kinase activity (but nevertheless has deficient BTK signaling function in B cells) (17) (Supplemental Figure 6B). This is in contrast to XLA (BTK-KO) mice, such as those used in the present study, which lack BTK expression (Supplemental Figure 3C), or to the great majority of humans with Bruton's agammaglobulinemia, such as those studied here (18). The presence of abnormal BTK activity in XID macrophages is not without consequences. Thus, when stimulated with LPS, BMDMs from XID mice exhibited increased NLRP3-BTK interaction as compared with BMDMs from WT mice; furthermore, although this interaction diminished after nigericin stimulation, it remained increased compared with that in WT cells (Supplemental Figure 6B). On the basis of the results of the studies reported below regarding the mechanism of BTK inhibition of the NLRP3 inflammasome, this enhanced and persistent NLRP3-BTK interaction led to decreased NLRP3 inflammasome oligomerization and activation, because

it facilitated persistent blockade of NLRP3 dephosphorylation. It therefore explains why NLRP3 inflammasome activation in macrophages derived from XID mice was low compared with that in macrophages derived from WT mice (Supplemental Figure 6A). Whether these findings indicating that NLRP3 inflammasome activity is decreased in XID mouse–derived macrophages in vitro are reflected in NLRP3 inflammasome activity in vivo will require studies of such activity in intact XID mice.

The studies above using murine BTK-KO cells suggest that the activation phase of NLRP3 inflammasome activity is enhanced in the absence of BTK, implying that this kinase is normally an inhibitor of this inflammasome. To test this latter possibility, we reconstituted BTK expression in BMDMs from BTK-KO mice by transduction of a lentiviral vector expressing BTK and found that after stimulation with LPS and nigericin, BTK-KO cells with BTK transduction now showed reduced NLRP3 inflammasome activity equivalent to that in WT cells, whereas cells transduced with an empty vector had enhanced inflammasome activity (Figure 1, F–H). These data further demonstrated that BTK plays an inhibitory role in NLRP3 inflammasome activation.

In yet another test of the idea that BTK is an inhibitor of the NLRP3 inflammasome in murine cells, we sought to determine whether the absence of BTK in mouse XLA cells is accompanied by increased generation of gasdermin D (GSDMD) and its downstream effect, pyroptosis, since cleavage of precursor GSDMD is a known consequence of NLRP3 inflammasome generation of mature caspase-1 (19, 20). Indeed, we found that BTK-KO cells stimulated with LPS and nigericin had increased generation of the cleaved (p30) form of GSDMD in BTK-KO BMDMs as compared with that generated in WT cells (Supplemental Figure 7A). In addition, reflecting an increase in resultant pyroptosis, BTK-KO cells also released increased amounts of lactate dehydrogenase (LDH) upon inflammasome activation compared with WT cells (Supplemental Figure 7B). These results were corroborated by the observation that BTK overexpression in HEK293T cells transfected with plasmids expressing each of the components of the NLRP3 inflammasome and cotransfected with a BTK-expressing plasmid exhibited decreased GSDMD cleavage and LDH release compared with similarly transfected cells cotransfected with a control plasmid (Supplemental Figure 7, C and D).

BTK regulation of human NLRP3 inflammasome activity. In studies complementary to those above, but focusing instead on human cells, we evaluated NLRP3 inflammasome function in XLA patients with verified BTK deficiency due to known BTK mutations as well as in healthy control individuals (see Methods for a description of the patients). As in the mouse studies, we found that stimulation of XLA patients' monocytes with LPS (200 ng/mL) plus nigericin or ATP led to enhanced NLRP3 inflammasome activation (IL-1β secretion), with no difference in IL-6 secretion (Figure 1, I and J). Again, in studies addressing the discrepant results from the Liu et al. study (10), we found that, whereas stimulation of XLA patients' PBMCs or monocytes with low concentrations of LPS (10 ng/mL) produced decreased levels of IL-1β compared with cells from control individuals, stimulation of cells with higher concentrations of LPS (50 ng/mL and 200 ng/mL) showed that XLA cells produced higher levels of IL-1β compared with levels in control cells (Supplemental Figure 5, A–D). In addition, low doses of LPS induced lower expression of NLRP3 and pro–IL-1β in patients' cells compared with control cells, and this difference vanished when the cells were stimulated with higher doses of LPS (Supplemental Figure 8, A–C). Stimulation of XLA patients' cells with low doses of LPS also induced higher IL-10 production than did stimulation of cells from healthy controls, and this difference disappeared when the cells were stimulated with higher doses (Supplemental Figure 5E). The fact that IL-10 is a potent NLRP3 inflammasome inhibitor is another possible reason that low-dose LPS stimulation of XLA patients' cells did not demonstrate that lack of BTK leads to increased NLRP3 inflammasome activation (21). Finally, we showed that stimulation of XLA patients' and control cells with varying doses of the TLR2 ligand Pam3CSK4 also led to enhanced NLRP3 inflammasome activation in XLA cells compared with control cells, and, again, this enhanced effect was observed at high but not low TLR2 ligand concentrations (Supplemental Figure 9). This indicates that BTK inhibition of the NLRP3 inflammasome is not specific to TLR4 activation of the responding cell.

Effect of BTK inhibitors on NLRP3 inflammasome activation. The results of the above studies prompted us to next explore how exposure of human cells to BTK inhibitors would influence NLRP3 inflammasome activation. We therefore stimulated human elutriated monocytes or human BMDMs under NLRP3 inflammasome activation conditions in the presence of various concentrations of the BTK inhibitors LFM-A13 and ibrutinib, the latter of which is a BTK inhibitor widely used in the treatment of chronic lymphocytic leukemia (CLL). We found that, whereas exposure to low concentrations of either inhibitor enhanced IL-1β production, high concentrations inhibited IL-1β production, and in neither case was there an effect on IL-6 production at any concentration (Supplemental Figure 10, A–D). In the case of LFM-A13, we observed similar results with human elutriated monocytes and human THP-1 monocytes (Supplemental Figure 10, E–H); in addition, we obtained similar results with culturing of WT and BTK-KO BMDMs (Supplemental Figure 10, I and J). It is important to note that the culturing of cells in the presence of either BTK inhibitor did not change the rate of transcription of pro–IL-1β or NLRP3 (Supplemental Figure 11); in addition, culturing of LPS-stimulated cells in the presence of either inhibitor did not cause significantly increased cell cytotoxicity as measured by LDH release (Supplemental Figure 12).

In further studies, we evaluated the effect of BTK inhibition by ibrutinib on patients with CLL who were on ibrutinib therapy. The mean blood ibrutinib concentration in appropriately treated patients with CLL is approximately 150 ng/mL (0.34 μM) (22). On the basis of the above data relating to concentration effects of the inhibitor, this concentration would be predicted to cause enhanced NLRP3 inflammasome–derived IL-1β production by patients' peripheral monocytes at least for a period of time after administration of the daily dose. Indeed, this prediction proved to be correct, given the actual NLRP3 inflammasome activation versus control activation observed in the patients studied (Supplemental Figure 13). Interestingly, IL-1β production by BMDMs from BTK-KO mice was inhibited by high concentrations of ibrutinib, without affecting IL-6 production (Supplemental Figure 10, I and J), strongly suggesting that inhibition of the NLRP3 inflammasome is BTK independent. In contrast, we found that the level

of enhancement of NLRP3 activation in BTK-KO cells was equal in cells not exposed to ibrutinib and in those exposed to low (ordinarily enhancing) doses of ibrutinib (Supplemental Figure 10, K and L). Such equality of results indicates that low-dose ibrutinib enhancement is BTK dependent.

Taken together, these studies of the effect of BTK inhibitors on human cells indicate that, like BTK deficiency caused by a genetic abnormality, BTK deficiency caused by exposure to low concentrations of an inhibitor leads to upregulation of NLRP3 inflammasome activity. In contrast, high concentrations of the inhibitor causes BTK-independent inhibition of NLRP3 inflammasome activity.

BTK regulates NLRP3 phosphorylation and oligomerization events initiating NLRP3 inflammasome activation. We next turned our attention to the specific molecular events accompanying BTK regulation of the NLRP3 inflammasome. In initial studies, we subjected lysates of BMDMs stimulated with LPS alone or LPS plus nigericin to immunoblotting and found that, in cells stimulated with LPS alone, NLRP3 interacted with BTK, whereas in cells stimulated with LPS and nigericin, such interaction was greatly reduced (Figure 2A). Importantly, we observed interaction in IP samples using an anti-BTK antibody and not in those in which an isotype control antibody was used (Supplemental Figure 14). These data indicate that the interaction of BTK with NLRP3, accompanied by the NLRP3 inflammasome activation noted previously (11), occurred during the "priming" phase of NLRP3 inflammasome activation and diminished during its subsequent activation phase.

The BTK interaction with NLRP3 described above suggested that BTK affected the level of tyrosine or serine phosphorylation (p-Tyr or p-Ser) of NLRP3 that, as indicated above, had previously been shown to accompany NLRP3 activation. To investigate this possibility, we transfected HEK293T cells with plasmids expressing the precursor form of IL-1β as well as plasmids expressing components of the NLRP3 inflammasome (as described above), with (or without) a BTK-expressing plasmid, and then after 24 hours we stimulated these cells with nigericin. Subsequently, we assessed the cells for the capacity to secrete IL-1β or, alternatively, stimulated the cells with nigericin and subjected them to lysis for immunoblotting with antibodies recognizing p-Tyr or p-Ser. We found that the transfected cells produced substantial amounts of IL-1β when stimulated with nigericin and that such IL-1β secretion was inhibited by the presence of BTK, as predicted from the studies using BTK-KO cells described above (Figure 2B). In addition, in the accompanying immunoblot studies, we found that the presence of BTK enhanced the level of Tyr phosphorylation of NLRP3 and inhibited the level of Ser phosphorylation of NLRP3 (Figure 2C). In reciprocal studies, we performed immunoblot analysis of WT and BTK-KO BMDMs stimulated with LPS and nigericin, again probing for phosphorylation using antibodies recognizing p-Tyr and p-Ser. These results corroborated the studies of transfected HEK293T cells described above, in that NLRP3 in BTK-KO cell lysates exhibited increased phospho-Ser and decreased phospho-Tyr signals (Figure 2D). It should be noted, however, that these BTK-related changes in phosphorylation reflect the sum total of changes occurring at many tyrosine and serine sites and thus do not necessarily reflect phosphorylation or dephosphorylation at any specific site (6).

Finally, we examined the effect of BTK on NLRP3 oligomerization, an initial event of NLRP3 inflammasome assembly. We found that NLRP3 oligomerization was increased in WT cells stimulated with LPS and nigericin (versus cells stimulated with LPS alone) and that such oligomerization was greatly increased in BTK-KO BMDMs (Figure 2E).

BTK regulates ASC activation accompanying NLRP3 inflammasome activation. In view of the effects of BTK on NLRP3 described above, we next determined the effect of BTK on NLRP3 interaction with apoptosis-associated speck-like protein containing a CARD (ASC) and ASC assembly. In the initial studies, we found that although NLRP3 interacted with ASC in HEK293T cells transfected with plasmids expressing NLRP3 or ASC, such interaction was diminished in cells also transfected with a plasmid expressing BTK (Figure 2F). In confirmation of these findings, we found that in WT BMDMs, NLRP3 interacted with ASC upon LPS stimulation; however, such interaction was more pronounced in BTK-KO cells (Figure 2G).

We then asked whether this increased NLRP3 interaction with ASC in BTK-KO cells is accompanied by effects on ASC speck formation and oligomerization, both of which are critical indicators of inflammasome assembly. We found that following LPS plus nigericin stimulation, ASC speck formation (as evaluated by imaging studies) and oligomerization (as evaluated by SDS-PAGE studies of cell lysates) were enhanced in BTK-KO cells compared with that seen in WT cells (Figure 2, H and I). Thus, studies of cells in which BTK levels were increased or decreased indicated that BTK downregulates NLRP3-ASC interaction and ASC assembly.

BTK inhibits NLRP3 and NEK7 interaction. Recent studies have shown that NLRP3 inflammasome activation requires interaction of NLRP3 with NEK7, a protein kinase previously shown to have a signaling function during mitosis (7, 8, 23). To determine whether BTK regulates NLRP3 interaction with NEK7, we first transfected NLRP3- and NEK7-expressing plasmids into HEK293T cells with or without a BTK-expressing plasmid and then performed immunoblot analysis of cell lysates to detect interaction. We found that NLRP3 did indeed interact with NEK7 but that this interaction was diminished in the presence of BTK (Figure 2J). Similarly, the interaction between NLRP3 and NEK7 could be observed in BMDMs, and this interaction was increased in BTK-KO cells compared with that in WT cells in response to LPS plus nigericin treatment (Figure 2K).

BTK regulates NEK7 phosphorylation and oligomerization. In previous studies, it has been shown that NEK7 kinase activity during mitosis is accompanied by phosphorylation and dimerization (24, 25). In addition, in Western blots of cells subjected to NLRP3 inflammasome activation, NEK7 was present in higher-molecular-weight bands, possibly reflecting dimerization or oligomerization of NEK7 or oligomerization of other NLRP3 components to which it was bound (7). Thus, BTK inhibition of NLRP3 inflammasome activity may involve effects on NEK7 phosphorylation and/or oligomerization. To examine this possibility, we transfected HEK293T cells with plasmids expressing NLRP3 and NEK7 with or without a plasmid expressing BTK and then performed immunoblot studies of lysates of the transfected cells. We found that the presence of BTK inhibited NEK7 tyrosine phosphorylation and had a marginally inhibitory effect on NEK7 p-Ser as well (Supplemental Figure 15A). These data correlated with studies

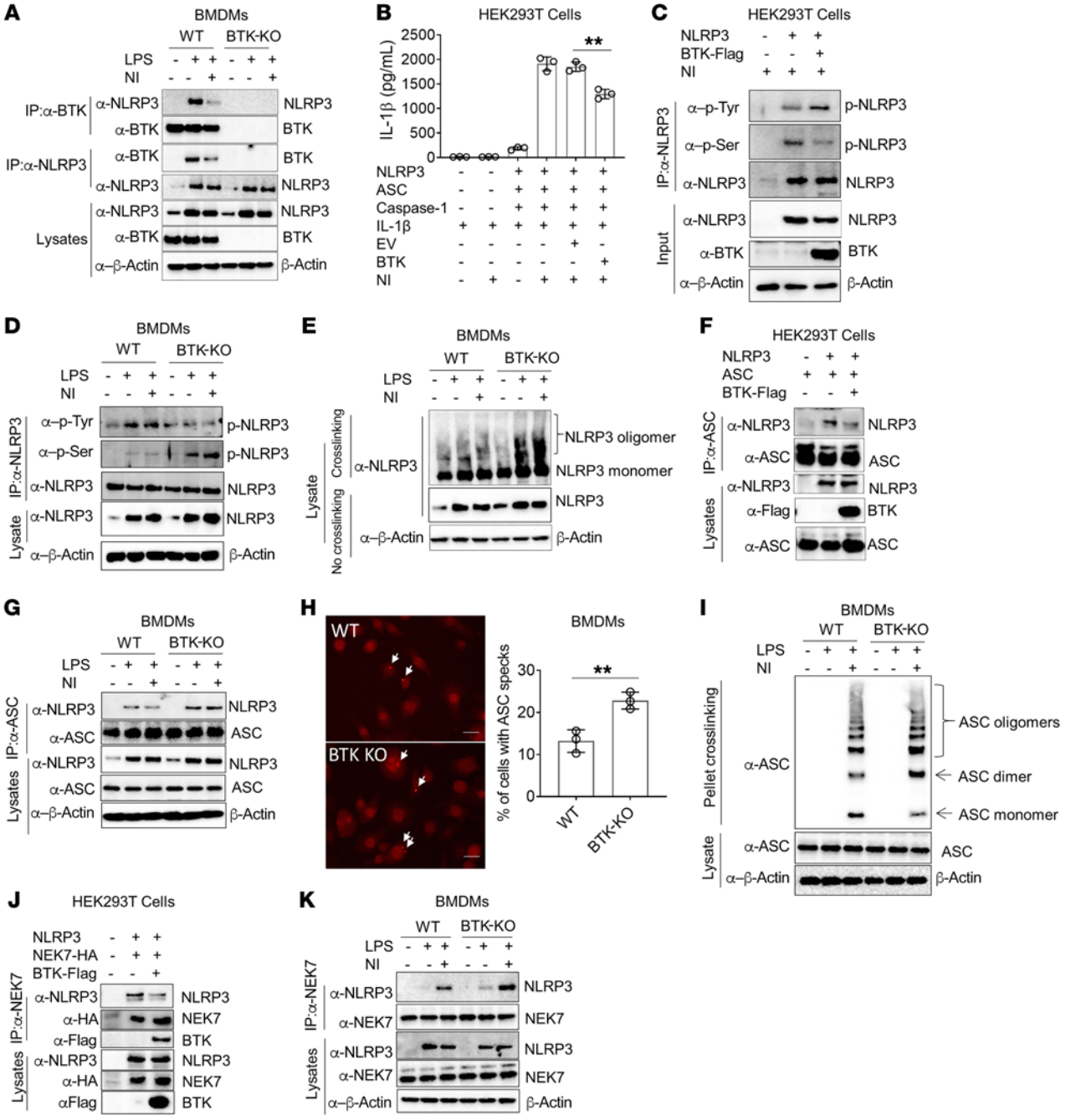

Figure 2. BTK regulates the consecutive stages of NLRP3 inflammasome activation. (**A**) BMDMs from WT or BTK-KO mice were primed with LPS (200 ng/mL) for 3 hours and stimulated with nigericin for 30 minutes; the cells were then lysed and the lysates subjected to IP and WB. (**B** and **C**) HEK293T cells were transfected with plasmids as indicated; 24 hours later, the cells were treated with nigericin as indicated for 30 minutes, and the culture supernatants were subjected to ELISAs for IL-1β (**B**). The cells were lysed and the lysates subjected to IP and WB (**C**). (**D** and **E**) BMDMs from WT or BTK-KO mice were treated as in **A**, and the cell lysates were subjected to IP and WB (**D**) or treated with DSS crosslinker and subjected to WB (**E**). (**F** and **J**) HEK293T cells were transfected with plasmids as indicated; after 24 hours, the cells were lysed and the lysates subjected to IP and WB. (**G**) BMDMs from WT or BTK-KO mice were treated as in **A**, and the cell lysates were subjected to IP and WB. (**H**) BMDMs from WT or BTK-KO mice were treated as in **A** and then subjected to immunocytochemistry for a ASC speck assay using anti-ASC antibody. Scale bars: 20 μm. (**I**) To examine ASC oligomerization, BMDMs were treated as described in Methods. (**K**) BMDMs from WT or BTK-KO mice were treated as in **A**, and cell lysates were subjected to IP and WB. $^{**}P < 0.01$, by 1-way ANOVA with multiple comparisons test (**B**) or 2-tailed Student's *t* test (**H**). Data are presented as the mean ± SD and are representative of 3 independent experiments.

of BMDCs isolated from WT or BTK-KO mice and stimulated with LPS and nigericin to activate the NLRP3 inflammasome, in which we found that NEK7 p-Tyr and p-Ser were increased in the absence of BTK (Supplemental Figure 15B).

In further studies examining whether BTK deficiency has an impact on NEK7 oligomerization, we subjected lysates of HEK293T cells transfected with plasmids expressing NLRP3 and NEK7 with or without transfection of a BTK-expressing plasmid

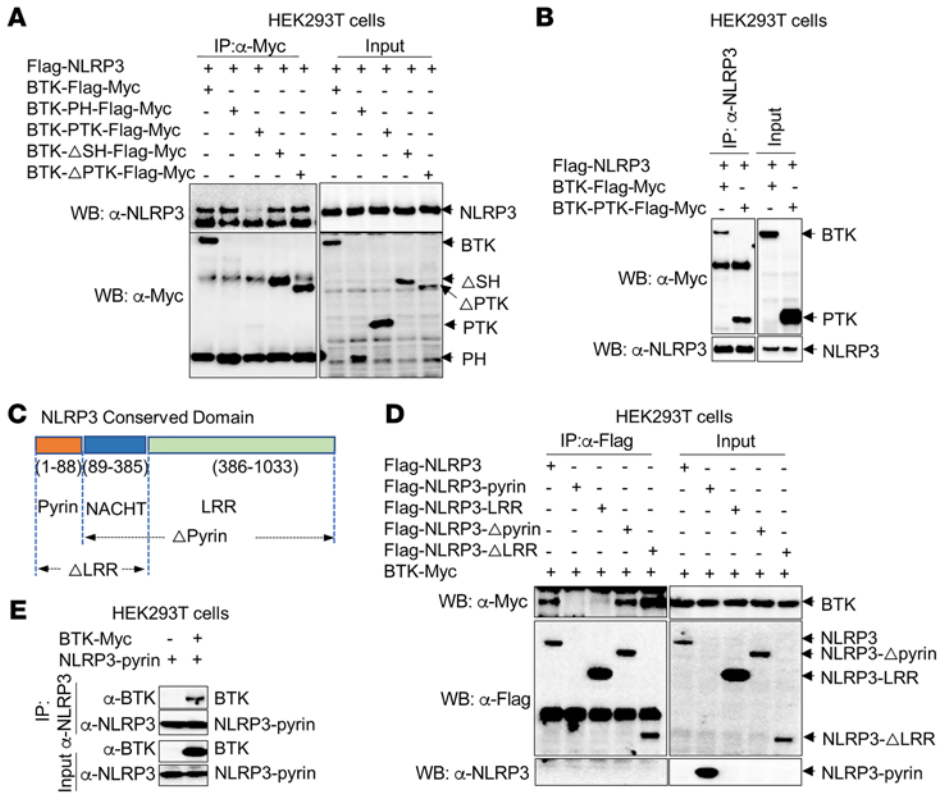

Figure 3. BTK interacts with the NLRP3 pyrin and NATCH domains via its PH and PTK domains. (**A**) HEK293T cells were transfected with plasmids expressing a full-length NLRP3 construct as well as one of various BTK constructs. After 24 hours, the cells were lysed and the lysates subjected to IP and WB. (**B**) HEK293T cells were transfected with plasmids expressing a full-length NLRP3 construct as well as constructs expressing the full-length or PTK domain of BTK. After 24 hours, the cells were lysed and lysates subjected to IP and WB. (**C**) Conserved domains of mouse NLRP3 were analyzed, and constructs containing the various domains were generated as indicated. (**D**) HEK293T cells were transfected with plasmids expressing a full-length BTK construct as well as one of various NLRP3 constructs. After 24 hours, the cells were lysed and lysates subjected to IP and WB. (**E**) HEK293T cells were transfected with plasmids expressing the NLRP3 pyrin domain with or without cotransfection of a construct expressing a full-length BTK construct. After 24 hours, the cells were lysed and lysates subjected to IP and WB. Data are representative of 2 independent experiments.

to SDS-PAGE and anti-NEK7 WB under nonreducing conditions. We found that in cells expressing NLRP3 and NEK7, NEK7 exhibited considerable dimerization, whereas in cells expressing NLRP3 and NEK7 and cotransfected with a BTK-expressing plasmid, such NEK7 dimerization was greatly reduced (Supplemental Figure 15C). Furthermore, we obtained a reciprocal result with cell lysates from WT and BTK-KO BMDMs treated with LPS plus nigericin that were subjected to SDS-PAGE and anti-NEK7 WB under nonreducing conditions. In this case, although lysates from both WT and BTK-KO BMDMs contained multiple high-molecular-weight bands indicative of dimer and oligomer formation, the intensity of the bands was much greater in lysates from BTK-KO cells than in those from WT cells (Supplemental Figure 15D). From these studies, it was thus apparent that NEK7 dimerization or oligomerization was enhanced in the absence of BTK.

Taken together, these studies suggest that Tyr and Ser phosphorylation of NEK7 is enhanced by interaction with NLRP3 during NLRP3 inflammasome assembly and that such phosphorylation is inhibited by BTK, most likely because the latter inhibits NLRP3-NEK7 interaction and, in turn, NEK7 oligomerization.

BTK regulates the NLRP3 inflammasome but not other inflammasomes. In studies to determine whether BTK regulation also affects inflammasomes other than NLRP3, we evaluated absent in melanoma 2 (AIM2) and NLR family, CARD domain–containing 4 (NLRC4) transcription induced by LPS and AIM2 and NLRC4 inflammasome function in LPS and poly(dA:dt)- or flagellin-stimulated WT and BTK-deficient mouse BMDMs. We found that BTK deficiency affected neither the transcription of these inflammasome components nor the function of their inflammasomes (Supplemental Figure 16, A–D).

The PH and PTK domains of BTK interact with the pyrin and NACHT domains of NLRP3. In parallel studies, we next sought to identify the NLRP3 and BTK domains that interact with one another. To this end, we transfected HEK293T cells with both plasmids expressing a full-length NLRP3 construct as well as plasmids expressing one of various BTK domains and after 24 hours subjected the cells to immunoblot analysis. We found that the pleckstrin homology (PH) domain of BTK as well as the BTK fragment with protein tyrosine kinase (PTK) or Src homology (SH) deletions interacted with NLRP3 (Figure 3A). The PTK domain also interacted with NLRP3, as shown in a study in which NLRP3 was immunoprecipitated with anti-NLRP3 and then subjected to WB with anti-Flag (Figure 3B).

In additional studies, we transfected HEK293T cells with a plasmid expressing a full-length NLRP3 construct or a truncated NLRP3 fragment construct (generated using the NCBI's Conserved Domains prediction tool; https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Figure 3C) along with a full-length BTK construct, and then subjected the cells to immunoblotting. We found that BTK bound to full-length NLRP3 as well as to NLRP3 with either pyrin or leucine-rich repeat (LRR) domain deletions but not to a LRR domain fragment (Figure 3D). In addition, using anti-NLRP3 to perform IP (because the Flag-tagged pyrin domain could not be recognized by anti-Flag), we found that the pyrin domain also bound to BTK (Figure 3E). It was thus clear that BTK binds to the pyrin and NACHT domains of NLRP3.

BTK domains involved in NLRP3 inflammasome inhibition. With the ultimate goal of understanding the mechanism of BTK regulation of the NLRP3 inflammasome, we performed further studies to define which domain of BTK is required for inflammasome inhi-

bition. To this end, we generated various truncated constructs of BTK (Figure 4A) using the NCBI's Conserved Domains prediction tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and then transfected plasmids expressing these constructs into HEK293T cells together with plasmids expressing NLRP3, ASC, pro–caspase-1, and pro–IL-1β. Twenty-four hours later, we harvested the cultured supernatants for ELISA assessment of IL-1β secretion and found that, as shown previously, the full-length BTK construct inhibited IL-1β production by the reconstituted NLRP3 inflammasome. In addition, we observed that the PTK construct that contained the kinase site of BTK showed even greater inhibition and that the BTK construct in which the PTK fragment had been deleted (ΔPTK) was not inhibitory (Figure 4B). In contrast, the PH domain construct was not inhibitory, and the BTK construct with deletion of the SH domain did not exhibit reduced inhibition (Figure 4B). These results showed that the PTK domain containing the BTK kinase site plays a critical role in BTK inhibition of the NLRP3 inflammasome.

In view of the above findings, we next asked whether kinase activity is required for the inhibitory function of BTK. To answer this question, we generated a kinase-dead mutant of the BTK construct (BTK K430E) that exhibits both defective Tyr223 phosphorylation and kinase activity (Supplemental Figure 17, A and B). We then transfected a plasmid expressing this construct into HEK293T cells together with plasmids expressing NLRP3 and other inflammasome components as described above. Next, we analyzed the culture supernatants by ELISA and found that the kinase-dead BTK-mutant construct failed to inhibit IL-1β production (Figure 4C), although it bound to NLRP3 to the same extent as intact BTK (Figure 4D). In addition, in lentiviral vector–mediated overexpression studies, we found that expression of WT BTK in BTK-KO cells inhibited IL-1β production, whereas kinase-dead BTK did not (Figure 4, E–G). These findings indicate that BTK inhibition of NLRP3 inflammasome activity requires its kinase activity. In addition, these observations suggest that initial activation of BTK by a TLR or other type of primary signaling is a necessary initial step in BTK regulation of the inflammasome (26).

BTK inhibits NLRP3 inflammasome activation by preventing protein phosphatase 2A activity. Recognizing that BTK inhibition of NLRP3 inflammasome activation is dependent on its kinase activity, we reasoned that BTK might negatively affect the function of another molecule (phosphatase or kinase) necessary for inflammasome activation. Using the STRING online tool (https://string-db.org/), which predicts the probability of protein interactions, we found that BTK was likely to have a strong association with protein phosphatase 2A (PP2A), a phosphatase previously shown to regulate NLRP3 dephosphorylation at Ser5 in the pyrin domain of NLRP3 and, thereby, its oligomerization (27).

To determine whether BTK affects NLRP3 inflammasome activation through PP2A, we examined IL-1β production in HEK293T cells transfected with constructs that allowed reconstitution of the NLRP3 inflammasome with and without cotransfection of plasmids expressing PP2A as well as with and without plasmids expressing BTK or fragments of BTK. We found that cells cotransfected with a PP2A plasmid had enhanced IL-1β production and that such enhancement was blocked by full-length BTK as well as the PTK fragment of BTK (Figure 4H); however, only the PTK fragment brought the IL-1β secretion down to a level below baseline. In a final study along these lines, we sought to determine whether BTK or the PTK fragment of BTK could block PP2A-mediated dephosphorylation of Ser5 in the pyrin domain of NLRP3 (the only p-Ser in this domain). Accordingly, we transfected HEK293T cells with plasmids expressing tagged pyrin and tagged PP2A along with plasmids expressing BTK, kinase-dead BTK, or PTK and then subjected the cell lysates to IP with anti-NLRP3 and to immunoblotting with anti–p-Ser. We found that, whereas the presence of PP2A led to serine dephosphorylation of pyrin expressing p-Ser, the presence of BTK and PTK blocked such dephosphorylation but kinase-dead BTK did not (Figure 4I). In addition, transfection of HEK293T cells with a plasmid expressing mutant full-length NLRP3 containing a pyrin domain in which Ser5 was replaced by Ala5 led to functionally impaired inflammasome activity that could not be blocked by cotransfection of a plasmid expressing BTK (Figure 4J). Finally, in formal studies of phosphatase activity, we found that the full-length BTK with intact kinase activity prevented PP2A phosphatase activity, whereas full-length BTK with a mutated kinase site lacking kinase activity did not (Figure 4K).

To investigate the mechanism by which BTK affects PP2A function, we first determined whether PP2A binds to BTK or any of its fragments in HEK293T cells transfected with plasmids expressing PP2A constructs and NLRP3 constructs as well as constructs of full-length and truncated BTK, and then subjected the cells to immunoblotting. We found that although full-length BTK or kinase-dead BTK bound to PP2A, the PH and PTK fragments of BTK did not (even though they bound to NLRP3) (Figure 5A). Interestingly, the strength of the NLRP3 band was enhanced in the presence of BTK, presumably because NLRP3 binds to both PP2A and BTK; in contrast, the strength of the NLRP3 band was diminished in the presence of the PH or PTK fragments, presumably because these fragments bind to NLRP3 but not PP2A and thereby block the binding of NLRP3 to PP2A.

In additional studies, we examined the relevant functional aspects of BTK interaction with PP2A, in this case in WT and BTK-KO BMDMs, to be sure of the physiological significance of this interaction. We found that LPS stimulation of WT BMDMs induced BTK interaction with PP2A but that this interaction was not detectable in WT BMDMs stimulated with LPS plus nigericin, i.e., when the NLRP3 inflammasome was activated (Figure 5B). Moreover, LPS stimulation of WT BMDMs was accompanied by increased PP2A p-Tyr307, whereas LPS stimulation of BTK-KO cells did not increase such phosphorylation above background levels (Figure 5B), the latter observation suggesting that BTK plays an essential role in the phosphorylation of PP2A in LPS-stimulated WT cells. Finally, we explored the impact of decreased PP2A activity in cells in which PP2A levels were reduced as a result of transfection of PP2A-specific siRNA on BTK inhibition of inflammasome activity (Figure 5C). We found that NLRP3 inflammasome activity was decreased (whereas IL-6 production was unaffected) by decreased PP2A expression in both WT and BTK-KO cells to an extent approximately commensurate with the level of decrease in PP2A expression (Figure 5, D and E). This result is consistent with and verifies studies of BTK-intact cells previously conducted by Stutz et al. establishing that PP2A phosphatase activity and

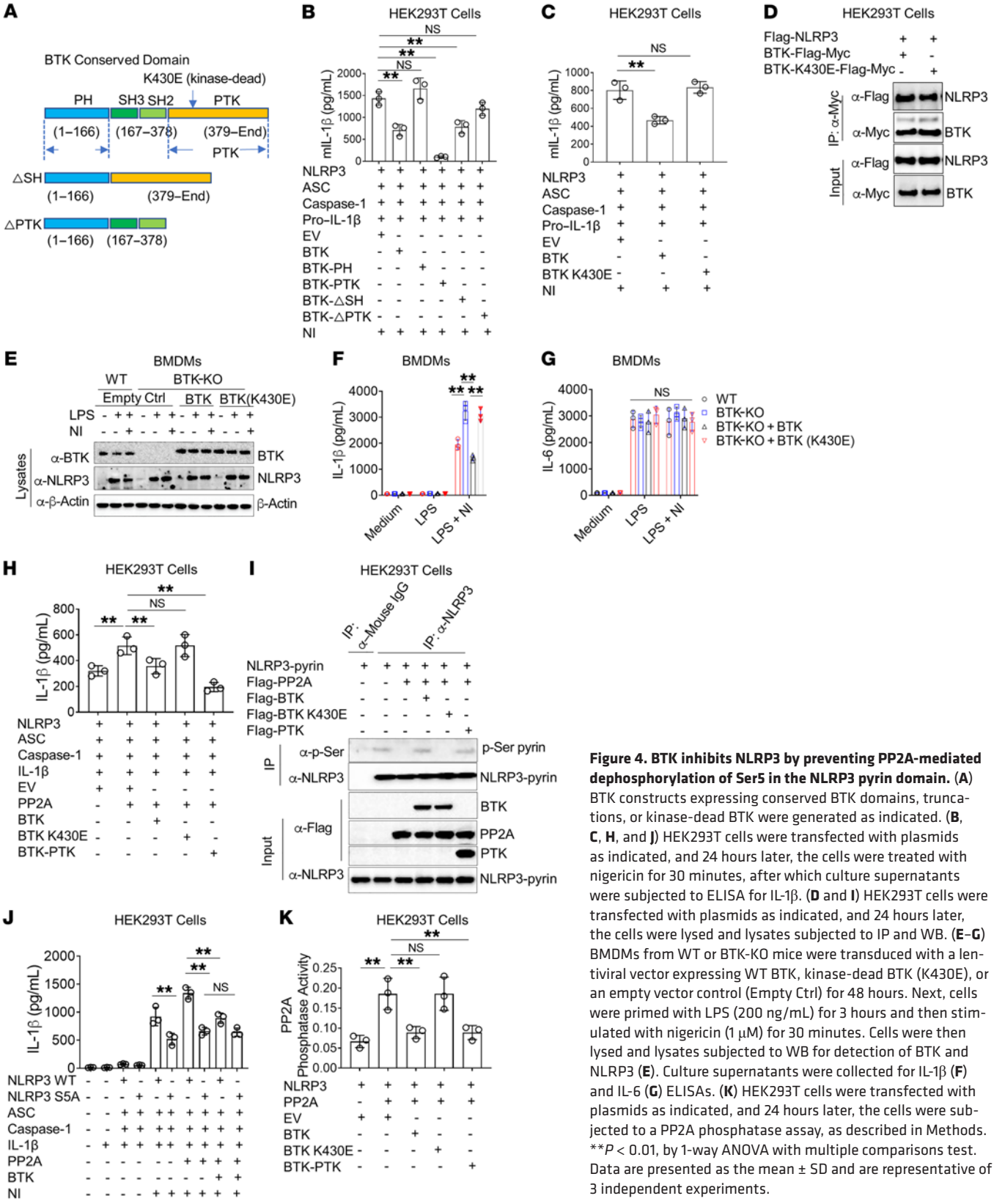

Figure 4. BTK inhibits NLRP3 by preventing PP2A-mediated dephosphorylation of Ser5 in the NLRP3 pyrin domain. (**A**) BTK constructs expressing conserved BTK domains, truncations, or kinase-dead BTK were generated as indicated. (**B**, **C**, **H**, and **J**) HEK293T cells were transfected with plasmids as indicated, and 24 hours later, the cells were treated with nigericin for 30 minutes, after which culture supernatants were subjected to ELISA for IL-1β. (**D** and **I**) HEK293T cells were transfected with plasmids as indicated, and 24 hours later, the cells were lysed and lysates subjected to IP and WB. (**E**–**G**) BMDMs from WT or BTK-KO mice were transduced with a lentiviral vector expressing WT BTK, kinase-dead BTK (K430E), or an empty vector control (Empty Ctrl) for 48 hours. Next, cells were primed with LPS (200 ng/mL) for 3 hours and then stimulated with nigericin (1 μM) for 30 minutes. Cells were then lysed and lysates subjected to WB for detection of BTK and NLRP3 (**E**). Culture supernatants were collected for IL-1β (**F**) and IL-6 (**G**) ELISAs. (**K**) HEK293T cells were transfected with plasmids as indicated, and 24 hours later, the cells were subjected to a PP2A phosphatase assay, as described in Methods. $^{**}P < 0.01$, by 1-way ANOVA with multiple comparisons test. Data are presented as the mean ± SD and are representative of 3 independent experiments.

dephosphorylation of Ser5 in the NLRP3 pyrin domain is a major initiator of NLRP3 inflammasome activation (27). As such, these studies indicate that blockade of PP2A function is a realistic mechanism of BTK-mediated inhibition of the NLRP3 inflammasome.

Overall, these data demonstrate that BTK inhibits NLRP3 inflammasome activation in large part by blocking PP2A dephosphorylation of Ser5 via its PTK domain and that such inhibition ceases with inflammasome activation and cessation of BTK bind-

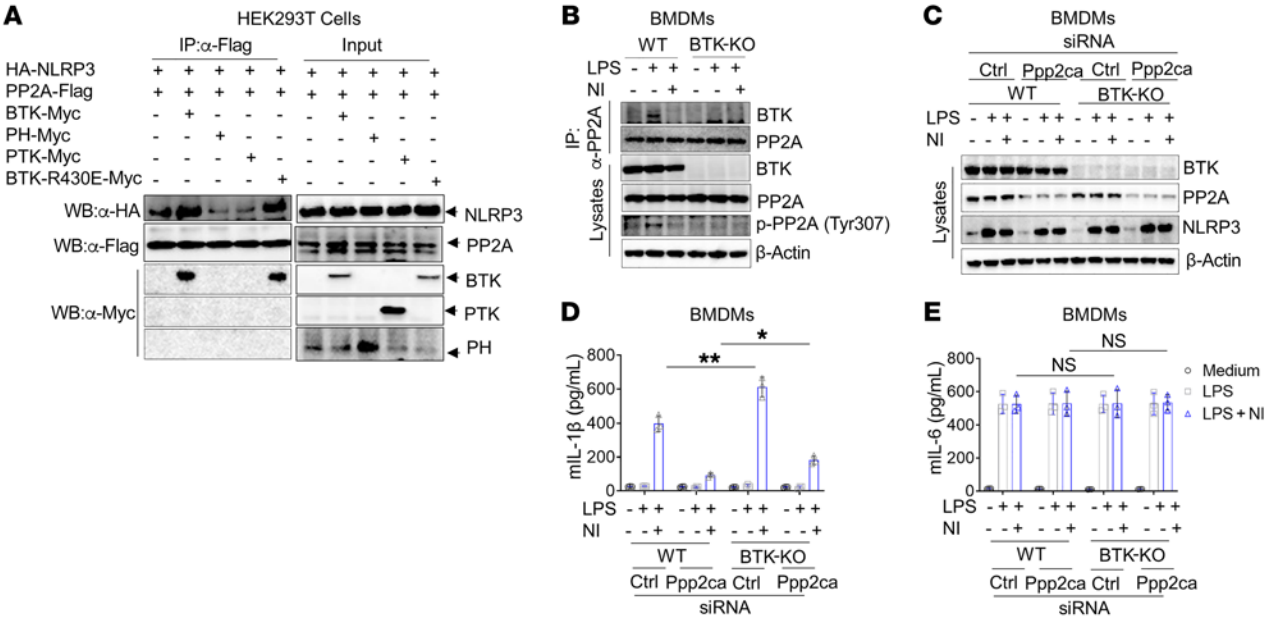

Figure 5. BTK interacts with PP2A and upregulates PP2A Tyr307 phosphorylation, and BTK-KO cells produce decreased amounts of IL-1β in cells with PP2A knockdown. (**A**) HEK293T cells were transfected with plasmids as indicated, and 24 hours later, the cells were lysed and lysates subjected to IP with anti-Flag antibody and then WB. Data are representative of 2 independent experiments. (**B**) BMDMs from WT and BTK-KO mice were stimulated with LPS (200 ng/mL) for 3 hours, after which the cells were lysed and lysates subjected to IP and WB. (**C**–**E**) BMDMs were transfected with siRNA specific for PP2A (Ppp2aca) as indicated, and 48 hours later, the cells were primed with LPS (200 ng/mL) for 3 hours and then stimulated with nigericin for 30 minutes. Cells were then lysed and lysates subjected to WB for BTK, PP2A, and NLRP3 (**C**); the above culture supernatants were collected for IL-1β (**D**) and IL-6 (**E**) ELISAs. $^*P < 0.05$ and $^{**}P < 0.01$, by 1-way ANOVA with multiple comparisons test. Data are presented as the mean ± SD and are representative of 2 independent experiments.

ing to NLRP3. Moreover, such inhibition requires BTK function allowing phosphorylation and thus temporary inactivation of PP2A.

Mechanism of high-dose BTK inhibitor–mediated inhibition of NLRP3 inflammasome activation. As noted above, although low doses of BTK inhibitors mimicked the enhancing effects of BTK deficiency on NLRP3 inflammasome activity, high doses of BTK inhibitors had the effect of inhibiting NLRP3 inflammasome activity. To investigate this paradoxical effect, we first set out to determine whether low and high doses of the BTK inhibitor (LFM-A13) differentially affects NLRP3 interaction with ASC and NEK7 in immunoblot studies of macrophages stimulated with LPS and nigericin. We found that, whereas low doses of LFM-A13 enhanced NLRP3 interaction with ASC and NEK7, high doses of this inhibitor abolished such interaction in a dose-dependent manner (Supplemental Figure 18A). Similarly, low and high doses of LFM-A13 upregulated both ASC and NEK7 oligomerization and inhibited ASC and NEK7 oligomerization, respectively (Supplemental Figure 18B). Finally, we assessed the dose-dependent effects on NLRP3 inflammasome generation of mature caspase-1 (caspase p20) and cleaved GSDMD in LPS-plus-nigericin–stimulated immortalized macrophages. In this case, we again found that although low doses of LFM-A13 enhanced expression of these downstream inflammasome components, high doses of LFM-A13 suppressed expression of these components (Supplemental Figure 18C). Thus, the differential effects of the LFM-A13 inhibitor on IL-1 secretion were reflected in the interaction of NLRP3 with downstream NLRP3 inflammasome components and in the generation of NLRP3 inflammasome products.

Given that BTK inhibition of the NLRP3 inflammasome is mediated by an effect on NLRP3 phosphorylation, it seemed likely that inhibition of the NLRP3 inflammasome by high doses of a BTK inhibitor would be caused by a similar (but different) effect on phosphorylation. One possibility is that high doses of BTK inhibitor inhibit serine phosphorylation of NLRP3 at Ser194 in the NACHT domain of NLRP3, a BTK-independent phosphorylation event that has recently been shown to be necessary for NLRP3 inflammasome activation (28). Ser194 phosphorylation is mediated by JNK, a kinase that has been shown to be inhibited by LFM-AI3 and perhaps by ibrutinib as well (29, 30). Thus, it is possible that inhibition of the NLRP3 inflammasome by high-dose BTK inhibitor is an off-target effect of inhibitors on JNK activity.

BTK deficiency exacerbates experimental colitis. TNBS-induced colitis (TNBS colitis) is a murine model of colonic inflammation widely used to study the pathogenesis of inflammatory bowel disease (31). It therefore presents an opportunity to determine how BTK expression and its possible effects on the NLRP3 inflammasome affect a clinically relevant mucosal inflammatory process.

In initial studies to explore this question, we first established that mouse small intestine and colon express considerable amounts of BTK at the protein level (Supplemental Figure 19). We then administered TNBS per rectum to age- and sex-matched C57BL/6 WT (or littermate) and BTK-KO mice on days 0 and 2, and then after 4 days assessed the features of the colitis thus induced. We found that BTK-KO mice had significantly greater body weight loss and colon shortening and a higher disease histological score than did WT mice (Figure 6, A–D). Accompanying studies of colonic tissue extracts subjected

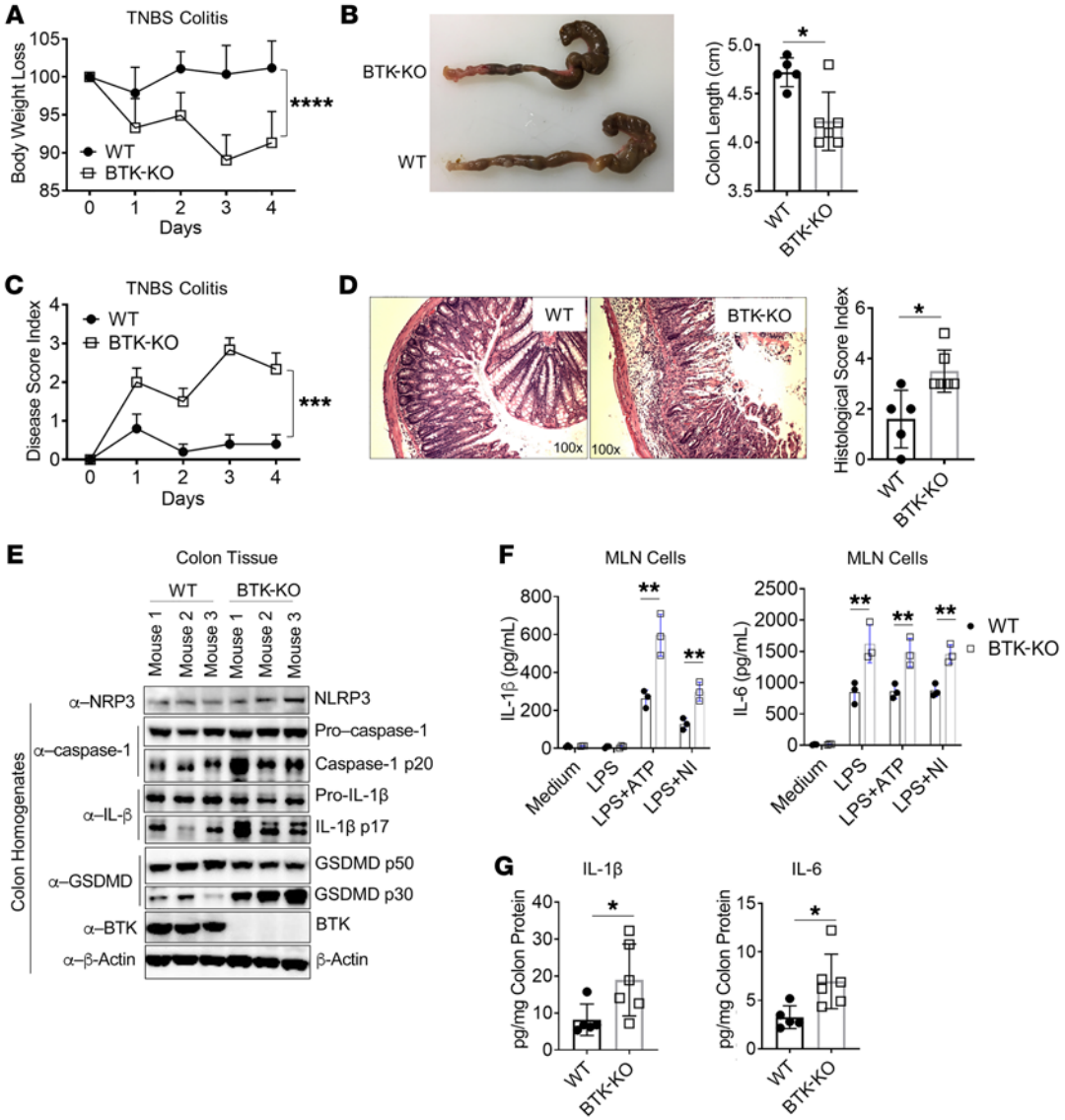

Figure 6. Mice with BTK deficiency exhibit more severe TNBS colitis than do WT mice. Male BTK-KO mice (n = 6) and their WT littermates (n = 5) were administered 3 mg TNBS per rectum on day 0 and day 2 to induce TNBS colitis. Mouse body weight loss after 4 days (**A**), colon length (**B**), disease score (**C**), and histological changes (**D**) due to colitis were measured. Original magnification, ×100. (**E**) Colonic tissue homogenates were subjected to WB. (**F**) Mononuclear cells from MLNs were primed with LPS (1 μg/mL) for 12 hours and then stimulated with ATP (5 mM, 30 minutes) or nigericin (1 μM, 30 minutes), and the culture supernatants were then subjected to ELISA for IL-1β and IL-6. (**G**) Colonic tissues were homogenized, and the homogenates were subjected to ELISAs for IL-1β and IL-6. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, by 2-tailed Student's t test (**B**, **D**, and **G**) or 1-way ANOVA with multiple comparisons test (**A**, **C**, and **F**). Data are presented as the mean ± SD and are representative of 3 independent experiments.

to WB revealed greater cleavage of IL-1β, caspase-1, and GSDMD in extracts from BTK-KO mice than in those from WT mice (Figure 6E). In addition, mesenteric lymph node (MLN) cells from BTK-KO mice produced larger amounts of IL-1β and IL-6 than did cells from WT mice (Figure 6F), and the IL-1β and IL-6 concentration was increased in colonic tissue homogenates from BTK-KO mice compared with those from WT mice (Figure 6G). This correlated with the fact that, although expression of NLRP3 inflammasome components in the intestinal tissue of BTK-KO mice was only mildly increased compared with that of WT mice, BTK-KO colonic tissue contained a clearly more active inflammasome as indicated by the presence of greater amounts of mature IL-1β, caspase-1, and GSDMD (Figure 6E).

In a further comparison of BTK-KO and WT mice subjected to TNBS colitis, we examined the expression of colonic tissue mRNA levels associated with inflammatory cell recruitment and function or epithelial cell barrier function. We found that colonic tissue from BTK-KO mice had increased expression of mRNA specific for CXCL1 and CCL2, chemokines involved in recruitment of neutrophils and monocytes to the colon (Supplemental Figure 20A). In addition, KO colonic tissue showed increased expression of mRNA specific for the neutrophil markers Ly6G and elastase (Supplemental Figure 20B) and the monocyte-associated proinflammatory proteins NLRP3, IL-1β, IL-18, IL-6, and TNF-α (Supplemental Figure 20C). Finally, we found that KO colonic tissue expressed

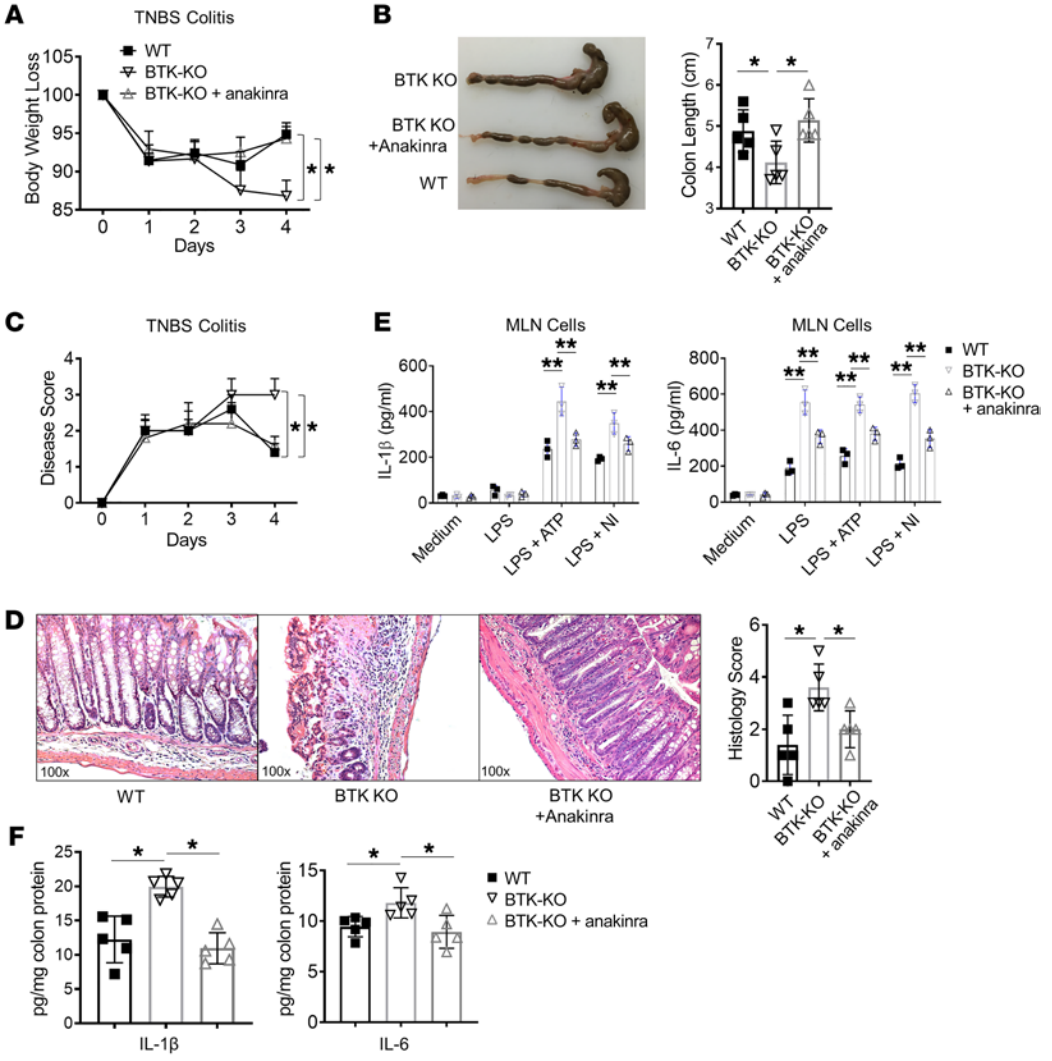

Figure 7. Inhibition of IL-1β signaling ameliorates TNBS colitis in BTK-KO mice. Male BTK-KO mice (n = 5) were administered anakinra i.p. (0.5 mg/mouse/day) on day –1 and then 3 mg TNBS per rectum on days 0 and 2. Male BTK-KO mice (n = 5) and WT mice (n = 5) administered TNBS per rectum and not treated with anakinra served as controls. Mouse body weight loss (**A**), colon length (**B**), disease score (**C**), and histological damage (**D**) due to colitis were measured. Original magnification, ×100. (**E**) Mononuclear cells from MLNs were primed with LPS (1 μg/mL) for 12 hours and then stimulated with ATP (5 mM, 30 minutes) or nigericin (1 μM, 30 minutes). The culture supernatants were then subjected to ELISA for IL-1β and IL-6. (**F**) Colonic tissues were homogenized and the homogenates subjected to ELISAs for IL-1β and IL-6. $^*P < 0.05$ and $^{**}P < 0.01$, by 1-way ANOVA with multiple comparisons test. Data are presented as the mean ± SD and are representative of 3 independent experiments.

increased mRNA specific for IFN-γ, a Th1 cytokine characteristic of TNBS colitis (Supplemental Figure 20D), and mRNA specific for claudin-2, a tight junction regulator associated with decreased epithelial cell barrier function (32) (Supplemental Figure 20E).

To provide additional proof of BTK's role in the development of colitis, we examined its role in dextran sodium sulfate (DSS) colitis. In parallel to the data obtained using the TNBS colitis model, the data from the study using this model showed that BTK-KO mice subjected to DSS colitis had a significantly greater body weight loss, colon shortening, and histological score than did WT mice (Supplemental Figure 21, A–C). In addition, MLN cells from BTK-KO mice produced more IL-1β and IL-6 than did cells from WT mice (Supplemental Figure 21D). These data therefore support the idea that BTK-KO mice are more susceptible to colitis than are WT mice.

Blockade of IL-1β signaling or IL-1β ameliorates the increased TNBS colitis occurring in BTK-KO mice. Since BTK inhibition or deficiency is associated with increased IL-1β production, we next asked whether blockade of IL-1β signaling could inhibit TNBS colitis induced in BTK-KO mice as demonstrated above. To this end, we induced TNBS colitis in 2 groups of BTK-KO mice, one treated with anakinra and one that did not receive this treatment, as well as in control WT mice not treated with anakinra. As shown previously, we found that BTK-KO mice had more severe colitis than did WT mice, as documented by various parameters of colon inflammation (Figure 7, A–D) and by increased IL-1β and IL-6 production by MLN cells subjected to NLRP3 inflammasome stimulation or IL-1 and IL-6 content of colonic tissue homogenates (Figure 7, E and F). In contrast, BTK-KO mice treated with anakinra (an agent that blocks IL-1β or IL-1α signaling) resulted in reduced colitis accompanied

by lower production of IL-1β and IL-6 by NLRP3 inflammasome–stimulated MLN cells and reduced IL-1β and IL-6 content in colonic tissue homogenates (Figure 7, A–F). In addition, anakinra treatment greatly reduced the higher levels of IL-1β, TNF-α, IFN-γ, CXCL1, CCL2, and claudin 2 mRNA present in BTK-KO colonic tissue (Supplemental Figure 22). These data demonstrated that anakinra treatment almost completely reduced TNBS colitis in BTK-KO mice to the level of colitis observed in WT mice and thus suggested that the increased colon inflammation in BTK-KO mice could be attributed to either increased IL-1β– or IL-1α–driven inflammation resulting from a dysregulated NLRP3 inflammasome. Finally, to distinguish between IL-1β and IL-1α proinflammatory effects, we conducted similar TNBS colitis studies in BTK-KO mice, in which colitis induction was accompanied by either administration of anti–IL-1β or anti–IL-1α antibodies that had been shown by WB to be specific for IL-1β and IL-1α, respectively (Supplemental Figure 23). We found that anti–IL-1β administration caused diminished colitis in BTK-KO mice, whereas anti–IL-1α had no such effect (Supplemental Figure 24, A–C). In addition, activation of the NLRP3 inflammasome in MLN cell populations from BTK-KO mice with TNBS colitis led to a robust secretion of IL-1α, IL-1β, and IL-6 in mice treated with an isotype control antibody or an anti–IL-1α antibody that was greatly reduced in mice treated with anti–IL-1β, indicating that administration of anti–IL-1β, but not anti–IL-1α, antibody had downregulated the number of cells migrating from the lamina propria to the MLNs that are capable of proinflammatory cytokine production (Supplemental Figure 24D). Taken together, these studies strongly suggest that the increased colitis in BTK-KO mice was largely due to excess IL-1β secretion arising from increased NLRP3 inflammasome activity. It should be noted, however, that in a separate study, administration of anti–IL-1β antibody to WT mice with TNBS colitis was found to decrease the severity of colitis. This suggests that the decrease in colitis in BTK-KO mice to the levels of colitis seen in untreated WT mice with anti–IL-1β antibody administration did not completely abrogate IL-1β–mediated inflammation in BTK-KO mice at the dose of antibody administered (Supplemental Figure 25, A–D).

BTK inhibition regulates NLRP3 inflammasome activation in vivo in a dose-dependent manner. The fact that BTK deficiency in vivo leads to increased susceptibility to experimental colitis suggested that a similar situation would occur in the case of BTK inhibition, but in this case the effect would be dose dependent, reflecting the dose-dependent effect of BTK inhibitor on the NLRP3 inflammasome in vitro.

In a study addressing this question, WT mice were i.p. injected with the BTK inhibitor LFM-A13 at various doses and were then i.p. administered alum crystals to induce peritonitis, an inflammatory response characterized by NLRP3 inflammasome–mediated recruitment of neutrophils into the peritoneal cavity (33). We found that the total numbers of peritoneal exudate cells (PECs) and $Gr1^+$ neutrophils were increased in mice treated with a low-dose inhibitor compared with those not treated with an inhibitor (Supplemental Figure 26, A and B). Consistent with this observation, IL-1β levels in the peritoneal lavage fluid (PLF) and the level of cleaved caspase-1 in PECs were also increased (Supplemental Figure 26, C and D). We observed the opposite result in mice injected with high doses of the inhibitor. Consistent with these data, BTK-KO mice phenocopied the mice injected with low doses of the BTK inhibitor, in that they had enhanced PECs, neutrophil infiltration, and IL-1β production in PLF (Supplemental Figure 26, E–G).

In a second study, we evaluated the effect of administration of the BTK inhibitor on BTK inhibitory function in the TNBS colitis model described above. In these studies, we preadministered a low dose (10 μg) of the BTK inhibitor LFM-A13 daily to C57BL/6 WT mice to inhibit BTK (based on the dose used in the peritonitis study), beginning on day –2 of the study. Then, on days 0 and 2 of the study, the mice were administered TNBS per rectum to induce colitis. Compared with the control mice, the BTK inhibitor–treated mice were more susceptible to TNBS colitis, as indicated by multiple clinical and immunologic parameters (Supplemental Figure 26, H–L). These data indicate that inhibition of BTK in mice by low doses of a BTK inhibitor confers susceptibility to colon inflammation.

Discussion

BTK has a key role in B cell signaling via the Ig receptor and is thus an essential component of B cell development and maturation (34). In addition, constitutive activation of BTK is an essential feature of B cell proliferation in chronic lymphocytic leukemia (CLL), and this characteristic of CLL has been successfully used in the treatment of this B cell leukemia with anti-BTK agents such as ibrutinib (22, 35). These facts tying BTK to B cell function, however, do not necessarily imply that BTK activity is exclusively related to humoral immunity; on the contrary, there is now emerging evidence that loss of BTK also influences cell-mediated (and innate) immunity via effects on DC and macrophage function (36). In this study, we investigated the effect of BTK on one such innate immune function: NLRP3 inflammasome activation in macrophages and DCs. We found that BTK had an inhibitory effect on the NLRP3 inflammasome, which was demonstrated by our experiment showing that low concentrations of ibrutinib (such as those occurring during CLL treatment) upregulated NLRP3 inflammasome activation. In addition, absent BTK expression in mice was marked by increased NLRP3 inflammasome activation that gives rise to increased proinflammatory responses in experimental models of colitis.

In studies in which we investigated the mechanism of BTK inhibition of the NLRP3 inflammasome, we first established that, although both the PH and PTK domains of BTK bound to NLRP3 in cotransfection studies, it was mainly the PTK domain that was responsible for inhibition of the NLRP3 inflammasome. In additional studies based on these findings, we used an online tool predictive of likely interactions between BTK and various kinases or phosphatases that led us to focus on possible interactions between BTK and PP2A, the latter being a phosphatase complex previously shown to induce NLRP3 activation by dephosphorylating Ser5 in the pyrin domain of NLRP3 and thereby promoting activation of the NLRP3 inflammasome (27). We found that BTK bound to PP2A and, via its kinase activity, induced phosphorylation at PP2A Tyr307, an established mechanism of PP2A inactivation (37). This correlates with the fact that in cotransfection studies, BTK and, more particularly, the PTK domain of BTK, inhibited the capacity of exogenous PP2A to augment NLRP3 inflammasome activation. Finally, and perhaps most important, BTK, via its PTK domain, inhibited PP2A dephosphorylation of Ser5 in the pyrin domain

of NLRP3. On the basis of these studies, we concluded that BTK inhibits NLRP3 inflammasome activation by binding to NLRP3 and, via its PTK domain, then inhibits PP2A phosphatase activity relating to dephosphorylation of Ser5 of the NLRP3 pyrin domain. However, in the presence of an NLRP3 activation signal (e.g., nigericin), the BTK interaction with NLRP3 diminishes, and BTK is no longer able to block PP2A initiation of inflammasome activation via dephosphorylation; hence, activation can proceed (see diagram in the Graphical Abstract).

A potential regulatory role of BTK in the NLRP3 inflammasome was proposed by Liu et al. (10) and Ito et al. (11), who showed that the BTK inhibitors ibrutinib and LFM-A13 prevented IL-1β production induced by NLRP3 activators in human monocytes and mouse macrophages carrying an XID mutation. Inhibition of the NLRP3 inflammasome at high doses of a BTK inhibitor was also observed by He et al., who found that immortalized, reconstituted *Nlrp3*-deficient macrophages cultured in the presence of LFM-A13 at a concentration of 20 μM or higher inhibited caspase-1 cleavage following NLRP3 inflammasome activation (7). Our findings are in agreement with these prior studies of the effects of BTK inhibitors on NLRP3 activation, but we add the important caveat that such inhibition is seen only with high and probably nonphysiologic concentrations of inhibitors. In addition, such high-dose inhibition was seen in cells lacking BTK expression, strongly suggesting it is mediated by a direct off-target effect on NLRP3 rather than on BTK itself.

On the other hand, our studies differ with those of Liu et al. and Ito et al. in showing that BTK enhances (rather than decreases) NLRP3 inflammasome activity. The reasons for this discrepancy are several-fold and are disclosed in a series of studies that mainly (but not solely) center around the fact that stimulation of BTK-KO cells with low doses of TLR4 ligand (LPS) such as that used by Liu et al. results in reduced cell activation compared with WT cells and thus results in decreased NLRP3 expression and BTK activation; this occurs because BTK-KO cells have demonstrably reduced TLR4-mediated activation (12). The stimulation of cells with higher doses of LPS (as in the present studies) results in equal levels of WT and BTK-KO cell activation and therefore overcomes this difficultly. A second reason for the discrepancy, in this case applying to the studies of Ito et al., involves the fact that in the their studies, cells with putatively absent BTK activity were from XID mice that actually expressed mutated BTK with retained kinase activity and that had an enhanced capacity to inhibit NLRP3 inflammasome activation; the use of these cells therefore masked the fact that a true absence of BTK, as in the XLA cells studied here, results in enhanced inflammasome activity.

The conclusion that BTK inhibits NLRP3 inflammasome activity was not only supported by direct studies of inflammasome activity in WT and BTK-deficient cells, as discussed above, but was also supported by studies in which the reciprocal effects of BTK deficiency or overexpression by plasmid transfection were explored with relation to each of the major steps of NLRP3 inflammasome assembly and activation. In addition, these findings were supported by our studies showing that BTK (or a fragment thereof) inhibited BTK-mediated inhibition of PP2A phosphatase activity, as discussed above. Finally, and perhaps most important, this conclusion was supported by the fact that the absence or inhibition of BTK in the whole animal rendered the latter susceptible to increased colitis (or peritonitis) mediated by the NLRP3 inflammasome and its production of IL-1β; this can only be true if BTK is an inhibitor, not an enhancer, of the NLRP3 inflammasome.

The inhibitory role of BTK on NLRP3 inflammasome activation in macrophages and DCs demonstrated here suggests that under normal circumstances, such inhibition has a physiologic ("gate-keeper") role in the regulation of this inflammasome. Recall that BTK bound to NLRP3 following primary NLRP3 inflammasome (LPS-only) activation, but that such binding was not observed (or was greatly reduced) after inflammasome activation signaling induced by nigericin. This suggests that binding of BTK to NLRP3 following LPS (or a similar primary stimulus) alone does not result in NLRP3 inflammasome activation at least in part because BTK binding to NLRP3 in the absence of an activation signal prevents Ser5 dephosphorylation. As already alluded to above, the mechanism by which this occurs is not simply physical blockade of PP2A with its potential dephosphorylation target in the NLRP3 pyrin domain; rather, it involves the kinase function of BTK and thus the ability of the latter to phosphorylate and thereby inactivate PP2A.

BTK inhibition of NLRP3 inflammasome activation, while partial in the in vitro studies presented here, was quite dramatically evident in studies of BTK-deficient mice subjected to TNBS- or DSS- induced colitis. In these studies, we showed that BTK-deficient mice had much more severe colitis than did WT mice. In addition, we demonstrated that the origin of this increased inflammatory response was due to NLRP3 inflammasome activation by showing that the inflammation was accompanied by elevated IL-1β production that was normalized by administration of anakinra, an agent that blocks IL-1β signaling, or by administration of anti–IL-1β itself.

The above studies of the relation of BTK expression to NLRP3 inflammasome–induced inflammation has several clinical ramifications. First, it provides an explanation for the observation that approximately one-third of patients with XLA have gastrointestinal abnormalities and approximately one-tenth of these patients have Crohn's disease (13). Second, patients with CLL being treated with ibrutinib frequently suffer from diarrhea (particularly early in the course of treatment), and it is reasonable to suggest that this may be related to increased gastrointestinal NLRP3 inflammasome activity arising from BTK inhibition by low concentrations of an inhibitor (38).

Methods

Complete details relating to the experimental materials and methods used in this study as well as a description of the patients are provided in the Supplemental Methods.

Human subjects. Peripheral blood from patients with XLA or CLL and healthy individuals was obtained.

Study approval. Peripheral blood from patients with XLA or CLL and healthy individuals was drawn for laboratory examination with written informed consent, in accordance with Declaration of Helsinki principles. All procedures involving patients and animal experiments in this study were approved by the ethics committees of the NIH.

Statistics. Data involving 2 groups of samples were analyzed using a 2-tailed Student's *t* test. Experiments involving more than 2 groups were analyzed using a 1-way ANOVA followed by Dunnett's or Tukey's post hoc multiple comparisons test. All data are presented as the mean ± SD unless otherwise indicated. A *P* value of 0.05 or less was considered statistically significant.

Author contributions

LM performed experiments and data analysis and wrote the manuscript. AK designed studies and analyzed data. EH conducted experiments. KMR collected specimens and provided clinical care. WZ performed bioinformatic analysis of BTK binding proteins. IF provided clinical care of patients, designed studies, and analyzed data. AW performed data analysis and provided patient care. WS designed studies, analyzed data, and wrote the manuscript.

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