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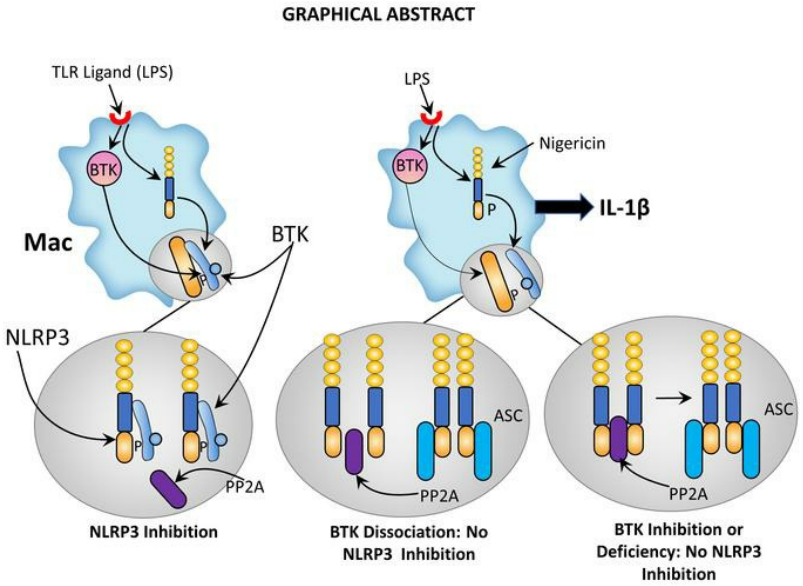
Graphical abstract



Bruton Tyrosine Kinase Deficiency Augments NLRP3 Inflammasome Activation and Causes IL-1β-mediated Colitis

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Abstract

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 NLRP3 inflammasome. We found that bone marrow-derived macrophages (BMDMs) from BTK-deficient mice or monocytes from X-linked agammaglobulinemia patients exhibit increased NLRP3 inflammasome activity; this was also the case with BMDMs exposed to low doses of BTK inhibitor such as ibrutinib and monocytes from chronic lymphocytic leukemia patients being treated with ibrutinib. In mechanistic studies, we found that BTK binds to NLRP3 during the priming phase of inflammasome activation and in doing so inhibits LPS/nigericin-induced assembly of the NLRP3 inflammasome during the activation phase of inflammasome activation. This inhibitory effect was caused by BTK inhibition of PP2A-mediated dephosphorylation of Ser5 in the pyrin domain of NLRP3. Finally, we showed that BTK-deficient mice are subject to severe experimental colitis and such colitis is normalized by administration of anti-IL-β or an inhibitor of IL-1β signaling, anakinra. Together, these studies strongly suggest that BTK functions as a physiologic inhibitor of NLRP3 inflammasome activation; they thereby explain the fact that XLA patients are prone to develop Crohn's disease.

Introduction

The 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 pro-inflammatory cytokines participating in host defense(1-3). However, this potentiality requires strict regulation if one is to suppress responses that lead inadvertently 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 co-factors 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 a molecule to NLRP3 that has the capacity to modify NLRP3 function, such as NEK7(7, 8) or CARD8 (9). 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 of NLRP3 and affects NLRP3 phosphorylation.

Previous investigations 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 wild type 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 wild type and BTK-KO cells we overcome this problem and show that absent BTK, NLRP3 inflammasome activity is augmented.

These studies were therefore in full accord with reciprocal (BTK over-expression) studies involving HEK293T cells transfected with plasmids allowing construction of the NLRP3 inflammasome in which we showed that inflammasome activity by the transfected cells is suppressed by co-transfection of BTK-expressing plasmids. Importantly, the negative regulation of the NLRP3 inflammasome by BTK in these *in vitro* studies was verified by *in vivo* studies that showed that BTK-KO mice exhibit increased severity of trinitrobenzene sulfonic acid (TNBS)-colitis and that the colitis is driven by IL-1β since it is attenuated by administration of an IL-1β inhibitors. Finally, in mechanistic studies we showed that BTK suppressive activity depends on its capacity to block NLRP3 Ser5 dephosphorylation and thus prevent NLRP3 oligomerization.

Overall, the studies reported here suggest that the activation of BTK in macrophages/dendritic cells by TLR ligands or other primary activation factors serves as a physiologic mechanism of NLRP3 inflammasome regulation in that it blocks the activation of the NLRP3 inflammasome activation in the absence of a co-stimulator. In addition, they 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 the activation of the NLRP3 inflammasome we evaluated inflammasome activation in BMDMs obtained from mice and humans with verified BTK deficiency (Supplemental Figure 1). In initial studies focused on murine cells we evaluated the priming phase of NLRP3 inflammasome stimulation in cells from BTK-KO mice by measuring transcription of NLRP3 and IL-1β by 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 WT cells (Figure 1A) whereas the secretion of IL-6 by these cells was equivalent (Figure 1B). Moreover, this enhanced IL-1β secretion was accompanied by increased cleavage of IL-1β and caspase 1 (Figure 1C). As expected, the enhanced production of IL-1β by BMDMs from BTK-KO mice was also observed in BMDCs from these mice (Figure 1D, 1E).

The above findings indicating that mouse cells lacking BTK activity manifest increased NLRP3 inflammasome activity are at odds with data from previous studies by Liu et al(10) and Ito et al(11) showing 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 been shown previously to have impaired TLR4-mediated responses(12). To examine this possibility, we stimulated WT and BTK-KO mouse BMDMs with 10ng/ml LPS (the dose used by Liu et al) and 200ng/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 increased in cells stimulated at the higher dose as compared to WT cells(Supplemental Figure 3A and 3B). 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 to WT cells stimulated with the lower dose of LPS whereas these parameters were equivalent in the two cell populations stimulated with the higher dose (Supplemental Figure 3C and 3D). These data strongly suggest that the previous data showing that BTK-KO cells exhibit decreased NLRP3 inflammasome activity compared to WT cells presumably due to lack of BTK enhancement of such activity is actually due to the fact that BTK-KO cells exhibit reduced TLR4 activation relative to WT cells when these cells are stimulated with low doses of LPS and thus generate reduced amounts of NLRP3 as compared to WT cells.

It should be noted that the problem introduced by reduced activation of BTK KO cells by TLR ligands such as LPS also applies on the basis of previous studies to stimulation by a TLR7/8 stimulant, R848, also utilized in the study by Liu et al. (14, 15). In addition, whereas Liu et al., found that presumably low dose stimulation of cells with LPS induced about equal amounts of pro-IL-1β 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 production of lesser amount of IL-1β protein.

Yet another potential problem arising from the use of the low dose of LPS employed in the Liu et al study to evaluate NLRP3 activation in WT vs. BTK-KO cells concerns whether this dose was sufficient to activate BTK. To evaluate this possibility we examined LPS dose effects on BTK Y223 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. (10ng/ml) induced a lower level of BTK Y223 phosphorylation (as evaluated by Western blot) than the higher dose (200ng/ml) employed in the present studies (Supplementary Figure 4). In 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 the BTK was not fully able to inhibit the NLRP3 inflammasome under these conditions and therefore supports a higher level of NLRP3 activity in sub-optimally activated WT cells than would be present in fully activated WT cells.

The discrepancy between the results in the Ito et al, paper (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 employed (50ng/ml). This follows from the fact that stimulation of mouse BTK-KO cells with this concentration of LPS again results in lower p-IκB and NLRP3 expression than similar stimulation of WT cells and thus an inappropriate comparison of NLRP3 activation in the two cell populations as noted in the discussion above (Supplemental Figure 3A-3B). It should be noted, however, that human XLA cells, in contrast to mouse cells BTK-KO cells, exhibit enhanced NLRP3 activity when stimulated with 50ng/ml of LPS, so that this concentration may be adequate for evaluation of BTK activity in human cells but not in mouse cells (Supplemental Figure 5A-D).

Another and more fundamental difficulty with the results reported by Ito et al. is that the latter authors used cells from CBA/N (XID) mice as a source of BTK-deficient with which to define the effect of BTK deficiency on the NLRP3 inflammasome. Using these cells one does observe that BTK deficiency cells exhibit lower inflammasome activity than WT cells as reported by Ito et al (Supplemental Figure 6A). However, this result is problematic because the R28C BTK mutation in such mice responsible for BTK deficiency results in B cells that express normal amounts of BTK that has 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 mice (BTK-KO mice) (such as those used in the present study) that lack BTK expression (Supplemental Figure 3C) or in the great majority of humans with Bruton's agammaglobulinemia such as those also studied here(18). The presence of the abnormal BTK in XID macrophages is not without consequences. Thus, when stimulated with LPS, BMDMs from XID mice exhibit increased NLRP3/BTK interaction as compared to BMDMs from WT mice; furthermore, while this interaction diminishes after nigericin stimulation it remains increased compared to that in WT cells (Supplemental Figure 6B). Based on the results of studies reported below concerning the mechanism of BTK inhibition of the NLRP3 inflammasome, this enhanced and persistent NLRP3/BTK interaction leads to decreased NLRP3 inflammasome oligomerization and activation because it facilitates persistent blockade of NLRP3 dephosphorylation. It thus

explains why NLRP3 inflammasome activation in macrophages derived from XID mice is low compared to that in macrophages derived from WT mice (Supplemental Figure 6A). Whether these studies indicating that NLRP3 inflammasome activity is decreased in XID mouse-derived macrophages *in vitro* is reflected in NLRP3 inflammasome activity *in vivo* awaits studies of such activity in intact XID mice.

The studies above suggesting that in the absence of BTK such as that occurring in murine BTK-KO cells the activation phase of NLRP3 inflammasome activity is enhanced, implies that BTK 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 lentivirus vector expressing BTK and found that after stimulation with LPS and nigericin BTK KO cells with BTK transduction now exhibited reduced NLRP3 inflammasome activity equivalent to that in WT cells whereas cells transduced with empty vector exhibit enhanced inflammasome activity (Figure 1F-1H). These data further demonstrated that BTK plays an inhibitory role for NLRP3 inflammasome activation.

In yet another test of the idea that BTK is an inhibitor of the NLRP3 inflammasome in murine cells, we determined if the absence of BTK in mouse XLA cells is accompanied by increased generation of gasdermin D and its downstream effect, pyroptosis, since cleavage of precursor gasdermin D is a known consequence of NLRP3 inflammasome generation of mature caspase I (19, 20). Indeed, we found that BTK-KO cells stimulated with LPS and nigericin exhibited 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 LDH upon inflammasome activation compared with WT cells (Supplemental Figure 7B). These results were corroborated by the observation that BTK over-expression in HEK293T cells transfected with plasmids expressing each of the components of the NLRP3 inflammasome and co-transfected with a BTK-expressing plasmid exhibited decreased GSDMD cleavage and LDH release compared to similarly transfected cells co-transfected with control plasmid (Supplemental Figures 7C-D).

BTK Regulation of Human NLRP3 Inflammasome Activity

In studies complementary to those above focusing in this case 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 description of patients). As in the mouse studies we found that stimulation of XLA patient monocytes with LPS (200ng/ml) plus nigericin or ATP led to enhanced NLRP3 inflammasome activation (IL-1β secretion) with no difference in IL-6 secretion (Figure 1I-1J). Again, in studies addressing the discrepant findings of Liu et al(10), we found that whereas stimulation of XLA patient PBMCs or monocytes with low concentrations of LPS (10ng/ml), produced decreased level of IL-1β as compared with cells from control individuals, stimulation of cells with a higher concentrations of LPS (50ng/ml and 200ng/ml) showed that XLA cells produced higher levels of IL-1β as compared to control cells (Supplemental Figure 5A-D). In addition, low doses of LPS induced lower expression of NLRP3 and pro-IL-1β in patient cells as compared to control cells, and this difference vanished when cells were stimulated with higher LPS doses (Supplemental Figure 8A-8C). Stimulation of XLA patient cells with low doses of LPS also induced more IL-10 production than stimulation of cells from healthy control and this difference disappeared when cells were stimulated with higher doses (Supplemental Figure 5E). Inasmuch as IL-10 is a potent NLRP3 inflammasome inhibitor, this is another possible reason that low dose LPS stimulation of XLA patient cells does not demonstrate that lack of BTK leads to increased NLRP3 inflammasome activation(21). Finally, we showed that stimulation of XLA patient and control cells with varying doses of the TLR2 ligand, Pam3CSK4 also led to enhanced NLRP3 inflammasome activation in XLA cells vs. 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 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 Figure10A-D). In the case of LFM-A13, similar results were observed with human elutriated monocytes and with THP-1 human monocyte cells (Supplemental Figure 10E-H); similar results were also obtained with culture of WT and BTK-KO BMDMs (Supplemental Figure 10I, J). It is important to note that the culture of cells in the presence of either BTK inhibitor did not change the rate of transcription of pro-IL-1β and NLRP3 (Supplemental Figure 11); in addition, culture 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 on ibrutinib therapy. The mean blood ibrutinib concentration in appropriately-treated CLL patients is approximately 150ng/ml (0.34μM)(22). On the basis of the above data relating to concentration effects of inhibitor, this concentration would be predicted to cause enhanced NLRP3 inflammasome-derived IL-1β production by patient peripheral monocytes at least for a period of time after administration of the daily dose. Indeed, this prediction proved to be correct based on actual NLRP3 inflammasome activation vs. control activation in the several patients studied (Supplemental Figure 13). Interestingly, IL-1β production by BMDMs from BTK-KO mice was inhibited by high concentrations by ibrutinib without affecting IL-6 production (Supplemental Figure 10 I-J), strongly suggesting that the 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 cells exposed to low (ordinarily

enhancing doses of ibrutinib) (Supplemental Figure 10K-L). Such equality of enhancement indicates that low dose ibrutinib enhancement is BTK dependent.

Taken together, these studies of the effect of BTK inhibitors on human cells indicate that as in the case of BTK deficiency caused by a genetic abnormality, BTK deficiency caused by exposure to low concentrations of inhibitor leads to up-regulation of NLRP3 inflammasome activity. In contrast, high concentrations of inhibitor cause 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 by LPS alone or LPS plus nigericin to immunoblotting and found that in cells stimulated by LPS alone NLRP3 interacts with BTK whereas in cells stimulated with LPS and nigericin such interaction is greatly reduced (Figure 2A); importantly, interaction was observed in IP samples using anti-BTK antibody and not in those using isotype control antibody (Supplemental Figure 14). These data indicate that BTK interaction with NLRP3 accompanying NLRP3 inflammasome activation noted previously(11) occurs during the "priming" phase of NLRP3 inflammasome activation and diminishes during its subsequent activation phase.

The BTK interaction with NLRP3 shown above suggested that BTK affects the level of tyrosine or serine phosphorylation of NLRP3 that, as indicated above, has previous been shown to accompany NLRP3 activation. To investigate this possibility, HEK293T cells were transfected with plasmids expressing the precursor form of IL-1β as well as plasmids expressing components of the NLRP3 inflammasome (as described above) plus (or minus) a BTK-expressing plasmid and then, after 24h, stimulated with nigericin; subsequently, the cells were assessed for capacity to secrete IL-1β or, alternatively, stimulated with nigericin and subjected to lysis for immunoblotting with antibodies

recognizing phosphorylated tyrosine or serine. We found that the transfected cells produce substantial amounts of IL-1β when stimulated with nigericin and that such IL-1β secretion is inhibited by the presence of BTK as predicted from studies of 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 tyrosine phosphorylation of NLRP3 and inhibited the level of serine phosphorylation of NLRP3 (Figure 2C). In reciprocal studies we performed immunoblots of WT and BTK-KO BMDMs stimulated with LPS and nigericin again probing phosphorylation with antibodies recognizing phosphorylated tyrosine and serine. These studies corroborated the studies of transfected HEK293T cells described above in that NLRP3 in BTK-KO cell lysates exhibited increased phosphoserine and decreased phosphotyrosine 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 is increased in WT cells stimulated with LPS and nigericin (vs. cells stimulated with LPS alone) and such oligomerization is 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 ASC and ASC assembly. In initial studies we found that whereas NLRP3 interacts with ASC in HEK293T cells transfected with plasmids expressing NLRP3 or ASC, such interaction is diminished in cells also transfected with a plasmid expressing BTK (Figure 2F). In confirmation of these findings, we found that in WT BMDMs NLRP3 interacts with ASC upon LPS stimulation; however, such interaction is more pronounced in BTK-KO cells (Figure 2G).

We then asked if 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) was enhanced in BTK-KO cells as compared to that occurring in WT cells (Figure 2H,2I). Thus, studies of cells in which BTK levels are increased or decreased indicate that BTK down-regulates 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 if 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 studies on cell lysates to detect interaction. We found that NLRP3 does indeed interact with NEK7 but this interaction was diminished in the presence of BTK (Figure 2J). Similarly, the interaction between NLRP3 and NEK7 can 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 is present in higher molecular weight bands, possibly reflecting dimerization or oligomerization of NEK7 or oligomerization of other NLRP3 components to which it is 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 serine phosphorylation as well (Supplemental Figure 15A). These data correlated with studies of BMDCs isolated from WT or BTK-KO mice and stimulated with LPS and nigericin to activate the NLRP3 inflammasome; here we found that NEK7 tyrosine and serine phosphorylation was increased in the absence of BTK (Supplemental Figure 15B).

In further studies examining if 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 plasmid expressing BTK to SDS PAGE and anti-NEK7 Western blotting under non-reducing conditions. We found that in cells expressing NLRP3 and NEK7, NEK7 exhibited considerable dimerization whereas in cells expressing NLRP3 and NEK7 and co-transfected with a BTK-expressing plasmid such NEK7 dimerization was greatly reduced (Supplemental Figure 15C). Furthermore, a reciprocal result was obtained with WT and BTK-KO BMDMs treated with LPS plus nigericin cell lysates subjected to SDS PAGE and anti-NEK7 Western blot analysis under non-reducing conditions. In this case, while 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 were much greater in lysates from BTK-KO cells than 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 both tyrosine and serine 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 examine if BTK regulation also effects inflammasomes other than NLRP3 we evaluated AIM2 and 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 affects neither transcription of these inflammasome components nor the function of their inflammasomes (Supplemental Figure 16A-D).

The PH and PTK domains of BTK interacts with the Pyrin and NACHT domains of NLRP3

In studies parallel to those described above we next determined the NLRP3 and BTK domains that interact with one another. In initial studies 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 studies. We found that the PH domain of BTK as well as the BTK fragment with PTK or SH deletions interact with NLRP3 (Figure 3A). The PTK domain also interacts with NLRP3 as shown in a study in which NLRP3 was immunoprecipitated with anti-NLRP3 and then subjected to Western blot 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 constructs [generated based on the NCBI conserved domain 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 binds to full length NLRP3 as well as to NLRP3 with either pyrin or LRR domain deletions but not to an LRR domain fragment (Figure 3D). In addition, using anti-NLRP3 to perform immunoprecipitation (because the flag-tagged pyrin domain could not be recognized by anti-flag) we found that the pyrin domain also binds 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

In further studies having the ultimate goal of understanding the mechanism of BTK regulation of the NLRP3 inflammasome we set out to define which domain of BTK is required for inflammasome inhibition. To this end we generated various truncated constructs of BTK (Figure 4A) based on the NCBI conserved domain 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; 24 hours later we harvested culture supernatants for assessment of IL-1β secretion by ELISA. We found that, as shown previously, the full length BTK construct inhibited IL-1β production by the re-constituted NLRP3 inflammasome; in addition, the PTK construct that contains the kinase site of BTK, displayed even greater inhibition and the BTK construct in which the PTK fragment had been deleted (△PTK) 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 quite clearly 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 if 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 Y223 phosphorylation and kinase activity (Supplemental Figure 17A-B). We then transfected a plasmid expressing this construct into HEK293T cells together with plasmids expressing NLRP3 and other inflammasome components as previously described; we then analyzed the culture supernatants by ELISA. We found that the kinase-dead BTK mutant construct failed to inhibit IL-1β production (Figure 4C) although it binds to NLRP3 to the same extent as intact BTK (Figure 4D). In addition, in lentiviral vector-mediated overexpression studies, we found that expression of wildtype BTK in BTK KO cells inhibited IL-1β production, whereas kinase-dead BTK did not so inhibit (Figure 4E-G). These findings indicate that BTK inhibition of NLRP3 inflammasome activity requires its kinase activity. In addition,

they suggest that initial activation of BTK by a TLR or another 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 (PP2A) activity

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

To examine if 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 co-transfection of plasmids expressing PP2A as well as with and without plasmids expressing BTK or fragments of BTK. We found that cells co-transfected with a PP2A plasmid exhibited enhanced IL-1β production and 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 determined if BTK or the PTK fragment of BTK could block PP2A-mediated dephosphorylation of Ser5 in the pyrin domain of NLRP3 (the only phosphorylated serine 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 cell lysates to immunoprecipitation with anti-NLRP3 and immunoblotting with anti-p-serine. We found that whereas the presence of PP2A led to serine dephosphorylation of pyrin expressing phosphorylated serine, the presence of BTK and PTK blocked such dephosphorylation whereas kinase-dead BTK did not so block (Figure 4I). In addition,

transfection of HEK293T cells with a plasmid expressing mutant full length NLRP3 containing pyrin domain in which Ser5 is replaced by Ala5 led to functionally impaired inflammasome activity that could not be blocked by co-transfection 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 prevents PP2A phosphatase activity, whereas full length BTK with a mutated kinase site lacking kinase activity does not (Figure 4K).

To investigate the mechanism by which BTK affects PP2A function, we first determined if 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 whereas full length BTK or kinase-dead BTK binds to PP2A, the PH and PTK fragments of BTK do not bind to PP2A (even though they bind 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 NLRP3 binding to PP2A.

In further studies we examined relevant functional aspects of the BTK interaction with PP2A, in this case in WT and BTK-KO BMDMs to be sure of their physiological significance. We found that LPS stimulation of WT BMDMs induces BTK interaction with PP2A but this interaction is not detectable in WT BMDM stimulated with LPS plus nigericin, i.e., when the NLRP3 inflammasome is activated (Figure 5B). Moreover, LPS stimulation of WT BMDMs is accompanied by increased PP2A Y307 phosphorylation whereas LPS stimulation of BTK-KO cells does not increase such phosphorylation above background levels (Figure 5B), the latter suggesting that BTK plays an essential role in 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 due to 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 5D-5E). This result is consistent with and verifies studies of BTK-intact cells previously conducted by Stutz et al establishing that PP2A phosphatase activity and dephosphorylation of Serine 5 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 it PTK domain and that such inhibition ceases with inflammasome activation and cessation of BTK binding to NLRP3. Moreover, such inhibition requires BTK kinase function allowing phosphorylation and thus temporary inactivation of PP2A.

Mechanism of High Dose BTK Inhibitor-Mediated Inhibition of NLRP3 Inflammasome Activation

As noted above, whereas 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 determined if low and high doses of BTK inhibitor (LFM-A13) differentially affected 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, the dose-dependent effects on NLRP3 inflammasome generation of mature caspase1 (caspase p20) and cleaved GSDMD was determined in LPS plus nigericin stimulated immortalized macrophages. In this case we again found that whereas low doses of LFM-A13 enhanced expression of these down-stream 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-1β secretion is reflected in NLRP3 interaction with down-stream 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 BTK inhibitor is caused by a similar (but different) effect on phosphorylation. One possibility is that high doses of BTK inhibitor inhibits 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 affects a clinically relevant mucosal inflammatory process.

In initial studies to examine this question we first established that mouse small intestinal 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 day 0 and day 2, and then, after 4 days assessed the features of the colitis thus induced. We found that BTK-KO mice exhibited a significantly greater body weight loss, colon shortening and higher disease histological score than WT mice (Figure 6A-D). Accompanying studies of colonic tissue extracts subjected to Western blotting disclosed more cleavage of IL-1β, caspase-1, and GSDMD in extracts

from BTK-KO mice than from wild type mice (Figure 6E). In addition, mesenteric lymph node (MLN) cells from BTK-KO mice produced greater amounts of IL-1β/IL-6 than cells from WT mice (Figure 6F) and IL-1β/IL-6 concentration was increased in colonic tissue homogenates from BTK-KO mice as compared with those from WT mice (Figure 6G). This correlated with the fact that while expression of NLRP3 inflammasome components in the intestine tissue of BTK-KO was only mildly increased compared to 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 of BTK-KO mice exhibited 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 expressed increased mRNA specific for the neutrophil markers Ly6G and elastase (Supplemental Figure 20B) and monocyte-associated proinflammatory proteins, NLRP3, IL-1β, IL-18, IL-6 and TNF-**α** (Supplemental Figure 20C); finally, KO colonic tissue expressed 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 the role for BTK 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 obtained from the study of this model showed that BTK KO mice subjected to DSS-colitis exhibit a significantly greater body weight loss, colon shortening and histological score than WT mice (Supplemental Figure 21 A-C). In addition, MLN cells from BTK KO mice produced more IL-1β and IL-6 than cells from WT mice (Supplemental Figure 21D). These data thus 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 Increased TNBS-Colitis occurring in BTK-KO Mice

Since BTK inhibition or deficiency is associated with increased IL-1β production we next asked if blockade of IL-1β signaling can inhibit TNBS-colitis induced in BTK-KO mice as demonstrated above. To this end, we induced TNBS-colitis in two groups of BTK-KO mice, one treated with anakinra and one not so treated as well as in control WT mice not treated with anakinra. As shown previously, we found that BTK-KO mice exhibited more severe colitis than wild type mice, as documented by various parameters of colon inflammation (Figure 7A-D) and by increased IL-1β/IL-6 production by MLN cells subjected to NLRP3 inflammasome stimulation or IL-1β/IL-6 content of colonic tissue homogenates (Figure 7E, F). In contrast, BTK-KO mice treated with anakinra (an agent the blocks IL-1β or IL-1α signaling) manifested a reduced level of colitis accompanied by reduced production of IL-1β/IL-6 by NLRP3 inflammasome-stimulated MLN cells and reduced colonic tissue homogenate content of IL-1β/IL-6 (Figure 7A-F). In addition, such treatment greatly reduced the higher level of colonic mRNA expression of IL-1β, TNF-**α**, IFN-**γ**, CXCL1, CCL2 and claudin 2 present in the BTK-KO colonic tissue (Supplemental Figure 22). These data demonstrated that anakinra treatment almost completely reduced the increased level of 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 can be attributed to ether increased IL-1β- or IL-1α-driven inflammation resulting from a dysregulated NLRP3 inflammasome. Finally, to distinguish between IL-1β and IL-1α pro-inflammatory 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 to be specific for IL-1β and IL-1α, respectively by Western blot (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 24A-C). In addition, activation of the NLRP3 inflammasome in mesenteric lymph node cell populations from BTK-KO mice with TNBS-colitis led to robust secretion of IL-1α, IL-1β and IL-6 in mice treated with isotype control antibody or anti-IL-1α that was

greatly reduced in mice treated with anti-IL-1β, indicating that administration of anti-IL-1β, but not anti-IL-1α, had down-regulated the number of cells migrating from the lamina propria to the mesenteric nodes that are capable of pro-inflammatory cytokine production (Supplemental Figure 24D). Taken together, these studies strongly suggest that the increased colitis in BTK-KO is 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 levels of colitis in untreated WT mice with anti-IL1β antibody administration does not completely abrogate IL-1β-mediated inflammation in BTK-KO mice at the dose of antibody administered (Supplemental Figure 25A-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 dependency of BTK effects on the NLRP3 inflammasome *in vitro*.

In a first study addressing this question, wild type mice were injected I.P. with the BTK inhibitor, LFM-A13, at various doses and were then administered alum crystals (I.P.) to induce peritonitis, an inflammatory response characterized by NLRP3 inflammasome-mediated recruitment of neutrophils into the peritoneal cavity (33). We found that the total peritoneal exudate cells (PECs) and Gr1+ neutrophils were increased in mice treated with low dose inhibitor compared with those not treated with inhibitor (Supplemental Figure 26A, B). Consistent with this, IL-1β levels in the peritoneal lavage fluid (PLF) and the level of cleaved caspase-1 in PECs were also increased (Supplemental Figure 26C, D). The opposite result was observed in mice injected with high doses of inhibitor. Consistent with these data, BTK-KO mice phenocopied the mice injected with low doses of BTK inhibitor

in that they exhibited enhanced PECs, neutrophil infiltration and IL-1β production in PLF (Supplemental Figure 26 E, F, G).

In a second study we evaluated the effect of administration of BTK inhibitor on BTK inhibitory function in the TNBS-colitis model described above. In these studies, we pre-administered a low dose of BTK inhibitor, LFM-A13 (10 μg) daily to C57BL/6 wild type mice to inhibit BTK (based on the dose used in the peritonitis study) beginning of day -2 of the study. Then on day 0 and day 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 26H-L). These data indicate that inhibition of BTK in mice by low doses of BTK inhibitor confers susceptibility to colon inflammation.

Discussion

Bruton tyrosine kinase (BTK) has a key role in B cell signaling via the Ig receptor and thus is 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 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 dendritic cell 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 dendritic cells. We found that BTK has an inhibitory effect on the NLRP3 inflammasome and this is manifested by the fact that low concentrations of ibrutinib (such as those occurring during CLL treatment) upregulate NLRP3 inflammasome activation. In addition, absent BTK expression or function in mice and humans with *BTK* mutations is marked by increased NLRP3 inflammasome activation that gives rise to increased pro-inflammatory 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 whereas both the PH and PTK domains of BTK bind to NLRP3 in co-transfection studies, it was mainly the PTK domain that is responsible for inhibition of the NLRP3 inflammasome. In further 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 protein phosphatase 2A (PP2A), the latter a phosphatase complex previously shown to induce NLRP3 activation by dephosphorylating Ser5 in the pyrin domain of NLRP3 and thereby promoting the activation of the NLRP3 inflammasome (27). We found that BTK binds to PP2A and via its kinase activity induces phosphorylation at PP2A Tyr307, an established mechanism of PP2A inactivation(37). This correlates with the fact that in co-transfection studies, BTK and, more particularly, the PTK domain of BTK, inhibits the capacity of exogenous PP2A to augment NLRP3 inflammasome activation. Finally, and perhaps most importantly, the BTK via its PTK domain inhibits 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 Graphical Abstract).

A potential regulatory role of BTK in NLRP3 inflammasome was proposed by Liu et al(10) and Ito et al(11) showing 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 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 20μM or greater inhibited caspase1 cleavage following NLRP3 inflammasome activation(7). Our studies are in an 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 non-physiologic 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 to 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 Ito et al. study cells with putatively absent BTK activity were cells from XID mice that actually express 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 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. It was also supported by studies in which the reciprocal effects of BTK deficiency or over-expression by plasmid transfection are explored with relation to each of the major steps of NLRP3 inflammasome assembly and activation. In addition, they were supported by studies that showed that BTK (or a fragment thereof) inhibits BTK-mediated inhibition of PP2A phosphatase activity, as already discussed above. Finally, and perhaps most importantly, this conclusion was supported by the fact that the absence or inhibition of BTK in the whole animal renders 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 dendritic cells demonstrated here suggests that under normal circumstance such inhibition has a physiologic ("gate-keeper") role in regulation of this inflammasome. Recall that BTK binds to NLRP3 following primary NLRP3 inflammasome (LPS-only) activation, but that such binding is not observed (or is greatly reduced) after inflammasome activation signaling 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 thus inactivate PP2A.

BTK inhibition of NLRP3 inflammasome activation, while partial in the *in vitro* studies presented here, is quite dramatically evident in studies of BTK-deficient mice subjected to induced colitis by administration of TNBS or DSS. In these studies, we showed that BTK-deficient mice exhibit much more severe colitis than 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 about one-third of XLA patients have GI abnormalities and about one tenth of these patients have Crohn's disease(13). Second, CLL patients being treated with ibrutinib therapy frequently suffer from diarrhea (particularly early in the course of treatment); it is reasonable to suggest that this may be related to increased

gastrointestinal NLRP3 inflammasome activity arising from BTK inhibition by low concentrations of inhibitor (38).

METHODS

Complete details relating to experimental materials and methods, as well as description of patients are provided in the Supplemental Materials.

Human subjects

Peripheral blood from patients with XLA or Chronic Lymphocytic Leukemia (CLL) and healthy individuals was obtained with written informed consent in accordance with the Declaration of Helsinki. All procedures in this study were approved by the ethics committees of National Institutes of Health.

Study Approval

Peripheral blood from patients with XLA or Chronic Lymphocytic Leukemia (CLL) and healthy individuals was drawn for laboratory examination with written informed consent in accordance with the Declaration of Helsinki. All procedures in this study were approved by the ethics committees of National Institutes of Health. All procedures of animal experiments were approved by the ethics committees of the National Institutes of Health.

Statistics

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

Author Contributions:

L.M. Performance of studies and data analysis; writing of MS; A.K. Planning and analysis of studies; E.H. Performance of some studies; K.M. Collection of specimens and clinical care; W.Z. Bioinformatic analysis of BTK binding proteins. I.F. Clinical care of patients

and planning/analysis of studies; A.W. Analysis of data and patient care; W.S. Planning and analysis of studies; writing of MS.

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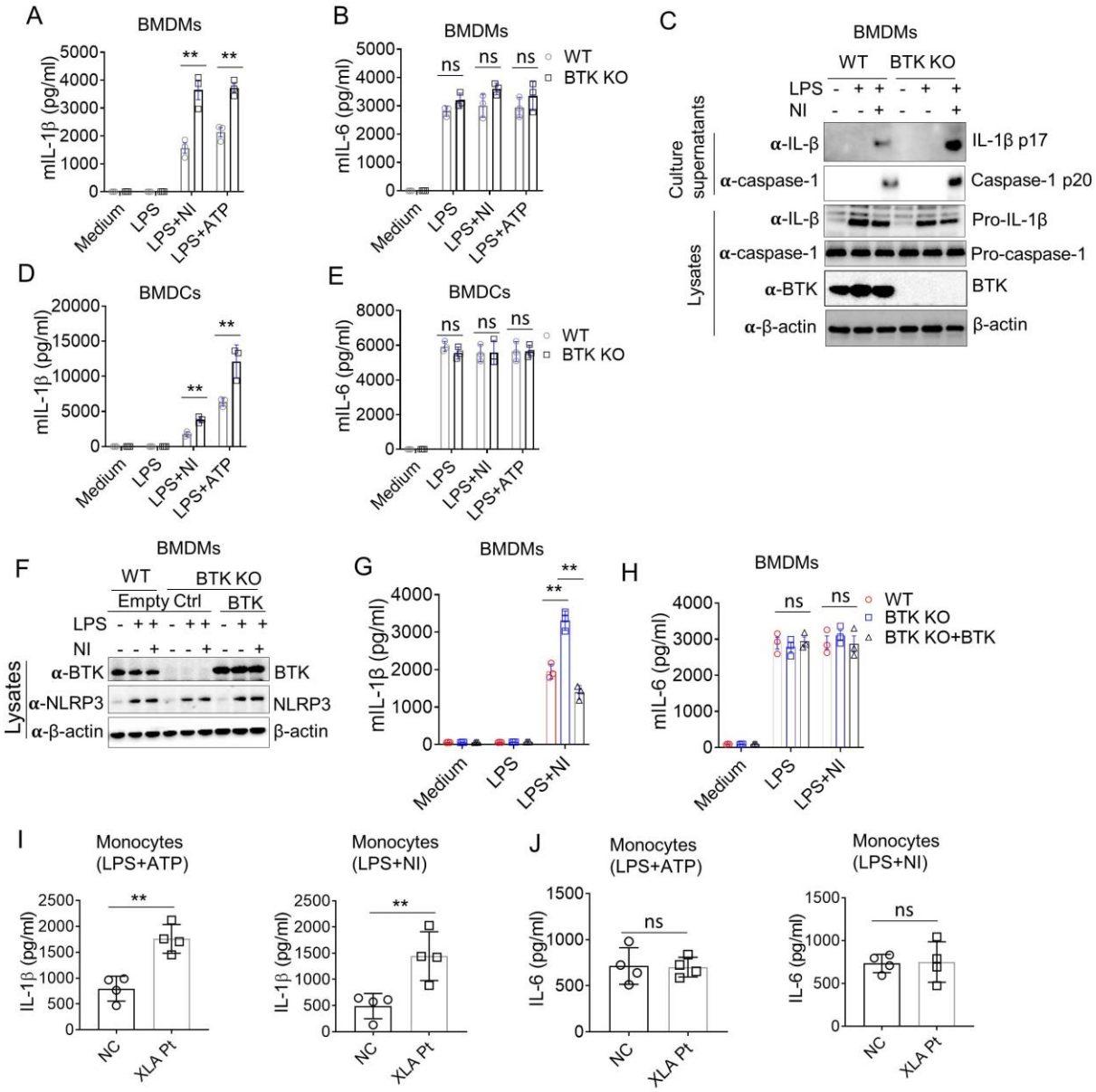

Figure 1. BTK deficiency Causes Increased NLRP3 Inflammasome Activation in Murine and Human Cells

(A, B, C) Mouse BMDMs were primed with LPS (200ng/ml) for 3 hours and then stimulated with nigericin (NI, 1uM) or ATP (5mM) for 30 minutes; the culture supernatants were then subjected to IL-1β (A) and IL-6 (B) ELISA assays and Western blots for mature IL-1β and caspase-1 (C). (D, E) Mouse BMDCs were treated as above and the culture supernatants were then subjected to IL-1β (D) and IL-6 (E) ELISA assays. (F, G, H) BMDMs from WT or BTK KO mice were transduced with a lentiviral vector expressing BTK or an empty vector control for 48 hours, the cells were then primed with LPS (200ng/ml) for 3 hours and then stimulated with nigericin (NI, 1uM) for 30 minutes; the cells were lysed and the cell lysates were subjected to Western blots for detecting BTK and NLRP3 (F); culture supernatants were collected for IL-1β (G) and IL-6 (H) ELISA assays. (I, J) Human monocytes from XLA patients (XLA Pts, n=4) and healthy individuals

(NCs, n=4) were primed with LPS (200ng/ml) for 3 hours, the cells were then stimulated with ATP (5mM) or nigericin (NI, 1uM) for 30 minutes; the culture supernatants were subjected to IL-1β (I) and IL-6 (J) ELISA assays. Data in A, B, D, E, G, H were analyzed using a one-way ANOVA with multiple comparisons, data in I and J were analyzed using two-tailed Student *t* test. Data were displayed as mean ± SD, **p<0.01; ns: not significant. All data are representative of three independent experiments.

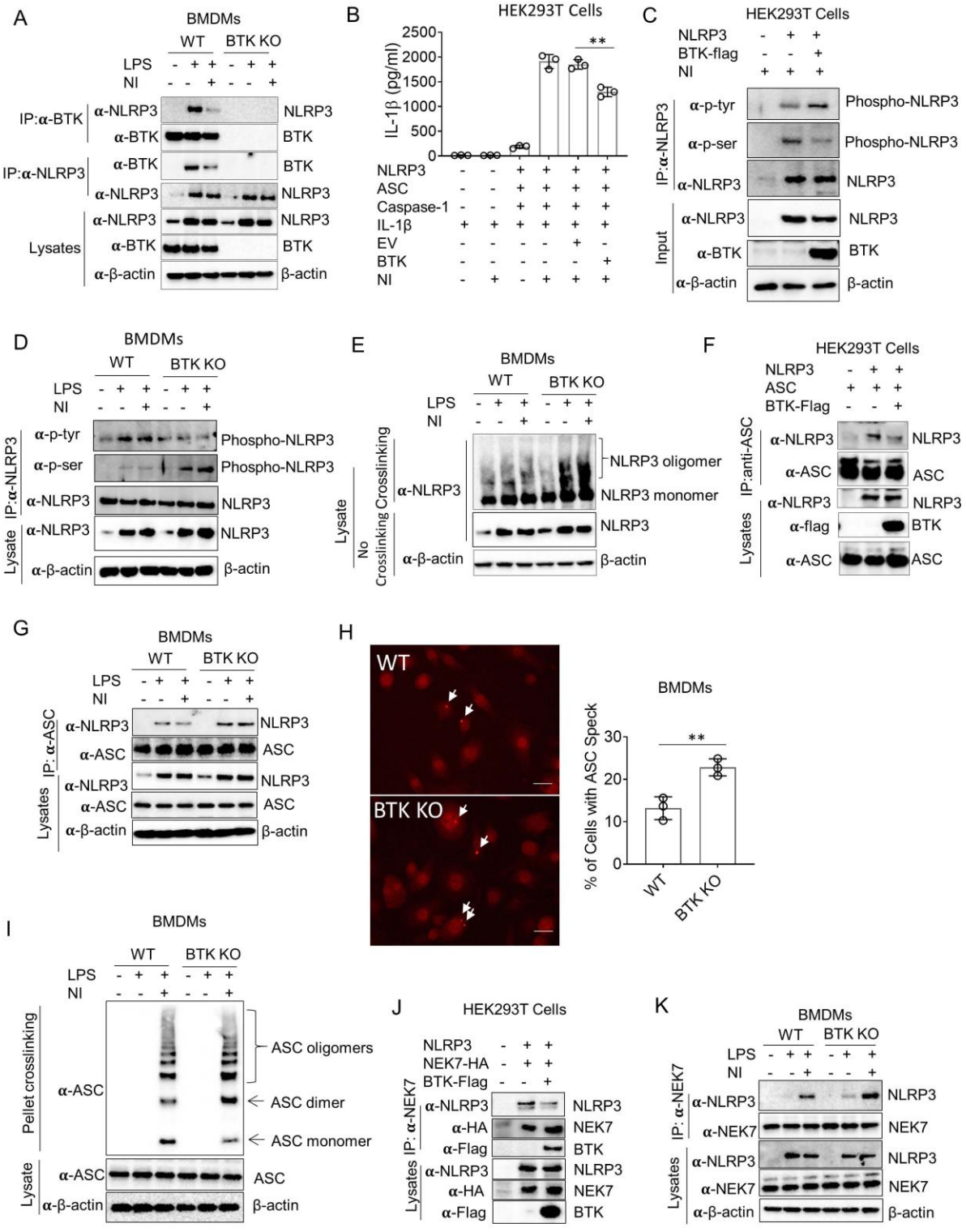

Figure 2. BTK Regulates the Consecutive Stages of NLRP3 Inflammasome Activation.

(A) BMDMs from wild type or BTK-KO mice were primed with LPS (200ng/ml) for 3 hours and stimulated with nigericin (NI) for 30 minutes; the cells were then lysed and the lysates

were subjected to IP and Western blotting. (B-C) HEK293T cells were transfected with plasmids as indicated; 24 hours later, the cells were treated with NI as indicated for 30 minutes and the culture supernatants were subjected to ELISA assays for IL-1β (B), the cells were lysed and the cell lysates were subjected to IP and Western blotting (C). (D, E) BMDMs from wild type or BTK-KO mice were treated as in (A) and the cell lysates were subjected to IP and Western blotting (D) or treated with DSS crosslinker and subjected to Western blotting(E). (F, J) HEK293T cells were transfected with plasmids as indicated; after 24h the cells were lysed and the lysates subjected to IP and Western blotting; (G) BMDMs from wild type or BTK-KO mice were treated as in (A); the cell lysates were subjected to IP and Western blotting; (H) BMDMs from wild type or BTK-KO mice were treated as in (A) and then subjected to immunocytochemistry for ASC speck assay using anti-ASC antibody. Scale bars, 20 μm. (I) To examine ASC oligomerization BMDMs were treated using the methods described in “Methods” section. (K) BMDMs from wild type or BTK-KO mice were treated as in (A); the cell lysates were subjected to IP and Western blotting. Data were analyzed using a one-way ANOVA with multiple comparisons (B) or two-tailed Student *t* test (H). Data were displayed as mean ± SD, $^{**}p<0.01$. All data are representative of three independent experiments.

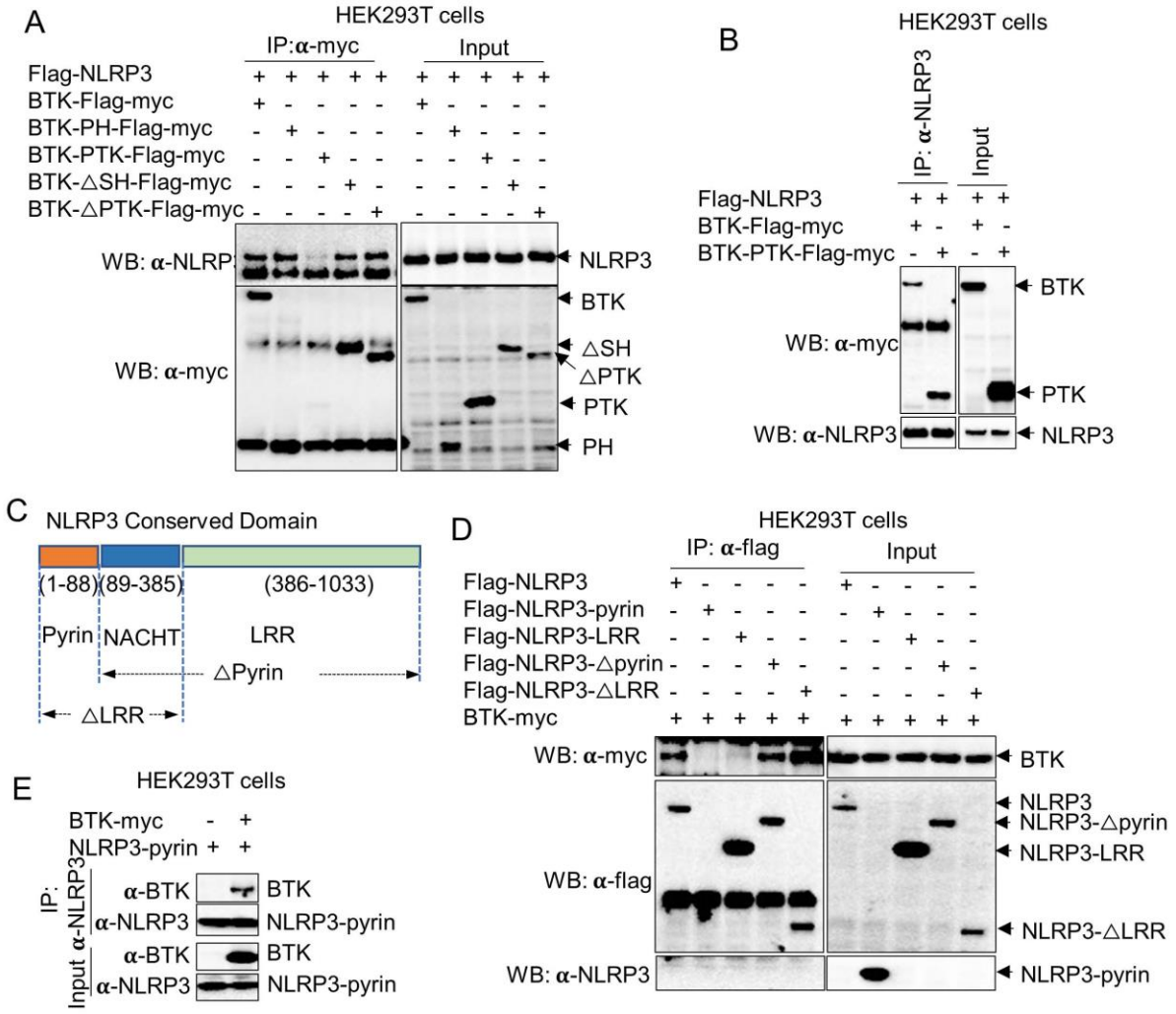

Figure 3. BTK Interacts with the NLRP3 Pyrin and NATCH Domains via Its PH and PTK Domains.

(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 cell lysates obtained were subjected to IP and Western blots; (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 the cell lysates obtained were subjected to IP and Western blot as indicated; (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; then, after 24 hours the cells were lysed and the cell lysates obtained were subjected to IP and Western blotting as indicated; (E) HEK293T cells were transfected with plasmids expressing the NLRP3 pyrin domain with or without co-transfection of a construct expressing a full length BTK construct; after 24 hours the cells were lysed and the cell lysates obtained were subjected to IP and Western blotting as indicated. All of the data are representative of two independent experiments.

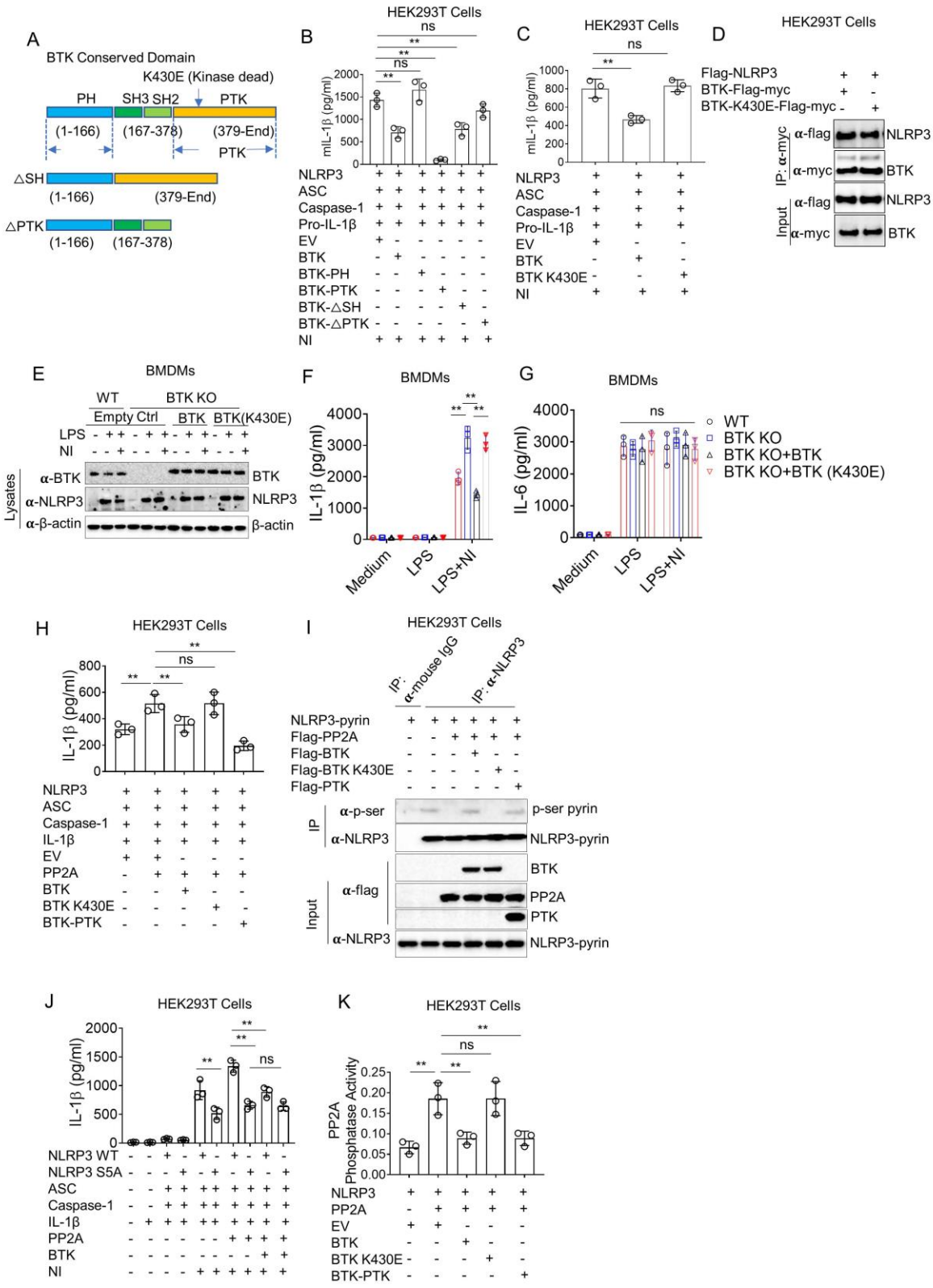

Figure 4. BTK Inhibits NLRP3 by Preventing PP2A-Mediated Dephosphorylation of Serine5 in the NLRP3 Pyrin Domain.

(A) BTK constructs expressing conserved BTK domains, truncations or kinase-dead BTK were generated as indicated. (B, C, H, J) HEK293T cells were transfected with plasmids as indicated; 24 hours later the cells were treated with nigericin for 30 minutes after which culture supernatants were subjected to ELISA assay for IL-1β. (D, I) HEK293T cells were transfected with plasmids as indicated; 24 hours later the cells were lysed and lysates were subjected to IP and Western blotting. (E,F,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 for 48 hours, the cells were then primed with LPS (200ng/ml) for 3 hours and then stimulated with nigericin (NI, 1uM) for 30 minutes; the cell were then lysed and cell lysates were subjected to Western blotting for detection of BTK and NLRP3 (E); the culture supernatants were collected for IL-1β (F) and IL-6 (G) ELISA assays. (K) HEK293T cells were transfected with plasmid as indicated; 24 hours later the cells were subjected to PP2A phosphatase assay using methods described in the “Methods” section. Data were analyzed using a one-way ANOVA with multiple comparisons and were displayed as mean ± SD, $^{**}p<0.01$; ns: not significant. All of the data are representative of three independent experiments.

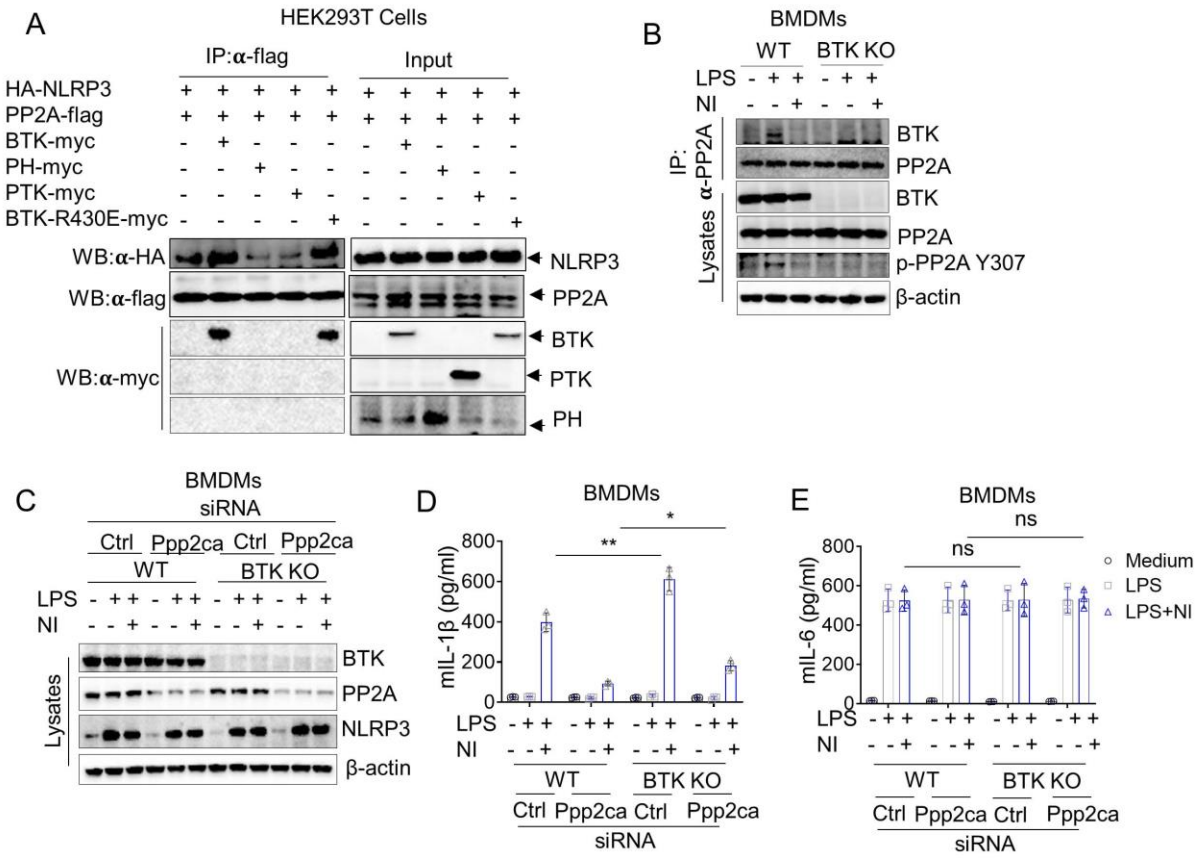

Figure 5. BTK Interacts with PP2A and Up-regulates PP2A Y307 Phosphorylation; BTK-KO Cells Produce Decreased Amounts of IL-1β in Cells with PP2A Knock-down.

(A) HEK293T cells were transfected with plasmids as indicated; 24 hours later the cells were lysed and lysates were subjected to IP with anti-flag antibody and then Western blotting. Data are representative of two independent experiments. (B) BMDMs from wildtype and BTK KO mice were stimulated with LPS (200ng/ml) for 3 hours after which the cells were lysed and the cell lysates were subjected to IP and western blot assay. (C-E) BMDMs were transfected with siRNA specific for PP2A as indicated, 48 hours later the cells were primed with LPS (200ng/ml) for 3 hours and the stimulated with nigericin for 30 minutes; the cells were then lysed and the cell lysates were subjected to Western blotting for BTK, PP2A and NLRP3 detection (A); the above culture supernatants were collected for IL-1β (B) and IL-6 (C) ELISA assays. Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean ± SD, *p<0.05; **p<0.01; ns: not significant. Data are representative of two independent experiments.

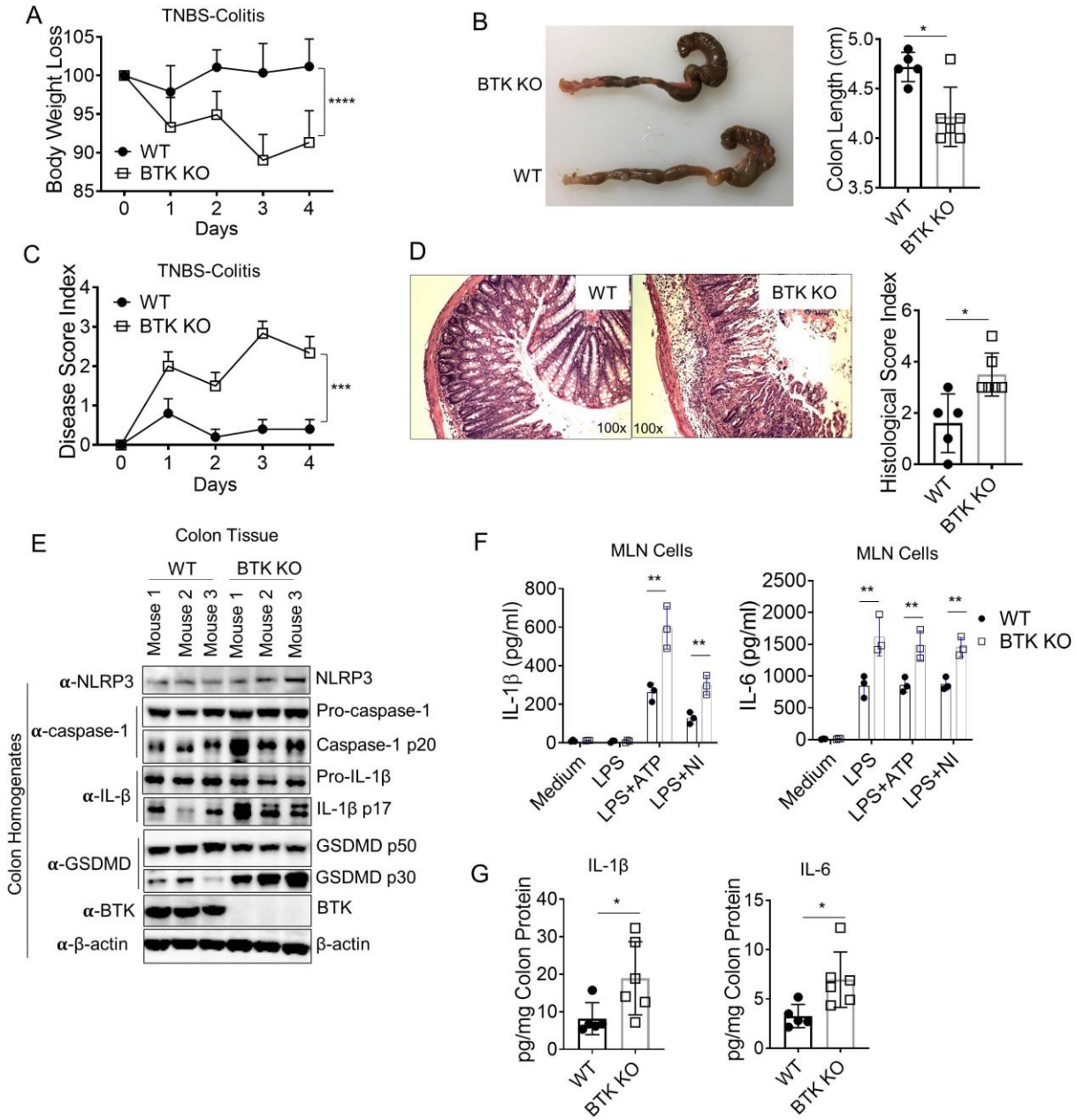

Figure 6. Mice with BTK Deficiency Exhibit More Severe TNBS-Colitis Than Wild Type Mice.

Male BTK-KO mice (n=6) and their wildtype 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, Original magnification, ×100) due to colitis were measured. (E) Colon tissue homogenates were subjected to Western blotting as indicated. (F) Mononuclear cells from mesenteric lymph nodes (MLN) were primed with LPS (1ug/ml) for 12 hours and then stimulated with ATP (5mM, 30 minutes) or NI (1μM, 30 minutes); the culture supernatants were then subjected to ELISA assay of IL-1β (upper panel) and IL-6 (lower panel). (G) Colonic tissues were homogenized and the homogenates were subjected to ELISA assays for IL-1β (left penal)

and IL-6 (right penal). Data were analyzed by two tailed Student's *t* test (B, D, G) or one-way ANOVA with multiple comparisons (A, C, F) and are displayed as mean ± SD, **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001. Data are representative of three independent experiments.

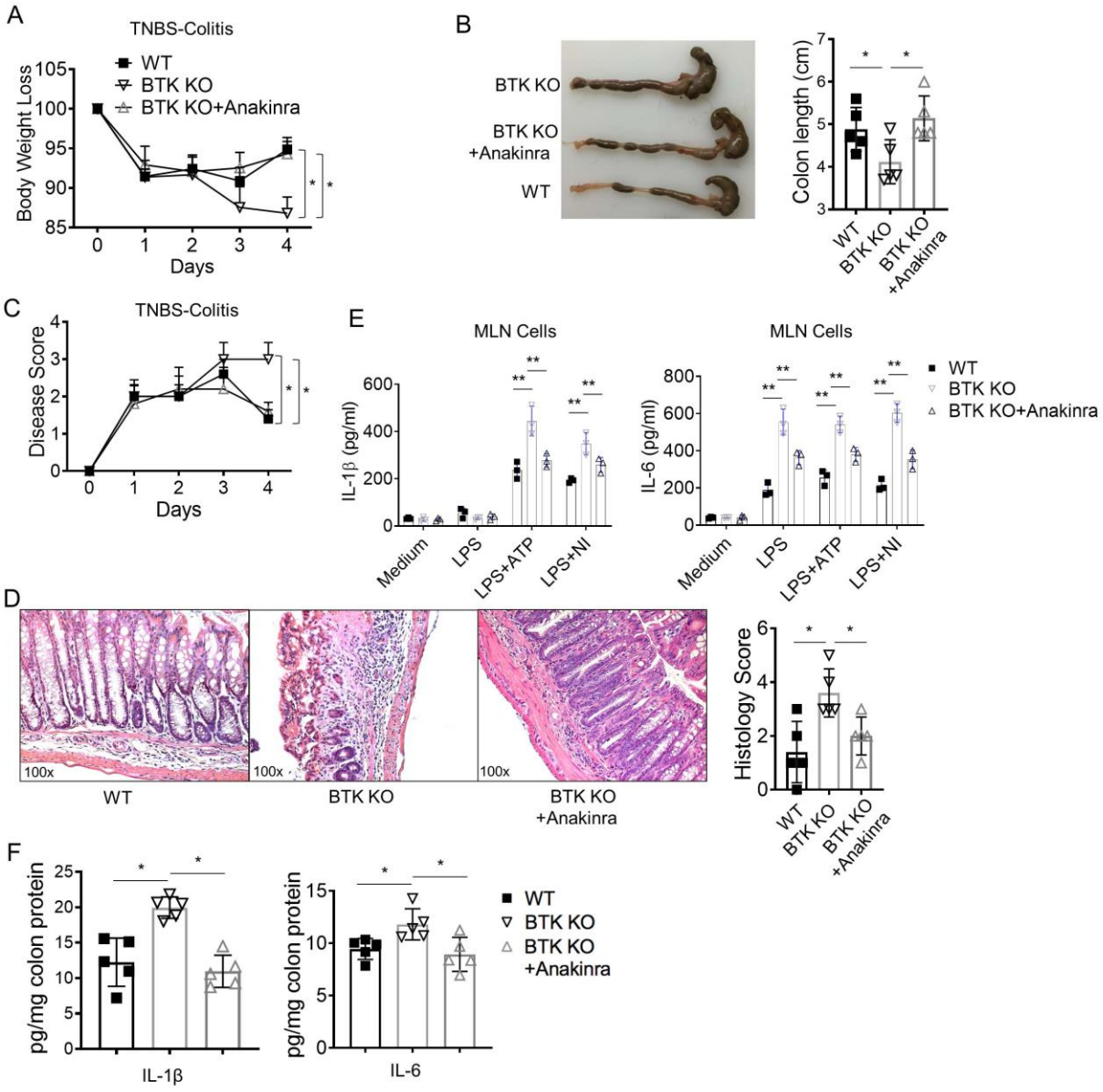

Figure 7. Inhibition of IL-1β Signaling Ameliorates TNBS-Colitis in BTK-KO Mice. Male BTK-KO mice (n=5) were administered anakinra IP (0.5mg/mouse/day) on day -1 and then administered 3 mg TNBS per rectum on days 0 and 2. Male BTK-KO mice (n=5) and wild type mice (n=5) administered TNBS per rectum and not treated with anakinra served as controls. Mouse body weight loss (A), colon length (B), disease score (C) and histological damage (D, Original magnification, ×100) due to colitis were measured. (E) Mononuclear cells from MLN were primed with LPS (1ug/ml) for 12 hours and then stimulated with ATP (5mM, 30 minutes) or NI (1μM, 30 minutes), the culture supernatants were subjected to ELISA assay of IL-1β (left penal) and IL-6 (right penal). (F) Colon tissues were homogenized and the homogenates were subjected to ELISA assays for IL-1β (left penal) and IL-6 (right penal). Data were analyzed using a one-way ANOVA with multiple comparisons and are displayed as mean ± SD, *p<0.05; **p<0.01. Data are representative of three independent experiments.