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Non-receptor tyrosine kinases ITK and BTK negatively regulate mast cell pro-inflammatory responses to lipopolysaccharide

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Abstract

Background—Mast cells are indispensible for LPS-induced septic hypothermia, in which TNF-α plays an essential role to initiate septic responses. ITK and BTK regulate mast cell responses to allergen, but their roles in mast cell responses in LPS-induced sepsis are unclear.

Objectives—We sought to investigate the roles of ITK and BTK in mast cell responses during LPS-induced septic inflammation.

Methods—Mice (genetically modified or BMMC-reconstituted *Sash*) were given LPS to induce septic hypothermia, in the presence or absence of indicated inhibitors. Flow cytometry was used to determine LPS-induced cell influx and TNF-α production in peritoneal cells. Microarray was used for genome-wide gene expression analysis on BMMCs. Quantitative PCR and multiplex were used to determine transcribed and secreted pro-inflammatory cytokines. Microscopy and western blotting were used to determine activation of signal transduction pathways.

Results—The absence of ITK and BTK leads to exacerbation of LPS-induced septic hypothermia and neutrophil influx. $Itk^{-/-}Btk^{-/-}$ mast cells exhibit hyperactive preformed and LPS-induced TNF-α production, and lead to more severe LPS-induced septic hypothermia when reconstituted into mast cell deficient *Sash* mice. LPS-induced NF-κB, Akt and p38 activation is enhanced in $Itk^{-/-}Btk^{-/-}$ mast cells, and blockage of PI3K, Akt or p38 downstream MNK1 activation significantly suppresses TNF-α hyper-production and attenuates septic hypothermia.

Conclusions—ITK and BTK regulate thermal homeostasis during septic response through mast cell function in mice. They share regulatory function downstream of TLR4/LPS in mast cells, through regulating the activation of canonical NF-κB, PI3K/Akt and p38 signaling pathways.

Keywords

Tec; Lipopolysaccharide; mast cell; septic hypothermia; TNF-α; NF-κB; MAPKs; PI3K/Akt

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INTRODUCTION

Sepsis is the systemic inflammatory response to infection and is the leading cause of in-hospital death. Although fever is a cardinal feature of sepsis[1, 2], hypothermia is more associated with severe or fatal sepsis[3, 4]. TLR4 ligation by bacterial endotoxin LPS activates mast cell production of pro-inflammatory cytokines including TNF-α and IL-6[5]. Administration of LPS to mice or humans causes sepsis-like symptoms, and LPS-triggered mast cell derived TNF-α is indispensible for septic hypothermia in mice[6]. TNF-α is central to this response, and when injected alone, symptoms resemble those in late phase LPS administration[7]. Mast cell derived TNF-α does play a protective role in a model of acute septic peritonitis (cecal ligation and puncture)[8], and in neutrophil influx during bacterial clearance[9]. While the crucial role of mast cell derived TNF-α in local infection, sepsis and thermal dysfunction is clear, how TNF-α production by these cells is modulated remains largely unexplained.

Tec family kinases ITK and BTK are non-receptor tyrosine kinases acting downstream of numerous receptors, and have been shown to modulate mast cell responses downstream of FcεRIα[10–14]. In mast cells, ITK and BTK have redundant functions[15], and although neither ITK nor BTK is required for mast cell development, the absence of both leads to impaired FcεRIα-mediated degranulation and cytokine secretion, including TNF-α[10–14]. However, the roles of ITK and BTK in mast cell responses in LPS-induced sepsis are unclear.

LPS triggers TLR4 on mast cells, activating MAPKs and NF-κB, the latter induced by PI3K/Akt[5, 16, 17]. BTK has been shown to interact with TLR4 and be involved in NF-κB activation[18]. Although some reports suggest that BTK is a positive mediator in TLR4 signaling[18–22], others suggest that BTK is dispensable, as LPS-induced TNF-α and IL-6 was slightly increased in 129/Sv mouse mast cells lacking BTK[23]. In murine and human monocytic cells, BTK has been shown to phosphorylate MyD88-adaptor like protein (MAL), an adaptor downstream of TLR4, leading to degradation of MAL[20, 24] and so attenuating TLR4 signaling activity. These findings suggest that the role of BTK in TLR4 signaling might be cell type specific or mouse strain dependent[23].

In this study, we investigated the function of ITK and BTK in LPS-induced mast cell-mediated septic hypothermic response. We report an unexpected regulatory function for these kinases in suppressing the inflammatory response to LPS though regulating NF-κB, PI3K/Akt, p38 and MNK1 signaling activity.

METHODS

Mice

Mice were on a C57BL/6 background. *Itk*$^{-/-}$*Btk*$^{-/-}$ mice were as described[14], *Tnfa*$^{-/-}$ (B6.129S-*Tnf*^{tm1Gkl}/J) and *Sash* (*Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ) mice were from The Jackson Laboratory (Bar Harbor, ME). IL-5 transgenic mice (gift from Drs. N.A. Lee and J.J. Lee[25]) were crossed to *Itk*$^{-/-}$*Btk*$^{-/-}$ mice as a source of eosinophils. All experiments were approved by IACUCs at The Pennsylvania State and Cornell Universities.

Generation of BMMCs and reconstitution of BMMCs in *Sash* mice

BMMCs were generated as previously described[14]. In brief, bone marrow cells were cultured in complete DMEM with 10 ng/ml recombinant murine (rm) IL-3 (Cell Sciences, Canton, MA) and 50 ng/ml rmSCF (Rocky Hill, NJ), and after 4 to 6 weeks, BMMCs with purity (c-Kit$^+$FcεRIα$^+$) > 96% were used. For *in vitro* stimulation, 2×10^6/ml BMMCs were starved in complete DMEM without IL-3/SCF overnight, followed by indicated stimulation. *Sash* mice received 5×10^6 BMMCs through retro-orbital (intravenous, i.v.) injection[26] and 5×10^6 BMMCs through intraperitoneal (i.p.) injection[27] to reconstitute mast cells, 12 weeks prior to experiments.

Generation and stimulation of CBMCs

CBMCs were generated as previous described[28]. In brief, CD34$^+$ human hematopoietic stem cells were enriched from cord blood (National Disease Research Interchange, Philadelphia, PA) using magnetic positive selection, cultured in serum-free medium (StemPro-34, Life Technologies, Grand Island, NY) with recombinant human (rh) SCF (100 ng/ml), rhIL-6 (50 ng/ml), and rhIL-3 (2 ng/ml) for 2 weeks, then with rhSCF and rhIL-6 only for 4 weeks. CBMCs with purity (c-Kit$^+$FcεRIα$^+$) > 95% were used. CBMCs were sensitized in 10 ng/ml IL-4 and 10% fetal bovine serum for 5 days[29] prior to LPS (1 μg/ml) stimulation.

LPS-induced hypothermia

Mice were i.p. injected with 1 mg/kg LPS, and core body temperature measured using an infrared thermometer (Reed ST-8812, Tequipment.NET, Long Branch, NJ). Cell influx into the peritoneum was determined by peritoneal lavage 4 hours post LPS injection and flow cytometry. Gating strategy for identification of primary cells in the peritoneal lavage by flow cytometry is depicted in Figure E2.

Microarray analysis

BMMCs were factor starved overnight, then treated with PBS or 100 ng/ml LPS for 1 hour. RNA was extracted and prepared for microarray as previously described[30], and data analyzed using GeneSpring GX (Agilent) as described in the Online Repository.

Detection of cytokine mRNA and secretion

Cytokine mRNA was detected as previously described[30]. Secreted cytokines were measured using a Milliplex MAP kit (Millipore, Billerica, MA) analyzed on a MAGPIX system (Luminex, Madision, WI).

Statistical analysis

Two-tailed Student's *t* test and two-way ANalysis Of Variance (ANOVA) between groups were performed using Prism (GraphPad, San Diego, CA), with $p < 0.05$ considered statistically significant. "NS" indicates differences that are not significant.

RESULTS

ITK and BTK suppress LPS-induced hypothermia, neutrophil influx and mast cell-derived TNF-α

Mast cells have been shown to be responsible for the LPS-induced hypothermic response, which is mediated by TNF-α[6]. To determine whether ITK and BTK play roles in mast cell response to LPS and thus regulate the hypothermic response, we injected WT, *Itk*$^{-/-}$, *Btk*$^{-/-}$ and *Itk*$^{-/-}$*Btk*$^{-/-}$ mice with LPS to induce septic hypothermia. We found no significant difference in the hypothermic response between WT, *Itk*$^{-/-}$ and *Btk*$^{-/-}$ mice (see Figure E1), however, *Itk*$^{-/-}$*Btk*$^{-/-}$ mice experienced significantly exacerbated hypothermia (Figure 1A), accompanied by significantly enhanced neutrophil influx in the peritoneum 4 hours post LPS administration (Figure 1B). Analysis of PBS or LPS treated peritoneal cells from these mice by flow cytometry (gating strategy shown in Figure E2), further showed that *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells from mouse peritoneum (PLMCs) produced higher levels of TNF-α in response to LPS (Figure 1C), whereas peritoneal dendritic cells and macrophages from *Itk*$^{-/-}$*Btk*$^{-/-}$ mice produced similar or less TNF-α than WT counterparts (Figure 1C). These data suggest that ITK and BTK limit mast cell-derived TNF-α and accompanying hypothermia, and neutrophil influx in response to septic LPS.

Itk$^{-/-}$Btk$^{-/-}$ BMMCs are hyper-responsive to LPS and exacerbate LPS-induced hypothermia

Mast cells have been shown to initiate LPS-induced hypothermia via TNF-α[6] and recruit neutrophils during T cell-mediated delayed-type hypersensitivity through TNF-α-mediated MIP-2 (CXCL2)[31] in murine models. To confirm the role of mast cell-derived TNF-α in LPS-induced hypothermic response in the absence of ITK and BTK, we generated bone marrow derived mast cells (BMMCs) and found that *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs carried significantly higher levels of preformed TNF-α transcripts (Figure 2A), and spontaneously generated and secreted TNF-α (Figure 2B). When stimulated with LPS, *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs rapidly produced significantly higher amounts of TNF-α, CXCL2, and IL-6 mRNA and secreted proteins in a dose dependent manner compared to WT BMMCs (Figure 2C, D). Interestingly, this behavior of ITK and BTK in TNF-α production was different in other myeloid innate immune cells (See Figure E3: A, BMDCs; B, BMDMs; C, eosinophils; and D, neutrophils). The hyperactive TNF-α response in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs is consistent with what we observed in primary PLMCs, suggesting that this was a conserved phenotype between mast cells differentiated *in vitro* and *in vivo*. We thus used BMMCs to reconstitute mast cell deficient *Sash* mice[26, 32], and used the "*Sash* + BMMC" model to examine the mast cell specific function of ITK and BTK in LPS-induced hypothermic response. In comparison to plain *Sash* mice, *Sash* mice reconstituted with WT BMMCs exhibited more severe LPS-induced hypothermia; this was further significantly exacerbated in *Sash* recipients of *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs (Figure 2E), despite a lower percentage of *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs in the peritoneal lavage of *Sash* recipients than WT BMMCs (Figure E4). These data suggest that ITK and BTK regulate murine thermal homeostasis in LPS-induced septic response through mast cell function.

ITK and BTK kinase activity is required for hypothermic responses to LPS challenge

ITK and BTK are involved in the development of lymphoma, autoimmunity and other inflammatory diseases, and inhibitors of these kinases are under intensive investigation as potential therapeutics (see review[33]). We therefore utilized an ITK/BTK cross-reactive kinase inhibitor, to determine whether ITK and BTK kinase activity is required to suppress the hyperactive TNF-α production in mast cells. WT murine BMMCs treated with ITK/BTK inhibitor up-regulated basal expression of TNF-α mRNA, with no response observed in $Itk^{-/-}Btk^{-/-}$ BMMCs (Figure 3A). Moreover, this ITK/BTK cross-reactive inhibitor enhanced LPS-induced TNF-α mRNA by WT BMMCs (Figure 3B) and TNF-α expression in PLMCs in mice (Figure 3C). When administered *in vivo*, this ITK/BTK inhibitor caused significant exacerbation of LPS-induced hypothermia in WT mice (Figure 3D). Furthermore, the ITK/BTK inhibitor significantly enhanced TNF-α production by human CBMCs in response to LPS (Figure 3E). The effect of the ITK/BTK inhibitor on enhancing LPS-induced mast cell-derived TNF-α and associated septic hypothermia suggests that ITK/BTK kinase activity is required to suppress the mast cell-derived TNF-α in septic response.

Unique mast cell transcriptomic profile in response to LPS in the absence of ITK and BTK

Given the unique LPS-induced response to LPS in $Itk^{-/-}Btk^{-/-}$ mice and mast cells, we compared the transcriptomic response in mast cells to LPS in the absence of ITK and/or BTK by stimulating BMMCs with LPS for 1 hour, followed by microarray analysis. After normalizing all LPS-induced responses to PBS-treated levels in each strain, we found that a high fraction of genes that exhibited significant changes (> 2- or > 4- fold change in at least one strain, false discovery rate corrected $P < .05$) were associated with the absence of both ITK and BTK (Figure 4A, Tables E1 & E2). Principal component analysis also revealed that in the absence of ITK and BTK, the genes that exhibited > 4-fold change in at least one strain shifted dramatically 1 hour post LPS stimulation (Figure 4B). Note that the PBS-treated $Itk^{-/-}Btk^{-/-}$ gene profile is closely clustered with the WT profile, without overlapping with the treated groups in the 95% confidence interval area (Figure 4B), suggesting that the difference is due to the differential response to LPS in the absence of ITK and BTK. The unique profile of $Itk^{-/-}Btk^{-/-}$ BMMC response to LPS also suggests redundant regulatory function of ITK and BTK in this process, and was only revealed in the simultaneous absence of both kinases.

ITK and BTK negatively regulate LPS-induced canonical NF-κB activation in mast cells

LPS/TLR4 can activate both canonical and non-canonical NF-κB pathways. The former is dependent on the phosphorylation and release of IκBα from p65, allowing its translocation to the nucleus, while the latter triggers the processing of p100 to generate p52 (see review[34]). To determine whether canonical NF-κB targets[35] are significantly altered by the absence of ITK and BTK in mast cell response to LPS, we clustered all targets and found a significantly altered gene expression profile (Figure E5A). Following LPS stimulation, a significantly higher number of NF-κB target genes exhibited significant fold changes in $Itk^{-/-}Btk^{-/-}$ BMMCs compared to WT cells (Figure E5B). Along with TNF-α, other NF-κB target genes are highly activated/suppressed in the absence of ITK and BTK (Figure 5A,

genes with > 4-fold change, $P < .05$). These data suggested a LPS-induced hyperactivity of canonical NF-κB pathway in the absence of ITK and BTK.

Murine mast cells express very low levels of TRIF and CD14, and use only MyD88 dependent signaling[36,37]. In monocytic cells, BTK has been suggested to regulate MAL stability downstream of TLR4 and MyD88 [20], however, in *Btk*$^{-/-}$ BMMCs, MAL stability remains similar to that in WT cells[23]. Consistent with this, we observed no change in MAL accumulation in LPS-stimulated *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs (Figure 5B, 1st panel), suggesting that the hyperactive pro-inflammatory response in mast cells lacking ITK and BTK is not due to the MAL stabilization. However, steady state MyD88 expression is higher in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs (Figure 5B, 2nd panel). We also found that the canonical NF-κB signaling is hyperactive in the absence of ITK and BTK: IκBα exhibited higher basal phosphorylation, which was increased and persisted in response to LPS; meanwhile, p65 phosphorylation is significantly higher than that in WT BMMCs in response to LPS (Figure 5B, 3rd to 6th panels). Furthermore, LPS-induced p65 nuclear translocation is more efficient in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs (Figure 5C & E6). In contrast, there was little evidence for activation of the non-canonical pathway, since there was little conversion of p100 into p52 (Figure 5B, 7th & 8th panels). This suggests that ITK and BTK function to negatively regulate the activation of the canonical NF-κB signaling pathway, and so suppress LPS-induced mast cell-derived TNF-α production in mast cells.

ITK and BTK regulate LPS-induced Akt and p38 signaling activity in mast cells

LPS-induced PI3K/Akt and MAPK activation has been shown to regulate the activity of NF-κB pathway in mast cells[38, 39]. We found that Akt phosphorylation was enhanced in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs stimulated with LPS (Figure 6, 1st & 2nd panels). The MAPK p38 also exhibited enhanced basal phosphorylation, and was further significantly induced by stimulation of LPS in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs. In contrast, activation of ERK and JNK was impaired (Figure 6, 3rd to 8th panels)[40]. Thus, ITK and BTK differentially regulate MAPKs in mast cell response to LPS, with regulatory role in p38 signaling activation.

PI3K/Akt and MNK1 signals are required for LPS-induced TNF-α hyper-production and exacerbated hypothermia in the absence of ITK and BTK

The lack of ITK and BTK expression in BMMCs led to hyperactive Akt in response to LPS. PI3K has been well characterized as a major activator of Akt[41]. To test whether hyperactive TNF-α production and associated septic hypothermia can be attributed to altered PI3K and Akt activation in the mast cell response to LPS in the absence of ITK and BTK, we used PI3K inhibitor LY294002[42] and Akt inhibitor Akti1/2[43] to treat BMMCs and PLMCs, and measured the LPS-induced TNF-α production. We found that blockade of PI3K or Akt activation resulted in impairment of LPS-induced TNF-α mRNA production in both WT and *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs. Furthermore and of note, the level of TNF-α transcripts in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs was restored to WT levels (Figure 7A (i)). WT PLMCs exhibited weak dependence on PI3K and Akt activation in early TNF-α production in response to LPS stimulation *in vitro*, however, *Itk*$^{-/-}$*Btk*$^{-/-}$ primary mast cells strongly depended on the PI3K and Akt activation for hyperactive TNF-α production induced by LPS (Figure 7A (ii)). Furthermore, we found that the inhibition of Akt or PI3K (using Akti1/2 and LY294002,

starting 30 minutes prior to LPS exposure) significantly attenuated LPS-induced hypothermia in *Itk*$^{-/-}$*Btk*$^{-/-}$ mice (Figure 7A (iii)). The requisite role of PI3K and Akt activation in LPS-induced hyper-production of mast cell-derived TNF-α and exacerbated hypothermic response suggests that ITK and BTK regulate PI3K and Akt activity to control LPS-induced mast cell-mediated pro-inflammatory response and associated thermal homeostasis in mice.

The rate of posttranscriptional TNF-α synthesis is mainly determined by the stability of mRNA and the rate of mRNA translation, both of which are regulated by p38[44, 45]. In mast cells, LPS-induced TNF-α is dependent on p38 and independent of TTP[40, 46], and if this p38/MK2/TTP axis played a role in the absence of ITK and BTK, then TNF-α mRNA should be significantly more stable in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs. However analysis of the stability/degradation rate of TNF-α mRNA between WT and *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMC following LPS stimulation (following blockade of mRNA transcription/production using Actinomycin D, starting 1 hour after LPS stimulation), revealed that the TNF-α mRNA degradation rate is higher in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs ($t_{1/2}$ for WT: 86.6 mins. vs. *Itk*$^{-/-}$*Btk*$^{-/-}$: 23.8 mins. Figure E7). This suggests that the hyperactive TNF-α production in the absence of ITK and BTK is not a result of p38 mediated TNF-α mRNA stabilization, but rather that the enhanced p38 activation in *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells contributes to TNF-α production through effects on mRNA translation via the activation of MNK1[47]. p38/MNK1-mediated initiation of TNF-α mRNA translation can be inhibited by a MNK1 selective inhibitor CGP57380[47], and we found that CGP57380 significantly reduced LPS-induced TNF-α mRNA production by *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs to level observed in WT cells (Figure 7B (i)). CGP57380 also suppressed LPS-induced TNF-α protein synthesis in primary *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells (Figure 7B (ii)). Targeting MNK1 *in vivo* also resulted in attenuation of the severe LPS-induced hypothermia in *Itk*$^{-/-}$*Btk*$^{-/-}$ mice (Figure 7B (iii)). These data suggest that MNK1-mediated TNF-α translation is required for the LPS-induced hyperactive TNF-α production in mast cells lacking ITK and BTK, which is likely mediated by p38 hyperactivity.

DISCUSSION

We show here that ITK and BTK share a regulatory role in mast cell-mediated inflammatory response to gram-negative endotoxin LPS. In the absence of both ITK and BTK, mice experienced exacerbated LPS-induced hypothermia. Mast cells lacking ITK and BTK exhibited elevated preformed and LPS-induced TNF-α production, and contributed to enhanced LPS-induced hypothermia in mice. ITK and BTK kinase activity is involved in executing this regulatory role. Mast cells lacking ITK and BTK also exhibited significantly enhanced LPS-induced signaling activity in canonical NF-κB, PI3K/Akt and p38 pathways. Blocking PI3K/Akt and p38-associated MNK1 activity dampened LPS-induced mast cell-derived TNF-α production and septic hypothermic response caused by the absence of regulation by ITK and BTK.

Unlike macrophages that use both MAL/MyD88 and TRAM/TRIF adaptor complexes downstream of TLR4, murine mast cells express very low levels of TRIF and CD14, and use only MyD88 dependent signaling[36]. Indeed in BMMCs, there was little change in production of IFNβ, a prominent downstream target of the TRAM/TRIF pathway

downstream of LPS[37]. In monocytic cells, BTK has been suggested to regulate the MyD88/MAL signaling axis downstream of TLR4 by phosphorylating MAL and inducing its degradation, thus reducing TLR4 downstream signaling[20]. However, in *Btk*$^{-/-}$ BMMCs, MAL stability remains similar to that in WT cells[23]. Consistent with this, we found no changes in the stability of MAL, suggesting that this pathway is not controlled by either ITK or BTK in mast cells.

We have noted enhanced and constitutive TNF-α production in mast cells lacking both ITK and BTK prior to LPS stimulation, thus it was possible that the hyper-active TNF-α production in response to LPS was the result of autocrine or paracrine actions of preformed TNF-α, which can also activate NF-κB and p38 and further induce TNF-α production (see review [48]). However we ruled out this possibility since the enhanced preformed and LPS-induced TNF-α production in *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells is independent of blockade of extracellular TNF-α, although the immediate (1 hour) LPS-induced TNF-α mRNA is partially dependent on this (See Figure E8). This data supports the conclusion that unlike their role in FcεRI mediated mast cell activation, ITK and BTK function downstream of LPS/TLR4 ligation to suppress mast cell pro-inflammatory response.

Phosphoinositide-mediated adaptor recruitment is critical for LPS/TLR4 signaling activation[49]. Although PI3K signaling has been reported as regulatory elements during dendritic cell and macrophage responses to LPS/TLR4 ligation (see review[50]), activation of the PI3K pathway increases TNF-α and IL-6 production in mast cells response to LPS[51]. ITK can interact with PI3K and both ITK and BTK are directly downstream of PI3K for their activation[52–54]. BTK and PI3K have been shown to differentially regulate B cell receptor signaling, but share a common target in activating NF-κB[55]. In IgE/FcεRI-mediated signaling in mast cells, Akt activation, which lies downstream of PI3K, was not affected by the absence of BTK, while blocking PI3K activity dampened BTK activation[54]. However, the relationship between ITK/BTK and PI3K in cellular response to LPS remained largely undefined. Our finding that LPS induced enhanced Akt activation in *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells suggests a reciprocal regulation between ITK/BTK and PI3K.

In the absence of BTK, LPS-induced p38 activity is similar to WT cells, with moderate increase in TNF-α and IL-6 production[23]. However, the additional absence of ITK leads to a significant enhancement in LPS-induced p38 activation, and TNF-α, CXCL2 and IL-6 production, suggesting that ITK and BTK share redundant function in negatively regulating LPS-induced p38 activation and associated pro-inflammatory cytokine production. Our previous work suggested that the absence of ITK and BTK resulted in enhanced ERK activation in mast response to IgE-mediated antigen, without affecting p38 activation[14]. However, we found that the *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells response to LPS is impaired activation of ERK and JNK, and enhanced p38 activation, suggesting that the function of ITK and BTK in mast cells might be pathway specific. This p38 activation pattern is consistent with the very recent findings that p38 critically regulates LPS-induced TNF-α production in BMMCs, while ERK activation is dispensable[40]. In *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells, p38 may function to promote TNF-α protein translation, as its downstream effector MNK1 is an essential component in the hyper-production of TNF-α in LPS-stimulated mast cells. Indeed, the rate of posttranscriptional TNF-α synthesis is mainly determined by the stability of mRNA and

the rate of mRNA translation, both of which are regulated by p38. TNF-α mRNA contains an Adenylate-uridylate-rich element (ARE) in its 3′ region, which can be bound by ARE-binding and -destabilizing factor tristetraprolin (TTP)[44]. In macrophages, p38 activates MK2, which further phosphorylates TTP to reduce TTP binding affinity for the ARE, thus stabilizing TNF-α mRNA[45]. However, in mast cells, LPS-induced TNF-α is dependent on p38 and independent of TTP[40, 46], suggesting that TNF-α mRNA stabilization by the p38/MK2/TTP signaling axis does not play a major role in TNF-α production by WT mast cells. p38 can also regulate TNF-α mRNA by activating MNK1, which phosphorylates and reduces the binding affinity of eukaryotic initiation factor 4E (eIF4E) to the 5′ cap structure of cytoplasmic mRNA, thus facilitating cap-dependent translation[56]. Our data suggest that hyperactive TNF-α production in the absence of ITK and BTK is not a result of p38 mediated TNF-α mRNA stabilization, but rather through effects on increased transcription via NF-κB, and increased mRNA translation via the activation of MNK1[47].

Activation of lymphocytes and certain myeloid lineages in autoimmune diseases and hypersensitivity requires the activation of ITK and BTK, making Tec kinase inhibitors promising selective targets for therapy[57–59]. However, given the overall regulatory role of ITK and BTK in the mast cell response to LPS, these same inhibitors might exacerbate mast cell-mediated diseases such as septic hypothermia. Due to the high homology structure of ITK and BTK, specific targeting may be difficult, hence mast cell function might be a critical issue in drug specificity and efficacy in therapeutically targeting Tec family kinases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

BMMC	Bone marrow derived mast cell
BMDC	Bone marrow derived dendritic cell
BMDM	Bone marrow derived macrophage
CBMC	Cord blood derived mast cell
IκBα	Inhibitor of NF-κB Alpha
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
MNK1	MAP kinase interacting serine/threonine kinase 1
NF-κB	Nuclear factor Kappa B
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLMC	Peritoneal lavage mast cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
WT	Wild-type

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Key Messages

- ITK and BTK share unique regulatory function in mast cell pro-inflammatory responses to septic LPS.
- ITK and BTK may be targets to modulate mast cell function during infection and allergy.

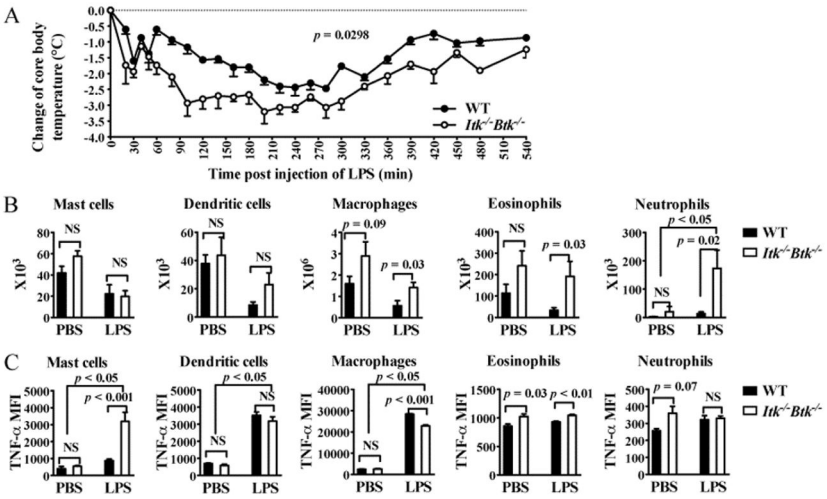

Figure 1. The absence of ITK and BTK enhances LPS-induced hypothermia, neutrophil influx, and mast cell-derived TNF-α

(**A**) Change in core body temperature induced by LPS. *p* value by ANOVA. (**B**) Number of indicated cells in peritoneal lavage. (**C**) MFI of TNF-α expression by peritoneal cells stimulated *in vitro* (gating strategy shown in Figure E2). *p* value by *t* test. n ≥ 3.

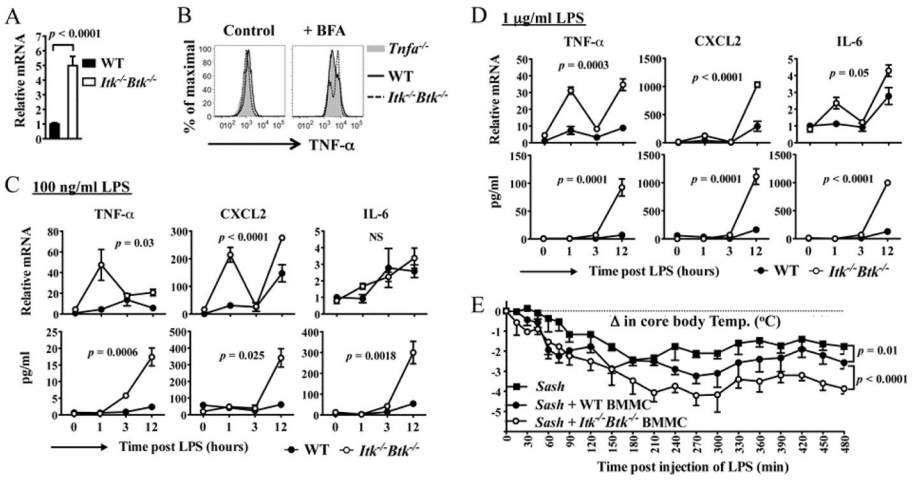

Figure 2. ITK and BTK negatively regulate LPS-induced hypothermia via mast cells

(**A**) Preformed TNF-α mRNA in BMMCs. *p* value by *t* test. (**B**) Steady state TNF-α synthesis by BMMCs. (**C, D**) Pro-inflammatory cytokine mRNA and secretion induced by (**C**) 100 ng/ml or (**D**) 1 μg/ml LPS. (**E**) LPS-induced hypothermia in *Sash* mice reconstituted with BMMCs. *p* values by ANOVA.

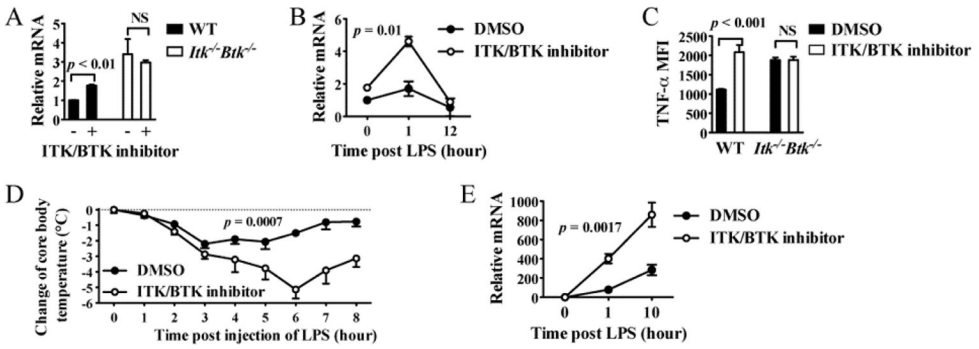

Figure 3. ITK/BTK kinase activity is required to attenuate LPS-induced TNF-α production in mast cells and hypothermia

Effect of ITK/BTK inhibitor on (**A**) preformed and (**B**) LPS-induced TNF-α mRNA in mouse BMMCs, (**C**) LPS-induced TNF-α expression in mouse PLMCs, and (**D**) LPS-induced murine hypothermia. (**E**) Effect of ITK/BTK inhibitor on LPS-induced TNF-α mRNA in human CBMCs. n ≥ 3, *p* by *t* test (columns) or ANOVA (curves).

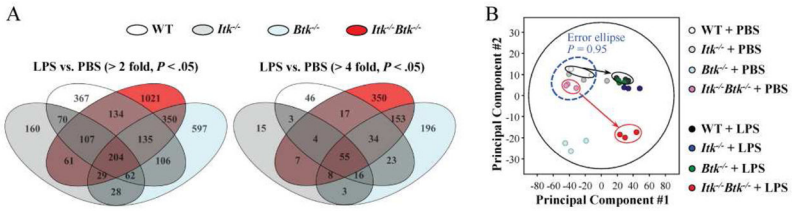

Figure 4. ITK and BTK differentially regulate LPS-induced gene expression in mast cells

LPS-induced transcriptomic profiles are normalized to control levels in WT, *Itk*$^{-/-}$, *Btk*$^{-/-}$ and *Itk/Btk*$^{-/-}$ BMMCs respectively. (**A**) Venn diagraphs of genes with > 2- or 4-fold LPS-induced changes. (**B**) Principal component analysis of genes with > 4-fold changes. Arrows indicate LPS-induced changes in WT (black) and *Itk/Btk*$^{-/-}$ (red) BMMCs. Ellipse (blue dashed) shows area with 95% confidence interval with WT and DKO controls.

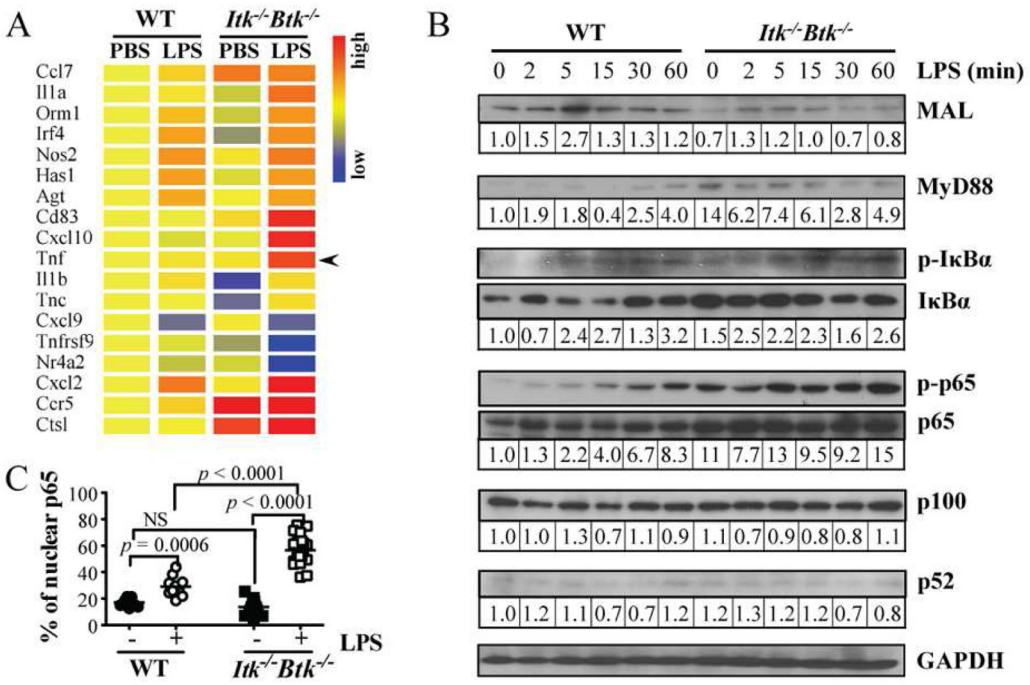

Figure 5. Hyperactive canonical NF-κB signaling by *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs in response to LPS

(**A**) Heat map of NF-κB target genes that changed > 4-fold in response to PBS or LPS (arrows indicate TNF-α). (**B**) Western blot analysis for the indicated components of the NF-κB pathway (representative results of ≥ 2 experiments). Fold changes below respective blots relative to WT at time 0 are shown. (**C**) Analysis for nuclear translocation of p65 in PBS or LPS treated BMMCs. *p* values by *t* test.

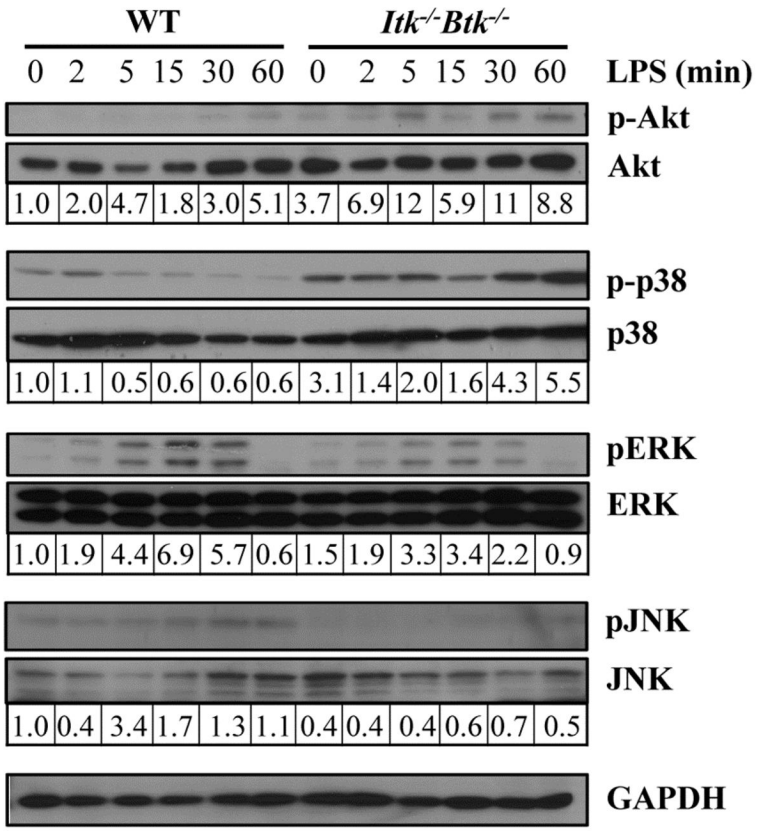

Figure 6. Hyperactive PI3K/Akt and p38 signaling by *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs in response to LPS

WT and *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs were stimulated with LPS and analyzed for the activation of Akt and the indicated MAPKs by western blotting. Fold changes below the respective blots compared to WT time 0 are shown. Data represent results from ≥ 2 independent experiments.

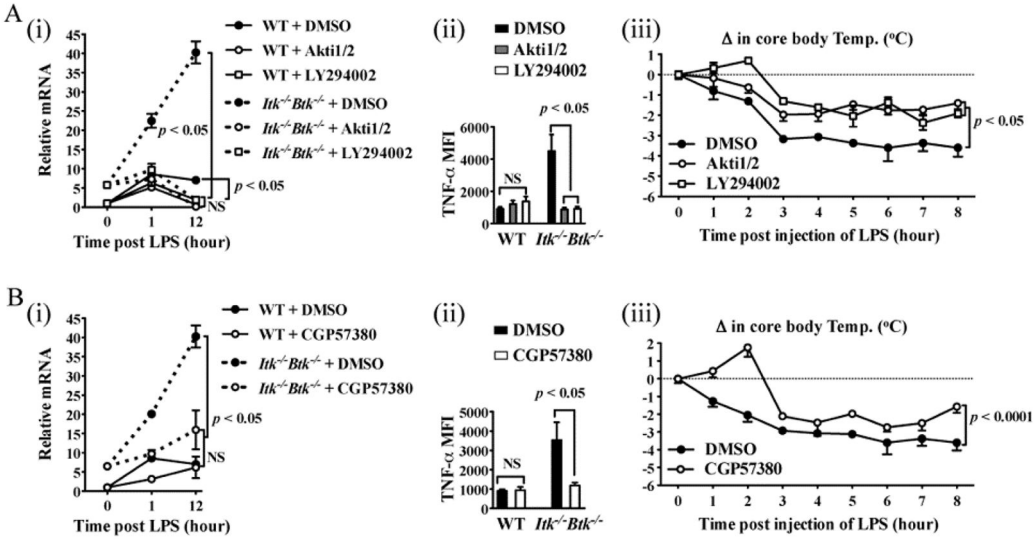

Figure 7. PI3K/Akt and MNK1 are required for hyperactive TNF-α production and enhanced hypothermia in the absence of ITK and BTK

Effectors of (**A**) PI3K/Akt and (**B**) MNK inhibitors on LPS-induced (**i**) TNF-α mRNA in BMMCs, (**ii**) TNF-α protein expression in PLMCs, and (**iii**) hypothermia in $Itk^{-/-}Btk^{-/-}$ mice. n ≥ 3, *p* by *t* test (columns) or ANOVA (curves).