

Nonreceptor tyrosine kinases ITK and BTK negatively regulate mast cell proinflammatory responses to lipopolysaccharide

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Background: Mast cells are indispensable 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 allergens, but their roles in mast cell responses in LPS-induced sepsis are unclear.

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

Methods: Mice (genetically modified or bone marrow–derived mast cell–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 bone marrow–derived mast cells. Quantitative PCR and multiplex were used to determine transcribed and secreted proinflammatory 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 nuclear factor kappa B, Akt, and p38 activation is enhanced in *Itk*^{-/-}*Btk*^{-/-} mast cells, and blockage of phosphatidylinositol-4,5-bisphosphate 3-kinase, Akt, or p38 downstream mitogen-activated protein kinase interacting serine/threonine kinase 1 activation significantly suppresses TNF- α hyperproduction 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 Toll-like receptor 4/LPS in mast cells, through regulating the activation of

canonical nuclear factor kappa B, phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt, and p38 signaling pathways. (J Allergy Clin Immunol 2015;■■■■:■■■■-■■■■.)

Key words: *Tec*, lipopolysaccharide, mast cell, septic hypothermia, TNF- α , NF- κ B, MAPKs, PI3K/Akt

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 associated more with severe or fatal sepsis.^{3,4} Toll-like receptor 4 (TLR4) ligation by bacterial endotoxin LPS activates mast cell production of proinflammatory cytokines including TNF- α and IL-6.⁵ Administration of LPS to mice or humans causes sepsislike symptoms, and LPS-triggered mast cell–derived TNF- α is indispensable 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 Although 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 nonreceptor tyrosine kinases acting downstream of numerous receptors, and have been shown to modulate mast cell responses downstream of Fc ϵ RI α .^{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 Fc ϵ RI α -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 mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF- κ B), the latter induced by phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt.^{5,16,17} BTK has been shown to interact with TLR4 and be involved in NF- κ B 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 were 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 specific to cell type or dependent on mouse strain.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

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Abbreviations used

ARE: Adenylate-uridylate-rich element
 BMBC: Bone marrow–derived mast cell
 CBMC: Cord blood–derived mast cell
 MAL: MyD88-adaptor like protein
 MAPK: Mitogen-activated protein kinase
 MNK1: Mitogen-activated protein kinase interacting serine/threonine kinase 1
 NF-κB: Nuclear factor kappa B
 PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase
 PLMC: Peritoneal lavage mast cell
 rh: Recombinant human
 TLR4: Toll-like receptor 4
 TTP: Tristetraprolin
 WT: Wild type

NF-κB, PI3K/Akt, p38, and mitogen-activated protein kinase interacting serine/threonine kinase 1 (MNK1) signaling activity.

METHODS**Mice**

Mice were on a C57BL/6 background. *Itk*^{-/-}*Btk*^{-/-} mice were as previously described,¹⁴ and *Tnfa*^{-/-} (B6.129S-*Tnfa*^{tm1Gkl/J}) and *Sash* (*Kit*^{W-sh}/HNIhrJaeBsmJ) mice were from The Jackson Laboratory (Bar Harbor, Me). IL-5 transgenic mice (gift from Drs N. A. Lee and J. J. Lee²⁵) were crossed to *Itk*^{-/-}*Btk*^{-/-} mice as a source of eosinophils. All experiments were approved by Institutional Animal Care and Use Committees at The Pennsylvania State and Cornell universities.

Generation of bone marrow–derived mast cells and their reconstitution in *Sash* mice

Bone marrow–derived mast cells (BMMCs) were generated as previously described.¹⁴ In brief, bone marrow cells were cultured in complete Dulbecco modified Eagle medium with 10 ng/mL recombinant murine IL-3 (Cell Sciences, Canton, Mass) and 50 ng/mL recombinant murine SCF (Peprotech, Rocky Hill, NJ), and after 4 to 6 weeks, BMMCs with more than 96% purity (c-Kit⁺FcεRIα⁺) were used. For *in vitro* stimulation, 2 × 10⁶/mL BMMCs were starved in complete Dulbecco modified Eagle medium without IL-3/SCF overnight, followed by indicated stimulation. *Sash* mice received 5 × 10⁶ BMMCs through retro-orbital (intravenous) injection²⁶ and 5 × 10⁶ BMMCs through intraperitoneal injection²⁷ to reconstitute mast cells, 12 weeks before experiments.

Generation and stimulation of cord blood–derived mast cells

Cord blood–derived mast cells (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 more than 95% purity (c-Kit⁺FcεRIα⁺) were used. CBMCs were sensitized in 10 ng/mL IL-4 and 10% FBS for 5 days²⁹ before LPS (1 μg/mL) stimulation.

LPS-induced hypothermia

Mice were intraperitoneally injected with 1 mg/kg LPS, and core body temperature was 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 after LPS injection and flow cytometry. Gating strategy used for the identification of primary cells in the peritoneal lavage by flow cytometry is depicted in Fig E2 in this article's Online Repository at www.jacionline.org.

Microarray analysis

BMMCs were factor starved overnight and 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 were analyzed using GeneSpring GX (Agilent, Santa Clara, Calif) as described in this article's Online Repository at www.jacionline.org.

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, Mass) analyzed on a MAGPIX system (Luminex, Madison, Wis).

Statistical analysis

Two-tailed Student *t* test and 2-way ANOVA between groups were performed using Prism (GraphPad, San Diego, Calif), with *P* < .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 wild-type (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 Fig E1 in this article's Online Repository at www.jacionline.org); however, *Itk*^{-/-}*Btk*^{-/-} mice experienced significantly exacerbated hypothermia (Fig 1, A), accompanied by significantly enhanced neutrophil influx in the peritoneum 4 hours after LPS administration (Fig 1, B). Analysis of PBS- or LPS-treated peritoneal cells from these mice by flow cytometry (gating strategy shown in Fig E2) further showed that *Itk*^{-/-}*Btk*^{-/-} mast cells from mouse peritoneum (peritoneal lavage mast cells [PLMCs]) produced higher levels of TNF-α in response to LPS (Fig 1, C), whereas peritoneal dendritic cells and macrophages from *Itk*^{-/-}*Btk*^{-/-} mice produced similar or less TNF-α than did WT counterparts (Fig 1, C). 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 hyperresponsive to LPS and exacerbate LPS-induced hypothermia**

Mast cells have been shown to initiate LPS-induced hypothermia via TNF-α⁶ 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 BMMCs and found that *Itk*^{-/-}*Btk*^{-/-} BMMCs carried significantly higher levels of preformed TNF-α transcripts (Fig 2, A), and spontaneously generated and secreted TNF-α (Fig 2, B). When stimulated with

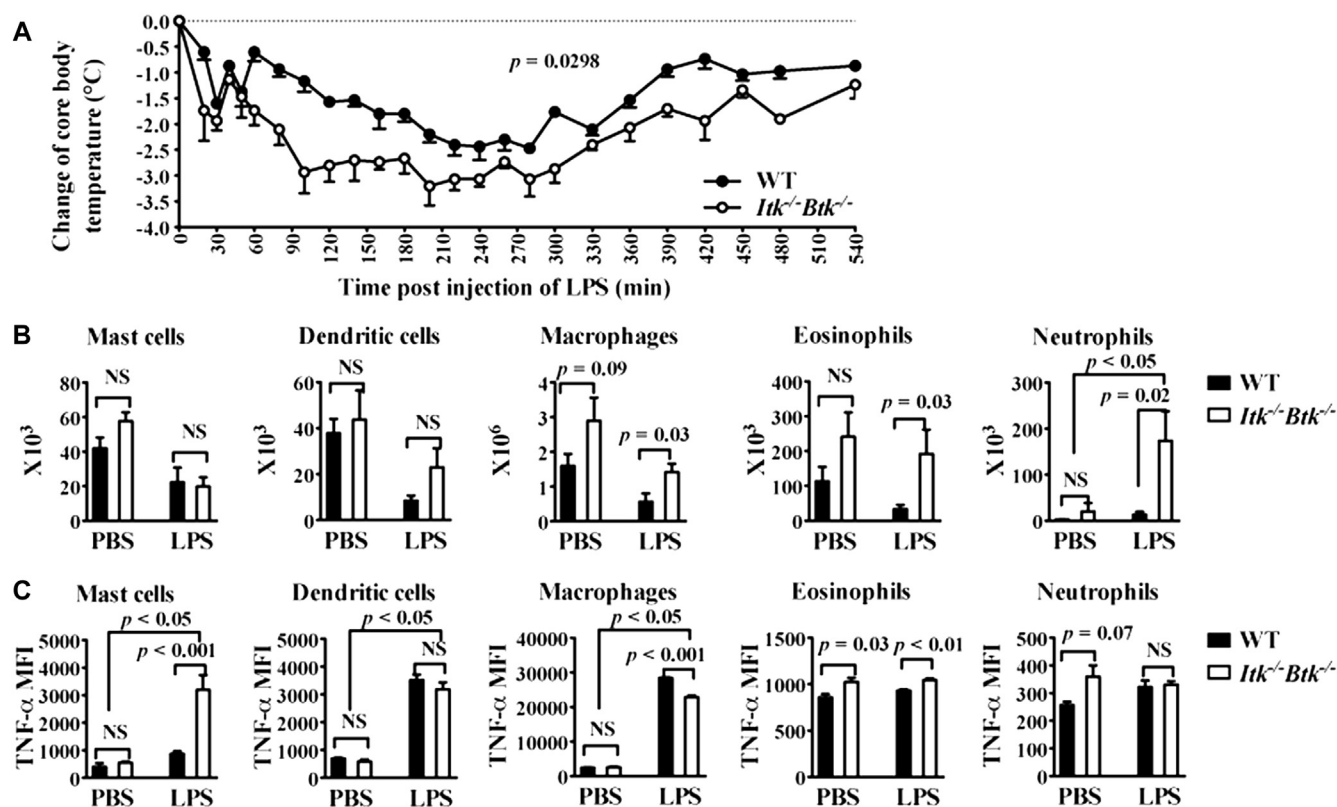


FIG 1. The absence of ITK and BTK enhances LPS-induced hypothermia, neutrophil influx, and mast cell-derived TNF- α . **A**, Change in core body temperature induced by LPS. P value by ANOVA. **B**, Number of indicated cells in peritoneal lavage. **C**, MFI of TNF- α expression by peritoneal cells stimulated *in vitro* (gating strategy shown in Fig E2). P value by t test. $n \geq 3$. MFI, Mean fluorescence intensity.

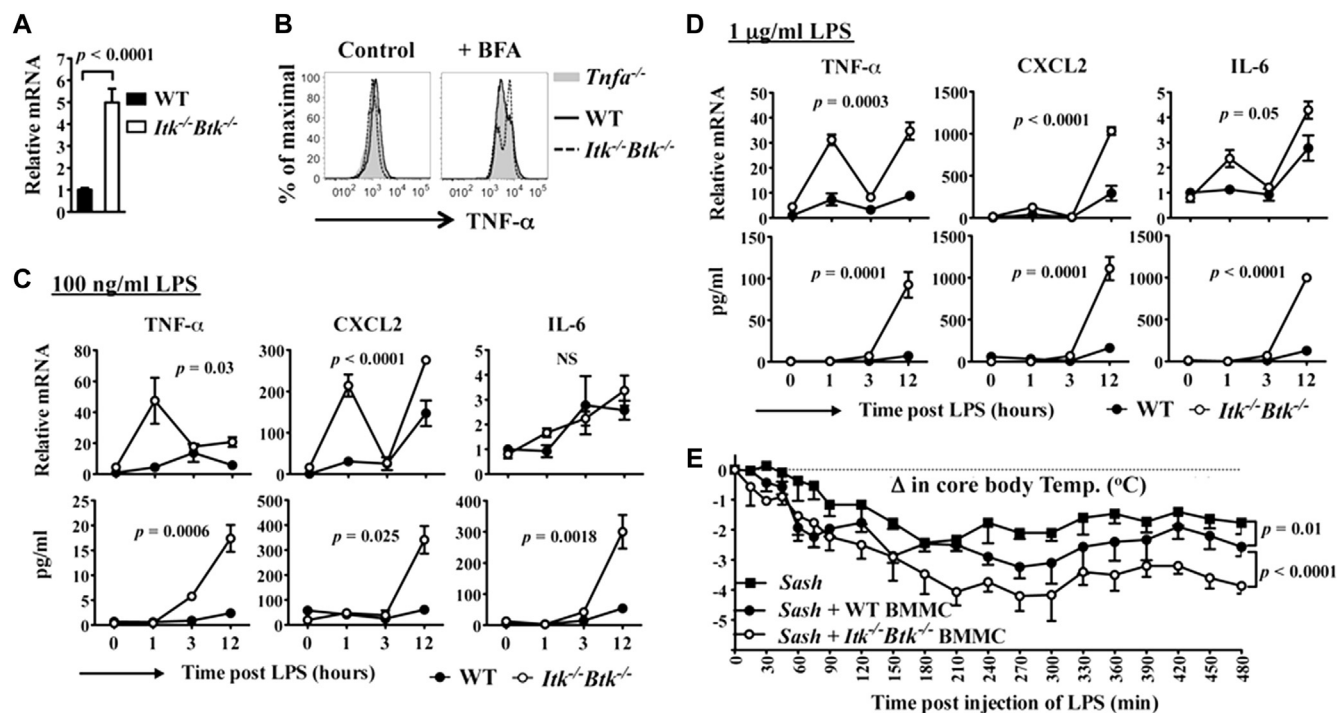


FIG 2. ITK and BTK negatively regulate LPS-induced hypothermia via mast cells. **A**, Preformed TNF- α mRNA in BMMCs. P value by t test. **B**, Steady-state TNF- α synthesis by BMMCs. **C** and **D**, Proinflammatory cytokine mRNA and secretion induced by (Fig 2, C) 100 ng/mL or (Fig 2, D) 1 μ g/mL LPS. **E**, LPS-induced hypothermia in *Sash* mice reconstituted with BMMCs. P values by ANOVA. BFA, Brefeldin A.

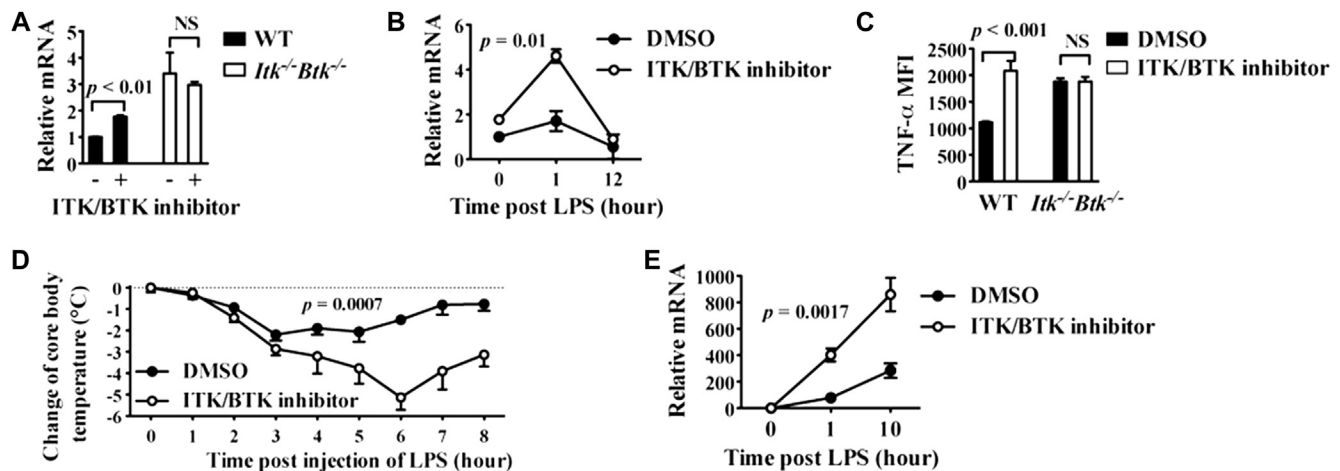


FIG 3. ITK/BTK kinase activity is required to attenuate LPS-induced TNF- α production in mast cells and hypothermia. Effect of ITK/BTK inhibitor on preformed (A) and LPS-induced TNF- α mRNA in mouse BMMCs (B), LPS-induced TNF- α expression in mouse PLMCs (C), and LPS-induced murine hypothermia (D). E, Effect of ITK/BTK inhibitor on LPS-induced TNF- α mRNA in human CBMCs. $n \geq 3$, P by t test (columns) or ANOVA (curves). DMSO, Dimethyl sulfoxide.

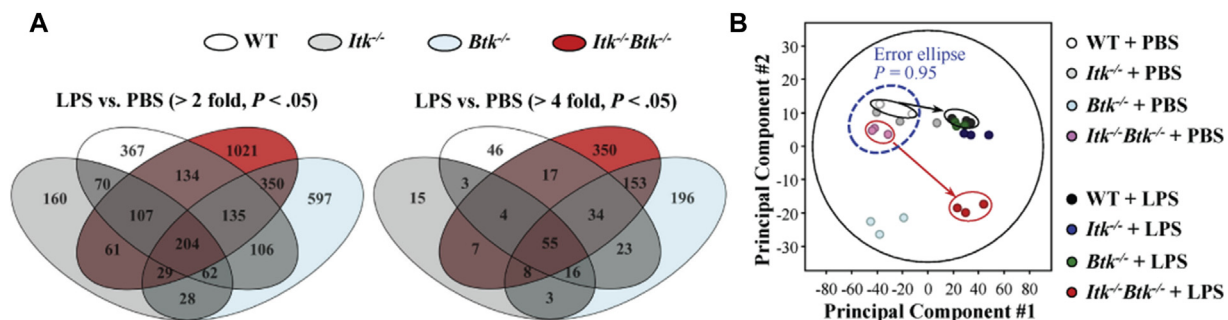


FIG 4. ITK and BTK differentially regulate LPS-induced gene expression in mast cells. LPS-induced transcriptomic profiles are normalized to control levels in WT, *Itk^{-/-}*, *Btk^{-/-}*, and *Itk^{-/-}Btk^{-/-}* BMMCs, respectively. A, Venn diagrams of genes with >2- or 4-fold LPS-induced changes. B, 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% CI with WT and *Itk^{-/-}Btk^{-/-}* controls.

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 with WT BMMCs (Fig 2, C and D). Interestingly, this behavior of ITK and BTK in TNF- α production was different in other myeloid innate immune cells (see Fig E3, A, bone marrow-derived dendritic cells; B, bone marrow-derived macrophages; C, eosinophils; and D, neutrophils, in this article's Online Repository at www.jacionline.org). 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 (Fig 2, E), despite a lower percentage of *Itk^{-/-}Btk^{-/-}* BMMCs in the peritoneal lavage of *Sash* recipients than in WT BMMCs (see Fig E4 in this article's Online

Repository at www.jacionline.org). 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 review³³). We therefore used 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 upregulated basal expression of TNF- α mRNA, with no response observed in *Itk^{-/-}Btk^{-/-}* BMMCs (Fig 3, A). Moreover, this ITK/BTK cross-reactive inhibitor enhanced LPS-induced TNF- α mRNA by WT BMMCs (Fig 3, B) and TNF- α expression in PLMCs in mice (Fig 3, C). When administered *in vivo*, this ITK/BTK inhibitor caused significant exacerbation of LPS-induced hypothermia in WT

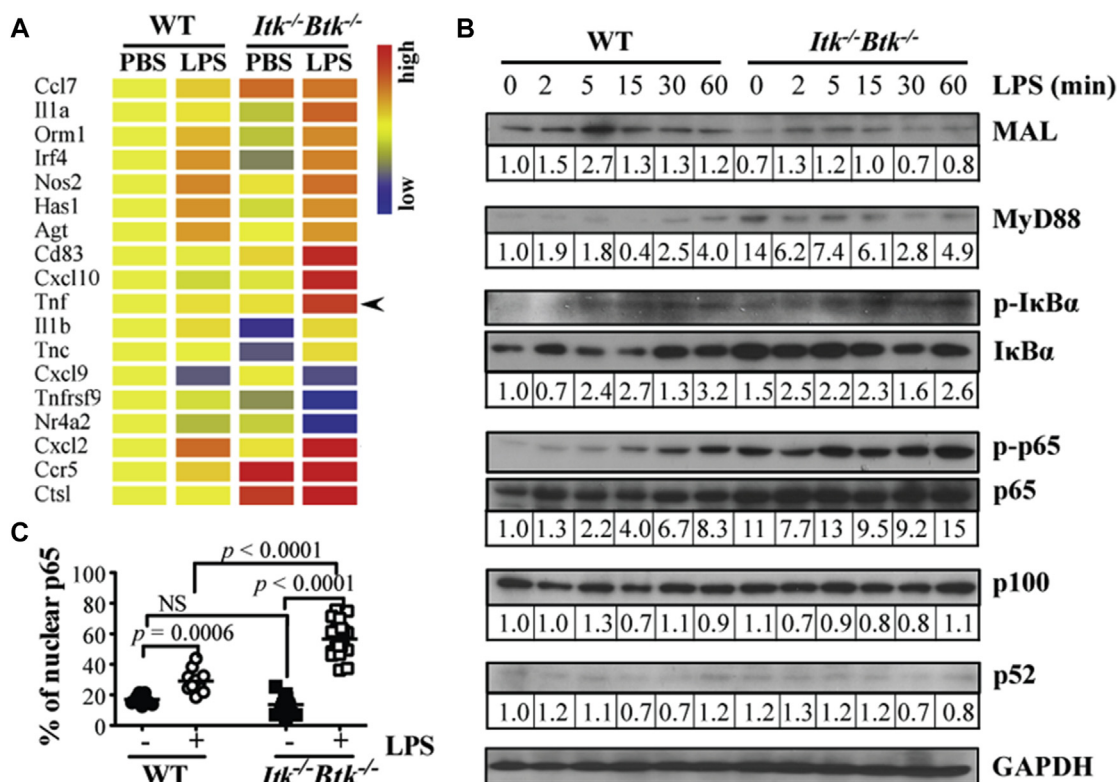


FIG 5. Hyperactive canonical NF-κB signaling by *Itk*^{-/-}*Btk*^{-/-} BMMCs in response to LPS. **A**, Heat map of NF-κB target genes that changed >4-fold in response to PBS or LPS (arrow indicates TNF-α). **B**, Western blot analysis for the indicated components of the NF-κB pathway (representative 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. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

mice (Fig 3, D). Furthermore, the ITK/BTK inhibitor significantly enhanced TNF-α production by human CBMCs in response to LPS (Fig 3, E). 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 1 strain, false-discovery rate corrected $P < .05$) was associated with the absence of both ITK and BTK (Fig 4, A; see Tables E1 and E2). Principal-component analysis also revealed that in the absence of ITK and BTK, the genes that exhibited more than 4-fold change in at least 1 strain shifted dramatically 1 hour after LPS stimulation (Fig 4, B). 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% CI area (Fig 4, B), 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 revealed only 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 noncanonical NF-κB pathways. The former is dependent on the phosphorylation and release of inhibitor of NF-κB alpha from p65, allowing its translocation to the nucleus, whereas the latter triggers the processing of p100 to generate p52 (see review³⁴). To determine whether canonical NF-κB targets³⁵ 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 (see Fig E5, A, in this article's Online Repository at www.jacionline.org). Following LPS stimulation, a significantly higher number of NF-κB target genes exhibited significant fold changes in *Itk*^{-/-}*Btk*^{-/-} BMMCs compared with WT cells (Fig E5, B). Along with TNF-α, other NF-κB target genes are highly activated/suppressed in the absence of ITK and BTK (Fig 5, A, genes with >4-fold change; $P < .05$). These data suggested an 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

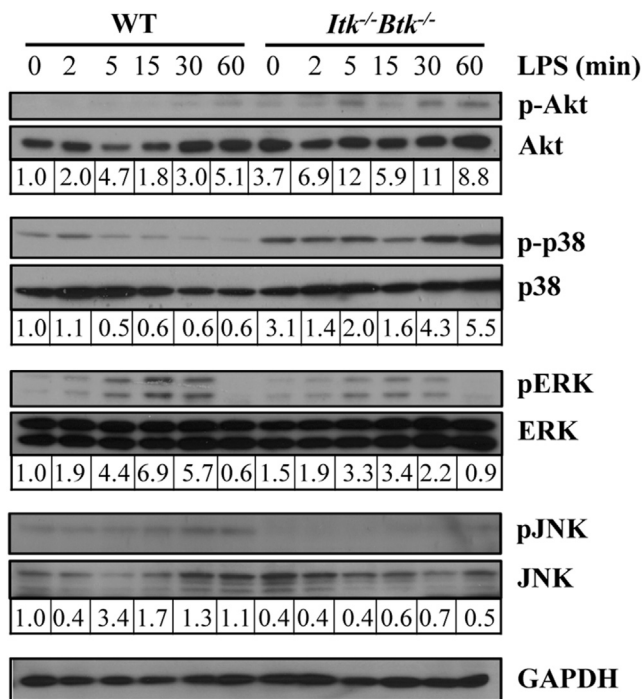


FIG 6. Hyperactive PI3K/Akt and p38 signaling by *Itk*^{-/-}*Btk*^{-/-} BMMCs in response to LPS. WT and *Itk*^{-/-}*Btk*^{-/-} BMMCs were stimulated with LPS and analyzed for the activation of Akt and the indicated MAPKs by Western blotting. Fold changes below the respective blots compared with WT time 0 are shown. Data represent results from ≥ 2 independent experiments. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

cells, BTK has been suggested to regulate MAL stability downstream of TLR4 and MyD88²⁰; 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 (Fig 5, B, first panel), suggesting that the hyperactive proinflammatory 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 (Fig 5, B, second panel). We also found that the canonical NF- κ B signaling is hyperactive in the absence of ITK and BTK: Inhibitor of NF- κ B α 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 (Fig 5, B, third to sixth panels). Furthermore, LPS-induced p65 nuclear translocation is more efficient in *Itk*^{-/-}*Btk*^{-/-} BMMCs (Fig 5, C; see Fig E6 in this article's Online Repository at www.jacionline.org). In contrast, there was little evidence for activation of the noncanonical pathway because there was little conversion of p100 into p52 (Fig 5, B, seventh and eighth panels). This suggests that ITK and BTK function to negatively regulate the activation of the canonical NF- κ B 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 the NF- κ B pathway in mast cells.^{38,39}

We found that Akt phosphorylation was enhanced in *Itk*^{-/-}*Btk*^{-/-} BMMCs stimulated with LPS (Fig 6, first and second panels). The MAPK p38 also exhibited enhanced basal phosphorylation, and was further significantly induced by the stimulation of LPS in *Itk*^{-/-}*Btk*^{-/-} BMMCs. In contrast, activation of ERK and JNK was impaired (Fig 6, third to eighth 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 hyperproduction 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 LY294002⁴² and Akt inhibitor Akti1/2⁴³ 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 (Fig 7, A [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 (Fig 7, A [ii]). Furthermore, we found that the inhibition of Akt or PI3K (using Akti1/2 and LY294002, starting 30 minutes before LPS exposure) significantly attenuated LPS-induced hypothermia in *Itk*^{-/-}*Btk*^{-/-} mice (Fig 7, A [iii]). The requisite role of PI3K and Akt activation in LPS-induced hyperproduction 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 proinflammatory 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 after LPS stimulation (after 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 minutes vs *Itk*^{-/-}*Btk*^{-/-}: 23.8 minutes; see Fig E7 in this article's Online Repository at www.jacionline.org). 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 an MNK1 selective inhibitor CGP57380,⁴⁷ and we

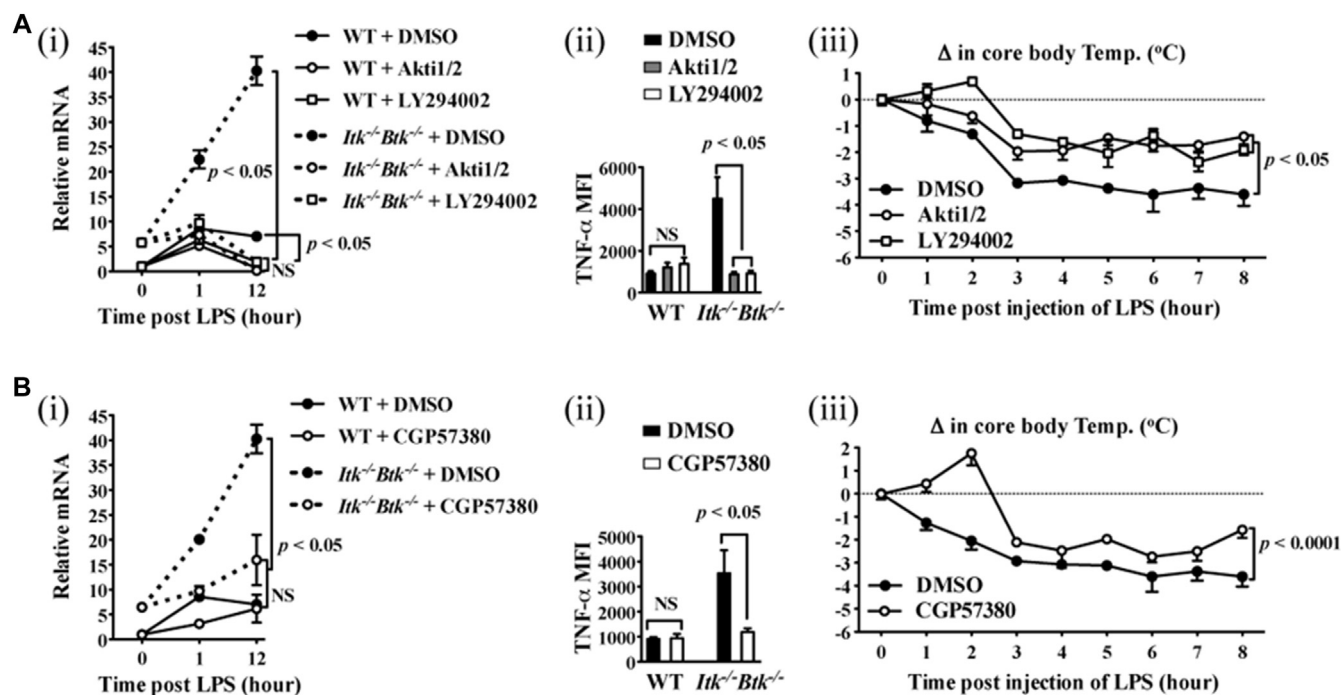


FIG 7. PI3K/Akt and MNK1 are required for hyperactive TNF- α production and enhanced hypothermia in the absence of ITK and BTK. Effectors of PI3K/Akt (**A**) and MNK inhibitors (**B**) on LPS-induced TNF- α mRNA in BMMCs (i), TNF- α protein expression in PLMCs (ii), and hypothermia in *Itk*^{-/-}*Btk*^{-/-} mice (iii). $n \geq 3$, P by t test (columns) or ANOVA (curves). DMSO, Dimethyl sulfoxide.

found that CGP57380 significantly reduced LPS-induced TNF- α mRNA production by *Itk*^{-/-}*Btk*^{-/-} BMMCs to the level observed in WT cells (Fig 7, B [i]). CGP57380 also suppressed LPS-induced TNF- α protein synthesis in primary *Itk*^{-/-}*Btk*^{-/-} mast cells (Fig 7, B [ii]). Targeting MNK1 *in vivo* also resulted in attenuation of the severe LPS-induced hypothermia in *Itk*^{-/-}*Btk*^{-/-} mice (Fig 7, B [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- κ B, 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 the production of IFN- β , a prominent downstream

target of the TRIF-related adapter molecule/TIR-domain-containing adapter-inducing IFN- β 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 before LPS stimulation; thus, it was possible that the hyperactive TNF- α production in response to LPS was the result of autocrine or paracrine actions of preformed TNF- α , which can also activate NF- κ B and p38 and further induce TNF- α production (see review⁴⁸). However, we ruled out this possibility because 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 Fig E8 in this article's Online Repository at www.jacionline.org). These data support the conclusion that unlike their role in Fc ϵ RI-mediated mast cell activation, ITK and BTK function downstream of LPS/TLR4 ligation to suppress mast cell proinflammatory response.

Phosphoinositide-mediated adaptor recruitment is critical for LPS/TLR4 signaling activation.⁴⁹ Although PI3K signaling has been reported as a regulatory element during dendritic cell and macrophage responses to LPS/TLR4 ligation (see review⁵⁰), 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 they share a common target in activating NF- κ B.⁵⁵ In IgE/Fc ϵ RI-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 that of 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 proinflammatory cytokine production. Our previous work suggested that the absence of ITK and BTK resulted in enhanced ERK activation in mast cell response to IgE-mediated antigen, without affecting p38 activation.¹⁴ However, we found that the *Itk*^{-/-}*Btk*^{-/-} mast cells' response to LPS is accompanied by 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 hyperproduction 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 ARE-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 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- κ B, 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. Because of 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.

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Key messages

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

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

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METHODS

Generation and stimulation of dendritic cells, macrophages, eosinophils, and neutrophils.

Bone marrow derived dendritic cells (BMDCs) were generated as previously described¹, and further purified using biotin conjugated anti-CD11c (N418, eBioscience) followed by magnetic positive selection. BMDCs with $> 95\%$ CD11c⁺CD11b⁺ are used for stimulation. Bone marrow derived macrophages (BMDMs) were generated as previously described² and BMDMs with $> 96\%$ F4/80⁺CD11b⁺ were used. Eosinophils were isolated from peritoneal lavage and spleens of the IL-5 transgenic mice using biotin conjugated anti-Gr1 (RB6-8C5), F4/80 (BM8), CD11c, DX5, TER119, B220 (RA3-6B2), and CD3 ϵ (eBio500A2) (from eBioscience) followed by negative magnetic selection, and eosinophils with $> 97\%$ Siglec-F⁺ were used. Neutrophils were induced by 10% thioglycollate (Becton Dickinson) and isolated as previously described³ and cells with $> 96\%$ Ly6G⁺CD11b⁺ are used. Cells were plated at $1-2 \times 10^6$ /ml, and stimulated with 100ng/ml LPS for indicated time.

Antibodies and reagents

Fluorescent anti-mouse antibodies:

FITC conjugated anti-c-Kit (2B8), Phycoerythrin (PE) conjugated anti-Fc ϵ RI α (MAR-1), Allophycocyanin (APC) conjugated anti-CD11c (N418), APC eFluor780 conjugated anti-F4/80 (BM8), PerCP-eFluor 710 conjugated anti-TNF- α (MP6-XT22), and Alexa Fluor 488 conjugated anti-Ly6G (1A8) antibodies were from eBioscience (San Diego, CA). PE conjugated anti-Siglec-F (E50-2440) was from BD Biosciences (San Jose, CA). Pacific Blue conjugated anti-Ly6G (1A8), APC conjugated anti-F4/80 (BM8), Alexa Fluor 700 conjugated anti-MHCII (M5/114.15.2), and PE-Cy7 conjugated anti-Fc ϵ RI α (MAR-1) antibodies were from BioLegend

(San Diego, CA). PE-Texas Red conjugated anti-CD11b (M1/70.15) was from Life Technologies (Grand Island, NY).

Anti-Human antibodies:

Biotin conjugated anti-CD34 (4H11), PE conjugated anti-Fc ϵ RI α (AER-37 (CRA1)), and APC conjugated anti-c-Kit (104D2) were from eBioscience (San Diego, CA).

Antibodies for western blotting:

Anti- p-p65 (Ser536, 93H1, #3033), p65 (E498, #3987), p-p38 (Thr180/Tyr182, #9211), p38 (#9212), p-ERK (Thr202/204, E10, #9106), ERK (137F5, #4695), p-JNK (Thr183/Tyr185, G9, #9255), JNK (#9252), p-Akt (Ser473, 587F11, #4051) and Akt (#9272), p-I κ B α (Ser32, 14D4, #2859), p100/p52 (#4882) and GAPDH (14C10, #2118) antibodies were from Cell Signaling (Danvers, MA). Anti-I κ B α (sc-847) antibody was from Santa Cruz. Anti-MyD88 (IMG-178) antibody was from Imgenex (San Diego, CA). Anti-MAL (TIRAP, Pearl-1, ALX-210-366) was from Enzo Life Sciences (Farmingdale, NY). Horseradish peroxidase (HRP) conjugated anti mouse/rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were detected by ECL system (GE Healthcare Biosciences, Pittsburgh, PA).

TaqMan probes for real-time quantitative PCR (RT-qPCR):

To measure mRNA production of relevant genes, the following probes from TaqMan (Life Technologies, Grand Island, NY) were used: mouse Gapdh (Mm99999915_g1), Tnf (Mm00443258_m1), Cxcl2 (Mm00436450_m1), Il-6 (Mm00446190_m1); human Gapdh (Hs02758991_g1) and Tnf (Hs01113624_g1).

Other reagents:

LPS (*Escherichia coli* 0111:B4) was from Sigma-Aldrich (St. Louis, MO). The protein transport blocker Brefeldin A was from Sigma, and used at 10 µg/ml. ITK/BTK cross-reactive inhibitor was a kind gift from Avila Therapeutics and was used at 5 nM for LPS induced BMMC TNF- α mRNA production, 200 nM for LPS induced PLMC and CBMC TNF- α production (*in vitro*), and 5 µmol/kg in LPS induced murine hypothermia (*in vivo*). Akt inhibitor Akti1/2 (Santa Cruz Biotechnology, Dallas, TX) was used at 10 or 20 µM *in vitro*, and 5.5 mg/kg *in vivo*. PI3K inhibitor LY294002 (Cayman Chemical, Ann Arbor, MI) was used at 10 or 20 µM *in vitro*, and 6.86 mg/kg *in vivo*. MAP-kinase interacting kinase-1 inhibitor CGP57380 (Tocris, Bristol, United Kingdom) was used in 20 µM *in vitro*, and 9.8 mg/kg *in vivo*. Actinomycin D (Sigma) was used at 10 µg/ml. Anti-TNF- α neutralizing antibody (AF-410-NA, neutralizing dose 0.1-0.4 µg/ml) was from R&D systems (Minneapolis, MN), and used at 0.8 µg/ml.

Microarray data analysis

Microarray data were analyzed using GeneSpring GX (Agilent). Quantile-normalized gene values were used to identify genes with significant change (false discovery rate $P < .05$) and analyzed for NF- κ B targets (listed <http://bioinfo.lifl.fr/NF-KB/NM-mouse.txt>, derived from⁴). To reduce the dimensionality of the complex datasets, principal component analysis (PCA) was performed on genes with ≥ 4 -fold change between at least two groups to identify the most dominant gene profiles in the datasets. To define the area with 95% confidence interval with the sample groups, PCA values were used to generate error ellipses in MATLAB (Natick, MA) based on the algorithm described by Johnson and Wichern⁵. In brief, error ellipse was defined as

$(x/\sigma_x)^2 + (y/\sigma_y)^2 = s$, where σ_x and σ_y correspond to the eigenvalues of the covariance matrix of the group data, and s satisfies probability $P(s) = 0.95$ for a 2-degree freedom (component #1 and #2 used for calculation) normally distributed case. The angle of the largest eigenvector towards the x -axis was used to define the orientation of the error ellipse. Microarray data are deposited at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE64287; the reviewer link is:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ytcdckqodxkfrkx&acc=GSE64287>.

Quantification of western blotting signal intensity

ImageJ⁶ (NIH) was used to quantify signal intensity in western blotting. For proteins that are compared in the expression level, signals were first normalized to loading control GAPDH, and then to WT time 0 control; for proteins that are compared in the phosphorylation level, phosphorylated protein signals were first normalized to total protein signals, and then to the WT time 0 control.

Confocal microscopy and flow cytometry

To determine p65 nuclear translocation, BMMCs were stimulated with 1 μ g/ml LPS for 15 minutes, fixed, permeabilized with FoxP3 staining buffer kit (eBioscience), and stained with Alexa Fluor 488 conjugated anti-p65 antibody (sc-8008 AF488, Santa Cruz Biotechnology, Santa Cruz, CA), in the presence of Fc block (anti-CD16/32, clone: 93, eBioscience, San Diego, CA). Following staining, cells were treated with Prolong Gold anti-fade reagent with DAPI (Invitrogen, Carlsbad, CA), and imaged on a TCS SP5 confocal microscope (Leica

115 Microsystems, Inc., Buffalo Grove, IL). Images were processed by ImageJ⁶ software (NIH) to
116 calculate the fraction of nuclear p65 fluorescence over total p65.
117 Surface staining for flow cytometry was done in 2% FBS/PBS buffer in the presence of Fc Block
118 (BioLegend) and indicated antibodies. To determine mast cell TNF- α production, after surface
119 staining, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield,
120 PA), permeabilized and stained with anti-TNF- α antibodies in PBS containing 0.4% saponin
121 (Sigma). All flow cytometry data were acquired on LSRII (BD Biosciences), and analyzed in
122 FlowJo (Tree Star, Ashland, OR).

123

Figures

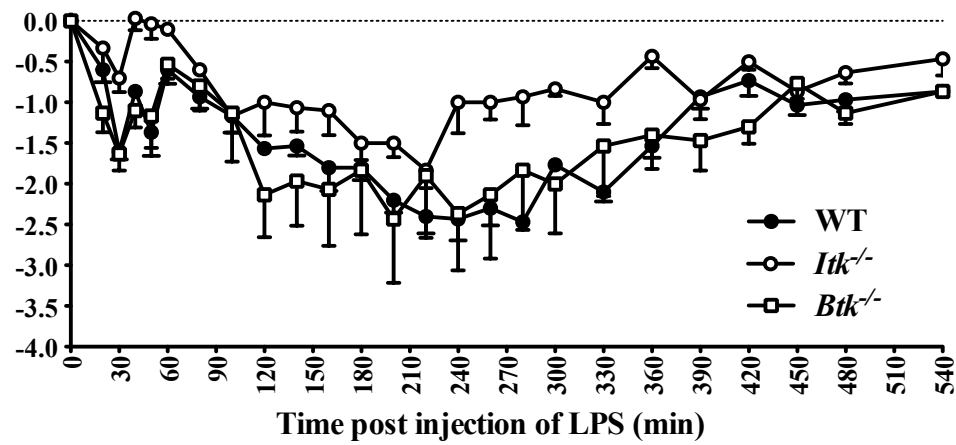


Figure E1. The absence of ITK or BTK does not affect LPS-induced hypothermia in mice.

Change in core body temperature induced by LPS. n = 3. Data represents result of two independent experiments.

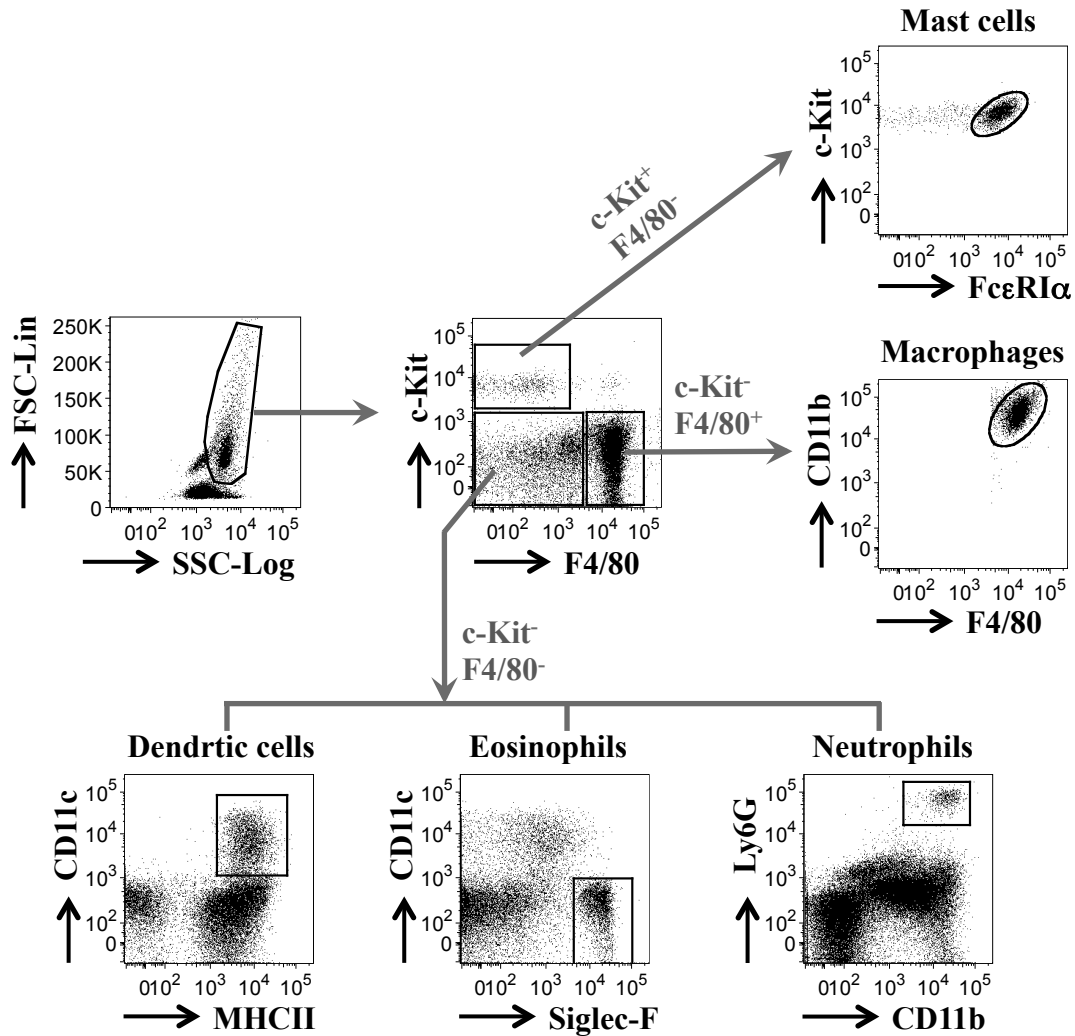


Figure E2. Gating strategy to identify cell types in peritoneal lavage. Leukocytes are gated and divided into c-Kit⁺F4/80⁻, c-Kit⁻F4/80⁺, and c-Kit⁻F4/80⁻ populations. Mast cells are defined as c-Kit⁺F4/80⁻FcεRIα⁺ and macrophages are defined as c-Kit⁻F4/80⁺CD11b⁺. c-Kit⁻F4/80⁻ cells are further analyzed to define MHCII⁺CD11c⁺ dendritic cells, Siglec-F⁺CD11c⁺ eosinophils, and CD11b⁺Ly6G⁺ neutrophils.

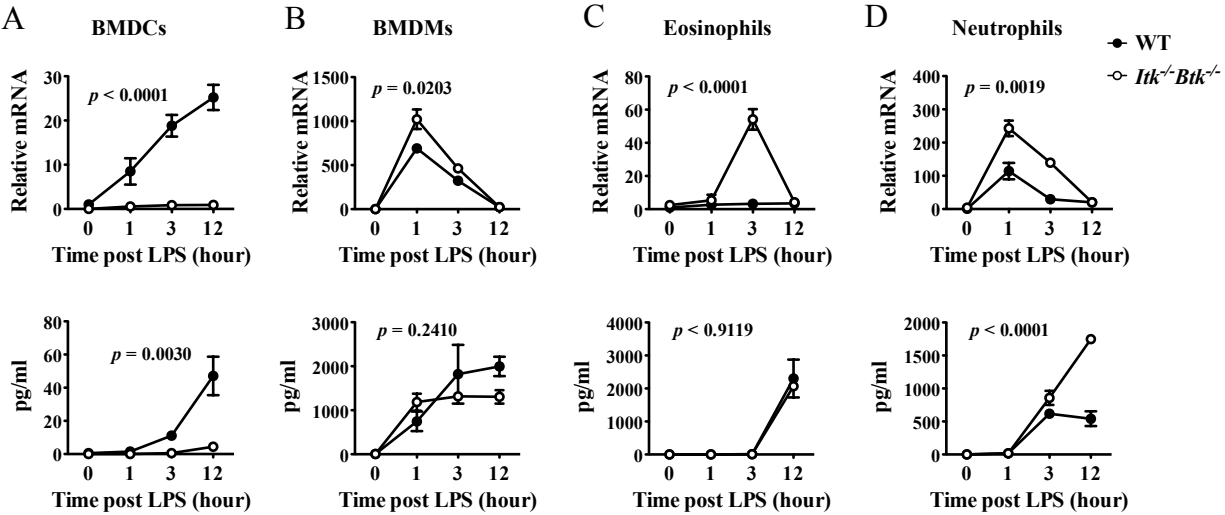


Figure E3. Differential roles of ITK and BTK in regulating TNF- α production by innate immune cells in response to LPS. LPS-induced TNF- α mRNA and secretion in (A) bone marrow derived dendritic cells (BMDCs), (B) bone marrow derived macrophages (BMDMs), (C) eosinophils recovered from IL-5 transgenic mice, and (D) thioglycollate-elicited peritoneal neutrophils. $n \geq 3$, p values by ANOVA.

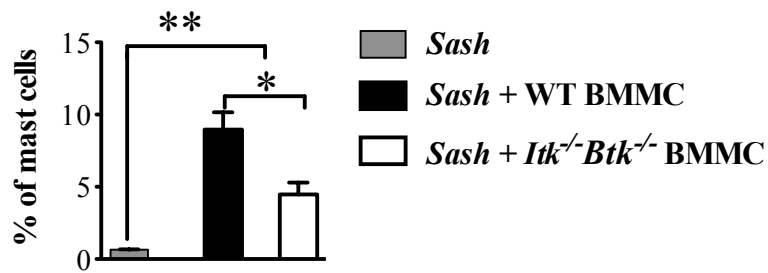


Figure E4. BMMC reconstitution in *Sash* recipients. BMMC reconstitution was performed as described in the Methods section, and peritoneal lavage was collected to determine the percentage of mast cells over total granulocytes as indicated by the gates in **Figure E2**. $n \geq 3$, p values by t test. $*p < 0.05$, $**p < 0.001$.

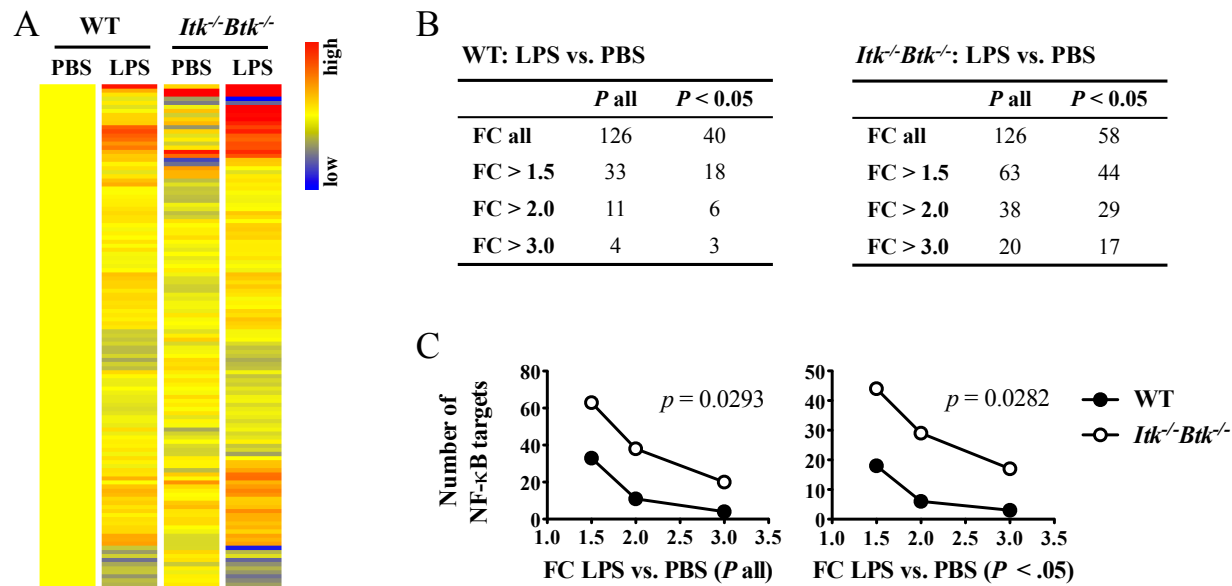


Figure E5. *Itk^{-/-}Btk^{-/-}* BMMCs exhibited significantly altered profile of NF-κB targets in response to LPS. BMMCs were stimulated with 100 ng/ml LPS (or PBS as control) for 1 hour and mRNA analyzed by microarray. (A) Clustering of all NF-κB targeted genes (average of levels in WT cells treated with PBS was set of 1). (B) LPS-induced fold changes (FC) of NF-κB targeted genes in WT and *Itk^{-/-}Btk^{-/-}* BMMCs (*P* is false discovery rate corrected *p* value). (C) Significantly more NF-κB targets were altered in *Itk^{-/-}Btk^{-/-}* BMMC response to LPS than in WT cells. *p* values were generated through two-way ANOVA.

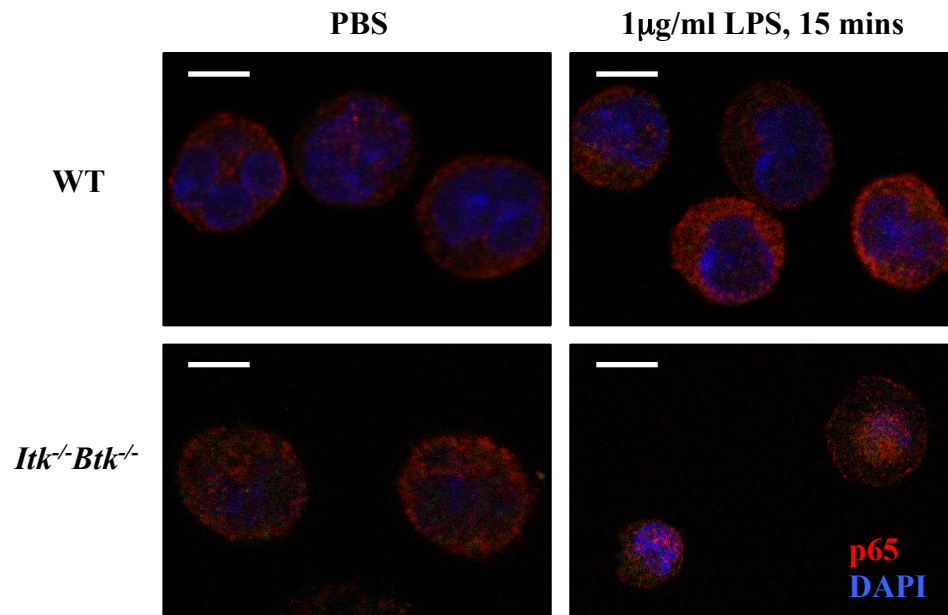


Figure E6. Representative images of p65 nuclear translocation in response to LPS. WT or *Itk*^{-/-}*Btk*^{-/-} BMMCs were treated with PBS or 1 μg/ml LPS for 15 mins, then stained for p65 (red) and DAPI (blue) followed by fluorescent imaging. Scale bar: 5 μm.

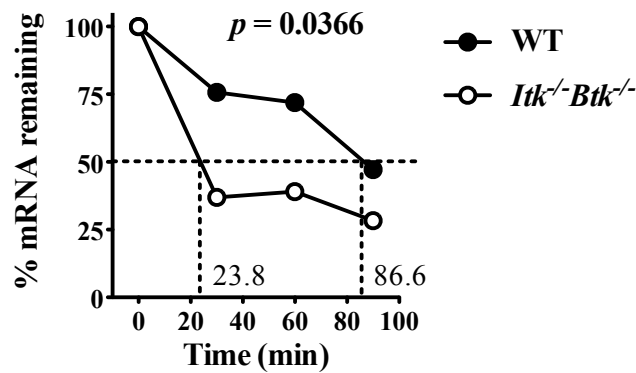


Figure E7. TNF- α mRNA is significantly less stable in *Itk*^{-/-}*Btk*^{-/-} BMMCs compared to WT cells. BMMCs were stimulated with LPS for 1 hour, followed by addition of Actinomycin D. TNF- α mRNA was measured every 0.5 hour, and normalized to the level at time 0 (1 hour post LPS). Half-life indicated was calculated based on segmented linear regression in the segment where 50% mRNA falls. *p* value was generated by two-way ANOVA comparing the LPS treated samples along the time course.

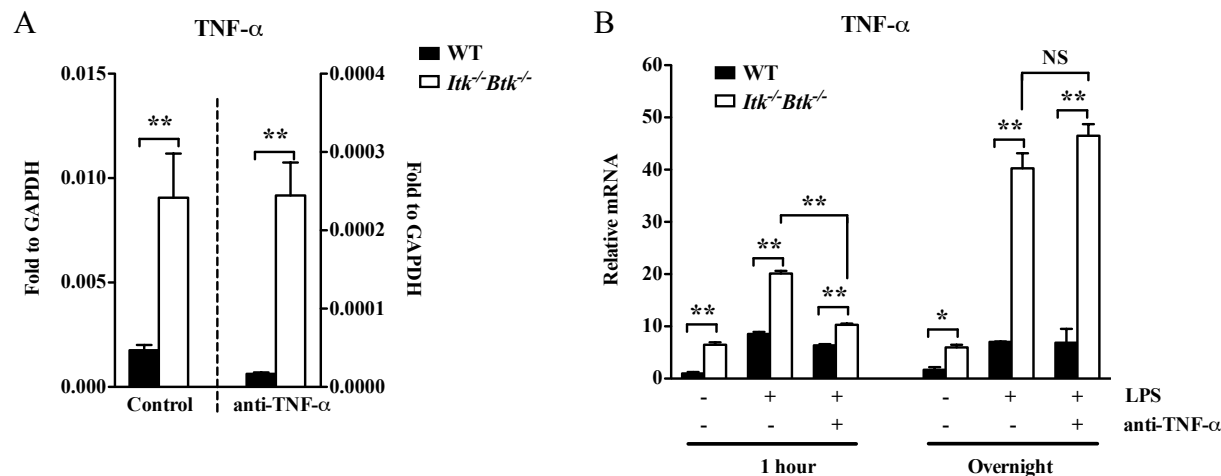


Figure E8. Hyper-production of TNF- α by *Itk*^{-/-}*Btk*^{-/-} BMMC is promoted by but does not depend on TNF- α auto/paracrine. (A) *Itk*^{-/-}*Btk*^{-/-} BMMCs are cultured in factor free medium for 12 hours in the absence or presence of TNF- α neutralizing antibody, and examined for preformed TNF- α mRNA (normalized to GAPDH mRNA levels). (B) *Itk*^{-/-}*Btk*^{-/-} BMMCs were factor starved for 12 hours, treated with control (-) or anti-TNF- α antibody (+) for 30 mins, followed by stimulation using PBS (-) or 100 ng/ml LPS (+) for indicated time, and examined for TNF- α mRNA production (normalized to WT basal level). * p < .05, ** p < .01 by t test.

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Table E1. List of most up-regulated genes by LPS stimulation in mast cells. 1 hour LPS-stimulated gene levels were normalized to average of levels in LPS-stimulated WT BMMCs, and genes (100) that are most up-regulated in *Itk^{-/-}Btk^{-/-}* mast cells are listed. n = 3, averages are shown.

GeneSymbol	GeneName	<i>Itk^{-/-}</i>	<i>Btk^{-/-}</i>	<i>Itk^{-/-}Btk^{-/-}</i>
Mrgprb1	MAS-related GPR, member B1	3.05	1069.65	449.33
Gdpd3	glycerophosphodiester phosphodiesterase domain containing 3	-1.06	314.44	418.80
Ccr5	chemokine (C-C motif) receptor 5	1.06	106.13	391.88
Hpgd	hydroxyprostaglandin dehydrogenase 15 (NAD)	15.03	13.07	178.94
Wfdc17	WAP four-disulfide core domain 17	1.24	43.12	100.75
Mmp12	matrix metalloproteinase 12	1.14	63.32	96.19
Gpnmb	glycoprotein (transmembrane) nmb	-1.13	39.51	86.30
Ctsl	cathepsin L	1.14	33.69	66.18
Ctss	cathepsin S	1.28	23.25	58.87
Mrc1	mannose receptor, C type 1	1.37	29.56	55.25
Bex4	brain expressed gene 4	1.49	18.69	42.92
Cxcl2	chemokine (C-X-C motif) ligand 2	-1.29	18.35	40.17
Fn3krp	fructosamine 3 kinase related protein	1.03	30.27	34.11
Ifi204	interferon activated gene 204	-1.34	1.85	34.02
Lyz1	lysozyme 1	-1.44	10.66	30.16
Cables1	CDK5 and Abl enzyme substrate 1	-1.14	1.30	28.51
Klk1b24	kallikrein 1-related peptidase b24	1.14	35.60	27.85
Lyz2	lysozyme 2	-1.42	10.44	27.16
Adamts1	a disintegrin-like and metalloproteinase (reprolysin type) with thrombospondin type 1 motif, 1	1.13	10.02	26.77
Mgst1	microsomal glutathione S-transferase 1	1.01	17.09	24.70
Ccr2	chemokine (C-C motif) receptor 2	1.08	20.90	23.76
Cd302	CD302 antigen	1.64	9.32	23.66
Ifi203	interferon activated gene 203	-1.45	-1.04	23.29
H2-Aa	histocompatibility 2, class II antigen A, alpha	-1.01	8.25	22.98
Pbx1	pre B cell leukemia homeobox 1	1.07	11.38	22.89
Bcl2a1c	B cell leukemia/lymphoma 2 related protein A1c	-1.33	5.39	22.10
Cxcl10	chemokine (C-X-C motif) ligand 10	1.07	14.32	21.86
AK044424	RIKEN full-length enriched library, clone:A930012B14	-1.10	19.76	21.78
Gm6166	fatty acid-binding protein, epidermal-like	-1.49	10.22	21.01
Twist2	twist basic helix-loop-helix transcription factor 2	1.36	1.99	20.04
Igf2r	insulin-like growth factor 2 receptor	-1.23	26.40	19.64
Fabp4	fatty acid binding protein 4, adipocyte	-1.05	8.06	19.62
Smoc2	SPARC related modular calcium binding 2	1.91	3.19	19.56
Myof	myoferlin	1.17	5.65	19.25
Lbh	limb-bud and heart	1.95	-1.12	18.78
Styk1	serine/threonine/tyrosine kinase 1	1.08	2.06	18.33
Lpl	lipoprotein lipase	1.03	13.35	18.10
Rbm45	RNA binding motif protein 45	1.02	10.38	18.04
Cd83	CD83 antigen	1.01	7.54	17.93
Hal	histidine ammonia lyase	-1.01	9.94	17.60
Hebp1	heme binding protein 1	1.06	-1.42	17.59
Nxpe5	neurexophilin and PC-esterase domain family, member 5	-3.12	-3.21	16.72
Mppe1	metallophosphoesterase 1	1.39	13.27	16.25
Slc6a12	solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	1.72	3.48	15.11
Spp1	secreted phosphoprotein 1	-1.63	9.76	15.01
Gm8579	spermatogenesis associated glutamate (E)-rich protein 4a pseudogene	-1.26	23.10	15.00

Ear10	eosinophil-associated, ribonuclease A family, member 10	1.06	3.03	13.99
Plk2	polo-like kinase 2	1.06	6.41	13.79
Cap1	CAP, adenylate cyclase-associated protein 1 (yeast)	1.54	8.84	13.36
2410003L11Rik	RIKEN cDNA 2410003L11 gene	1.64	16.54	12.39
Mmp2	matrix metalloproteinase 2	5.96	56.09	12.38
Nov	nephroblastoma overexpressed gene	-2.10	19.64	12.30
Tesc1	tescalcin-like	-1.70	-1.76	12.08
Lrrc4	leucine rich repeat containing 4	1.32	1.61	12.07
Irg1	immunoresponsive gene 1	-1.02	9.73	12.04
Gbp4	guanylate binding protein 4	1.14	1.60	11.68
Fstl1	folliculin-like 1	-1.11	6.82	11.51
Fxyd6	FXYD domain-containing ion transport regulator 6	1.07	4.49	11.19
Cxcl1	chemokine (C-X-C motif) ligand 1	1.24	3.40	11.12
St6galnac2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylglucosaminide alpha-2,6-sialyltransferase 2	-1.64	3.62	10.99
Dixc1	DIX domain containing 1	-1.29	2.73	10.97
Speer4b	spermatogenesis associated glutamate (E)-rich protein 4b	-1.14	14.55	10.92
Il1rn	interleukin 1 receptor antagonist	-1.14	5.52	10.88
Mnda	myeloid cell nuclear differentiation antigen	-2.32	1.98	10.79
D130051D11Rik	RIKEN cDNA D130051D11 gene	-1.06	8.96	10.60
Tpsab1	tryptase alpha/beta 1	-1.14	26.80	10.56
Zfp30	zinc finger protein 30	1.19	-1.32	10.50
Dusp4	dual specificity phosphatase 4	4.08	3.14	10.49
Trem2	triggering receptor expressed on myeloid cells 2	-1.13	5.74	10.38
Speer4d	spermatogenesis associated glutamate (E)-rich protein 4d	-1.10	15.79	10.20
Syn2	synapsin II	-1.01	2.00	10.15
Casc4	cancer susceptibility candidate 4	1.90	1.66	9.98
Cdk5rap1	CDK5 regulatory subunit associated protein 1	2.94	7.24	9.68
Arl5c	ADP-ribosylation factor-like 5C	1.14	5.16	9.68
Add2	adducin 2 (beta)	-1.13	23.88	9.65
Anxa3	annexin A3	-1.22	3.68	9.31
Igf1	insulin-like growth factor 1	-1.19	4.94	9.22
Slc4a11	solute carrier family 4, sodium bicarbonate transporter-like, member 11	2.79	3.52	9.12
Mrgprb3	MAS-related GPR, member B3	1.24	14.55	9.03
Fam81b	family with sequence similarity 81, member B	1.22	8.31	8.79
Cuedc1	CUE domain containing 1	-1.12	5.62	8.64
Nrp2	neuropilin 2	1.16	4.52	8.58
Gpr176	G protein-coupled receptor 176	1.11	-1.59	8.56
Rimk1a	ribosomal modification protein rimK-like family member A	2.15	3.94	8.55
F13a1	coagulation factor XIII, A1 subunit	2.46	2.67	8.53
Eml5	Echinoderm Microtubule Associated Protein Like 5	1.16	1.23	8.52
Tdrp	testis development related protein	1.88	1.97	8.50
Gbp6	guanylate binding protein 6	1.09	1.75	8.40
9230110C19Rik	RIKEN cDNA 9230110C19 gene	1.36	4.43	8.38
P2ry6	pyrimidinergic receptor P2Y, G-protein coupled, 6	-1.10	3.93	8.27
Kalrn	kalirin, RhoGEF kinase	3.10	3.98	8.22
Tnf	tumor necrosis factor	1.01	4.60	8.19
Sqrd1	sulfide quinone reductase-like (yeast)	3.03	1.97	8.09
4930444K16Rik	RIKEN cDNA 4930444K16 gene	-1.11	-1.19	8.06
AK046777	RIKEN full-length enriched library, clone:B830003K07	1.03	1.14	8.03
Gdf6	growth differentiation factor 6	2.89	6.47	7.91
Thsd4	thrombospondin, type I, domain containing 4	2.14	4.15	7.85
Orm3	orosomucoid 3	1.19	3.75	7.78
Tcf4	transcription factor 4	1.22	7.40	7.62
Fut10	fucosyltransferase 10	1.10	-1.16	7.60

Table E2. List of most down-regulated genes by LPS stimulation in mast cells. 1 hour LPS-stimulated gene levels were normalized to average of levels in LPS-stimulated WT BMMCs, and genes (80) that are most down-regulated in *Itk^{-/-}Btk^{-/-}* mast cells are listed. n = 3, averages are shown.

GeneSymbol	GeneName	<i>Itk^{-/-}</i>	<i>Btk^{-/-}</i>	<i>Itk^{-/-}Btk^{-/-}</i>
Trim12a	tripartite motif-containing 12A	1.05	-57.03	-51.46
Il1r2	interleukin 1 receptor, type II	1.11	-10.43	-44.12
Cd59a	CD59a antigen	-1.54	1.34	-38.02
Rps6	ribosomal protein S6	-1.03	-2.36	-36.29
Cd59b	CD59b antigen	-1.11	-1.11	-36.13
Imp4	IMP4, U3 small nucleolar ribonucleoprotein, homolog (yeast)	-1.11	-28.84	-32.86
Stmn2	stathmin-like 2	-2.18	-18.03	-26.01
Avil	advillin	1.04	-16.39	-24.40
Iqgap2	IQ motif containing GTPase activating protein 2	-1.19	-20.15	-23.95
Zfp125	zinc finger protein 125	1.10	-17.08	-22.97
4932438H23Rik	RIKEN cDNA 4932438H23 gene	1.79	-1.23	-21.84
Gdf15	growth differentiation factor 15	2.09	-1.27	-21.18
Fndc5	fibronectin type III domain containing 5	1.34	-12.92	-18.39
Eps8l1	EPS8-like 1	-1.30	-14.78	-18.15
AI462493	expressed sequence AI462493	-1.04	-1.22	-18.05
Mcpt4	mast cell protease 4	1.70	-1.30	-16.60
Fam213a	family with sequence similarity 213, member A	1.11	-1.05	-16.14
Mcts1	malignant T cell amplified sequence 1	-1.03	-1.09	-16.08
Plac8	placenta-specific 8	-18.05	-39.93	-15.67
C5ar2	complement component 5a receptor 2	-1.25	-3.56	-14.73
Cd300c	CD300C antigen	1.50	-15.03	-14.20
Trf	transferrin	-1.07	-1.13	-13.82
AK077492	RIKEN full-length enriched library, clone:5730420P06	-1.09	-14.01	-13.82
Ctsg	cathepsin G	4.59	-4.31	-12.55
Gpr183	G protein-coupled receptor 183	-2.50	-8.18	-12.49
Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	-1.17	-4.09	-12.01
Cma2	chymase 2, mast cell	1.75	-1.41	-11.49
Ifi2712a	interferon, alpha-inducible protein 27 like 2A	-3.41	-9.33	-11.16
Oasl1	2'-5' oligoadenylate synthetase-like 1	1.10	-9.29	-10.45
Fhl2	four and a half LIM domains 2	-2.07	-6.48	-9.65
0610007N19Rik	RIKEN cDNA 0610007N19	1.26	-10.02	-9.57
Kazald1	Kazal-type serine peptidase inhibitor domain 1	-1.56	-1.79	-9.24
Il7	interleukin 7	1.22	-8.08	-9.14
AK078692	RIKEN full-length enriched library, clone:7530414M05	1.16	-12.44	-9.07
Fhdc1	FH2 domain containing 1	-1.23	-3.90	-8.89
Ppcs	phosphopantothencysteine synthetase	-1.05	-1.04	-8.81
Mcpt8	mast cell protease 8	-1.11	-2.94	-8.68
Mcpt1	mast cell protease 1	1.75	-1.45	-8.55
Vcan	versican	-2.39	-6.48	-8.51
Itgae	integrin alpha E, epithelial-associated	-1.31	-2.00	-8.44
Zfp874a	zinc finger protein 874a	1.08	-7.52	-8.41
Creg2	cellular repressor of E1A-stimulated genes 2	-1.34	-8.25	-8.23
Jmjd7	jumonji domain containing 7	1.11	-1.18	-8.19

Prss12	protease, serine, 12 neurotrypsin (motopsin)	-1.26	-6.57	-8.16
Calca	calcitonin/calcitonin-related polypeptide, alpha	-1.06	-3.83	-8.07
2310069B03Rik	RIKEN cDNA 2310069B03 gene	1.38	-6.36	-7.88
Pga5	pepsinogen 5, group I	1.65	-7.41	-7.86
AK034318	RIKEN full-length enriched library, clone:9330176N07	-1.14	-4.80	-7.83
Slc16a4	solute carrier family 16 (monocarboxylic acid transporters), member 4	-2.67	-6.02	-7.81
2610034M16Rik	RIKEN cDNA 2610034M16 gene	1.15	-8.32	-7.73
Hspa1a	heat shock protein 1A	2.53	-10.61	-7.73
Pstpip1	proline-serine-threonine phosphatase-interacting protein 1	-1.40	-1.47	-7.66
Ankrd50	ankyrin repeat domain 50	-1.24	-7.53	-7.55
Hspb1	heat shock protein 1	2.29	-7.38	-7.31
Ramp3	receptor (calcitonin) activity modifying protein 3	-1.19	-5.46	-7.27
Cercam	cerebral endothelial cell adhesion molecule	-1.05	-1.74	-7.21
Xaf1	XIAP associated factor 1	-5.38	-6.46	-7.10
Pttg1	pituitary tumor-transforming gene 1	-6.29	-4.71	-7.10
Rtn4r1l	reticulon 4 receptor-like 1	-1.77	-7.23	-7.03
IgMk	IgMk rearranged heavy-chain variable region (V-D-J) anti-DNA autoantibody	-1.39	-15.65	-7.02
Cnm2	cyclin M2	1.08	-1.29	-6.97
Mcpt2	mast cell protease 2	2.03	-2.54	-6.93
Meis2	Meis homeobox 2	3.61	3.60	-6.92
Antxr1	anthrax toxin receptor 1	-2.26	-3.82	-6.82
4930515G01Rik	RIKEN cDNA 4930515G01 gene	-7.46	-7.93	-6.79
Gpr160	G protein-coupled receptor 160	1.14	-8.98	-6.75
4933436C20Rik	RIKEN cDNA 4933436C20 gene	1.03	-6.45	-6.73
Pde1c	phosphodiesterase 1C	-1.04	-5.43	-6.48
LOC100862620	zinc finger protein 41-like	1.63	-5.93	-6.28
Kif23	kinesin family member 23	-1.80	-3.51	-6.23
AF210281	B880 immunoglobulin heavy chain variable region	-1.11	-9.90	-6.21
Lzts2	leucine zipper, putative tumor suppressor 2	-1.40	-2.40	-6.16
X12388	IgM(b) heavy chain variable region V(H)-J(H)2	-1.25	-9.02	-6.16
Xpo7	exportin 7	-1.00	1.29	-6.11
Trim30d	tripartite motif-containing 30D	-1.01	-8.20	-6.08
AY173987	BaPB1-92 immunoglobulin heavy chain variable region	-1.28	-8.08	-6.05
Rsad1	radical S-adenosyl methionine domain containing 1	1.10	-7.75	-5.95
Slco2b1	solute carrier organic anion transporter family, member 2b1	-1.37	-3.21	-5.79
Rab40b	Rab40b, member RAS oncogene family	2.12	-10.50	-5.64
Tgif1	TGFB-induced factor homeobox 1	1.07	-3.03	-5.63