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The Tec Family Kinase, IL-2-Inducible T Cell Kinase, Differentially Controls Mast Cell Responses

Archana S. Iyer*† and Avery August2*†

The Tec family tyrosine kinase, IL-2-inducible T cell kinase (Itk), is expressed in T cells and mast cells. Mice lacking Itk exhibit impaired Th2 cytokine secretion; however, they have increased circulating serum IgE, but exhibit few immunological symptoms of allergic airway responses. We have examined the role of Itk in mast cell function and FcεRI signaling. We report in this study that Itk null mice have reduced allergen/IgE-induced histamine release, as well as early airway hyperresponsiveness in vivo. This is due to the increased levels of IgE in the serum of these mice, because the transfer of Itk null bone marrow-derived cultured mast cells into mast cell-deficient W/Wv animals is able to fully rescue histamine release in the W/Wv mice. Further analysis of Itk null bone marrow-derived cultured mast cells in vitro revealed that whereas they have normal degranulation responses, they secrete elevated levels of cytokines, including IL-13 and TNF-α, particularly in response to unliganded IgE. Analysis of biochemical events downstream of the FcεRI revealed little difference in overall tyrosine phosphorylation of specific substrates or calcium responses; however, these cells express elevated levels of NFAT, which was largely nuclear. Our results suggest that the reduced mast cell response in vivo in Itk null mice is due to elevated levels of IgE in these mice. Our results also suggest that Itk differentially modulates mast cell degranulation and cytokine production in part by regulating expression and activation of NFAT proteins in these cells. The Journal of Immunology, 2008, 180: 7869–7877.

mast cells and IgE play an important role during allergic responses, including allergen-induced airway hyperreactivity and allergic asthma (1, 2). Cross-linking of IgE bound to high-affinity FcεRI with the cognate Ag releases proinflammatory mediators, which can result in activation and recruitment of other innate inflammatory cells and aid with the development of adaptive immune responses (1–5). Due to the importance of IgE in allergic asthma, there is significant interest in understanding the interplay of signals downstream of the FcεRI pathway. The allergic airway response follows a typical biphasic course: an early or immediate phase, which appears within minutes of exposure and resolves in 1–2 h (6). This is caused by allergen-induced cross-linking of the IgE molecules bound to FcεRI on mast cells, resulting in the release of preformed inflammatory mediators such as histamine, serotonin, and tryptase (2, 3). These vasoactive amines cause vasodilation, mucous secretion, and bronchoconstriction, which present as immediate airway hyperreactivity (AHR). The late-phase allergic responses begin 3–4 h after Ag provocation and peak at ~9 h, and the airway obstruction resolves within 12–24 h. T lymphocytes, basophils, and eosinophils are thought to be responsible in inducing the late-phase response, although the role of mast cells may not be ruled out (4, 7).

The FcεRI is a tetrameric receptor composed of an IgE-binding α subunit and β,γ signaling subunits. Engagement of FcεRI results in phosphorylation of Src kinase Lyn, followed by subsequent activation of Syk and tyrosine phosphorylation of adapter proteins such as linker for activation of T cells and non-T cell activation linker (8). These adapter proteins act as scaffolds to organize other signaling proteins such as PI3K, Tec kinases, SLP76, Gads, and Vav-1, resulting in the activation of phospholipase C (PLC) γ, protein kinase C, and Ras/MAPK pathway, and release of intracellular Ca2+ reserves (8, 9). PI3K plays a critical role in mast cell signaling and is required for the production of phosphoinositides, leading to the recruitment of Tec kinases and PLCγ to the FcεRI-activated signaling complex (9, 10). Thus, the receptor-proximal events are amplified by the sequential activation of Src, Syk, and Tec family of tyrosine kinases and adapter molecules, resulting in a multimolecular signalosome that further activates multiple pathways leading to mast cell degranulation, activation of transcription factors, and cytokine production (8, 10).

The Tec family of protein tyrosine kinases is an important component of this multimolecular signalosome complex. Studies in T cells, B cells, and mast cells have shown that Tec kinases play a role in regulating the calcium pathway (10, 11). Three Tec family kinases, Bruton’s tyrosine kinase (Btk), IL-2-inducible T cell kinase (Iκk), and Tec, have been reported to be expressed in mast cells (12, 13). Of these, Btk and Iκk have been shown to be activated downstream of the FcεRI pathway (14–16). Btk and Iκk are also major players in BCR and TCR signaling, respectively. Absence of Btk leads to defective B cell development and a reduction in B cell numbers (17–22). Similarly, absence of Iκk in T cells leads to defects in T cell development and function (11). In T cells, Iκk is required for Ca2+ mobilization, activation of PLCγ, MAPK, and NFAT family of transcription factors (20–23). T cells from Iκk−/− mice exhibit impaired cytokine secretion and reduced or absent Th2 differentiation (20, 22–24). This results in Iκk−/− mice

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being significantly resistant toward developing allergic asthma in an OVA/Alum murine model (25, 26).

The importance of Btk in mast cells has been well established through a number of studies by Kawakami and colleagues (14, 16, 27–29). Btk−/− mice have reduced anaphylaxis, and Btk-deficient cultured mast cells reveal defects in degranulation and cytokine production due to reduced PLCγ activation, Ca2+ mobilization, and activation of the JNK pathway. By contrast, the role of Itk in mast cells is largely unknown. Itk is expressed and phosphorylated upon FcεRI triggering in mast cells (15). Recently, Forsell et al. (30) suggested that Itk−/− mice have defective mast cell degranulation in vivo. They also compared both Btk−/− and Itk−/− mice and found that the absence of Itk led to a more severe defect in mast cell degranulation in vivo than the absence of Btk. However, the use of Itk−/− mice and the OVA/Alum model to assess mast cell function complicates the interpretation of the results given the fact that these mice are known to have a well-established defect in Th2 cell effector functions that affects their response in this model (23–26). In addition, it should be noted that there is less of a role for mast cells in the OVA/Alum model, with T cells playing a major role in the development of airway infiltration and hyperresponsiveness (31, 32).

In light of these recent findings and to understand the role of Itk in mast cells during allergic asthma (without interfering with T cell functions), we adopted a model to study allergen-induced mast cell-deficient early AHR. We report that Itk-deficient mice have reduced IgE/allergen-induced AHR and histamine release to system anaphylaxis. However, Itk−/− mast cells are not defective in degranulation because transfer of Itk−/− bone marrow-derived cultured mast cells (BMMCs) into mast cell-deficient W/W+v mice can rescue the histamine responses to Ag challenge. Our results support the idea that the impaired degranulation observed in vivo in Itk−/− mice is due to high levels of IgE observed in these mice. Further investigation of FcεRI signaling in Itk−/− BMMCs revealed that whereas they exhibit similar levels of degranulation, they secrete elevated levels of cytokines, including IL-13 and TNF-α. This split response in degranulation and cytokine secretion suggests that Itk may differentially regulate FcεRI signals in mast cells.

Materials and Methods

Mice

Wild-type (WT) C57BL/6, WBB6F1/J-Kiwi/W (W/Wv), congenic WBB6F1, and B6.Cg-KiwiW−/− mice were obtained from The Jackson Laboratory. Itk−/− mice backcrossed to C57BL/6 background for greater than 10 generations were used for these experiments. Mice were kept in microisolator cages and provided with food and water ad libitum. All experiments were approved by the Office of Research Protection’s Institutional Animal Care and Use Committee at Pennsylvania State University.

Determination of AHR

Airway responsiveness was assessed using a Buxco whole body plethysmograph (Buxco Electronics). Five to 8-wk-old WT and Itk−/− mice were injected i.p. with 1 μg of murine IgE anti-DNP (SPE-7; Sigma-Aldrich), 12 h before the i.v. administration of DNP-HSA (50 μg). Three minutes later, animals were sacrificed, and serum was collected and analyzed for histamine content using an ELISA (Beckman Coulter). Cultured BMMCs from WT and Itk−/− (5 × 106) were transferred i.v. into mast cell-deficient W/W+v mice, and after 9 wk the mice were analyzed for systemic anaphylaxis, as described above.

Analysis of degranulation in vitro

WT and Itk−/− BMMCs (1 × 106/ml) were factor starved overnight and sensitized in complete RPMI 1640 with 1 μg/ml anti-DNP IgE overnight. Cells were washed in Tyrode buffer (112 mM NaCl, 2.7 mM KCl, 0.4 mM NaH2PO4, 1.6 mM CaCl2, 1 mM MgCl2, 10 mM HEPES (pH 7.5), 0.05% gelatin, and 0.1% glucose) and resuspended at 2 × 106/ml. BMMCs were stimulated with varying amounts of DNP-HSA (0–100 ng/ml) for 1 h at 37°C. For the hexosaminidase release assay, aliquots of supernatant and cell pellets were assayed for hexosaminidase.

Analysis of skin mast cells

Skin sections from WT and Itk−/− mice were fixed overnight in 2% glutaraldehyde in 0.1 M cacodylate buffer. Samples were treated with 1% osmium tetroxide in 0.1 M cacodylate buffer and 2% uranyl acetate and infiltrated with epon resin. Ultrathin sections of the samples were placed on uncoated copper grid, treated with uranyl acetate and lead citrate, and analyzed with a transmission electron microscope at the Electron Microscope Facility of Pennsylvania State University. For toluidine blue staining, skin samples were stained with 0.1% toluidine blue for 1–2 min, washed, and observed under an Olympus BX51 microscope.

Analysis of surface IgE expression on mast cells in vivo

Splenocytes or peritoneal cells were stained with anti-c-Kit and anti-IgE (to detect surface-bound IgE and thus FcεRI). Mast cells were identified by gating on c-Kit/IgE-reactivity. To assess complete occupancy of IgE on mast cells, peritoneal cells were incubated with excess of IgE for 1 h. Samples were washed and stained with anti-c-Kit and anti-IgE before and after the treatment.

Analysis of B cells

Spleens were collected from WT and Itk−/− mice and analyzed by flow cytometry for expression of B220, IgM, and IgE using specific Abs.

Analysis of IgE levels

Dilutions of sera were analyzed for total IgE by IgE-specific ELISA (Southern Biotechnology Associates).

Cytokine analysis

BMMCs were factor starved and sensitized by overnight incubation with 1 μg/ml anti-DNP IgE mAb (SPE-7; Sigma-Aldrich). Cells were washed once in Tyrode buffer, resuspended in complete medium to 2 × 106 cells/ml. Cells were stimulated with DNP-HSA and supernatants collected after 8 h for TNF-α, or 24 h for IL-13 and analyzed by ELISA (R&D Systems). For analysis of stimulation with IgE alone, cells were treated as indicated above, and supernatants were collected after overnight incubation. IL-2, IL-4, TNF-α, and GM-CSF were analyzed using a Luminex multiplex system (Bio-Rad).

Calcium analysis

BMMCs were sensitized by an overnight incubation with 1 μg/ml anti-DNP IgE, washed in Tyrode buffer, and loaded with 2 μM fura 2-AM (Invitrogen). Bulk intracellular calcium levels were monitored with a Hi-Tachi F-2000 spectrophotometer. Briefly, the fluorescence emission at 510
nm was recorded at excitation wavelengths of 340 and 380 nm. Intracellular Ca\(^{2+}\) was calculated from the 340/380 ratio for the fura 2-Ca\(^{2+}\) complex. Analysis at baseline was acquired for 50–100 s before FCRI cross-linking with 0–100 ng/ml DNP-HSA. Calcium analysis was continued for 300 s, followed by addition of 1 \(\mu\)M ionomycin (Sigma-Aldrich) to determine the peak population response using an additional 60-s data acquisition. Extracellular calcium response was determined by stimulating BMMCs with DNP-HSA in the absence of extracellular calcium at 50 s, followed by the addition of 2 mM Ca\(^{2+}\) at 100 s and 1 \(\mu\)M ionomycin at 400 s.

**In vitro FCRI stimulation**

BMMCs were factor starved and sensitized by overnight incubation with 1 \(\mu\)g/ml anti-DNP IgE. Cells were washed once in Tyrode buffer, resuspended (2 \(\times\) 10\(^6\) cells/ml) and stimulated with Ag (100 ng/ml DNP-HSA) for the indicated time intervals. Cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Igepal CA-630, 50 mM Na pyrophosphate, 2 mM sodium vanadate, 50 mM sodium fluoride, 1 mM PMSF, 1 \(\mu\)g/ml leupeptin, and 2 \(\mu\)g/ml aprotinin) immediately after stimulation. Lysates were centrifuged at 4°C for 10 min. Cleared lysates were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (NEN Life Science Products). Membranes were blocked and incubated consecutively with primary Ab and HRP-conjugated secondary Ab, and immunoreactive proteins were visualized by ECL reagents (Amersham). The following Abs were used: anti-phospho-ERK, anti-ERK, anti-phospho-p38, and anti-p38 (Cell Signaling Technology); anti-phosphotyrosine (Santa Cruz Biotechnology); anti-actin (Sigma-Aldrich); and anti-NFAT1/NFATc2 and NFAT2/NFATc1 (Santa Cruz Biotechnology).

**Real-time PCR analysis**

Total RNA was extracted from BMMCs using TRIzol (Invitrogen), and cDNA was synthesized. Quantitative RT-PCR was performed in triplicate...
A–C. In addition, mast cell-deficient mice (KitW−/− mice) did not develop AHR over time compared with control mice exposed to PBS alone (Fig. 1A).

We adopted a model of passive sensitization with Ag-specific IgE to monitor early AHR after the first exposure of allergen (33). In this model, mice are injected with Ag-specific IgE (anti-DNP IgE) to coat slides. Cells were permeabilized in a buffer containing 0.1% Triton X-100 and 2% FBS in PBS for 30 min and stained with anti-NFAT1/2, anti-NFATc2, and TOPRO-3 (Molecular Probes). Samples were visualized using confocal microscopy Olympus FV300.

Data analysis

Statistical evaluation was conducted using Student’s t test with a probability value, \( p \leq 0.05 \), considered statistically significant.

Results

Itk−/− mice exhibit reduced responses to allergen-induced mast cell-dependent early AHR

We adopted a model of passive sensitization with Ag-specific IgE to monitor early AHR after the first exposure of allergen (33). In this model, mice are injected with Ag-specific IgE (anti-DNP IgE) and then administered the allergen (DNP-HSA) intranasally. This should lead to activation of mast cells in the airways, resulting in altered airway responsiveness to methacholine. Mice were exposed to an aerosol dose of methacholine at 10-min intervals after Ag challenge over a 1-h time period, and AHR was determined using a noninvasive whole body plethysmograph. The results show that WT mice respond to allergen exposure with an increase in AHR over time compared with control mice exposed to PBS alone (Fig. 1A). In addition, mast cell-deficient mice (KitW−/− mice) did not exhibit any increase in AHR upon exposure to Ag, confirming that the AHR response in this model is indeed mast cell dependent (data not shown).

To understand the role of Itk in this mast cell-dependent physiological response during early AHR, we examined Itk−/− mice in this model (Fig. 1B). We found that Itk−/− mice did not exhibit any changes in AHR upon Ag exposure, suggesting that Itk−/− mice fail to generate an acute airway response despite the presence of circulating Ag-specific IgE.

Reduced histamine release in Itk−/− mice in response to Ag challenge

The reduced AHR observed in Itk−/− mice to intranasal challenge of Ag suggested that Itk may regulate the release of pharmacological mediators from mast cells in response to FcεRI triggering. Because histamine is a major pharmacological mediator secreted by mast cells, we determined histamine release in WT and Itk−/− mice following injection of anti-DNP IgE and systemic Ag challenge i.v. Fig. 1C demonstrates that whereas WT mice respond to Ag challenge with increased histamine levels in sera, Itk−/− had significantly reduced levels of histamine following Ag challenge. This result supports the observation of Forssell et al. (30) that Itk−/− mice exhibit reduced mast cell degranulation in vivo.

Itk−/− mast cells have normal tissue mast cells, but higher surface expression of FceR

Examination of Itk−/− mice for mast cell numbers and morphology in skin, peritoneum, and lungs by toluidine blue and ultrastructural morphology by transmission electron microscopy (data shown for skin in Fig. 2, A–C) revealed no difference in mast cell distribution (WT = 40/mm² vs Itk−/− = 38/mm², \( p = 0.239 \), not statistically significant) within the tissue or ultrastructural morphology. Similar analysis of dissociated lungs from WT and
FIGURE 5. Normal degranulation response, but enhanced cytokine secretion by Itk-/- BMCMs in response to FcεRI triggering. A, WT or Itk-/- BMCMs were left untreated (control) or coated with anti-DNP IgE (IgE), followed by stimulation with increasing doses of DNP-HSA. Supernatants were analyzed for the indicated cytokines (data not shown). Mast cells from Itk-/- mice, with similar results observed in D. peritoneal mast cells (Fig. 2D), were analyzed for the indicated cytokines (n = 3; *, p < 0.05 vs WT). B and C, WT or Itk-/- BMCMs were left untreated (control), or coated with anti-DNP IgE (IgE), followed by stimulation with 100 ng/ml DNP-HSA (B and C) or anti-IgE (C). Supernatants were analyzed by ELISA for IL-13 (n = 3; *, p < 0.05 vs WT) or TNF-α (n = 3; *, p < 0.05 vs WT). D, WT or Itk-/- BMCMs were incubated overnight with anti-DNP IgE alone, and supernatants were analyzed for the indicated cytokines (n = 3; *, p < 0.05 vs WT). E, FcεRI expression on BMCMs after overnight incubation with IgE.

Itk-/- mice suggested normal numbers of mast cells (data not shown). To determine the expression of FcεRI on the mast cells in Itk-/- mice, we analyzed the levels of IgE occupied FcεRI on peritoneal mast cells (Fig. 2D) with similar results observed in splenic mast cells (data not shown). Mast cells from Itk-/- mice were found to have higher levels of IgE occupying the FcεR than mast cells from WT mice, suggesting higher levels of FcεR on these cells. These results rule out a role for Itk in mast cell development, or that the lack of response in the Itk-/- mice was due to reduced mast cells or FcεR expression.

**Itk-/- mice have increased levels of serum IgE leading to almost complete occupancy of IgE on FcεR in mast cells**

We and others have noted that Itk-/- mice have increased levels of serum IgE, and that these mice can mount a normal Ag-specific IgE response in the OVA/Alum-induced model of allergic asthma (Fig. 3A) (24, 25, 30). Indeed, analysis of B cells in the spleen of Itk-/- mice for surface expression of IgM and IgE revealed that a large percentage of the B cells has undergone class switch to express IgE (IgE/IgM-), WT = 1.01% vs Itk = 35.28%, p < 0.05; Fig. 3B shows a representative analysis). Although the reason for this increased IgE level is unknown, it has been well documented that IgE binding to FcεRI enhances mast cell FcεRI expression both in vivo and in vitro (34–36). This might explain why we observe higher levels of IgE on the cell surface of Itk-/- mast cells in Fig. 2D. It also suggests that the high-level occupancy of FcεRI by the IgE molecules in the Itk-/- mast cells may preclude the newly delivered Ag-specific IgE from binding to the cell surface. To test this possibility, we analyzed FcεR occupancy on peritoneal mast cells from WT and Itk-/- mice before and after incubation with an excess of IgE in vitro. This should allow us to determine whether the FcεRIs on Itk-/- mast cells are occupied/saturated with IgE and thus are unavailable for binding to newly delivered Ag-specific IgE, provided to them in the experiments shown in Fig. 1. The results demonstrate that whereas WT peritoneal mast cells showed an increase in IgE binding after incubation, there was no difference in Itk-/- peritoneal cells (Fig. 3C). These data support the idea that in Itk-/- mice, the FcεRIs on mast cells are saturated with IgE, precluding the binding of new IgE and thereby reducing their responses to Ag-specific allergen challenge in vivo.

Adoptive transfer of Itk-/- BMCMs into W/Wv mice rescued mast cell degranulation in these mice

The interaction of IgE with FcεRI is very stable (35–37). Indeed, Kubo et al. (36) have shown that IgE can be maintained on the surface of mast cells for several weeks. Because the high levels of
IgE in Itk−/− may interfere with mast cell responses in vivo, we cultured BMMCs from WT and Itk−/− mice for further analysis. Examination of these cells for expression of FcεRI did not reveal any difference in IgE binding or FcεRI expression (Fig. 4, A and B), suggesting that BMMCs from Itk−/− develop normally. To analyze these cells in an in vivo context devoid of extraneous IgE, we transferred WT and Itk−/− BMMCs into mast cell-deficient W/Wv mice (38, 39). Nine weeks posttransfer (to allow for complete reconstitution of mast cells), we challenged the mice with Ag-specific IgE (anti-DNP IgE) and analyzed histamine release upon systemic Ag challenge with DNP-HSA. In contrast to our results with the Itk−/− mice, we found that Itk−/− BMMCs in W/Wv mice were fully able to respond and release histamine similar to WT BMMCs (Fig. 4C). These data indicate that Itk−/− BMMCs are capable of responding to IgE/FcεRI triggering in vivo, and that the high levels of IgE seen in these mice may be interfering with the passive sensitization process, leading to the observed apparent reduction in mast cell responses.

Itk−/− BMMCs have normal degranulation, but enhanced cytokine secretion

We next analyzed these BMMCs for their responses to Ag-FcεRI triggering in vitro. Itk−/− BMMCs responded to FcεRI stimulation with similar levels of degranulation to WT BMMCs (Fig. 5A), confirming the in vivo data with the W/Wv mice that degranulation is not affected in the absence of Itk. Ag-IgE stimulation of mast cells results in secretion of a number of cytokines, including TNF-α and IL-13 (2, 34, 40). To determine whether cytokine secretion is altered in the absence of Itk, BMMCs were incubated overnight with IgE and then stimulated with Ag, and the supernatants were analyzed for IL-13 and TNF-α. The results show that Itk−/− BMMCs secreted more IL-13 and TNF-α than WT BMMCs (Fig. 5B). We also compared the cytokine responses upon stimulation with Ag to that seen upon stimulation with anti-IgE. In both cases, the Itk−/− BMMCs secreted more IL-13 and TNF-α compared with the WT BMMCs (Fig. 5C).

Several studies have indicated that IgE/FcεRI interaction on mast cells in the absence of Ag can also result in the production of cytokines (41–45). We therefore examined cytokine secretion under conditions in which the BMMCs were exposed to IgE alone in the absence of Ag, and analyzed the supernatants for a number of cytokines. We found that Itk−/− BMMCs secreted elevated levels of IL-2, IL-4, TNF-α, and GM-CSF, whereas these cytokines were not detected from WT BMMCs under these conditions (Fig. 5D). These data indicate that unliganded IgE can trigger Itk−/− BMMCs for cytokine secretion, which is more pronounced than that seen in WT BMMCs. Unliganded IgE alone can also cause increased expression of FcεRI on BMMCs, and mast cells that have undergone this process are more sensitive to increased cytokine production and degranulation in response to sensitization, in part due to the elevated levels of FcεRI (45). We therefore examined the expression of FcεRI on the surface of the BMMCs after incubation with anti-IgE-DNP overnight. There was no difference in the expression of FcεRI between WT and Itk−/− BMMCs under these conditions, ruling out the possibility that increased expression of FcεRI might contribute to increased sensitivity (Fig. 5E). These observations indicate that the absence of Itk makes BMMCs more responsive to IgE/FcεRI-stimulated cytokine secretion, suggesting that Itk may play a negative role in modulating cytokine responses through the FcεRI on mast cells.
in the activation of these kinases (Fig. 7, Erk/MAPK and p38 MAPK pathways also revealed no difference in overall activation pattern (Fig. 7). Further analysis of the phosphorylated proteins revealed that BMMCs from WT and Itk$^{-/-}$ BMMCs express higher levels of this transcription factor (Fig. 8). These data suggest that contrary to T cells, in mast cells Itk is not essential for calcium response through the FcRI.

### FcεRI-mediated signaling in Itk$^{-/-}$ BMMCs

To determine the molecular basis for this response, we analyzed select biochemical responses to FcεRI triggering in BMMCs. Itk-deficient T cells have reduced calcium mobilization upon TCR stimulation (20), and so we analyzed WT and Itk$^{-/-}$ BMMCs for the calcium response to FcεRI triggering at various concentrations of Ag DNP-HSA (5, 10, and 100 ng/ml) (Fig. 6A). Our data show that Itk$^{-/-}$ BMMCs exhibit slightly higher calcium response than WT BMMCs upon stimulation with various concentrations of Ag. However, analysis of peak calcium responses revealed no significant difference between the WT and Itk$^{-/-}$ BMMCs (Fig. 6C). These data suggest that contrary to T cells, in mast cells Itk is not essential for calcium response through the FcεRI.

Analysis of FcεRI-triggered tyrosine phosphorylation of cellular proteins revealed that BMMCs from WT and Itk$^{-/-}$ showed similar overall activation pattern (Fig. 7A). Further analysis of the Erk/MAPK and p38 MAPK pathways also revealed no difference in the activation of these kinases (Fig. 7, B and C). Thus, the absence of Itk does not grossly affect signaling pathways downstream of the FcεRI.

### Elevated NFAT expression and activation in Itk$^{-/-}$ BMMCs

The NFAT family of transcription factors plays a critical role in inducing cytokine synthesis. Indeed, NFATs 1 and 2 (NFATc2 and c1) play important roles in mast cell cytokine production of IL-13 and TNF-α triggered by the FceRI (40, 46, 47). We therefore analyzed mRNA levels of various NFAT isoforms in the WT and Itk$^{-/-}$ BMMCs by quantitative RT-PCR. We found that Itk$^{-/-}$ BMMCs have higher levels of NFAT1/NFATc2 and NFAT2/NFATc1 compared with WT BMMCs (Fig. 8A, top). Analysis of NFAT2/NFATc1 expression by Western blot revealed that Itk$^{-/-}$ BMMCs express higher levels of this transcription factor (Fig. 8A, bottom). To determine whether the NFAT in Itk$^{-/-}$ BMMCs was activated, we examined WT and Itk$^{-/-}$ BMMCs for NFAT nuclear localization. We found that in unstimulated WT cells, NFAT1/NFATc2 was largely cytoplasmic, whereas in Itk$^{-/-}$ cells, there was significant nuclear localization of NFAT (Fig. 8B). These data suggest that the observed higher levels of cytokine secretion in the absence of Itk may be due to elevated NFAT expression and activation.

### Discussion

In this study, we used a mast cell-specific early airway responsiveness model and found that Itk$^{-/-}$ mice exhibit reduced AHR and histamine secretion in response to Ag challenge. However, these initial results are in agreement with the published report by Forsell et al. (30), suggesting that Itk$^{-/-}$ mice may be defective in mast cell degranulation following passive sensitization. Our data reported in this study have determined that Itk$^{-/-}$ mast cells are unable to respond in vivo due to saturating levels of IgE occupancy on FcεRI, because transferring Itk$^{-/-}$ BMMCs into mast cell-deficient W/Wv mice resulted in normal levels of histamine release. In addition, in vitro experiments using different concentrations of Ag reveal no difference in degranulation between WT and Itk$^{-/-}$ BMMCs. These experiments strongly suggest that Itk may not be essential for FcεRI-induced degranulation. Previously, Forsell et al. (30) compared WT, Btk$^{-/-}$, and Itk$^{-/-}$ mice using a mast cell-dependent passive cutaneous sensitization model, and found reduced mast cell degranulation in Itk$^{-/-}$ mice, suggesting that in
ERK/MAPK activation. Itk is also critical for T cell development, activation, and cytokine production, particularly IL-2 and Th2, such as cytokines IL-4 (11). By contrast, we have shown that Itk does not affect these pathways in mast cells, but may play a negative role in the production of cytokines in these cells, without affecting the ability of these cells to degranulate. It is possible that Itk plays a more critical role in T cell function due to levels of expression in these cells, whereas in mast cells, it may play a more specialized role. Alternatively, Itk may play negative roles for cytokine production in both cell types, but this role is more dominantly revealed in mast cells, where the absence of Itk results in deregulation of transcription factors such as NFAT, which set the stage for deregulated expression of these cytokines. These results also suggest that signaling pathways regulated by Itk have cell type-specific functions.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


vivo, Itk deficiency is more detrimental to mast cell degranulation than Btk deficiency. Itk−/− mice have elevated levels of IgE, and the binding of IgE to FcεRI on mast cells is very stable interaction and lasts for several weeks (25, 36). Our observation of elevated FcεRI occupancy on Itk-deficient mast cells in vivo thus supports the idea that the Itk-deficient mast cells have reduced degranulation in vivo because the FcεRIIs are already occupied/loaded with saturating levels of IgE, which interferes with the binding of newly synthesized IgE or passive administration of IgE.

Analysis of the role of Btk in mast cells using BMMCs has shown that the absence of Btk leads to reduced calcium responses to FcεRI triggering in mast cells (28, 29, 48, 49). We do not observe any difference in both intracellular calcium response and extracellular calcium influx in the Itk−/− BMMCs, suggesting that Btk, but not Itk, is critical for this process in mast cells. Further biochemical analysis showed that the absence of Itk leads to normal ERK and p38 MAPK activation, suggesting that contrary to T cell signaling, the activation of these pathways may be independent of Itk in mast cells (20, 21, 29, 49).

Interestingly, we find increased cytokine secretion of TNF-α and IL-13 by Itk−/− BMMCs, suggesting that Itk may play a negative role in FcεRI regulation of these cytokines. The absence of Btk has been reported to cause defective or reduced histamine release as well as secretion of cytokines like TNF-α, IL-6, and GM-CSF. We find increased expression as well as nuclear localization of NFAT in the Itk−/− BMMCs, suggesting a mechanism for the observed increase in cytokine secretion. It is well established that NFAT is a major transcription factor that regulates the expression of TNF-α and IL-13 (40, 46, 49). Two isoforms of NFAT, NFAT1/NFATc2 and NFAT2/NFATc1, have been shown to regulate transcription of TNF-α and IL-13 in mast cells both individually or in combination, and mast cells deficient in NFAT1/NFAT2 and NFAT2/NFATc1 have been shown to be severely impaired in the production of TNF-α and IL-13 (47). These data support the idea that increase in NFAT levels may contribute to a mechanism for increased cytokine.

It should be noted that retroviral mediated expression of Itk into Btk-deficient mast cells has been shown to partially rescue histamine, TNF-α, IL-6, and GM-CSF secretion in these cells (16). We confirmed that Itk−/− mast cells express equivalent levels of Btk to WT mast cells (data not shown). These data therefore raise the question as to whether the two Tec family kinases differentially regulate signaling pathways in response to FcεRI triggering in mast cells. Altogether, these experiments indicate that Btk may play a more important role in amplifying signals from FcεRI in mast cells; however, the increased cytokine response in Itk−/− BMMCs implies that Itk may have a role in either enhancing or regulating the FcεRI signaling. Alternatively, because both Itk and Btk share similar substrates, it is possible that in the absence of Itk, Btk has less competition for substrates and is therefore more effective at signaling, leading to elevated cytokine secretion.

Our data also suggest that Itk−/− mice behave in part similar to mice lacking the Src kinase Lyn with regard to mast cell responses. Lyn was originally reported to be a positive regulator of mast cells because Lyn−/− mice were hyporesponsive to FcεRI stimuli in vivo (51, 52). However, it was subsequently found that Lyn−/− mice have high levels of IgE, and that Lyn−/− BMMCs were hyperresponsive to FcεRI triggering (53, 54). It is now well established that Lyn regulates FcεRI signaling both positively and negatively, by activating Syk, and recruiting the phosphatase SHIP, respectively (55).

In summary, our data indicate that whereas Itk is expressed in both T cells and mast cells, it functions differentially in these cells. In T cells, Itk−/− T cells exhibit defects in calcium increase and