

Interleukin-2-Inducible T Cell Kinase Regulates Mast Cell Degranulation and Acute Allergic Responses

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Bruton's tyrosine kinase (Btk) is thought to positively regulate mast cell activation, implying a role in allergic responses. We have compared acute and late phase allergic airway reactions in mice lacking either Btk or interleukin-2-inducible T cell kinase (Itk), another Tec kinase expressed in mast cells. Btk^{-/-} mice showed minor protection against allergic symptoms when challenged with allergen via the airways. In sharp contrast, both acute and late phase inflammatory allergic responses were markedly reduced in Itk^{-/-} mice. Notably, airway mast cell degranulation in Itk^{-/-} mice was severely impaired, despite wild-type levels of allergen-specific IgE and IgG₁. The degranulation defect was confirmed in DNP-conjugated human serum albumin-challenged mice passively sensitized with anti-DNP IgE antibodies, and was also observed after direct G-protein stimulation with the mast cell secretagogue c48/80. Moreover, late phase inflammatory changes, including eosinophilia, lymphocyte infiltration, and Th₂ cytokine production in the lungs, was eliminated in Itk^{-/-} mice. Collectively, our data suggest a critical role of Itk in airway mast cell degranulation *in vivo* that together with an impaired T cell response prevents the development of both acute and late phase inflammatory allergic reactions.

Keywords: allergy and immunology; asthma; signal transduction

Allergic asthma and rhinitis are common inflammatory airway diseases with increasing prevalences (1). The response to allergens is generally divided into an acute, largely mast cell-derived response, and a late phase inflammation promoted by T cells (2). The acute reaction is initiated by the release of stored mast cell mediators such as histamine, which induce an immediate plasma extravasation, hypersecretion, and bronchoconstriction (3). Antigen recognition by mast cells through IgE and aggregation of surface-bound FcεRI-IgE complexes leads to activation of non-receptor tyrosine kinases of the Src, Syk, and Tec family, as well as lipid kinases including phosphatidylinositol-3-kinase (PI3K), the combined action of which orchestrate the release of mast cell mediators (4).

Among the Tec family of kinases, Bruton's tyrosine kinase (Btk) has been shown to positively regulate FcεRI-mediated mast cell activation (5–7). Interleukin (IL)-2-inducible T cell kinase (Itk), another member of the Tec family known to be involved in T cell regulation, is also expressed in mast cells (8) and activated following FcεRI crosslinking *in vitro* (9). However,

contrary to Btk, the biological consequences of this observation have remained unknown.

Development of a late phase inflammation (e.g., eosinophilia, goblet cell hyperplasia, and airway hyperresponsiveness) involves complex interactions between many types of immune cells. In this regard, CD4⁺ T cells, through differentiation into Th₂ cells and release of Th₂ cytokines (e.g., IL-4, IL-5, IL-13), have a pivotal role in regulating the inflammatory process (10–12). Recent findings have revealed a critical role for Itk in the establishment of a Th₂ phenotype (13, 14), and Itk deficiency is associated with an attenuation of chronic/late phase inflammatory responses (15). Hence, while Btk through its regulatory role in mast cells has been implicated in the acute allergic response, Itk may, by affecting the Th balance, influence the late phase inflammatory response. However, it cannot be excluded that Btk can also affect the late phase inflammatory allergic reaction. Similarly, Itk is expressed in mast cells and this may well have functional consequences for the development of an acute allergic response.

To address whether lack of Btk or Itk affect the outcome of an allergen challenge *in vivo*, we have made direct comparisons between the acute and late phase inflammatory allergic reactions in wild-type mice (C57/BL6) and in mice that carry targeted Btk^{-/-} or Itk^{-/-} alleles on the same genetic background. Using validated *in vivo* models of allergic airway inflammation, we found that Btk^{-/-} mice were only moderately protected against acute (i.e., plasma extravasation and mast cell degranulation) and late phase inflammatory responses. In sharp contrast, despite normal IgG₁ and IgE levels, Itk^{-/-} mice had a significantly attenuated airway mast cell degranulation and plasma extravasation response. A degranulation defect in Itk^{-/-} mice was also observed after passive sensitization or after challenge with the mast cell secretagogue compound 48/80 (c48/80). These findings, and the fact that late phase inflammation was absent in Itk^{-/-} mice, point out Itk as an attractive target for simultaneous therapeutic manipulation of both acute and late phase inflammatory allergic reactions.

MATERIALS AND METHODS

Animals

The capacity to develop acute and late phase allergic inflammation was tested in wild-type mice (C57/BL6) and age-matched mice that carry targeted Btk^{-/-} (16) and Itk^{-/-} (17) alleles backcrossed for at least eight generations to C57/BL6. Animals were kept in a barrier facility with a 12-h light-dark cycle and were allowed water and food *ad libitum*. All animal procedures were approved by the local ethics committee, Lund/Malmö, Sweden.

Immunizations

Animals were actively immunized against ovalbumin (OVA, grade III; Sigma, St Louis, MO) at Day 1 by an intraperitoneal injection (7.5 μg OVA + 1.5 mg Al(OH)₃) (18). For evaluation of acute-phase parameters, an identical booster injection was given at Day 7 (19).

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For anti-IgD immunizations, animals were injected intravenously with goat anti-mouse IgD (concentrated plasma; kindly provided by Dr. Finkelman, University of Cincinnati, OH) or normal goat serum (DAKO A/S, Glostrup, Denmark). Blood was collected 8 d later for analysis of total mouse IgE.

Plasma Levels of IgE and IgG1

Heparinized blood from immunized animals were centrifuged and plasma was collected and stored at -70°C until analysis. Plasma levels of total IgE were determined by ELISA using commercial kits (BD Biosciences Pharmingen, San Diego, CA).

To determine OVA-specific IgE and IgG1, 96-well flat bottomed plates (Nunc, Roskilde, Denmark) were incubated overnight with OVA (Sigma) at a concentration of 100 $\mu\text{g}/\text{ml}$, followed by incubation with 3% bovine serum albumin. Mouse plasma was serially diluted, followed by application of biotin-conjugated rat anti-mouse IgE (R35-72; BD Biosciences Pharmingen) or biotin-conjugated rat anti-mouse IgG1 (A85-1; BD Biosciences Pharmingen). Bound R35-72 and A85-1 were detected with horseradish peroxidase-conjugated ExtrAvidin (Sigma). Plates were developed for 30 min using TMB peroxidase substrate (KPL, Gaithersburg, MD) and read at 450 nm. A plasma pool from OVA-sensitized mice was used as an internal laboratory standard. The relative titer of allergen-specific antibodies was expressed as arbitrary units/ml plasma.

Allergen Challenge, Plasma Extravasation, and Termination of Acute Allergen Responses

Mice were immunized to OVA by intraperitoneal administration of OVA + $\text{Al}(\text{OH})_3$ on Days 1 and 7. Acute phase parameters were assessed on Day 14. To measure allergen-induced acute extravasation of plasma, Monastral blue particles (Sigma) (a marker that is trapped between endothelial cells and the basement membrane of hyperpermeable blood vessels [20]) or in separate experiments, Evans blue dye (Sigma) were administered by intravenous injection (80 μl). Next, the airways of sensitized animals were subjected to local allergen challenge by a 10-min exposure in aerosolized ovalbumin (3% OVA in 0.9% NaCl; separate control animals received saline). The aerosol was generated by a micronebulizer (Bird, 500 ml, Inline micronebulizer; Bird Co., Palm Springs, CA) using an air pressure of 4 bar. Animals were killed by an intraperitoneal injection of pentobarbital sodium. The chest was carefully opened and blood was collected from the still-beating heart. Tissue samples of the trachea and lungs were immediately placed in fixatives for histologic analysis of mast cell degranulation (*see below*). A central part of the trachea was stretched out on Sylgard-coated petri dishes as whole-mount preparations and immersed in fixative. For quantification of Monastral blue-positive blood vessels, high-resolution digital images were obtained from each tracheal whole-mount preparation using an Olympus digital camera. The area of blue-labeled vessels was identified using an image analysis system (Viewfinder Lite, v1.0, 2000, Pixera Co and Image-Pro Plus v 4.5, 2000, Media Cybernetica, Inc., Silver Spring, MD) and the extent of extravasation was expressed as mean blue-stained pixels/area unit. For each sample a total mucosal area of $8 \times 10^6 \mu\text{m}^2$ was analyzed.

In separate animals, the mid part of the trachea and the main bronchi were gently excised and analyzed for tissue content of Evans blue tracer. The tracheal tissue sample was weighed and the Evans Blue dye extracted in 500 μl formamide overnight. Tissue content of Evans Blue was determined by comparing absorbance at 620 nm against a standard curve, and the amount of plasma tracer was expressed as $\mu\text{g}/\text{mg}$ tracheal tissue.

Passive Immunization and DNP Challenge

Passive immunizations were performed by an intravenous injection of monoclonal anti-DNP IgE antibodies (13 μg IgE in 100 μl 0.9% NaCl; Clone SPE-7; Sigma). Twenty-four hours after IgE administration, animals were given plasma tracer (100 μl 1% Evans blue solution) and 1 min later challenged under light Efrane anesthesia by intranasal administration of 5 μg DNP-HSA (Sigma). Animals were killed 8 min later by an overdose of pentobarbital sodium, and blood and tissue samples (trachea and lungs) were collected for analysis of plasma extravasation and mast cell degranulation.

Systemic Challenge with c48/80 and Determination of Histamine Levels

Separate animals were given 5 μg of compound 48/80 intravenously and killed 4 min later. Mast cell degranulation of both airway and ear mast cells were analyzed as described below, and levels of systemic histamine was analyzed.

Plasma levels of histamine were determined using an assay based on radioactive labeling of histamine by histamine methyl transferase. Samples and histamine standard (Sigma) were incubated with THG buffer, pH 7.4 (NaCl 8 $\mu\text{g}/\text{ml}$, HEPES 2.4 $\mu\text{g}/\text{ml}$, D-glucose 1 $\mu\text{g}/\text{ml}$, gelatin type B 1 $\mu\text{g}/\text{ml}$, 0.4 M NaH_2PO_4 , 2.7 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2), histamine methyl transferase (purified from male rat kidneys) and S-Adenosyl-L-(methyl- ^3H) methionine (Amersham Biosciences, Uppsala, Sweden) for 1 h at 37°C . The reaction was stopped by addition of 10 M NaOH. To extract methyl histamine, toluene/isoamyl alcohol (4:1) was added, the tubes were shaken vigorously, and samples were centrifuged to separate phases. The organic phase was collected into 96-well plates and dried before counting.

Mast Cell Degranulation

Mast cell degranulation was assessed in 1- μm Toluidine blue-stained plastic sections. Tissue samples were placed in fixative overnight (buffer supplemented with 1% glutaraldehyde and 3% formaldehyde), rinsed in buffer, postfixed in 1% osmium tetroxide for 1 h, and dehydrated in graded acetone solutions. The specimens were embedded in Polarbed 812, and 1- μm -thin plastic sections were cut on an ultratome (Ultracut E; Leica, Wetzlar, Germany), stained with toluidine blue and examined at $\times 1,000$ in a bright-field microscope (Axioscop; Zeiss, Oberkochen, Germany). The granules of normal, nondegranulated mast cells were identified by their characteristic dark blue-purple color. Degranulated mast cells were identified by the altered staining properties of the granules resulting in the appearance of large pink granules (21, 22). The pink granules correspond to granules that have decreased electron density and are devoid of histamine, as demonstrated by combined transmission electron microscopy and enzyme-affinity cytochemistry (21).

For each mast cell, the numbers of normal and altered granules were identified and the percentage of altered granules was calculated (on average 1,100 granules were analyzed from each sample). In a separate transmission electron microscopic analysis, we confirmed that blue versus pink stained granules represented resting and degranulating granules, respectively.

Transmission Electron Microscopy

From the 1- μm -thin plastic sections representative areas were selected for further transmission electron microscopic analysis. Ultrathin sections (90 nm) were cut and placed on 200-mesh, thin-bar copper grid before staining with uranyl acetate and lead citrate. The specimens were examined in a Philips CM-10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Late-Phase Inflammatory Allergen Responses

In the present study, the expression late phase inflammation is used to denote the cellular inflammation occurring 24 h after the repeated allergen challenges. Late phase inflammatory responses were evaluated in animals separate from those used for acute phase experiments. At Days 21–28 after intraperitoneal immunization they were exposed daily to allergen (30 min 1% aerosolized OVA). Animals were killed 24 h after final challenge by an intraperitoneal overdose of pentobarbital sodium, and bronchoalveolar lavage (BAL) samples were collected for determination of luminal cells and cytokines (*see below*). The trachea, main bronchi, and lung tissue samples were excised and immediately immersed in fixative.

BAL

BAL was performed by cannulation of the trachea via a midcervical incision, and 1 ml PBS was introduced to the lungs over a period of 2 min using 10 cm H_2O filling pressure. The lavage fluid was centrifuged (10 min, 4°C , $160 \times g$) and the supernatant stored at -70°C for cytokine analysis. Total numbers of BAL cells was determined using a hemacytometer. Differential cell counts were obtained from May-Grünwald-stained cytospin slides (Shandon, Cheshire, UK), and the proportion of

neutrophils, lymphocytes, eosinophils, and large mononuclear cells was determined.

Cytokine Levels in BAL Fluid

The amount of cytokines in the BAL fluid supernatant was determined by commercially available ELISA kits according to manufacturers' instructions (Endogen Inc., Woburn, MA for IL-4; R&D Systems Inc., Minneapolis, MN, for IL-5 and IL-13). Detection limits are 5 pg/ml for IL-4, 7 pg/ml for IL-5, and 1.5 pg/ml for IL-13.

Eosinophil Peroxidase Staining

The distribution of eosinophils in cryo sections was assessed by histochemical staining of cyanide-resistant eosinophil peroxidase (EPO) (18). Briefly, the tissue was placed in fixative overnight, rinsed in 10% sucrose buffer, and frozen in Tissue Tek freezing medium. Sections were exposed to 100 μ l incubation solution (PBS buffer pH 7.6 containing 3.3 diaminobenzidine tetrahydrochloride [75 mg/100 ml; Sigma], H_2O_2 [100 μ l/100 ml], and NaCN [50 mg/100 ml]) for 7 min at room temperature. After rinsing in tap water, samples were mounted in Kaisers medium. For quantification, lung sections were digitalized and the EPO-reactivity was captured using a digital image analysis package (Viewfinder Lite, v1.0, 2000; Pixera Co., and Image-Pro Plus v 4.5, 2000; Media Cybernetica, Inc.). The extent of tissue eosinophilia was expressed as EPO-positive pixels/area unit lung tissue. In separate experiments we demonstrated that the present method of EPO assessment correlates well with actual tissue numbers of eosinophils in the same section.

Goblet Cell Hyperplasia

The number and distribution of goblet cells was assessed by Periodic Acid Schiff (PAS) staining of mucin granules. Briefly, 8 μ m cryo sections were PAS stained, rinsed in tap water, counterstained with methyl green, and mounted. Goblet cells in medium-size and large bronchi were quantified at $\times 400$ magnification. The length of the epithelial lining examined in each cross-sectioned bronchi (i.e., the epithelial perimeter) was calculated using digital image analysis. The extent of PAS-stained goblet cells was expressed as goblet cell numbers/mm epithelium.

Quantifications and Statistics

All quantifications were performed in a blinded manner using coded samples. The statistical analysis, one-way ANOVA according to Bonferroni, was performed using Astute 1.5, a statistics Add-in for Microsoft Excel (DDU Software, Leeds, UK). *P* values < 0.05 (*), 0.01 (**), or 0.001 (***) were considered statistically significant. Asterisks in brackets within the figures indicate differences between saline and OVA groups within the same genotype.

RESULTS

OVA-Induced Acute Phase Plasma Extravasation

Plasma extravasation is a hallmark of the acute allergic reaction and is generally believed to be dependent on degranulation of IgE-bearing mast cells. The extravasation was assessed 10 min after OVA challenge using Monastral Blue (19) or Evans Blue (5) as plasma tracers. Upon challenge with OVA, wild-type mice developed a significant increase in occurrence of hyperpermeable (i.e., Monastral blue-positive) blood vessels as well as tissue content of extravasated Evans blue (Figure 1). *Btk*^{-/-} mice had a somewhat reduced plasma extravasation, although this reduction was not significant (Figure 1). In contrast, in *Itk*^{-/-} mice both the numbers of extravasated blood vessels and tissue content of Evans blue were markedly and significantly reduced compared with wild-type positive controls (Figures 1E–1F).

Numbers, Distribution, and Ultrastructural Morphology of Airway Mast Cells

Both *Btk*^{-/-} and *Itk*^{-/-} mice were found to have normal numbers of mast cells in the tracheobronchial airways (Figure 2A and data not shown), and no difference in mast cell distribution

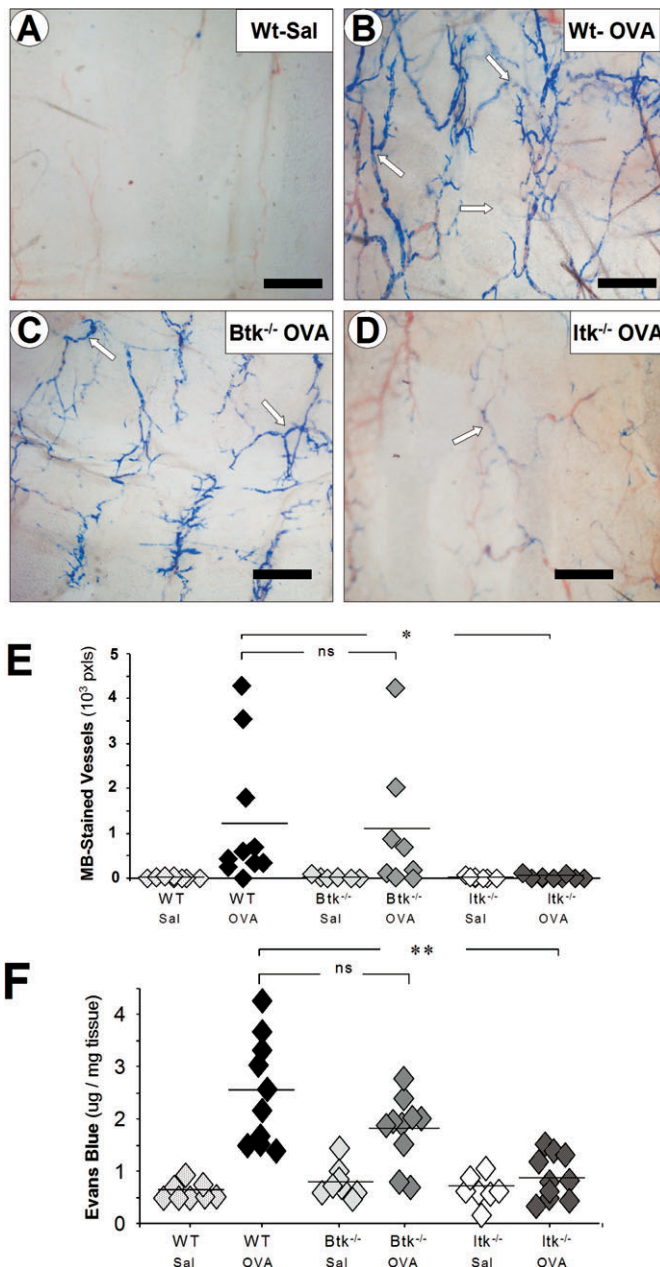


Figure 1. Decreased allergen-induced acute phase plasma extravasation in *Itk*^{-/-} mice. Panels A–D depict the density of Monastral blue-labeled vessels in representative tracheal whole-mount preparations (scale bars: 200 μ m). Labeled vessels were quantified by digital image analysis and expressed as blue-stained pixels/area unit (E). In separate animals the tissue content of the vessel permeable plasma marker Evans blue was analyzed spectrophotometrically after dye elution in formamide (F). The data presented are individual values from experiments with 10–12 animals per group. Horizontal bars represent mean values. Asterisks denote statistical differences between allergen-exposed genotypes.

within the tissue was observed. Furthermore, no ultrastructural differences between tissue mast cells in wild-type and any of the mutant mice were detected when analyzed by transmission electron microscopy. Hence, mast cells from both *Btk*^{-/-} and *Itk*^{-/-} mice contained normal granules with characteristic ultrastructural features, indistinguishable from wild-type mast cells.

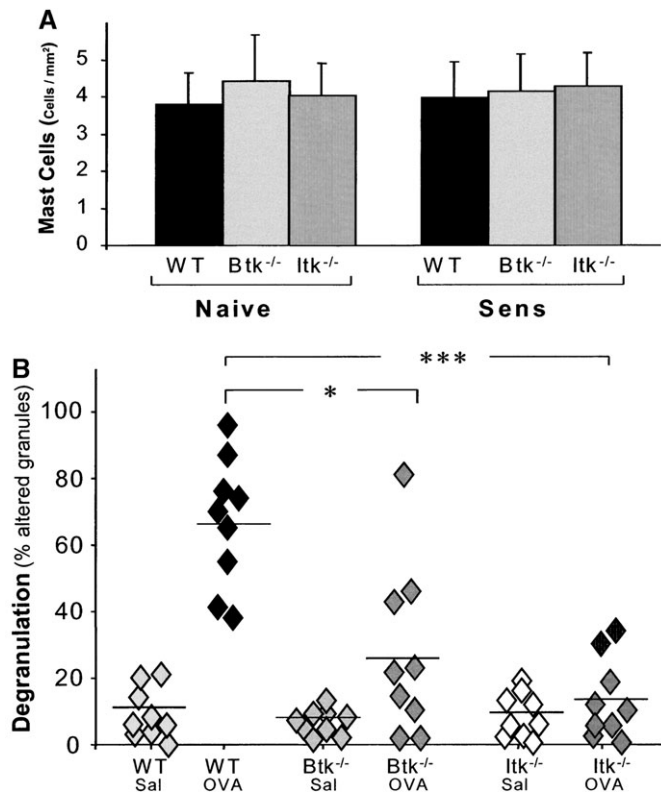


Figure 2. Normal numbers but impaired degranulation abilities of airway mast cells in *Itk*^{-/-} mice after allergen exposure. (A) Quantification of mast cells in tracheobronchial airways revealed no differences in cell density between the three genotypes. (B) In separate, sensitized animals the degranulation status of tissue mast cells was assessed in tracheobronchial airways 10 min after challenge with aerosolized allergen (OVA). In individual mast cells the percent granules altered due to activation (i.e., granules with degranulation-induced shift in the metachromatic staining) was calculated (21). To view examples of the typical color change occurring in activated mast cells, see Figures 6A–6C. Each data point represents the mean of individual cells from one animal. Bars represent mean values in each group of 8–10 mice. Asterisks denote statistical differences between groups.

Degranulation of Airway Mast Cells after OVA Challenge

To assess mast cell activation, we performed a detailed quantification of the metachromatic shift that occurs in mast cell granules during degranulation (21, 22). The pink granules correspond to granules with decreased electron density and are devoid of histamine, as previously demonstrated by combined transmission electron microscopy and enzyme-affinity cytochemistry (21). Moreover, in a separate transmission electron microscopic analysis we confirmed that blue versus pink granules represent resting and degranulating granules, respectively.

A moderate reduction in mast cell degranulation, compared with wild-type controls, was evident in *Btk*^{-/-} mice (Figure 2B). In comparison, examination of mast cells in *Itk*^{-/-} mice revealed a more pronounced and significant degranulation defect (Figure 2B).

Development of Late Phase Inflammatory Allergic Reactions

Determination of Th₂ cytokines in the BAL fluid revealed low levels of IL-4 in allergen-challenged mice lacking either *Btk* or *Itk*. In both mutant mice this was 5- to 10-fold below IL-4 BAL levels in wild-type mice (Figure 3A). While a significant allergen-induced increase of IL-13 and IL-5 was observed in *Btk*^{-/-} mice,

although moderately reduced compared with wild-type mice, neither of these cytokines were induced in *Itk*^{-/-} mice (Figures 3A–3C).

IL-13, IL-4, and IL-5 are known to be important for the local recruitment of immune cells during the development of an allergic inflammation. Accordingly, there was a significant cellular content in the BAL fluid of allergen provoked *Btk*^{-/-} mice, with moderate reductions compared with wild-type mice (Figures 3D–3H). In the lungs of *Btk*^{-/-} mice, eosinophil infiltration into the peribronchial tissue was similar to wild-type positive controls (Figures 4A–4C and 4I). In contrast, as for most cytokines examined, the cellular infiltration into the BAL fluid of allergen-challenged *Itk*^{-/-} mice was essentially eliminated. Thus, *Itk*^{-/-} mice had markedly reduced numbers of eosinophils, neutrophils, monocytes/macrophages, and lymphocytes in the BAL fluid (Figures 3D–3H). Histologic examinations of *Itk*^{-/-} mice further revealed a markedly diminished peribronchial tissue eosinophilia (Figures 4D and 4I). Finally, in allergen-challenged wild-type and *Btk*^{-/-} mice, a similar increase in mucus-producing goblet cells occurred (Figures 4E–4H and 4J). Again, in *Itk*^{-/-} mice, this response was significantly reduced, although a goblet cell hyperplasia also developed in *Itk*^{-/-} mice (Figures 4E–4H and 4J).

Production of Anaphylactic Antibodies in Response to OVA Sensitization

Given the dramatic effect of *Itk* deficiency on both acute and late phase inflammatory responses, we decided to focus further experimental work toward the *Itk*-deficient mice. In that respect, the impaired mast cell degranulation was considered of particular importance. As immunologic mast cell degranulation is dependent on the presence of anaphylactic antibodies, blood plasma titers of OVA-specific IgE and IgG₁ antibodies were assessed. Despite reduced levels of Th₂ cytokines, *Itk*-deficient mice had normal levels of OVA-specific antibodies of both subclasses (Figures 5A and 5B). *Btk*^{-/-} mice also mounted a normal IgE and IgG₁ response (data not shown).

The production of IgE in *Itk*^{-/-} mice was further tested in a model in which an IL-4-dependent polyclonal IgE response was induced by immunization with monoclonal goat anti-mouse IgD (23). In this model, *Itk*^{-/-} mice produced increased levels of IgE compared with wild-type positive controls (Figure 5C).

Degranulation of Airway Mast Cells after Passive Immunization

To confirm a direct role for *Itk*^{-/-} mast cells to the reduced plasma extravasation response, we investigated the effect of *Itk* deficiency after challenge of mice that were passively immunized with anti-DNP monoclonal IgE. This is a well-characterized model of direct IgE-mediated mast cell degranulation *in vivo* and should exclude any non-mast cell-related immunization effects as cause to the reduced mast cell degranulation and plasma extravasation. As depicted in Figure 6, mast cell degranulation and a plasma extravasation response developed acutely in wild-type mice after local challenge with DNP-HSA. In contrast, both mast cell degranulation and associated extravasation of plasma were significantly reduced in DNP-challenged *Itk*^{-/-} mice (Figure 6). Plotting of the degranulation status for individual mast cells revealed a heterogeneous degranulation within each genotype (Figure 6I).

Mast Cell Degranulation in Response to Nonimmunologic Secretagogues

Having established an impaired response of airway mast cells in allergen-challenged *Itk*^{-/-} mice following both passive and

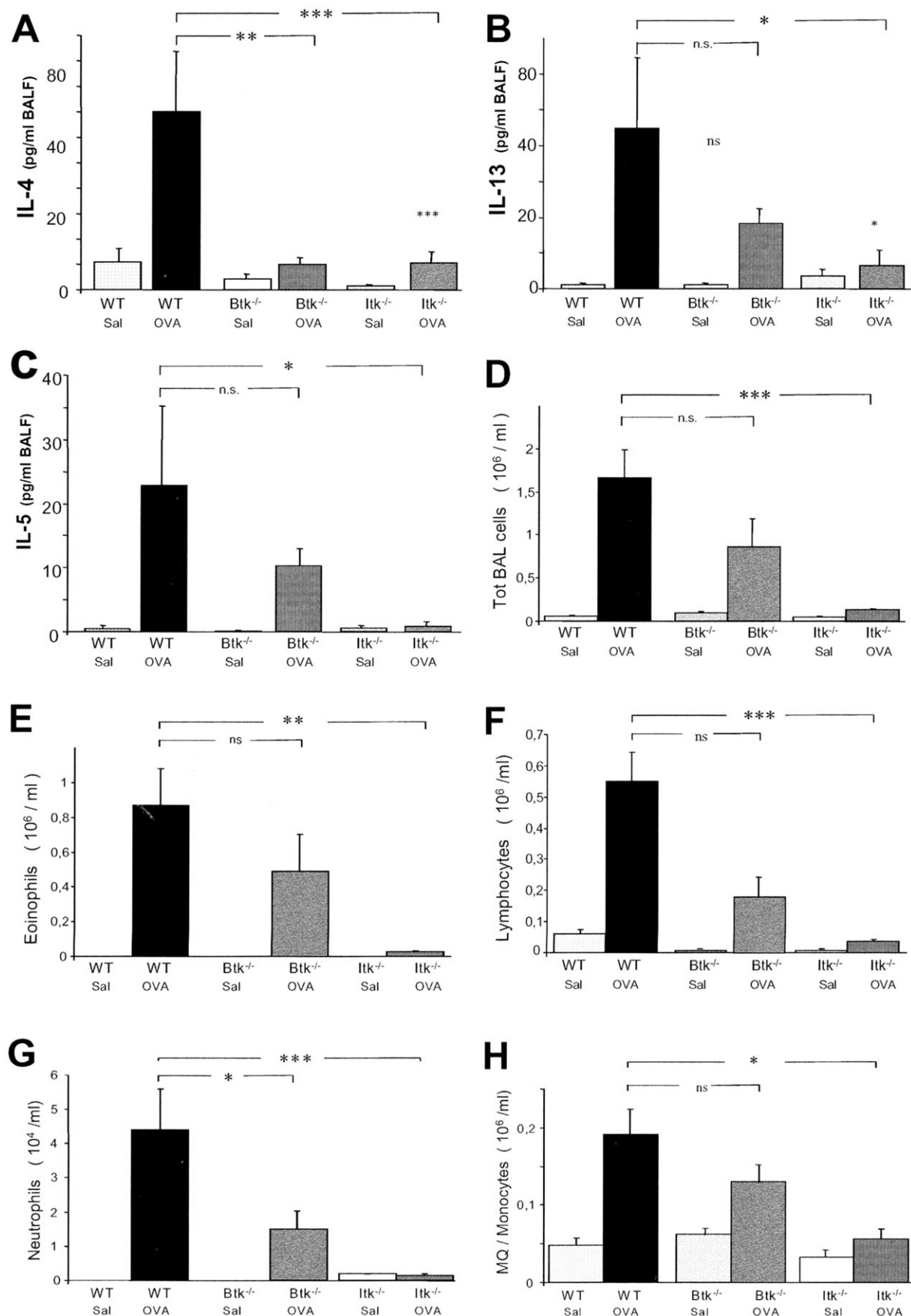


Figure 3. Attenuated Th₂-mediated allergic inflammation in sensitized Itk^{-/-} mice after allergen challenge. Levels of Th₂ cytokines (A–C) and immune cells (D–H) in BAL samples collected 24 h after 1 wk repeated allergen exposures (A–C). Data are presented as mean values $\pm$ SEM for 8–10 animals per group. Asterisks denote statistical differences between groups.

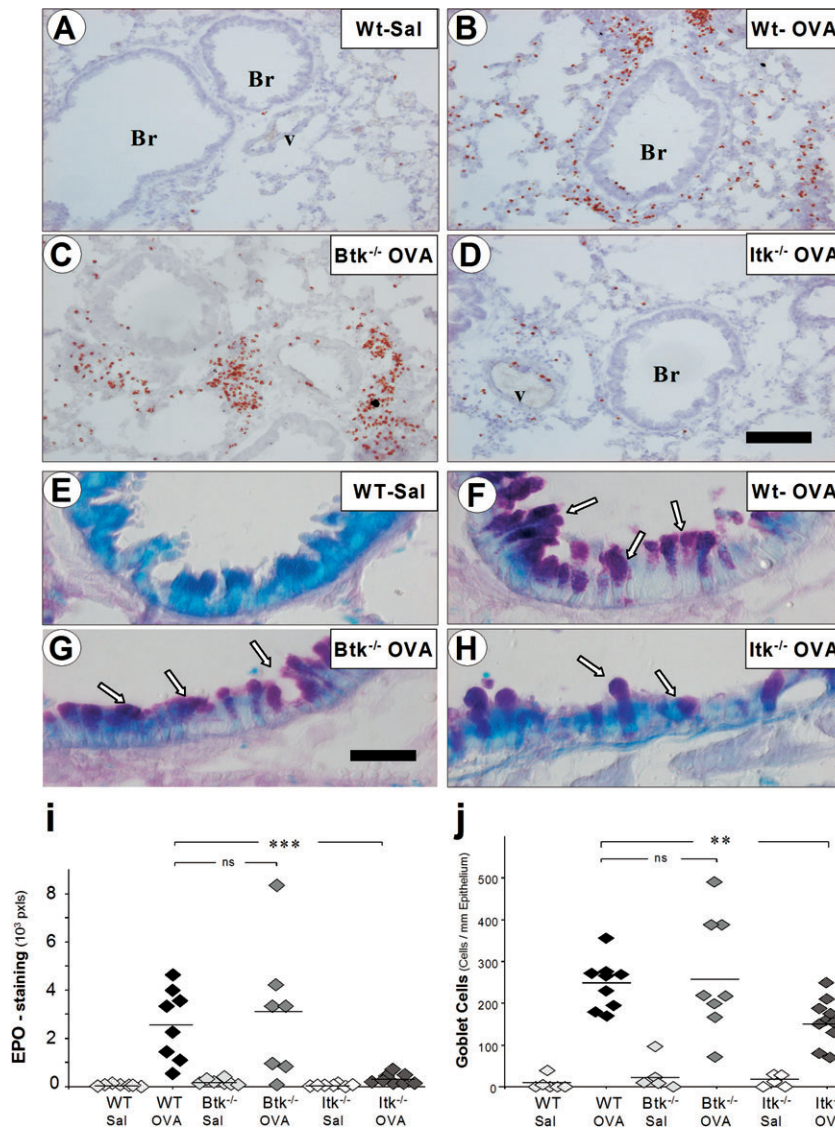


Figure 4. Reduced eosinophilic inflammation in *Itk*^{-/-} mice. Representative brightfield images showing EPO-stained eosinophils (A–D) and PAS-stained goblet cells (E–H). Lungs were excised 24 h after seven daily allergen exposures, fixed in 4% formaldehyde, and processed for (A–D) eosinophil staining (EPO histochemistry) and (E–H) detection of goblet cell mucin by PAS staining. Scale bars: D, 250 μ m; G, 60 μ m. Mean values of eosinophil and goblet cell numbers are presented in *i* and *j*, respectively. Bars represent mean values for each group $\pm$ SEM. Asterisks denote statistical differences between groups. Br = bronchus, V = vessel.

active sensitization, we next compared the systemic degranulation response in *Itk*^{-/-} and wild-type mice after intravenous administration of the mast cell secretagogue c48/80. This compound is a synthetic polycationic secretagogue acting directly on pertussis toxin-sensitive G_i proteins as a receptor mimetic agent and produces a strong histamine release when given *in vivo* (24). This fact allowed us to assess systemic histamine release not easily determined during the previously described local challenge. Histologic analysis of mast cells in the ears and tracheobronchial airways revealed a reduced c48/80-induced degranulation in *Itk*^{-/-} mice (Figure 7B), although less dramatic than that following Fc ϵ RI challenge (Figure 7B). The roughly 50% reduction in mast cell degranulation was also reflected by similarly reduced levels of circulating histamine in c48/80-challenged *Itk*^{-/-} mice (Figure 7A), demonstrating that the mast cell histology used herein correlates well with histamine release. Collectively, these findings provide evidence that mast cells in *Itk*-deficient mice are affected in their ability to degranulate following both Fc ϵ RI-dependent and G protein-dependent activation mechanisms.

DISCUSSION

As transducers and regulators of signals from receptors on hematopoietic cells, Tec kinases have emerged as important regulatory elements for a variety of immunologic processes. Among these are events that could contribute to the onset of allergic traits. By the use of mainly *in vitro* strategies, Btk has previously been shown to positively regulate mast cell activation and has thus been implicated in allergic responses. *Itk*, on the other hand, has a well-documented role in T cell function and is implicated in late phase inflammatory responses. However, the role *Itk* in non-T cells and acute phase responses has remained unexplored, despite early observations of *Itk* expression in mast cells (8).

We could now, by direct comparisons of *Itk*^{-/-}, *Btk*^{-/-}, and wild-type mice, establish that *in vivo* it is deficiency of *Itk*, rather than *Btk*, that leads to a significant reduction in airway mast cell degranulation and subsequent plasma extravasation. Hence, the present work extends the functional repertoire of *Itk* by suggesting a critical role for this kinase as a regulator of airway mast cell activation *in vivo*. This observation may be of direct relevance to acute allergic reactions occurring in the airways in

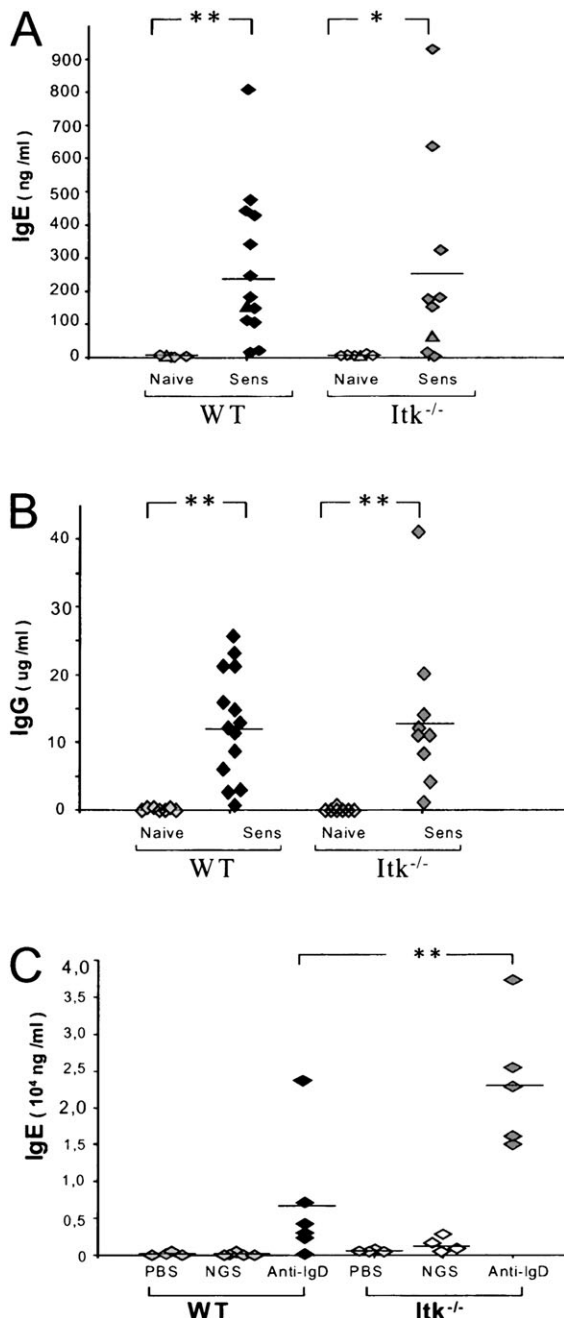


Figure 5. Maintained production of anaphylactic antibodies in sensitized *Itk*^{-/-} mice. Levels of antibodies are expressed as OVA-specific IgE and IgG₁ (A and B) and of total mouse IgE (C). Regardless of sensitization regime, *Itk*^{-/-} mice produced IgE at least to the same extent as wild-type mice. Data presented are representative individual values from repeated experiments with 10–12 animals per group. Bars represent mean values ± SEM. Asterisks denote statistical differences between groups. NGS = Normal Goat serum.

asthma and allergic rhinitis, but may also have bearings on other allergic diseases or conditions where mast cells may be pathogenic (e.g., in arthritis and cardiac diseases [25–27]).

While a role for Itk in late phase inflammatory responses could be envisioned based on its role in Th₂ responses, the drastically impaired acute phase responses in *Itk*^{-/-} mice as revealed herein was somewhat unexpected, as was the minimally reduced

acute phase responses in *Btk*^{-/-} mice. In both genotypes, the plasma extravasation correlates well with degranulation of airway mast cells. Thus, the slight reduction in airway mast cell degranulation in *Btk*^{-/-} mice translated into a tendency toward a reduced plasma extravasation, although it did not reach significance. However, in *Itk*^{-/-} mice, both plasma extravasation and airway mast cell degranulation was severely attenuated compared with OVA-treated positive controls. These phenomena were not caused by poor sensitization, as both *Btk*^{-/-} and *Itk*^{-/-} mice had a full capacity to produce allergen-specific anaphylactic antibodies. More significantly, the impaired degranulation was also observed in passively DNP-IgE sensitized *Itk*^{-/-} mice, or after direct activation with the mast cell secretagogue c48/80. These findings and our demonstration of normal distribution, numbers, or structure of airway mast cells in *Itk*^{-/-} mice strongly suggest that Itk deficiency is associated with an impaired degranulation of airway mast cells, despite proper extrinsic stimulation of FcεRI receptors and G proteins.

Theoretically, the reduced degranulation abilities could be caused by an altered route of mast cell differentiation due to the lack of Itk. However, despite careful examination we have been unable to detect any morphologic aberrations of *Itk*^{-/-} mast cells. In addition, human blood basophils as well as human lung mast cells treated with low molecular Itk-inhibiting compounds display an impaired IgE-induced degranulation (patent: WO 2,004/016270 and our unpublished observations). These observations not only argue in favor of a direct signaling role for Itk in mast cells, but also indicate that this role might be extended to humans. Therefore, the role of Itk in mast cells at the molecular level emerges as an important future research line. Based on our *in vivo* data, it would appear that Itk positively regulates, but is not absolutely required for, mast cell degranulation. The scattergrams (see Figure 6), showing degranulation changes in individual mast cells reveal that the absence of Itk does not automatically render mast cells incapable of degranulation, as some cells respond. It is possible that Itk acts as an amplifier rather than an on-off switch of FcεRI signaling, thus reducing the probability for a given mast cell to overcome the signaling threshold required for degranulation. Such a role was previously proposed for Tec-family kinases in signal transduction from the T cell receptor (28).

Notably, in this study direct injection of c48/80 also led to significantly reduced degranulation of airway mast cells and lower levels of circulating histamine in *Itk*^{-/-} mice. Compound 48/80 does not activate mast cells via the FcεRI receptor, but instead acts through G proteins (29), which in turn leads to activation of phosphatidylinositol kinases and production of phosphatidylinositol-tri-phosphate (PIP₃). This opens a potential site of action for Itk, as Itk can be activated in a process involving both G_{βγ} subunits (29) and PI3K (30). Activation through PI3K and G proteins appears to be a general feature of Tec kinases and has also been demonstrated for Btk (30–33). Interestingly, *Btk*^{-/-} mice also respond with a similar reduction in systemic histamine release following challenge with c48/80 (data not shown). The fact that both *Itk*- and *Btk*-deficient mice show impaired histamine release upon challenge with c48/80 suggests an important role for Tec kinases to amplify G protein-dependent degranulation responses.

The observations herein of reduced late phase inflammatory responses in *Itk*^{-/-} mice confirm results from another inflammatory model reported recently (15), and are also in accordance with recent findings using low molecular Itk inhibitors (34). In these studies, *Itk*-deficient T cells were considered to account for the reduced late phase inflammation (15). Our data certainly do not exclude T cells from playing an important, or perhaps even the major role in the reduced late phase inflammatory

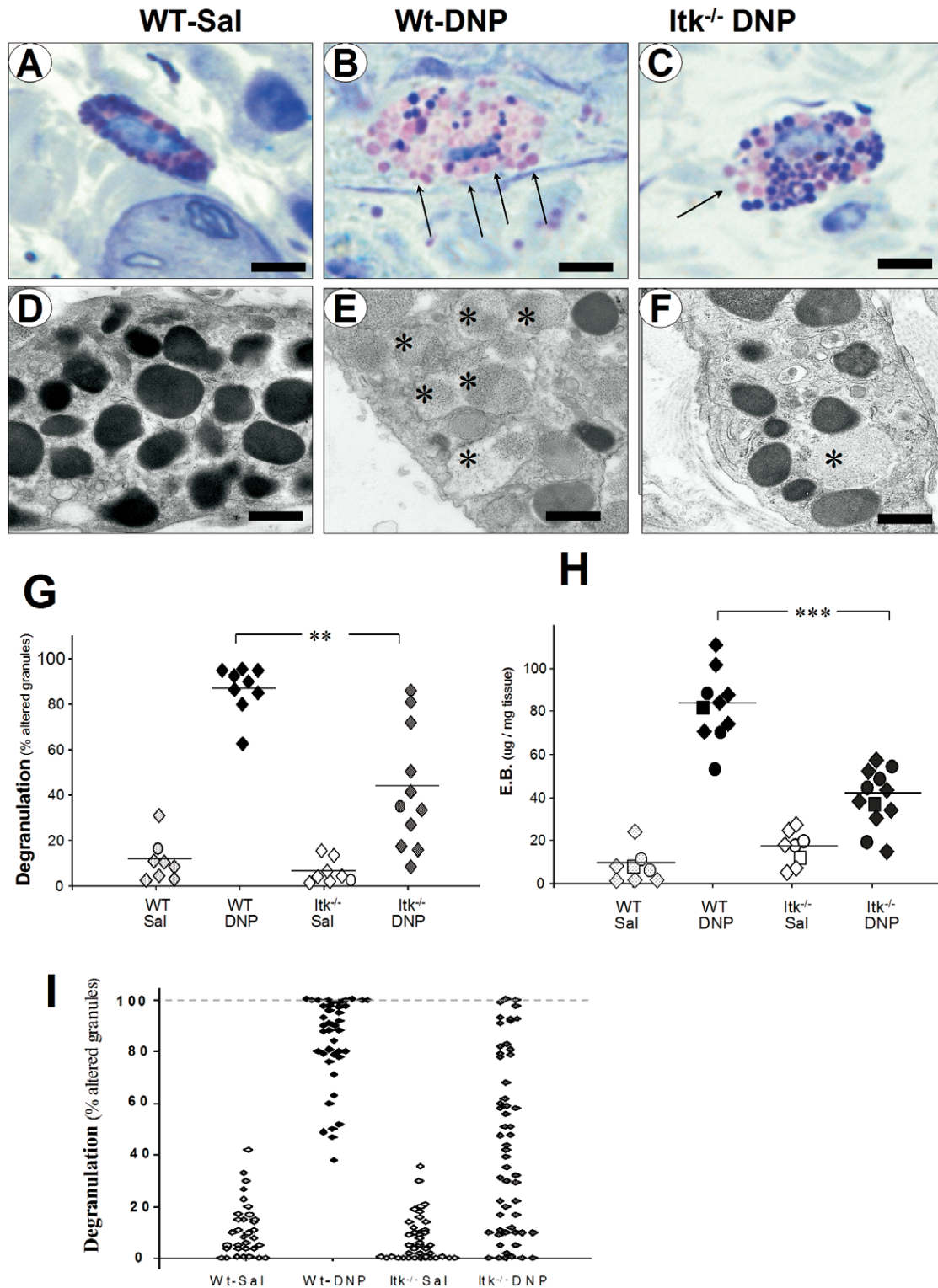


Figure 6. Mast cells from *Itk^{-/-}* mice have impaired degranulation upon direct stimulation of FcεRI. Mast cell degranulation after DNP challenge of mice passively sensitized mice with monoclonal anti-DNP IgE (10–12 animals per group). (A–C) Brightfield images illustrating typical mast cells in the tracheobronchial airways of wild-type and *Itk^{-/-}* mice. (D–F) Details of granule alterations are further illustrated by transmission electron microscopy. Upon DNP challenge, *Itk^{-/-}* mice had a significantly reduced mast cell degranulation as well as plasma extravasation compared with wild-type mice (G and H). Representative scattergram of individual cells within the same tissue region is presented in I.

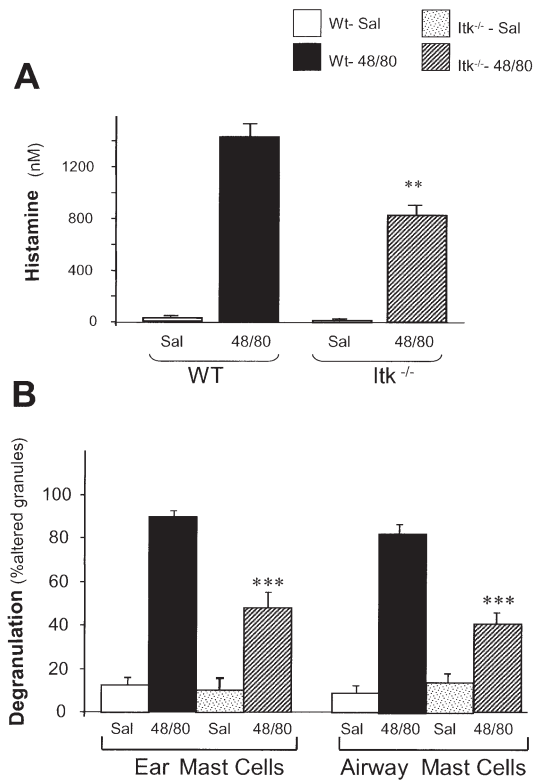


Figure 7. Mast cells from *Itk*^{-/-} mice have impaired degranulation upon direct stimulation with the nonimmunologic mast cells secretagogue c48/80. Following stimulation with the nonimmunologic mast cells secretagogue c48/80, *Itk*^{-/-} mice had significantly lower plasma levels of histamine compared with similarly treated wild-type controls (A). Histologic analysis of toluidine blue-stained plastic section revealed a reduced degranulation of both ear and tracheobronchial *Itk*^{-/-} mast cells, compared with wild-type controls (B). Data presented are representative mean values $\pm$ SEM from 10–12 animals per group. Asterisks denote statistical differences between OVA-challenged Wt and *Itk*-deficient groups.

responses of the *Itk*^{-/-} mice. For example, our failure to detect an allergen-induced increase of cytokine levels in the BAL fluid (Figure 3) could be a reflection of the fact that *Itk*^{-/-} T cells produce reduced amounts of Th₂ cytokines, including IL-4, IL-13, and IL-5 (14, 15, 35). However, as the ability to respond to these cytokines was apparently retained (35) and as we detected few lymphocytes in the BAL fluid of allergen-challenged *Itk*^{-/-} mice, an alternative possibility is that some factors needed for recruitment of T cells to the lungs were not produced in *Itk*^{-/-} mice. It is becoming increasingly clear that mast cells may influence adaptive and innate immunity, for example by recruitment of other immune cells, like T cells, to the site of challenge (25, 36, 37). Therefore, we favor the presently speculative hypothesis that mast cells can amplify the late phase inflammatory state during establishment, whereas it is executed and perpetuated mainly by T cells. However, although our data are fully compatible with the idea of mast cells affecting late phase responses, establishment of such a concept will require further investigation and additional mice models, such as the mast cell-deficient mice.

In summary, we evaluated whether the reported impairment in FcεRI degranulation of *Btk*-deficient mast cells would also result in reduced allergic responses using an *in vivo* model for allergic asthma. We detected a moderate reduction in airway mast cell degranulation and acute phase responses, whereas most

late phase inflammatory parameters examined were essentially normal in allergen-challenged *Btk*^{-/-} mice. We also explored the role of *Itk* in mast cell activation and allergic responses. In contrast to *Btk*^{-/-} mice, our findings revealed a profound defect on the acute phase allergic response in mice lacking *Itk*. We propose that this phenomenon is due to an impaired degranulation of airway mast cells. These findings, together with the observed lack of late phase inflammatory responses in *Itk*^{-/-} mice, highlight *Itk* as an attractive target for antiallergic therapy to obstruct both acute and late phase inflammatory allergic reactions.

Conflict of Interest Statement: J.F. has no declared conflict of interest; P.S. has no declared conflict of interest; C.E. is an employee of AstraZeneca; M.M.E. has no declared conflict of interest; K.R.-T. has no declared conflict of interest; P.-O.E. is an employee of AstraZeneca; and J.S.E. has no declared conflict of interest.

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