**Triβ3 is regulated by IL-3 and affects bone marrow-derived mast cell survival and function**

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**ABSTRACT**

Mast cells are the principal effectors of IgE-mediated immune responses, including allergic reactions. Triβ3 homolog 3 (Triβ3) encodes a pseudokinase implicated in the cellular stress response and has been linked to inflammation in certain situations. Here we report the role of Triβ3 in mouse bone marrow-derived mast cells (BMMCs). Our results show that Triβ3 mRNA expression in BMMCs is positively regulated by the growth factor interleukin (IL)-3. BMMCs originating from Triβ3 knockout mice demonstrate unaltered differentiation kinetics and cell surface expression of mast cell markers. When challenged with transient IL-3 deprivation, Triβ3-deficient BMMCs display delayed recovery, and during prolonged IL-3 starvation, cell death is accelerated in Triβ3-null cultures. IgE-dependent and pharmacologically induced degranulation is impaired in Triβ3-deficient BMMCs, as is activation-induced cytokine mRNA expression. Thus, Triβ3 contributes to the survival and activity of primary cultured mast cells, which suggests a role for Triβ3 in the modulation of the immune response.

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**1. Introduction**

Triβ3 (also known as TRB3, NIPK, SKIP3 and SINK) is a mamma-
lian homolog of the *Drosophila* gene *tribbles*. The TRB3 protein con-
tains a protein kinase-like domain which is predicted to lack kinase
activity due to mutations in critical amino acid residues in the ki-
nase active site, and it is therefore designated a pseudokinase [1].
Through protein–protein interactions, TRB3 is able to inhibit the
activity of several basic region-leucine zipper transcription factors,
namely CCAAT/enhancer binding protein β (C/EBPβ), C/EBP homol-
ogous protein (CHOP) and activating transcription factor 4 (ATF4)
[2–5], and, via these interactions, TRB3 is involved in the regu-
lation of the cellular stress response, cell viability and differentia-

Through binding of the protein kinase Akt, TRB3 has been found to
participate in the control of insulin signaling [6] and autophagy [7].
Additionally, TRIB3 has been reported to interact with and inhibit
NF-κB [8], a transcription factor which plays an important role in
inflammation and immune cell survival [9], and the silencing of
Triβ3 expression was found to ameliorate inflammation in an ani-
mal model of diabetic cardiomyopathy [10].

Mast cells are the key effector cells in immunoglobulin E
(IgE)-mediated immune responses and also have a role in innate
immunity [11]. When activated, mast cells rapidly release pro-
inflammatory mediators from intracellular granules, and degranulation is followed by the de novo synthesis and secretion of
cytokines and lipid-derived mediators. Mast cells are crucial
for providing defense against parasitic infections, such as helminth
infections in the gut, but also participate in the development of
autoimmune and allergic disorders [12]. Mast cells arise from pre-
cursors in the bone marrow and complete their maturation after
migration into peripheral tissues, under the influence of local
growth factors [13]. Several hematopoietic growth factors, includ-
ing IL-3 and stem cell factor (SCF), participate in the development
and maturation of mast cells, and the differentiation of mast cells
can also be performed in vitro from extracted bone marrow cells.
For the transcriptional control of hematopoietic differentiation,
members of the C/EBP transcription factor family serve important
roles in several lineages [14], including the specification of mast
cells [15]. By forming heterodimeric complexes with C/EBP family

**Abbreviations:** ATF, activating transcription factor; BMMCs, bone mar-
nor-derived mast cells; BSA, bovine serum albumin; C/EBP, CCAAT/enhancer
binding protein; CHOP, C/EBP homologous protein; DPAT, 4,6-diamino-
die-pheylindole; DNP, dinitrophenyl; Epo, erythropoietin; ER, endoplasmic
reticulum; ES, embryonic stem; GM-CSF, granulocyte-macrophage colony-
stimulating factor; IgE, immunoglobulin E; IL, interleukin; PCA, passive cuta-
aneous anaphylaxis; PMA, phorbol 12-myristate 13-acetate; Rgp74, ribosomal
protein L7a; RT-qPCR, reverse transcription-quantitative PCR; SCF, stem cell
factor; TNF, tumor necrosis factor; TRIB, tribbles homolog; UPR, unfolded protein
response.

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members, ATF4 and CHOP have also been shown to contribute to the regulation of myeloid-specific gene expression [16,17]. Trib3 has been found to be highly expressed in bone marrow [18] and has previously been implicated in the signaling of the hematopoietic cytokine erythropoietin (Epo) in erythroid progenitors [19].

Here we report the role of Trib3 in mast cells by creating and characterizing BMMCs from wild type and Trib3 knockout mice. We find that the expression of Trib3 mRNA is regulated by IL-3 in BMMCs. The lack of Trib3 affects several properties of BMMCs, such as cell viability when challenged with growth factor (IL-3) deprivation, and degranulation and cytokine gene expression in response to stimulation.

2. Materials and methods

2.1. Generation of Trib3 knockout mice

To obtain Trib3-deficient mice, a targeting construct was designed to replace the protein coding region of the Trib3 gene (spanning from exon 2 to exon 4) with a neomycin resistance gene driven by the phosphoglycerate kinase promoter. The construct was transfected into mouse embryonic stem (ES) cells by electroporation, and neomycin resistant ES cell clones were analyzed by PCR to verify the occurrence of homologous recombination. C57BL/6 donor blastocysts were injected with recombinant ES cells to obtain chimeras, which were crossed with wild type C57BL/6 mice to obtain heterozygous F1 mice. These mice were used to further propagate the Trib3 null allele. All experiments were performed using mice that were between 2 and 6 months of age and originating from litters produced by crossing mice heterozygous for Trib3. Mice were genotyped for wild type and null Trib3 alleles by multiplexed allele-specific PCR.

All animal procedures were performed in compliance with the EU guidelines (Directive 86/609/EEC) and approved by the Estonian National Board of Animal Experiments.

2.2. Generation and cultivation of BMMCs

Bone marrow cells were flushed out of the femurs of Trib3+/- and Trib3-/- mice, and seeded in BMMC medium consisting of RPMI 1640, 15% heat-inactivated fetal calf serum, 1% penicillin–streptomycin, 4 mM L-glutamine, 1% MEM non-essential amino acids, 1 mM sodium pyruvate (all purchased from PAA, Austria), 50 µM β-mercaptoethanol (Sigma–Aldrich) and 10 ng/ml recombinant murine IL-3 (Peprotech, Rocky Hill, NJ). Cells were grown under standard conditions (37°C and 5% CO2) and the culture medium was changed twice per week.

Experiments with BMMCs were started when more than 95% of cells were double-positive for cell surface expression of FcεRI and c-Kit, as evaluated by flow cytometry. This level was achieved after approximately 4 weeks of cultivation, and experiments were carried out within the following 5 weeks. At the beginning of each experiment, cells were placed in fresh medium at a uniform density (1 × 106 cells/ml, unless specified otherwise). To perform IL-3 deprivation studies, BMMCs were washed twice with IL-3-free growth medium before being seeded into BMMC medium without IL-3 or with 10 ng/ml IL-3 (control).

2.3. Flow cytometry

Expression of FcεRI and c-Kit on the surface of BMMCs was determined using fluorescein isothiocyanate (FITC)-conjugated armenian hamster anti-mouse FcεRI monoclonal antibody and phycoerythrin (PE)-conjugated rat anti-mouse CD117 (c-Kit) monoclonal antibody, respectively. FITC-labeled armenian hamster IgG and PE-labeled rat IgG2b were used as the corresponding isotype controls. The antibodies for flow cytometry were purchased from ebioscience (San Diego, CA) and cells were stained according to the manufacturer’s recommendations. To allow determination of cell viability, cells were incubated in 0.5 µg/ml DAPI (4′,6-diamidino-2-phenylindole; Roche) in PBS for 10 min on ice. Flow cytometric analysis was performed with a FACSaria cytometer and FACSdiva software (both from BD Biosciences, San Jose, CA).

For cell cycle phase analysis, cells were fixed with 70% ethanol and stained with 10 µg/ml propidium iodide (Sigma–Aldrich) in PBS containing 0.1% Triton X-100 for 30 min at room temperature. Flow cytometry data was analyzed with ModFit LT software (version 3.2; Verity Software House).

2.4. Trypan blue exclusion assay

Exclusion of trypan blue dye (Sigma–Aldrich) was used to determine cell viability as described previously [4]. Cells were counted in a hemocytometer. To assess the effect of IgE on cell survival, 5 µg/ml anti-dinitrophenyl (anti-DNP) monoclonal IgE antibody (clone SPE-7; Sigma–Aldrich) was used.

2.5. Sensitization and activation of BMMCs

BMMCs were sensitized by overnight incubation in BMMC medium containing 0.5 or 1 µg/ml anti-DNP IgE, depending on the experiment. Sensitized BMMCs were washed once with buffer H consisting of Hank’s balanced salt solution with calcium and magnesium (PAA) supplemented with 0.1% bovine serum albumin (BSA) fraction V (PAA). After washing, the cells were suspended in buffer H at a density of 106 cells/ml. To initiate antigen-dependendent activation, 500 ng/ml (or as specified) DNP-BSA conjugate (Calbiochem) was added to the cell suspension. To pharmacologically induce activation, cells unexposed to IgE were used in the procedure described above, and 1 µM iomycin and 1.6 nM phorbol 12-myristate 13-acetate (PMA) (both from Sigma–Aldrich) were added to the cells. BMMCs were activated in buffer H to study β-hexosaminidase release. To analyze mRNA expression in activated BMMCs, IgE-sensitized BMMCs were washed once with BMMC medium, resuspended in BMMC medium, and activated by adding DNP-BSA.

2.6. Determination of β-hexosaminidase release

To determine the degree of BMMC degranulation, β-hexosaminidase release was analyzed from activated BMMCs after 30 min incubation at 37°C and 5% CO2. The cell suspension was centrifuged at 450g for 5 min and the supernatant was collected. The cells were lysed with buffer H supplemented with 0.1% Triton X-100. To measure β-hexosaminidase activity, 45 µl of supernatant or cell lysate was mixed with an equal volume of 1 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG; Calbiochem) in 0.2 M sodium citrate (pH 4.5) in a black 96-well microtiter plate well. After 30 min incubation at 37°C, the reaction was stopped by adding 30 µl of 1 M solution of Tris base (Sigma–Aldrich). The fluorescence of 4-methylumbelliferyl, the compound released by β-hexosaminidase activity, was measured with a GENios Plus microplate reader (Tecan, Austria) using λex = 360 nm and λem = 465 nm. The percentage of β-hexosaminidase release was calculated using the formula 100 × (supernatant content)/(supernatant content + lysate content).

2.7. RNA isolation and quantification by RT-qPCR

Total RNA was isolated from 2 × 106 BMMCs using the RNeasy Mini kit (Qiagen) and quantified spectrophotometrically with
NanoDrop 1000 (Thermo Scientific). To minimize contamination with genomic DNA, RNA was treated with RNase-free DNase I (Fermentas, Lithuania). The synthesis of first strand cDNA was performed with RNase H-minus M-MLV reverse transcriptase (Solis BioDyne, Estonia) using 925 ng of DNase I-treated total RNA in a final volume of 20 μl. Random hexamer and oligo(dT)18 primers were used in combination.

Real-time PCR was performed on an Applied Biosystems 7900HT thermal cycler using EvaGreen qPCR master mix (Solis BioDyne). Reactions were carried out in triplicate, with each 10 μl reaction containing 1× master mix, cDNA equivalent to 12.5 ng of total RNA (0.03 ng for 28S rRNA) and primers (200 nM each). The amplification program was 95°C 15 min, 40 repetitions: 95°C 15 s, 60°C 60 s. Melting curves were analyzed at the end of each run. Quantification was performed relative to standard curves generated from serial dilutions of PCR product or plasmid DNA containing the target sequence. Template DNA for standard curves was purified using the QiAquick PCR purification kit (Qiagen) and quantified by spectrophotometry. The quantity of target amplicon in each sample was normalized to the level of the endogenous reference (28S rRNA or ribosomal protein L7a (Rpl7a) mRNA, as specified). Sequences of the primers used for RT-qPCR are as follows: Trib3 mRNA: 5′-TGGCTGGCAGATACCCATT-3′ (forward) and 5′-CAAGTGCCTTCAGAGTCCTCTATT-3′ (reverse), Trib3 precursor mRNA: 5′-GGCTTTATATGCTTGGAGAAAG-3′ (forward) and 5′-AGAACCTCAATGGTCCTTTTAGGA-3′ (reverse), TNF mRNA: 5′-CATCTTCTCAAAITATGAGTACA-3′ (forward) and 5′-TGGAGTAGACAAAGGTTCAACC-3′ (reverse), IL-6 mRNA: 5′-GAGGATACACCTCACAAGGCC-3′ (forward) and 5′-AAGTGCATCATCGTTGTTCATACA-3′ (reverse), Rpl7a mRNA: 5′-CCACAGGAACATGCGCACCC-3′ (forward) and 5′-CAGCGAGTTTCTCCATTTTGGC-3′ (reverse), 28S RNA: 5′-TGGAAATTCCCGGGGAGAG-3′ (forward) and 5′-ACATTGTTCCAACATGCCAG-3′ (reverse).

2.8. Passive cutaneous anaphylaxis (PCA)

For sensitization, mice were anesthetized with isoflurane and injected with 0.5 μg of anti-DNP IgE in 20 μl PBS intradermally into the left ear, using a 30G insulin syringe. The right ear was injected with 0.5 μl PBS intradermally into the left ear, using a 30G insulin syringe. After 45 min, the mice were sacrificed by cervical dislocation and blue dye (Sigma–Aldrich) in PBS intravenously into the tail vein. The results demonstrate that the re-exposure of BMMCs to IL-3 after 7 h of deprivation rapidly increases the amount of Trib3 mRNA precursor (approximately 4-fold within 2 h), indicating that IL-3 induces the expression of Trib3 mRNA by upregulating its synthesis (Fig. 1C).

In addition to cytokine signaling, important determinants of mast cell gene expression include sensitization by the binding of IgE to its cell surface receptor, and antigen-dependent activation. In the case of Trib3 mRNA expression, the exposure of BMMCs to IgE does not result in a significant change (Supplementary Fig. S1A) and IgE receptor-mediated activation results in a slight (approximately 2-fold) decrease after 75 or 240 min (Supplementary Fig. S1B). Therefore, out of the conditions investigated, the regulation of Trib3 by IL-3 is the most substantial.

3.2. IL-3-induced maturation of Trib3 knockout BMMCs is comparable to the wild type

We have generated a novel transgenic mouse line that carries a targeted deletion of Trib3, and verified that homozygous knockout (Trib3−/−) individuals lack TRIB3 coding sequence in the genome (Supplementary Fig. S2A) and do not express Trib3 mRNA (Supplementary Fig. S2B). To study whether Trib3 influences the ability of bone marrow cells to differentiate into BMMCs in response to IL-3, we monitored the development of BMMCs from bone marrow that was extracted from littermate Trib3−/− and Trib3+/+ mice, and subjected to a culture protocol in which 10 ng/ml recombinant murine IL-3 is added as a growth factor. Starting from a population of approximately 1 × 10^7 femur bone marrow cells, the change in the overall number of viable cells follows a similar pattern for both genotypes, increasing mildly (to approximately 2 × 10^7 cells) over the first two weeks in culture, and expanding at an accelerated pace over the subsequent two weeks (reaching approximately 8 × 10^7 cells) (Fig. 2A). The progress of in vitro mast cell differentiation can be followed by analyzing the proportion of cells displaying mast cell surface markers such as the high-affinity IgE receptor (FcsRII) and the receptor for SCF (c-Kit). After 3 weeks of cultivation, the cell populations derived from wild type and Trib3 knockout mice contained on average 73% and 58% cells double-positive for FcsRII and c-Kit expression, respectively, and after being cultivated for an additional 1 or 2 weeks, the proportion of cells expressing both markers reached more than 95% for both genotypes (Fig. 2B). Concurrently, the percentage of non-viable cells in culture declined steadily from 24% and 16% at week 3 for Trib3−/− and Trib3+/+, respectively, to fewer than 5% by week 5 for both genotypes (Fig. 2C). The differences between genotypes at week 3 in Fig. 2B and C were not statistically significant. Additionally, as shown in Fig. 2D, the magnitude of IgE receptor and c-Kit expression on the cell surface of mature (week 5) BMMCs is unaltered by Trib3 deletion. Thus, Trib3 appears dispensable with regard to the dynamics of mouse mast cell development from bone marrow-resident progenitors in IL-3-rich medium in vitro.

3. Results

3.1. Trib3 mRNA expression is regulated by IL-3 in bone marrow-derived mast cells

The addition of IL-3 to in vitro culture of mouse bone marrow cells leads to the differentiation and proliferation of mast cells [20]. In order to investigate the effect of IL-3 on Trib3 expression, mouse BMMCs were deprived of IL-3 for up to 10 h, and the expression level of Trib3 mRNA was determined by RT-qPCR. As shown in Fig. 1A, Trib3 mRNA expression is downregulated approximately 4-fold in response to 5 h incubation in medium without IL-3, and no further change is revealed after an additional 5 h of IL-3 starvation. The sensitivity of Trib3 expression to IL-3 is also observed in BMMCs re-exposed to IL-3 after 7 h of IL-3 deprivation, resulting in approximately 2- and 4-fold increases in Trib3 mRNA expression at 2 and 12 h after the re-addition of IL-3, respectively (Fig. 1B). Previous studies have shown that IL-3 affects the level of several mRNAs at the post-transcriptional level, altering their stability [21]. To examine whether the Trib3 mRNA is regulated transcriptionally or post-transcriptionally, we utilized quantification of the unspliced precursor mRNA to indicate transcription rate [22].
3.3 Trib3-deficient BMMCs display increased sensitivity to IL-3 deprivation

BMMCs respond to IL-3 deprivation by blocking the cell cycle in G1-phase [23], and after an extended period of time, undergo apoptotic cell death [24]. To investigate whether the deletion of Trib3 affects the resilience of BMMCs to a short-term IL-3 deprivation challenge, cell cycle phase analysis was performed on BMMCs incubated for 24 h in cell culture medium without IL-3, and 48 h after being re-transferred into complete medium to allow for recovery. As depicted in Fig. 3A, a large proportion of BMMCs (nearly 90%) have accumulated in G1-phase after 24 h of IL-3 deprivation, and the fraction in S-phase is marginal (less than 1%), for Trib3+/+ as well as Trib3−/− cultures. The subsequent re-addition of IL-3 into the culture medium reverses the cell cycle arrest, however, the response is more pronounced in wild type BMMCs than in Trib3 knockout cells, with approximately 9% and 4% of cells in S-phase after 48 h, respectively (Fig. 3A). This is accompanied by a difference between the genotypes in the fraction of cells in G1 (75% for wild type and 80% for Trib3−/−) but not in G2/M-phase (approximately 16% for both genotypes) (Fig. 3A). In the continuous presence of IL-3, the proliferation rate of BMMCs appears unaltered by the absence of Trib3 (Supplementary Fig. S3), indicating an effect of Trib3 specifically on the response to growth factor deprivation.

To study whether Trib3 affects the survival of BMMCs subjected to growth factor withdrawal, cell viability analysis of BMMCs originating from Trib3+/+ and Trib3−/− mice was performed over the course of prolonged IL-3 deprivation. The results shown in Fig. 3B demonstrate that while 24 h of IL-3 deprivation only...
slightly reduces cell culture viability, the anti-apoptotic effect of IL-3 becomes readily apparent after 48 h in IL-3-deficient growth medium, which decreases the viability of wild type BMMCs to 65%. At this time-point, the viability of Trib3−/− BMMCs is significantly lower (53%), and the accelerated rate of cell death in Trib3-deficient BMMCs is also evident following 3 days of IL-3 starvation, by which time only 22% of Trib3−/− BMMCs remain viable, compared to 36% for Trib3+/+ cultures (Fig. 3B). The binding of monomeric IgE to the IgE receptor on the surface of mast cells can increase resistance to cytokine deprivation [25]. The addition of IgE into the IL-3-deficient growth medium increased the survival of both wild type and Trib3 knockout BMMCs, however, similarly to the results without IgE, the progression of cell death was accelerated in Trib3−/− BMMCs (Fig. 3B). Thus, Trib3 deficiency renders BMMCs more susceptible to the growth-inhibitory as well as the pro-apoptotic effects of IL-3 deprivation.

3.4. Trib3 deletion impairs BMMC degranulation and cytokine induction but PCA in mice is unaffected

The activation of mast cells through cross-linking of the high affinity IgE receptor by multivalent antigen leads to the release of the granular content of the cells into the surrounding environment [13]. To study the effect of Trib3 deletion on the ability of BMMCs to degranulate, we sensitized the cells with anti-DNP IgE, followed by stimulation with DNP-BSA, and measured the release of β-hexosaminidase, an enzyme stored in mast cell granules [26]. Trib3−/− BMMCs demonstrate significantly reduced degranulation compared to Trib3+/+ BMMCs, releasing on average 14% and 30% of total β-hexosaminidase, respectively (Fig. 4A). The impairment of degranulation is observed over a wide range of antigen concentration, extending from 10 to 1000 ng/ml of DNP-BSA (Fig. 4B). For both genotypes, BMMCs exposed to DNP-BSA without previous sensitization with anti-DNP IgE released less 2% of total β-hexosaminidase (Fig. 4A). Degranulation can also be initiated by pharmacological agents that bypass early IgE receptor-mediated signals. When treated with ionomycin, a calcium ionophore, in conjunction with PMA, Trib3−/− BMMCs display significantly reduced degranulation compared to their wild type counterparts, releasing 45% and 63% of total β-hexosaminidase, respectively (Fig. 4C). In vivo, the activation-induced extrusion of mast cell granule-associated mediators leads to an immediate inflammatory response, including tissue swelling due to increased vascular permeability [11]. When subjected to PCA, vascular permeability indicated by Evans blue extravasation was on average 1.2-fold lower in Trib3−/− mice compared to Trib3+/+ mice, however, the difference did not reach statistical significance (Fig. 4D).

4. Discussion

Cultivation of mouse bone marrow cells in the presence of the hematopoietic cytokine IL-3 gives rise to a primary culture of mast cells which are dependent on IL-3 for growth and survival. In this article, we study the expression of Trib3 in mouse BMMCs and characterize mast cells generated in vitro from mice with a targeted deletion of Trib3.

Our results show that IL-3 upregulates Trib3 mRNA level in BMMCs, a novel facet of Trib3 gene expression regulation. IL-3 signals through a heterodimeric receptor that consists of a cytokine-specific alpha chain, and a beta chain which is involved in signal transduction [28]. Two different beta subunits exist in mice: one functions as a common beta chain for the IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF) receptors, and the other only forms a high-affinity receptor with the IL-3-specific alpha subunit [29]. IL-3 exerts its biological effects by modulating at least three signaling pathways (Jak/STAT, MAPK and PI3 K), enabling it to regulate cellular responses such as growth, differentiation and survival [28]. Few previous reports have described Trib3 expression to be regulated by growth factors. In the cytokine-dependent hematopoietic cell line TF-1, Trib2 mRNA level was shown to be downregulated following GM-CSF deprivation [30]. As mentioned above, GM-CSF signaling is closely related to that of IL-3, and the TF-1 cell line can be grown similarly using either IL-3 or GM-CSF [31]. Which signal transduction pathways modulate Trib3 expression downstream of the IL-3 or GM-CSF receptors is currently not known. For the induction of Trib3 by Epo in erythroid progenitor cells, STAT5 was implicated as a proximal signaling component downstream of the Epo receptor [19].
STAT5 is also a critical STAT family member for IL-3 signal transduction in mast cells [32]. In addition, Trib3 expression is induced in response to insulin in hepatocyte and adipocyte cell lines via a mechanism that requires PI3 K [33], a pathway targeted also by IL-3 and related cytokines. Numerous studies have documented the upregulation of Trib3 in response to different forms of cellular stress, including essential amino acid deprivation, glucose deprivation, endoplasmic reticulum (ER) stress and oxidative stress [3,4,34–37]. It appears that the upregulation of stress-responsive genes by hematopoietic growth factors is not limited to Trib3. For instance, genes induced along with Trib3 by amino acid deprivation via the eIF2α-ATF4 cellular stress response pathway include Atf3 and Atf5 [38–41]. Atf3 is also upregulated by IL-3 in mouse BMMCs [42], and Atf5 is downregulated in response to IL-3 deprivation in the IL-3-dependent cell line FL5.12 and in mouse bone marrow cells [43]. Moreover, recently Kurata et al. [44] reported that IL-3 treatment of pro-B cell line BaF3 enhanced the transcription of Xbp1 and induced the phosphorylation of the ER stress sensor protein IRE1, resulting in the activation of the IRE1-XBP1 branch of the unfolded protein response (UPR) pathway. Trib3 is also part of the UPR program, as a downstream target of the ER stress sensor protein PERK [45]. It is conceivable that in hematopoietic cells exposed to IL-3, moderate expression of stress response genes facilitates general cell functions, owing to the adaptive nature of the stress response, or that these genes are used more broadly to regulate processes

Fig. 4. The influence of Trib3 deficiency on mast cell functions. (A) Trib3−/− BMMCs demonstrate diminished ability to degranulate in response to FcεRI cross-linking, compared to Trib3+/+ BMMCs. Cells were sensitized overnight with 1 μg/ml anti-DNP IgE or left untreated, and exposed to DNP-BSA conjugate (500 ng/ml). The amount of β-hexosaminidase released into the supernatant is presented as percent of the total amount. The mean ± SEM is shown from six pairs of Trib3−/− and Trib3+/+ BMMC cultures derived from individual mice. (B) Degranulation of Trib3−/− BMMCs in response to FcεRI cross-linking is defective over a wide range of antigen concentration. Cells from a representative pair of Trib3+/+ and Trib3−/− BMMCs were sensitized with anti-DNP IgE (0.5 μg/ml) and treated with the indicated concentrations of DNP-BSA. The percentage of β-hexosaminidase released into the supernatant is shown. (C) Trib3-deficient BMMCs display decreased degranulation in response to treatment with ionomycin in combination with PMA. Degranulation was quantified by measuring β-hexosaminidase release. The mean ± SEM of five independent pairs of Trib3+/+ and Trib3−/− BMMC cultures is shown. (D) Analysis of IgE-mediated passive cutaneous anaphylaxis by Evans blue dye extravasation. Trib3+/+ and Trib3−/− mice (n = 7 for each genotype) were injected intradermally with anti-DNP IgE in the left ear and with vehicle alone (PBS) in the right ear. After 20 h, DNP-BSA and Evans blue dye were injected intravenously. Extravasated dye was extracted from tissue into formamide, and the mean absorbance at 620 nm ± SEM is shown. (E and F) Expression of TNF (E) and IL-6 (F) mRNA in response to FcεRI cross-linking is diminished in Trib3−/− BMMCs compared to Trib3+/+ BMMCs. Cells were sensitized overnight with anti-DNP IgE (0.5 μg/ml) and exposed to DNP-BSA (200 ng/ml) for the time indicated. Gene expression was analyzed by RT-qPCR, using Rpl7a mRNA as the endogenous reference. The means ± SD from a representative pair of Trib3+/+ and Trib3−/− BMMCs are shown. Expression levels are presented relative to the level of the respective gene in unstimulated (0 min DNP-BSA) wild type cells. *P < 0.05 comparing genotypes. n.s., not significant.
such as cell viability. The results presented in this paper suggest that Trib3 expression is induced by IL-3 at the transcriptional level, however, the precise transcription factors involved remain unknown. Possible candidates include ATF and C/EBP family transcription factors, which bind as heterodimers to the C/EBP-ATF composite sites that reside in both the Trib3 and Atf3 promoters and are necessary to activate the transcription of the genes during cellular stress [3,35,39].

When challenged with IL-3 deprivation, Trib3−/− BMMCs demonstrate reduced viability compared to wild type BMMCs. Trib3 has been previously implicated as a regulator of cell viability in response to nutritional and ER stress. As in the case of BMMCs deprived of IL-3, Trib3 acts as a pro-survival factor in glucose-starved PC-3 prostate carcinoma cells [37] and in nutrient-starved SaOS2 osteosarcoma cells [4]. The pro-survival effects of Trib3 during glucose and essential amino acid deprivation can be attributed to inhibition of protein–protein interactions with ATP4 and with CHOP, a transcriptional target of ATF4 [3,4]. In line with our IL-3 deprivation results, Trib3 also acts as an anti-apoptotic factor in erythroid progenitor cells starved of Epo [19], however, the mechanisms by which Trib3 regulates hematopoietic cytokine deprivation-induced cell death remain unknown. In mast cells, pro-apoptotic Bcl-2 family members Bim and Bax are essential for IL-3 deprivation-induced cell death [46,47]. Recently, Altman et al. [48] reported that CHOP is induced after IL-3 withdrawal and is required for triggering cell death, and mediates the upregulation of Bim and Bax expression. Thus, the diminished survival of Trib3−/− BMMCs during IL-3 deficiency might be a result of increased CHOP activity due to the lack of Trib3 inhibitory effect.

When subjected to analysis of mast cell immunological functions, Trib3-deficient BMMCs revealed a reduced ability to degranulate and induce cytokine (TNF and IL-6) mRNA expression in response to stimulation. Interestingly, PCA in the mouse ear was not significantly affected by Trib3 deletion, highlighting the possibility of compensatory mechanisms being activated in dermal mast cells in vivo or the diversity of mast cell subtypes. In Trib3−/− BMMCs, degranulation evoked by FceRI aggregation is markedly impaired, and, additionally, degranulation in response to treatment with calcium ionophore and PMA is also reduced, although to a slightly lesser degree. Since the pharmacological manipulation of intracellular calcium levels bypasses the IgE receptor and the signaling immediately downstream of it, this result indicates that the degranulation defects caused by the absence of Trib3 are not attributable to an altered signaling at the IgE receptor. In Trib3−/− BMMCs treated with anti-tumor compound, Trib3 has been shown to reduce the activity of mTORC1 by inhibiting the activity of the protein kinase Akt, leading to increased autophagy [7]. In BMMCs, autophagy appears to be constitutively induced, as determined by the lipidification of LC3, and required for mast cell function [49]. However, the disruption of autophagy via the genetic deletion of Atg7 results in defective IgE-dependent degranulation but not cytokine induction [49], a notable difference from the effects of Trib3 deficiency. The multitude of functional defects in Trib3−/− BMMCs (encompassing IgE-dependent and -independent degranulation, and cytokine induction) suggests a suppression of activation signaling in general. The ablation of a central downstream component of the signaling cascade, such as protein kinase Cζ [50], or the impairment of calcium mobilization, for example by the deletion of the calcium sensor STIM1 [51], produces a similar broadly defective BMMC phenotype, although with increased severity. Growth factors also influence the properties of immune cells, and the activity of different types of mast cells has been shown to be enhanced by the presence of IL-3 [52,53]. Since Trib3 deficiency renders mast cells more sensitive to IL-3 withdrawal, it is possible that certain aspects of growth factor signaling are attenuated in Trib3−/− BMMCs in the presence of IL-3 as well, leading to decreased responsiveness to stimulation. Taking into account that Trib3 mRNA expression is upregulated by IL-3, the attenuation of IL-3-dependent processes in Trib3−/− BMMCs implies that Trib3 acts as a mediator of a subset of IL-3 functions, including the preservation of cell function and viability. Further studies are needed to assess the effector pathways of IL-3 signaling with regard to the role and regulation of Trib3.  

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Appendix A. Supplementary data  
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2012.11.011.

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