

Transcription factor E3, a major regulator of mast cell-mediated allergic response

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Background: Microphthalmia transcription factor, an MiT transcription family member closely related to transcription factor E3 (TFE3), is essential for mast cell development and survival. TFE3 was previously reported to play a role in the functions of B and T cells; however, its role in mast cells has not yet been explored.

Objective: We sought to explore the role played by TFE3 in mast cell function.

Methods: Mast cell numbers were evaluated by using toluidine blue staining. FACS analysis was used to determine percentages of Kit and FcεRI double-positive cells in the peritoneum of wild-type (WT) and TFE3 knockout (TFE3^{-/-}) mice. Cytokine and inflammatory mediator secretion were measured in immunologically activated cultured mast cells derived from either knockout or WT mice. *In vivo* plasma histamine levels were measured after immunologic triggering of these mice.

Results: No significant differences in mast cell numbers between WT and TFE3^{-/-} mice were observed in the peritoneum, lung, and skin. However, TFE3^{-/-} mice showed a marked decrease in the number of Kit⁺ and FcεRI⁺ peritoneal and cultured mast

cells. Surface expression levels of FcεRI in TFE3^{-/-} peritoneal mast cells was significantly lower than in control cells. Cultured mast cells derived from TFE3^{-/-} mice showed a marked decrease in degranulation and mediator secretion. *In vivo* experiments showed that the level of plasma histamine in TFE3^{-/-} mice after an allergic trigger was substantially less than that seen in WT mice.

Conclusion: TFE3 is a novel regulator of mast cell functions and as such could emerge as a new target for the manipulation of allergic diseases. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Allergy, mast cells, degranulation, histamine, cytokine secretion, transcription factor E3 knockout mice

Mast cells, the primary effectors of immediate-type allergic reactions, reside in peripheral tissues that directly associate with the external environment. In most allergic reactions the allergen is recognized by IgE, which is bound to the surface high-affinity receptor for IgE (FcεRI) on mast cells. Aggregation of this receptor initiates the intracellular signals that lead to the release of multiple granular mediators, as well as cytokines that are responsible for the immediate hypersensitivity reaction.

Kit/CD117 (Kit) is another cell-surface receptor on mast cells that is crucial for mast cell differentiation, proliferation, and survival. The expression of Kit is mainly regulated by microphthalmia transcription factor (MITF), a member of the MiT family of transcription factors. The MiT family of transcription factors is composed of 4 closely related members: MITF, transcription factor E3 (TFE3), TFEB and TFEC.¹

It is well established that MITF, the most investigated protein of this family, plays a critical role in the regulation of mast cell differentiation.²⁻⁴ Genetic and biochemical studies have revealed a functional overlap of MiT activity in certain developmental lineages.⁵⁻⁸ Specifically, Jenkins' group⁷ demonstrated, by using knockout mice, that although mutation of either MITF or TFE3 in mice does not disrupt osteoclast development, mutation of both genes or the presence of a dominant negative MITF resulted in severe osteopetrosis. Phylogenetic analysis shows that of the 4 MiT family members (see Fig E1 in this article's Online Repository at www.jacionline.org), TFE3 is the closest family member to MITF. In light of the previous observations regarding MITF and TFE3 redundancy⁷ and the similarity between MITF and TFE3, the role played by TFE3 in mast cell function was explored in this work.

TFE3 has been shown to play a major role in the immune system because of its regulation of CD40 ligand expression in

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Abbreviations used

BMMC: Bone marrow–derived mast cell
 ChIP: Chromatin immunoprecipitation
 Gzmb: Granzyme B
 Mcpt4: Mast cell protease 4
 MITF: Microphthalmia transcription factor
 PE: Phycoerythrin
 siRNA: Small interfering RNA
 TFE3: Transcription factor E3
 Tpsb2: Mast cell protease 6
 WT: Wild-type

T cells.⁹ In addition, a considerable decrease in the expression of the low-affinity receptor for IgE (CD23) on B cells derived from stem cells deficient in TFE3 was observed.¹⁰ This receptor has an important role in the control of allergic diseases.¹¹ It was previously reported that TFE3 compensated for MITF deletion in the development of osteoclasts in MITF-deficient mice⁷ and that these 2 transcription factors have overlapping roles in their functions as transcriptional mediators in the macrophage colony-stimulating factor pathway.^{7,12}

Using TFE3-deficient mice and knocking down TFE3 in mast cells derived from MITF-deficient mice, we explored the specific role played by TFE3 in mast cell function. The results of these 2 combined approaches suggest that TFE3 compensates for the lack of MITF by means of regulation of MITF target genes. Furthermore, TFE3 was found to be a novel regulator of the α subunit of the high-affinity IgE receptor (Fc ϵ RI α). Together, these findings indicate that TFE3 has a critical role in mast cell function.

METHODS**Mice**

VGA-9^{tg/+} and TFE3^(fcr) mice were kindly provided by H. Arnheiter (National Institutes of Health, Bethesda, Md). Mice carrying the *tg/tg* mutation have an insertion of approximately 50 copies of a transgene integrated inside the MITF-M promoter and are unable to express MITF.¹³ Normal littermates of *tg/tg* and TFE3^(fcr) mice were determined by means of genomic DNA extraction from tails and subsequent PCR analysis. Primers for detection of the transgenes are specified in the Methods section in this article's Online Repository at www.jacionline.org.

Cells

For bone marrow–derived mast cells (BMMCs), femoral bone marrow cells derived from wild-type (WT) C3H, TFE3^(fcr), *tg/tg*, and *tg/+* mice were cultured for 4 to 8 weeks to generate BMMCs, as previously described (see additional information in the Methods section in this article's Online Repository).¹² The level of differentiation to BMMCs was verified by using toluidine blue staining.

For details about resident peritoneal mast cells, see additional information in the Methods section in this article's Online Repository.

Gene silencing

TFE3 siGENOME SMARTpool small interfering RNA (siRNA) oligomers were purchased from Dharmacon (Thermo Fischer, Waltham, Mass). SiRNA was delivered by means of electroporation with Amaxa nucleofection solution V, program T-17 (Amaxa Biosystems, Cologne, Germany). BMMCs (1.5×10^6) were electroporated with either 0.2 nmol/L siRNA (approximately 3 μ g) or scrambled nonrelevant siRNA as control. Protein levels were monitored 24,

48, and 72 hours after transfection to evaluate silencing of TFE3 protein. The levels of TFE3 silencing were determined for each biological repeat independently by using real-time PCR and varied from 50% to 70%.

Antibodies

Anti-TFE3 H-300 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif); clone MAR-1 of phycoerythrin (PE)–conjugated anti-Fc ϵ RI and clone 2B8 of PE-CY5–conjugated anti-Kit antibodies were purchased from eBioscience (San Diego, Calif).

Immunological activation

BMMCs were treated with 1 μ g/mL IgE anti-DNP (Sigma-Aldrich, St Louis, Mo) for 2 hours and then washed 3 times in serum free medium and incubated with DNP-albumin (Sigma) for 2 to 8 hours. Cells were then collected and analyzed for the various procedures.

PCR and real-time PCR

BMMCs were immunologically activated, and then RNA was extracted with the RNeasy Kit (Qiagen GmbH, Hilden, Germany), followed by DNase treatment (Qiagen). RNA was quantified with a Nanodrop spectrophotometer and reverse transcribed with random primers (Promega, Madison, Wis) and MMLV reverse transcriptase (Invitrogen, Carlsbad, Calif).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) from *tg/tg* BMMCs was performed as previously described.¹⁴ For further details, see the Methods section in this article's Online Repository.

Measurements of secreted mediators

BMMCs (3×10^6) were treated with 1 μ g/mL IgE anti-DNP (Sigma-Aldrich) for 4 hours and then washed 3 times in PBS and incubated with 50 ng/mL DNP-albumin (Sigma) for 20 hours in complete growth medium. Supernatants were collected, and IL-4, IL-6, granzyme B (Gzmb), and TNF- α content was measured by using ELISA kits (eBioscience). Cells were counted by using flow cytometry, and readings were normalized to total protein levels of the cell lysates. The BD cytometric bead array (BD Biosciences, San Jose, Calif) was also used as a second method to measure secreted mediator levels by means of flow cytometry.

In vivo histamine release assay

TFE3^{-/-} mice and WT control animals were sensitized with 3 μ g of the murine IgE anti-DNP mAb SPE-7 by means of intravenous injection in the tail vein. Twenty-four hours later, the mice were challenged with intravenous injection of DNP-BSA (250 μ g per mouse). Blood histamine levels were determined by using competitive ELISA (IBL International GmbH, Hamburg, Germany) on approximately 100 μ L of blood 1.5 minutes after antigen challenge.

Further method details

Other methods used were flow cytometry and immunofluorescence; histological analysis of mast cells; and the b-Hexosaminidase release assay. The details of these methods can be found in the Methods section in this article's Online Repository.

Statistical analysis

Statistical analysis was conducted with Microsoft Excel 2007 software (Microsoft, Redmond, Wash). Mutant mice and their normal littermates were compared by using 2-tailed Student *t* tests. Data are reported as means $\pm$ SEs.

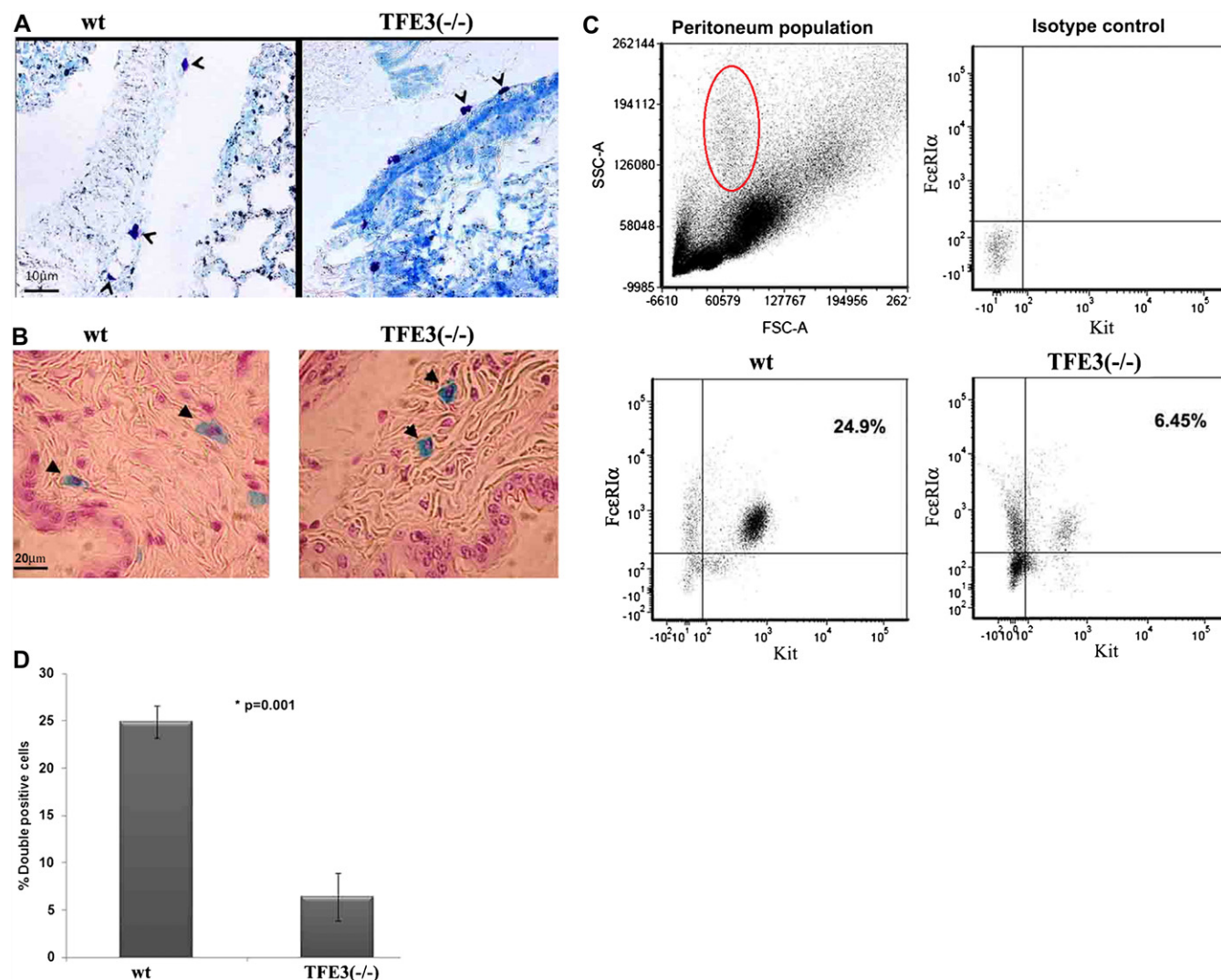


FIG 1. Evaluation of mast cell numbers in WT and TFE3^{-/-} mice. **A** and **B**, Toluidine blue (Fig 1, **A**) and Alcian blue (Fig 1, **B**) staining of lung (Fig 1, **A**) and skin (Fig 1, **B**) sections of WT and TFE3^{-/-} mice. Mast cells are indicated with black arrows. One representative result of 7 experiments is shown. **C**, Flow cytometry of peritoneal mast cells. Dot plot analysis of FcεRIα and Kit staining of the peritoneal cell population of TFE3^{-/-} and WT mice is shown. One representative result of 7 experiments is shown. **D**, Bar diagram of the mean percentage of cells double stained with FcεRIα and Kit in the peritoneal population derived from WT and TFE3^{-/-} mice. Results represent means ± SEMs (n = 7, P ≤ .001).

RESULTS

Evaluation of mast cell numbers in TFE3^{-/-} mice

The number of mast cells in the lung, skin, and peritoneal cavity derived from WT and TFE3^{-/-} mice was determined to evaluate whether TFE3 is involved *in vivo* in regulating mast cell numbers.

No significant decrease in mast cell numbers was observed in lung and skin sections of TFE3^{-/-} compared with WT mice (63.7 ± 9.3 vs 53.5 ± 7.1 mast cells/mm² and 13.8 ± 1.4 vs 16.3 ± 1.5 mast cells/mm² for lung and skin sections, respectively; Fig 1, **A** and **B**). Next, the peritoneal populations of both WT and TFE3^{-/-} mice were stained with toluidine blue. No significant difference was observed in the percentage of positive stained cells between WT ($2.5\% \pm 0.3\%$) and TFE3^{-/-} ($2.3\% \pm 0.2\%$) mice. Further characterization of peritoneal mast cells was carried out by

staining for the mast cell markers FcεRIα and Kit. Analysis of double-stained cells was determined by using flow cytometry. Because in adult mice only about 2% of the population of the peritoneal cavity is mast cells (see Fig E2, **A**, in this article's Online Repository at www.jacionline.org), the granular cell population was first defined by using high side scatter and low forward scatter values and verified by means of reverse gating of the Kit and FcεRIα double-stained population (see Fig E2, **C**).

As shown in Fig 1, **C** and **D**, in WT peritoneum about 25% of the high side scatter and low forward scatter population of cells were stained for both Kit and FcεRIα. However, in TFE3^{-/-} peritoneum a considerable decrease of about 4-fold in the double-stained population was noted. Fig 1, **D**, shows a bar diagram of the percentage of double-stained cells in WT and TFE3^{-/-} mice.

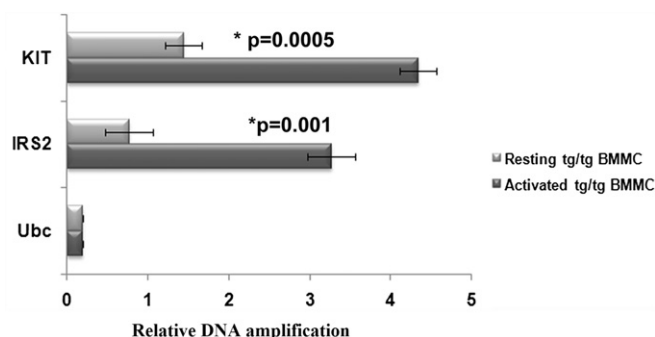


FIG 2. TFE3 directly associates with the Kit promoter. ChIP of BMMC lysate from quiescent or 4-hour-activated WT and *tg/tg* BMMCs with TFE3 antibody is shown. The extracted DNA was quantified, equalized, and used as a template for real-time PCR with specific primers to detect the Kit promoter. Results represents means $\pm$ SEMs ($n = 3$, $P \leq .001$).

TFE3 directly associates with Kit promoter *in vivo*

The Kit tyrosine kinase receptor plays an essential role in the development and survival of mast cells.² It was shown that MITF binds to the E-box motif in the promoter of the Kit gene and initiates its transcription.^{2,15} Because MITF/TFE3 heterodimers are thought to form *in vivo*, where they might regulate cell type-specific genes,^{7,15,16} a method to discriminate TFE3 from MITF binding was needed. Therefore we used *tg/tg* mutant mice, which are a virtual null mutation of MITF (see the **Methods** section). Determination of the direct interaction of TFE3 with the Kit promoter was carried out by using ChIP from immunologically activated BMMCs derived from both *tg/tg* and WT mice (see Fig E3 in this article's Online Repository at www.jacionline.org). The immunoprecipitated DNA fragment was amplified by using specific primers flanking the E-box element on the Kit promoter. As shown in Fig 2, TFE3 binds the Kit promoter in the absence of MITF. Basal binding of the Kit promoter was observed in resting cells, whereas a considerable 4-fold increase in the PCR product was observed after immunologic activation. Primers to detect TFE3 recognition sites in the insulin receptor substrate 2 (IRS2) promoter, a known TFE3 target gene, were used as a positive control. No amplification of nonspecific DNA was observed (ubiquitin C).

Redundant role for TFE3 and MITF in cultured mast cells

A redundant role for MITF and TFE3 in osteoclast development was reported previously.⁷ BMMCs derived from WT and MITF knockout (*tg/tg*) mice were used as an *in vitro* model for studying possible redundancy to further study the relations between these 2 transcription factors in mast cells. Initially, we studied the effect of IgE-antigen on TFE3 mRNA (Fig 3, A). It was found that TFE3 mRNA was upregulated 2-fold following immunologic triggering.

TFE3 was silenced by 70% by using specific siRNA in *tg/tg* BMMCs to further evaluate the role of TFE3 in mast cell function (Fig 3, B). The transcript levels of Kit and 3 other MITF target genes, *Gzmb*, mast cell protease 4 (*Mcp4*), and mast cell protease 6 (*Tpsb2*), were determined by using real-time PCR 48 hours after silencing. As shown in Fig 3, C, even in the absence of MITF, mRNAs of all these 4 MITF target genes were detected in *tg/tg* BMMCs. However, 48 hours after silencing of TFE3, a decrease

in the expressions of Kit, *Gzmb*, and *Tpsb2* were observed. When *tg/tg* BMMCs were immunologically stimulated, MITF target genes were induced by 2-fold (Fig 3, D). However, after TFE3 silencing, there was a considerable decrease in the response to activation (Fig 3, D). Thus in the absence of MITF, TFE3 can modulate the expression of MITF target genes.

TFE3 transcriptionally regulates FcεRIα expression

The flow cytometric results of FcεRI staining in TFE3^{-/-} mice suggested that TFE3 is not only a coregulator of MITF target genes but might also affect FcεRI expression (see Fig E2, D). FcεRIα transcript expression was monitored 48 hours after TFE3 silencing to further explore the possible effect of TFE3 on FcεRI expression in WT BMMCs. After TFE3 silencing, a decrease of 65% $\pm$ 15.4% was observed in FcεRIα transcript expression (Fig 4, A). Furthermore, in BMMCs derived from TFE3^{-/-} mice, a decrease of 47% $\pm$ 6.6% in FcεRIα transcript expression was observed compared with the amount of this transcript detected in the WT BMMCs (Fig 4, B). Similar results were obtained with peritoneally derived cells (results not shown).

Previously, 2 canonical E-boxes (CAGCTG) were identified in the FcεRIα gene and were shown to be used for regulation of FcεRIα by a complex of the upstream stimulatory factors 1 and 2.¹⁶ Because it is well established that TFE3 can bind E-box elements, TFE3's direct interaction with the FcεRIα promoter was evaluated by using ChIP in immunologically activated BMMCs derived from both *tg/tg* and WT mice. As shown in Fig 4, C, TFE3 binds the FcεRIα promoter in WT BMMCs and also binds the FcεRIα promoter in *tg/tg* BMMCs (Fig 4, D). Hence MITF is not essential for TFE3 binding to the FcεRIα promoter.

Effect of TFE3 on BMMC differentiation

Our accumulated data showed that TFE3 can not only compensate for MITF's absence by regulating MITF target genes but also is itself a regulator of FcεRI expression. BMMCs were cultivated from WT and TFE3^{-/-} mice and stained for both differentiation markers to further investigate the role of TFE3 in the expression of both FcεRIα and Kit during *in vitro* differentiation from bone marrow. Fig 5, A, shows flow cytometric analysis of BMMCs double stained with Kit and FcεRI antibodies. In WT cultures 70.3% $\pm$ 5.8% of the cells were double positive, whereas 25.4% $\pm$ 5.1% of TFE3^{-/-} derived cells were double positive. In addition, in BMMCs derived from *tg/tg* mice, the percentage of double-positive cells was found to be 44.1% $\pm$ 7.3%. The higher percentage than in the TFE3^{-/-} cells suggests an important role for TFE3 in the regulation of mast cell differentiation. It is important to note that more than 90% of the cells from WT, TFE3^{-/-}, and *tg/tg* mice stained positive with toluidine blue after 3 weeks in culture (results not shown). We have been using this method to identify mast cells for the last 30 years.¹⁷

The role played by TFE3 in Kit and FcεRI regulation in MITF-deficient cells was then studied. TFE3 expression was silenced in BMMCs derived from *tg/tg* mice by using specific siRNA. A minor effect on the expression of Kit/FcεRI was detected in BMMCs derived from WT mice (a decrease of 23.3% $\pm$ 8.2%), whereas a considerable decrease of 41.6% $\pm$ 7.4% of double-positive cells was monitored in *tg/tg* BMMCs (Fig 5, B). These results can be explained by both an incomplete effect of TFE3 silencing (50% to 70%) and the presence of sufficient MITF to

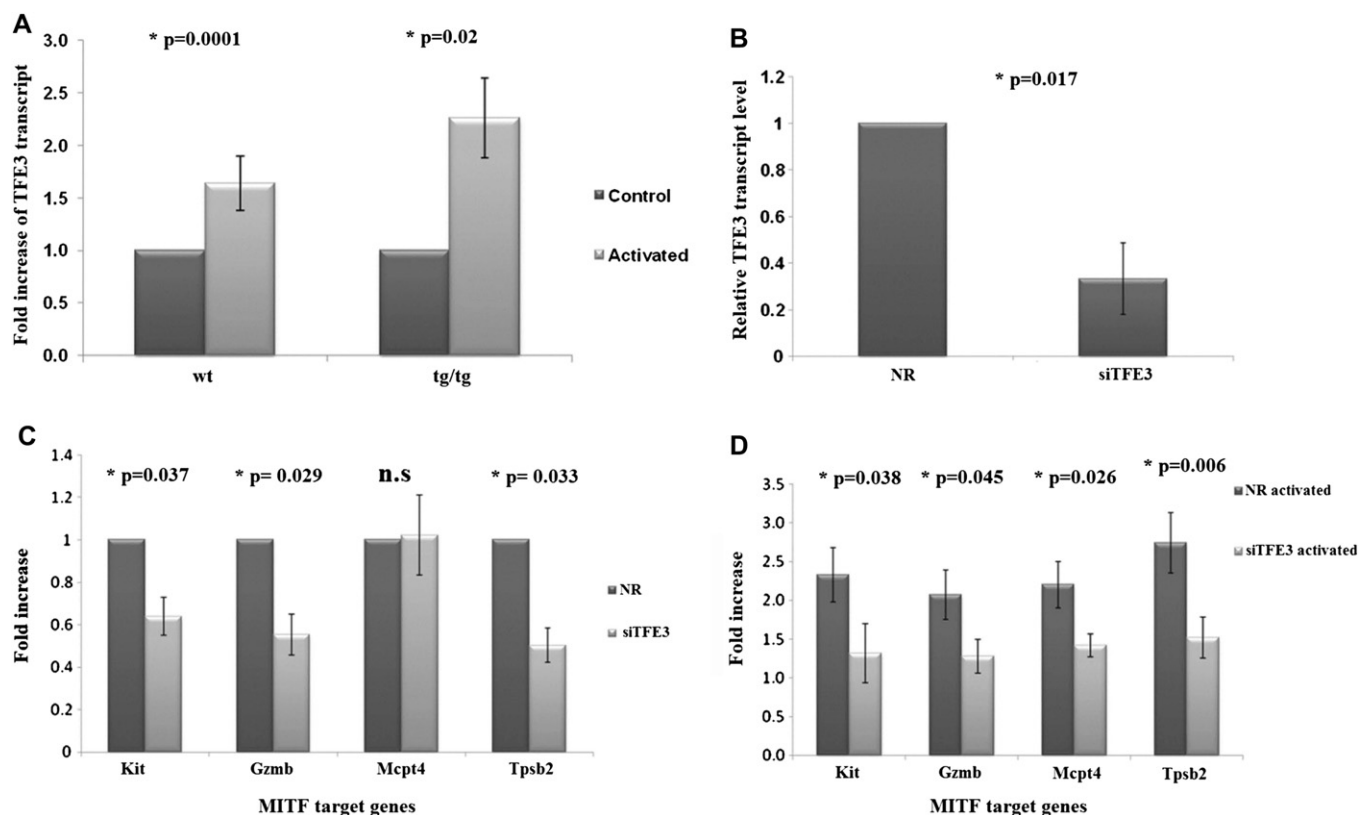


FIG 3. Redundant effect between TFE3 and MITF. **A**, Effect of mast cell activation on expression of TFE3. A marked increase in TFE3 transcript was observed in BMMCs derived from both WT and *tg/tg* mice (1.64 ± 0.26 - and 2.26 ± 0.54 -fold increase, respectively [means $\pm$ SEMs]; $n = 5$). **B**, Quantification of TFE3 transcript in nonrelevant siRNA (NR) and TFE3-specific (*siTFE3*) treated *tg/tg* BMMCs ($n = 6$, $P \leq .017$). **C**, Effect of TFE3 silencing on MITF target genes in *tg/tg* BMMCs. Forty-eight hours after TFE3 silencing, the expression of Kit, Gzmb, Mcpt4, and Tpsb2 transcripts was monitored by means of real-time PCR. Results were normalized to ubiquitin C. Results represent means $\pm$ SEMs ($n = 4$, $P \leq .037$). **D**, Effect of TFE3 silencing on MITF target genes after activation of BMMCs. Results were normalized to ubiquitin C and represent means $\pm$ SEMs ($n = 4$, $P \leq .05$).

maintain Kit expression. The finding that TFE3 silencing affects both Kit and FcεRIα expression in the absence of MITF provides further support to the notion that TFE3 is a regulator of both of these critical molecules in mast cells.

TFE3 and mast cell degranulation

Degranulation was examined in BMMCs derived from TFE3^{-/-} mice and compared with that seen in BMMCs derived from WT mice. Cells were sensitized with IgE for 2 hours and then challenged for 30 minutes with DNP-BSA. Degranulation was evaluated by means of measurement of β-hexosaminidase activity. As shown in Fig 6, A, a considerable decrease in degranulation rate was observed in BMMCs from TFE3^{-/-} mice compared with that seen in BMMCs from WT mice. Although in WT cultures the maximal percentage of degranulation was $31.2\% \pm 4.1\%$, in BMMCs from TFE3^{-/-} mice, the maximal percentage of degranulation decreased to $16.4\% \pm 3\%$. Mast cell mediator secretion 20 hours after an immunologic trigger was also measured in these BMMCs by using 2 methods, as described in the Methods section. Substantial decreases in the levels of secreted IL-6, TNF-α, and Grzb was observed in BMMCs from TFE3^{-/-} mice compared with those seen in BMMCs from WT mice (Fig 6, D). Similar results were obtained by using both methods.

In vivo experiments

The response to allergic stimulation was evaluated in WT and TFE3^{-/-} mice by using the passive systemic IgE-mediated anaphylaxis method to investigate TFE3's role *in vivo*. Mice were intravenously injected with IgE anti-DNP and were challenged 24 hours later with DNP-BSA for 1.5 minutes. Plasma was collected, and histamine levels were measured by using competitive ELISA. A substantial decrease was observed in histamine levels secreted from TFE3^{-/-} mice compared with those from WT mice (Fig 7). These results demonstrate the critical role of TFE3 in mast cell-dependent allergic reactions.

DISCUSSION

Kitamura et al^{2,4} firmly established the critical role played by MITF in mast cell differentiation using MITF mutated mice. This group demonstrated that the absence of MITF resulted in a significant decrease in mast cell number, as assessed based on metachromatic staining. However, mast cell precursors still exist in these MITF-mutated mice because mast cells could be obtained *in vitro* from bone marrow in the presence of IL-3.^{2,3} Mice of the *tg/tg* strain, which were used in our current study, harbor a transgene incorporated into the MITF gene that prevents MITF expression.¹⁸ Previously, it has been shown that members of this

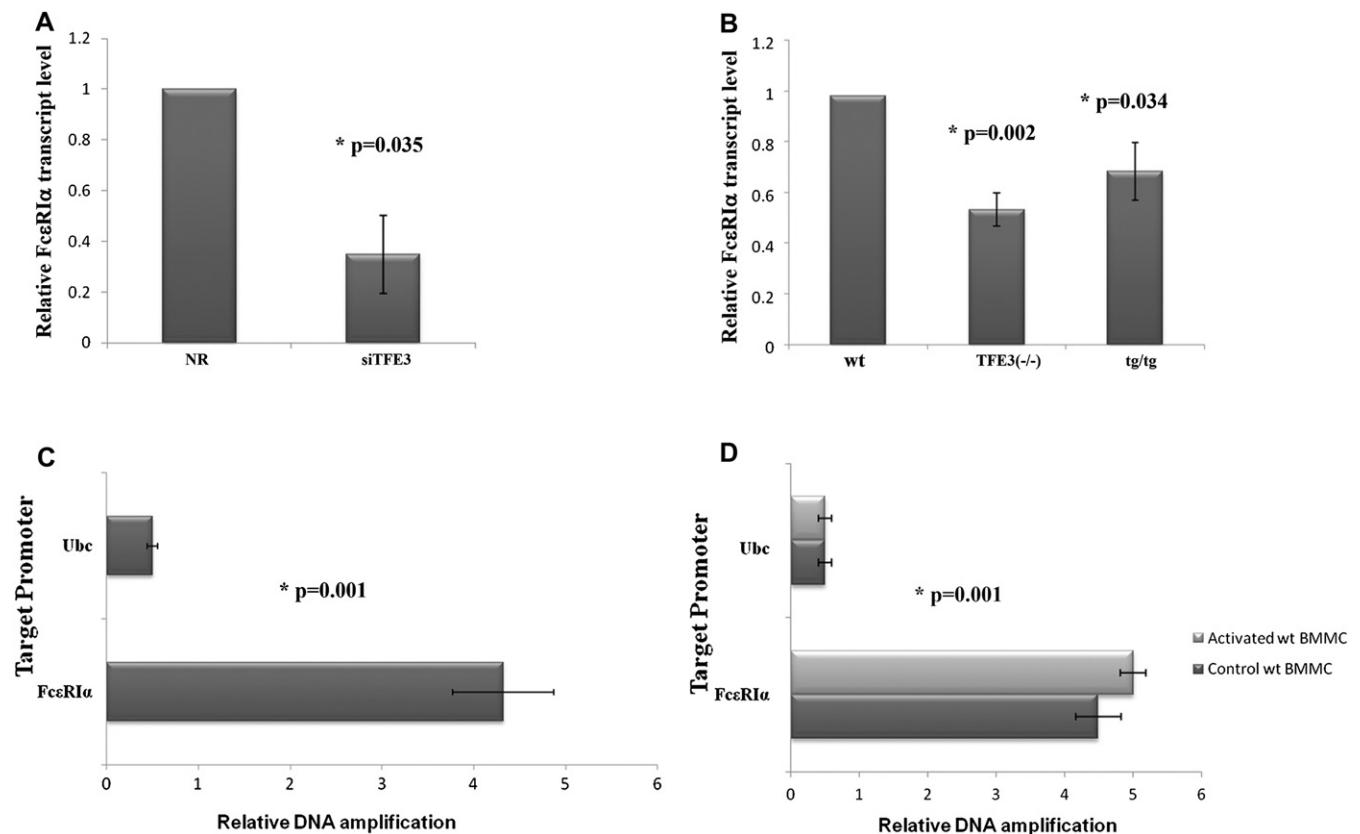


FIG 4. TFE3 directly regulates FcεRIα expression. **A**, Effect of TFE3 silencing on FcεRIα expression in *tg/tg* BMMCs. BMMCs were cultured for 28 days and then treated with nonrelevant siRNA (*NR*) or TFE3 siRNA for 72 hours. Results represent means $\pm$ SEMs ($n = 4$, $P \leq .035$). **B**, FcεRIα transcript levels in BMMCs derived from knockout mice. Results represent means $\pm$ SEMs ($n = 3$, $P \leq .034$). **C**, ChIP of BMMC lysate from WT BMMCs with TFE3 antibody. The extracted DNA was quantified and used as the template for real-time PCR with specific primers to detect FcεRIα promoter. Results represent means $\pm$ SEMs ($n = 3$, $P \leq .001$). **D**, ChIP of BMMC lysate from *tg/tg* BMMCs with TFE3 antibody. Results represents means $\pm$ SEMs ($n = 3$, $P \leq .001$). *Ubc*, Ubiquitin C.

transcription factor family have unique nonredundant roles, yet some of their functions can be replaced by other family members.^{7,19,20} Here we studied both the specific roles of TFE3 using mice lacking the TFE3 gene and the functions of TFE3 in MITF knockout mice. Using these 2 different approaches, we were able to thoroughly explore the role played by TFE3 in mast cells.

We noted a substantial induction in TFE3 mRNA levels after mast cell IgE-antigen stimulation both in normal mast cells and in MITF-deficient mast cells (Fig 3, A). In contrast, we found previously that MITF mRNA is not affected by immunologic activation.²¹ These results indicate that the MITF and TFE3 genes are regulated differently in mast cells.

No change in mast cell numbers, as measured by using toluidine blue staining, was observed in the lungs, skin, and peritoneal cavities of TFE3^{-/-} mice compared with numbers seen in WT mice. Similar results were previously reported by others without mentioning the source of mast cells analyzed.²² However, flow cytometric analysis revealed a substantial decrease in the levels of Kit⁺ and FcεRIα⁺ cells (Fig 2, C), and similar results were obtained by using BMMCs derived from TFE3^{-/-} mice (Fig 5, A).

Kit, a known MITF-regulated gene, serves as a critical receptor for mast cell function and development.^{4,23} Because

of the fact that MITF is known to heterodimerize with TFE3¹ and that the bHLH region of TFE3 plays a role in DNA binding,²⁴ we analyzed whether TFE3 by itself can bind to the Kit promoter using the ChIP experimental approach and found that TFE3 bound specifically to this promoter. Furthermore, this binding was induced by IgE-antigen stimulation. Regulation of Kit is critical for mast cell development, survival, and activity.²⁵ The finding that TFE3 has a critical role in the regulation of Kit, especially in MITF-depleted cells, might have implications for physiologic situations in which MITF is downregulated. It was recently published that TFE3 can regulate GPNMB, a known MITF target gene that is involved in cancer cell metastasis.²⁶ These pathologic circumstances, in which MITF or TFE3 expression is distorted, might reflect the biological significance of the redundancy between the 2 transcriptional factors.

There are numerous mechanisms that regulate MITF expression. These include silencing by specific microRNA^{27,28} and inhibition by cellular repressors, such as protein inhibitor of activated STAT3 (PIAS3)²⁹ and Hint-1.³⁰ Also, regulation of its expression could occur by means of rapid degradation of the protein after its activation by phosphorylation.^{31,32} These mechanisms could be different in the case of TFE3, which differs from MITF in its 3

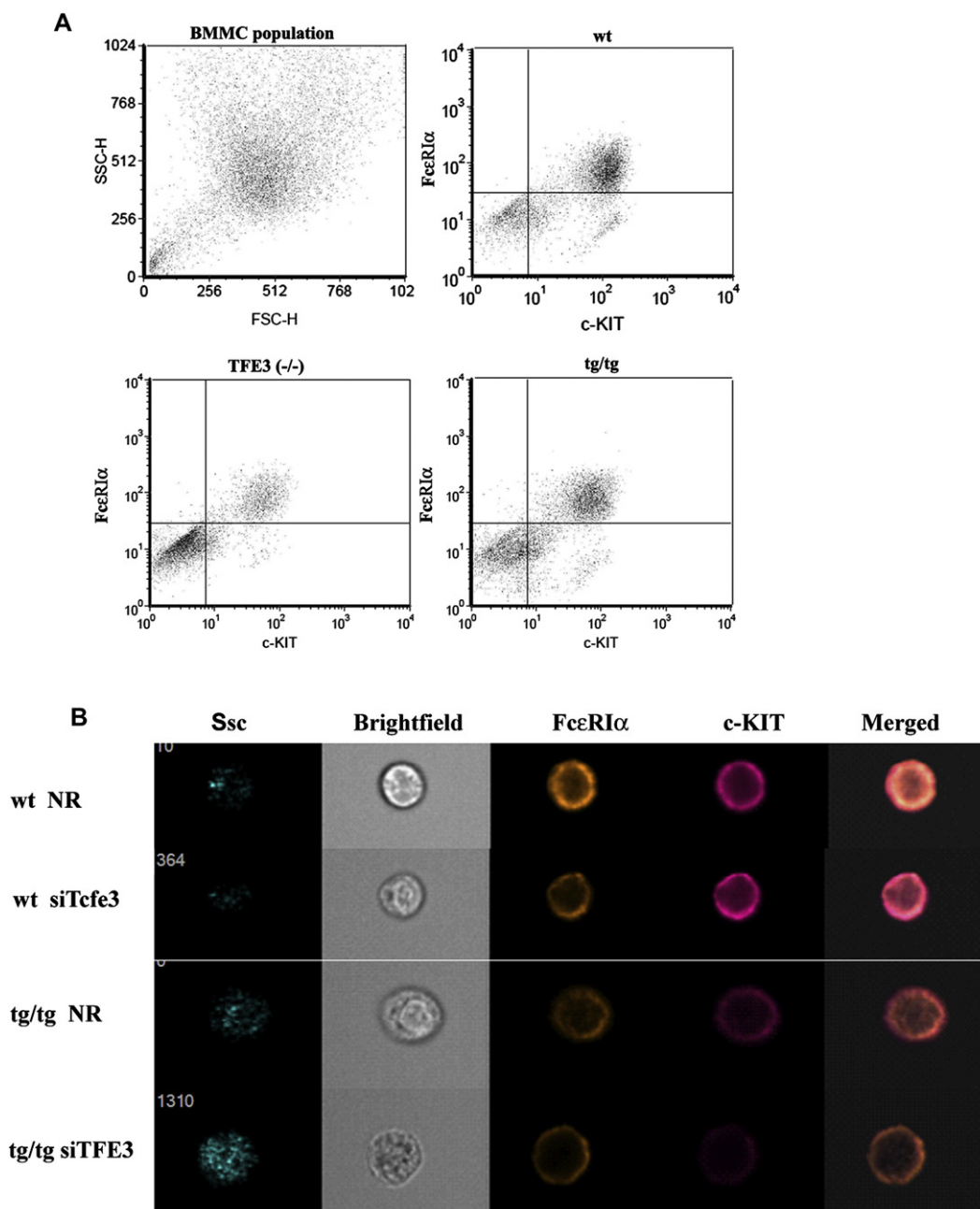


FIG 5. Effect of TFE3 on BMMC markers. **A**, Flow cytometric analysis of BMMCs derived from WT, *tg/tg*, and *TFE3*^{-/-} mice. Cells were stained for FcεRI and Kit and analyzed by using flow cytometry. A dot plot of FcεRI- and Kit-stained cells is shown. One representative result of 5 experiments is shown. **B**, Effect of TFE3 silencing on WT and *tg/tg* BMMC numbers. Cells were double stained with FcεRI and Kit and analyzed by using ImageStream flow cytometry (n = 3, *P* ≤ .05). NR, Nonrelevant siRNA; Ssc, side scatter.

prime mRNA and in the sequence outside the bHLH zipper motif. Moreover, as mentioned above, TFE3 but not MITF²¹ mRNA is induced by IgE-antigen (Fig 3, A). An example of these divergent regulatory mechanisms has been recently described. The kinase Cdc25C-associated kinase 1 (C-TAK1) was shown to interact with MITF but not with TFE3.³³ It also might be expected that TFE3 can regulate genes, even when MITF expression is downregulated.

Thus redundancy between similar transcription factors in the regulation of critical target genes might have physiologic

significance and is probably a general phenomenon. Further research should be carried out to define the circumstances under which this occurs.

Our studies suggest a novel role of TFE3 in the regulation of FcεRIα. The aggregation of this receptor on mast cells initiates the intracellular signals that lead to the release of multiple granular mediators, as well as cytokines that are responsible for immediate hypersensitivity reactions and might result in acute anaphylaxis. In this work we have shown for the first time the regulation of the α subunit of the FcεRI receptor by TFE3.

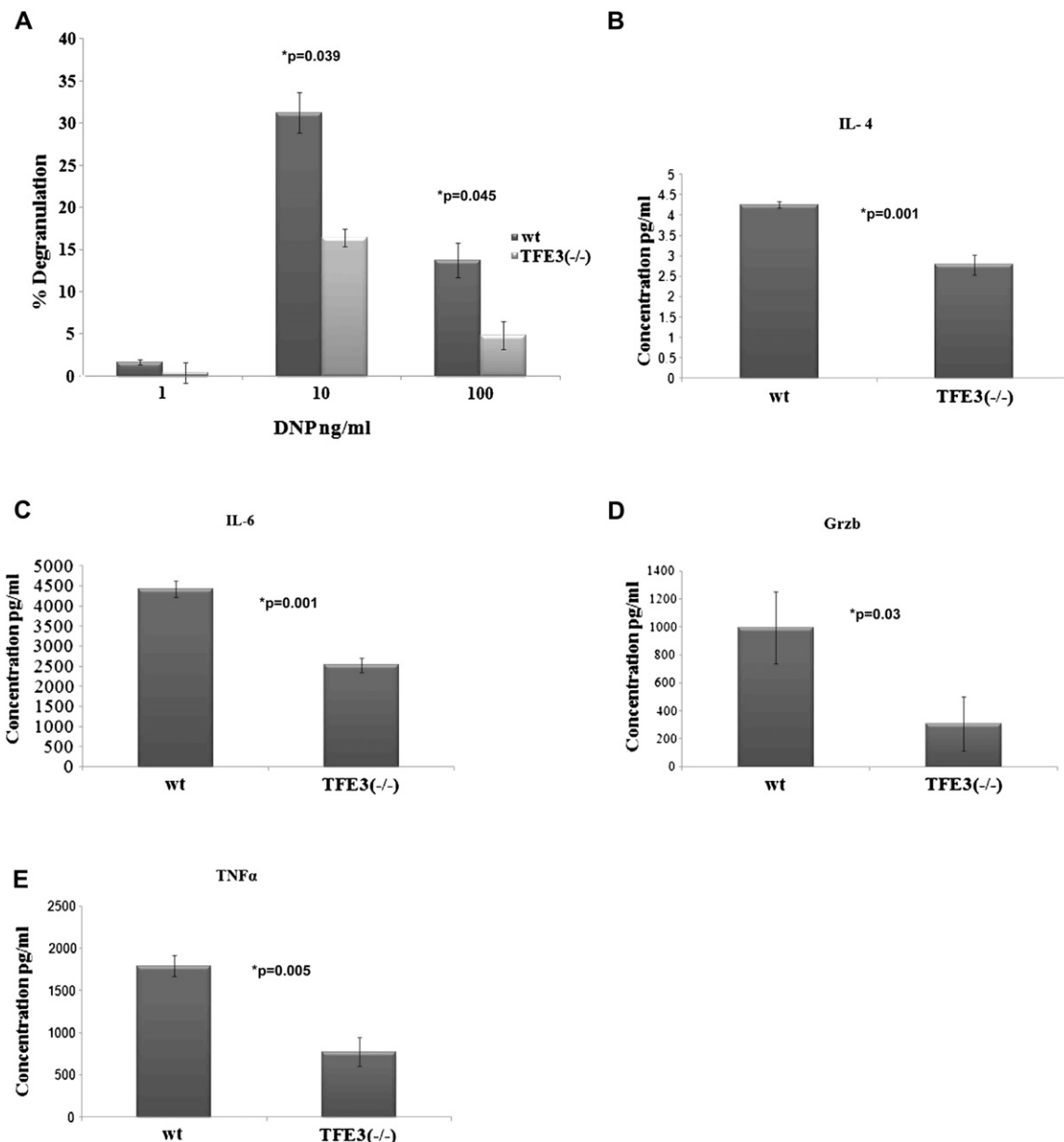


FIG 6. Functional analysis of BMMCs from TFE3^{-/-} mice. **A**, Degranulation assay. Cells were sensitized to IgE/DNP and analyzed for β -hexosaminidase activity ($n = 3$). **B-E**, Analysis of BMMC-secreted mediators. Twenty hours after an immunologic trigger, BMMC supernatants were subjected to ELISA for analysis of mast cell-secreted mediators. The secreted concentrations of IL-4, IL-6, Grzb, and TNF- α were calculated as described in the [Methods](#) section. Results are shown as means $\pm$ SEMs ($n = 3$, $P \leq .03$).

Furthermore, BMMCs cultured from TFE3^{-/-} mice showed decreased cytokine and inflammatory mediator release in response to immunologic stimulus (Fig 6). *In vivo* studies using a previously established acute systemic anaphylaxis model showed a marked decrease in histamine secretion by TFE3^{-/-} mice compared with that seen in WT mice (Fig 7). Therefore our data indicate a novel and important role for TFE3 in mast cell function. As such, TFE3 could serve as a potential target for therapeutic interventions.

TFE3 has been previously shown to have an important regulatory role in B and T cells. In addition, it was found to be a major

regulator of the low-affinity IgE receptor (Fc ϵ R2).¹⁰ Activity of both Fc ϵ R2 and Fc ϵ R1 α has significant effects on IgE-related diseases, such as asthma.³⁴ Its ability to regulate both of these receptors suggests that TFE3 is a critical transcription factor in the regulation of the immune response.

Our findings have revealed a critical role for TFE3 in both MITF-deficient and normal mast cells. The fact that TFE3 is emerging as a major transcription factor in the regulation of mast cell activity has great significance for the field because it provides direct evidence for another target that could be regulated in mast cell-associated diseases.

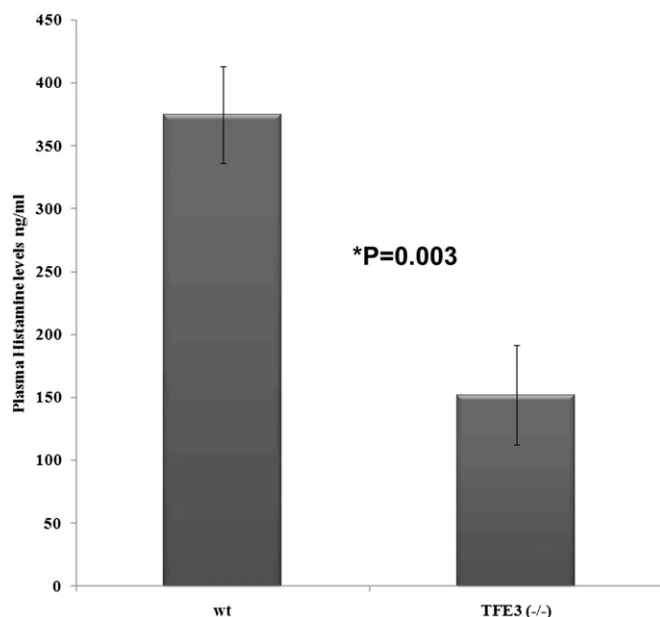


FIG 7. *In vivo* allergic response assay. Mice were sensitized with IgE anti-DNP through injection to the tail vein. Twenty-four hours later, mice were challenged with DNP-BSA for 1.5 minutes. Plasma was collected, and histamine levels were measured by using competitive ELISA. The histamine level in plasma from WT mice was 749.6 ± 76.5 ng/mL compared with 304.2 ± 78.8 ng/mL in plasma cells from TFE3^{-/-} mice ($n = 6$, $P \leq .003$).

We thank H. Arnheiter (National Institutes of Health) for providing TFE3 and VGA9 mutant mice, E. Steingrimsdottir (University of Iceland) for help in mutant analysis, L. Weiss (Department of Bone Marrow Transplantation and Cancer Immunotherapy, Hadassah Hebrew University Medical Center, Jerusalem, Israel) for animal experiments, and D. Lehmann (Hebrew University) for FACS analysis and discussion.

Key messages

- TFE3 regulates major genes in mast cells.
- Mast cells derived from mice lacking TFE3 have significant reduction in their response to immunologic triggers.
- TFE3^{-/-} mice have a reduced response to IgE-induced histamine release.
- TFE3 is potentially a target for antiallergic manipulations.

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METHODS

Mice

VGA-*g^{fl/+}* and TFE3^(fcr) mice were kindly provided by H. Arnheiter (National Institutes of Health). Mice carrying the *tg/tg* mutation have an insertion of approximately 50 copies of a transgene integrated inside the MITF-M promoter and are unable to express MITF.^{E1} Normal littermates of *tg/tg* and TFE3^(fcr) mice were determined by means of genomic DNA extraction from tails and subsequent PCR analysis. Primers for detection of the *tg/tg* transgene were 5' GAA AGC TGG CTG GAG TGC 3' and 5' CCG TAATGG GAT AGG TCA CG 3'. Primers for detecting the WT of the TFE3 allele were TFE3 3GT_F1 5' CGG TTC CCC TGC GTC CTT TTG TTT TGA 3' and TFE3 3GT_R1 5' AGC GCC CCT GAT CCT CTC CCT TCC T 3'. Primer sequences for detecting the knockout allele were kindly provided by E. Steingrimsen (University of Iceland, Reykjavik, Iceland): Neo7 5' GCA TCG CAT TGT CTG AGT AGG TGT C 3' and Ko2 5' GAC TGT AGC TTA CCT ATG GCC 3'. All experiments were performed in compliance with the Israeli Prevention of Cruelty to Animals Law and were approved by the Hebrew University Animal Care and Use Committee.

Cell culture

For BMMCs, femoral bone marrow cells derived from WT C3H, TFE3^(fcr), *tg/tg*, and *tg/+* mice were cultured in RPMI 1640 growth medium containing 10% FCS, 2 mmol/L L-glutamine, 2 mmol/L nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco Invitrogen, Grand Island, NY), and 50 µmol/L β-mercaptoethanol (Fisher Scientific, Medford, Mass). Medium was supplemented with IL-3 collected from B653 supernatant, as previously described.^{E2} Cells were cultured for 4 to 8 weeks to generate BMMCs, as previously described.^{E3} BMMCs were defined when 90% of the cells were stained positively with toluidine blue. All the cells were grown in a humidified incubator at 37°C in a 5% CO₂ atmosphere.

Resident peritoneal mast cells

Mice were killed by means of inhalation of CO₂. Fifteen milliliters of FACS buffer (PBS, 0.5% FCS, and 0.05% sodium azide) was injected into the mice intraperitoneally, and the abdomen was massaged for 0.5 minutes. The buffer containing resident cells was extracted with an 18-gauge needle and syringe. The cells were washed once with FACS buffer and then stained for immunofluorescence analysis.

PCR and real-time PCR

BMMCs were immunologically activated, as mentioned above, and then RNA was extracted with the Rneasy kit (Qiagen GmbH), followed by DNase treatment (Qiagen). RNA was quantified with a Nanodrop spectrophotometer and reverse transcribed with random primer (Promega) and MMLV reverse transcriptase (Invitrogen). cDNA served as a template for both PCR and real-time PCR reactions. All PCR reactions were normalized to ubiquitin C levels. The PCR primers that served to detect TFE3 were as follows: TEF3—5' primer CCT GAA GGC ATC TGT GGA TT and 3' primer CGA TAC ATG GAA CGT TGT GC. The primers used for real-time PCR were as follows: TFE3—5' primer ATT GGG TCC AGC TCA GAA AA and 3' primer TGA TAG CTG GGG TAG CCA CT; ubiquitin C—5' primer CGT CGA GCC CAG TGT TAC CAC CAA GAA GG and 3' primer CCC CCA TCA CAC CCA AGA ACA AGC ACA AG.

Real-time PCR primers for c-KIT, Gzmb, Mcpt4, and Tpsb2 were as follows: c-KIT—5' primer GCC ACG TCT CAG CCA TCT G and 3' primer GTC GCC AGC TTC AAC TAT TAA CT; Gzmb—5' primer CCA CTC TCG ACC CTA CAT GG and 3' primer GGC CCC CAA AGT GAC ATT TAT T; Mcpt4—5' primer AGA AAA GAT CGG CAT ACA AGG G and 3' primer TCT CCG CGT CCA TAA GAT ACA; and Tpsb2—5' primer CTG GCT AGT CTG GTG TAC TC and 3' primer CAG GGC CAC TTA CTC TCA GAA.

ChIP

ChIP from *tg/tg* BMMCs was performed, as described previously.^{E4} In brief, 4×10^6 cells were cultured for 45 days and then immunologically activated, as described above. Cells were collected, washed in PBS, and fixed in

1% formaldehyde for 10 minutes. Samples were sonicated and subjected to immunoprecipitation with TFE3-specific antibody (Santa Cruz Biotechnology). Samples were digested with proteinase K (Roche Applied Science, Mannheim, Germany), and DNA was extracted by means of chloroform precipitation. DNA samples were then quantified, and similar amounts were used as a template for real-time PCR reaction with specific primers that flank the E-box region on c-KIT promoter as follows: 5' primer GGC AGG TGC TCT CAT GAC AAT and 3' primer CCG CAA TCC CGA CTA GTA AC. Specific primers that flank the E-box region of the FcεRIα promoter were as follows: 5' primer ATGGTCACTGGAAGGTCTGC and 3' primer GGGGCAGAGAATAGCATGAA. Ubiquitin C primers (sequence as above) were used to exclude nonspecific DNA precipitation.

Flow cytometry and immunofluorescence

BMMCs or peritoneal cells were washed once in PBS and then blocked for 10 minutes in PBS 0.5% BSA followed by means of incubation with IgG block (eBioscience). Cells were then incubated with PE-conjugated anti-FcεRIα antibody and PE-cy5-conjugated anti-c-KIT antibody (eBioscience) at a 1:300 dilution for 30 minutes on ice.

Stained cells were analyzed by means of flow cytometry (LSRII Flow Cytometer, BD Biosciences). Analysis was performed with the FCS Express V3 software (De-novo Software, Los Angeles, Calif); 1×10^4 BMMCs and 1×10^6 peritoneal cavity cells were collected for each analysis.

For ImageStream analysis (Amnis Corp, Seattle, Wash), BMMCs were fixed in 10% formaldehyde, permeabilized with PBS 0.1% triton, and blocked in PBS 0.5% BSA. Antibody concentrations were used as recommended by the manufacturers. Fluorescence imaging and FACS analysis were carried out with the ImageStream flow cytometer (Amnis Corp). Data analysis was performed with IDEAS software version 1.10, with compensation according to the software standards.

β-Hexosaminidase release assay

BMMCs (3×10^6) were immunologically activated, as described above. After IgE incubation, the cells were washed 3 times with 5 mL of Tyrode buffer (NaCl, 130 mmol/L; KCl, 5 mmol/L; glucose, 5.6 mmol/L; MgCl₂, 1 mmol/L; CaCl₂, 1.2 mmol/L; HEPES [pH 7.4], 10 mmol/L; and BSA 0.1%) and resuspended in 1 mL of the same buffer with various concentrations of DNP-albumin for 30 minutes.

The release of β-hexosaminidase was then determined in triplicate in a 96-well plate. Aliquots (20 µL) of supernatants and cell lysates were incubated for 30 minutes with 100 µL of substrate solution (1.3 mg/mL *p*-nitrophenyl-*b*-D-2-acetamido-2-deoxyglucopyranoside in 0.1 mol/L citrate [pH 4.5]). The reaction was stopped by the addition of 200 µL of 0.2 mol/L glycine (pH 10.7).

The plate OD was read in an ELISA reader at a wavelength of 405 nm. Percentage release values for each experimental condition were calculated by using the following formula:

$$[S/(S+P)] \times 100,$$

where S and P are the β-hexosaminidase contents of the supernatant (S) and pellet (P) from each sample. The net percentage release values were obtained by subtracting the percentage release of sensitized cells incubated in IgE alone from that of replicate cells sensitized with IgE and challenged with cross-linking antibodies.

Histologic analysis of mast cells

Paraffinized lung samples were cut into 4.5-µm sections and stained with toluidine blue. Briefly, lung sections were dewaxed with HistoClear (National Diagnostics, Atlanta, Ga) for 10 minutes and then rehydrated in a series of ethanol dilutions (100%, 90%, and 70%). Rehydrated lung sections were washed in deionized water and stained in 0.1% toluidine blue or Alcian blue solution for 5 minutes. Next, the sections were washed under running tap water for 10 minutes. Lastly, sections were dehydrated in a series of ethanol dilutions (70%, 90%, and 100%) and cleaned in HistoClear for another 10 minutes. Lung sections were left to dry at room temperature and mounted with HistoMount medium. The stained sections were

examined under a Nikon microscope (model Eclipse 80i) at $\times 200$ magnification. The number of mast cells per square millimeter in the lung and skin was calculated by counting mast cells in 4 different sections derived from each murine organ. Mast cells with greater than 10% extrusion of granules are defined as degranulated mast cells.

Peritoneal cells were extracted as mentioned above and resuspended in 500 μ L of PBS. Twenty microliters of cell solution was dried as individual drops on slides and stained for 30 seconds with toluidine blue and then washed with 70% ethanol. Two hundred fields were counted to the total of approximately 2000 cells per mouse. The percentage of mast cells from the whole population was then calculated. Three mice from each strain aged 6 to 8 weeks were used for this experiment.

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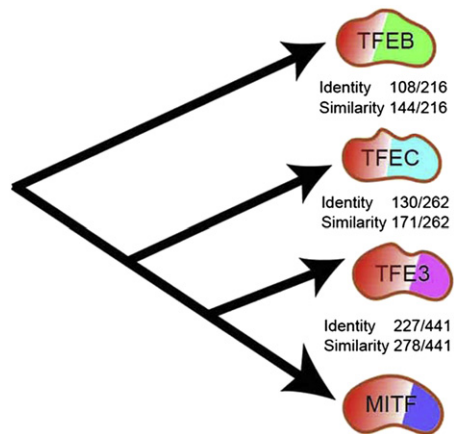
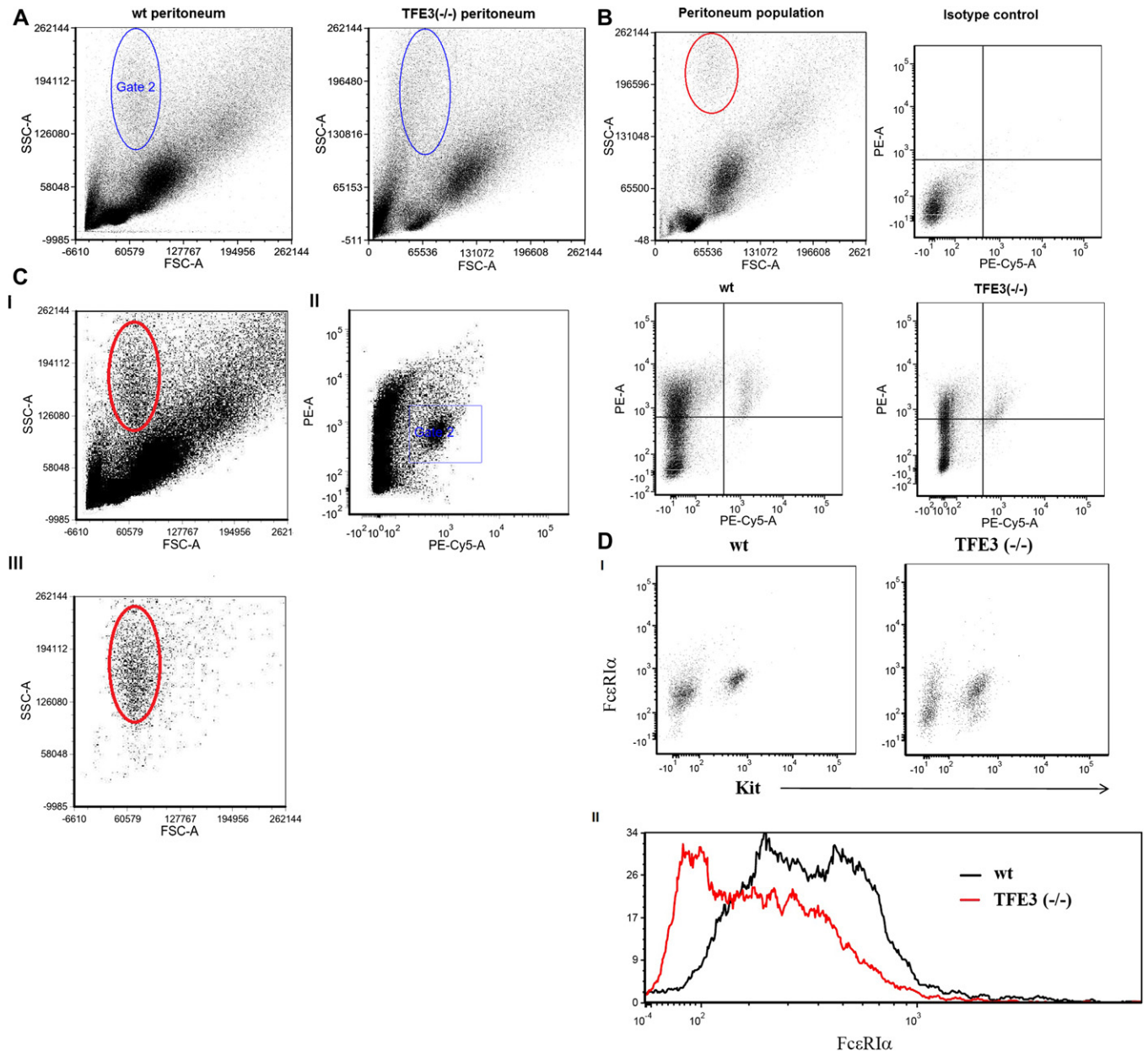


FIG E1. Phylogenetic tree of MiT family members. Protein sequences of the MiT family members were retrieved from GenBank: MITF (Q08874), TFE3 (NP_766060), TFEB (NP_035679), and TFEC (Q9WTW4). Multiple sequence alignment was performed by using the National Center for Biotechnology Information's Constraint-based Multiple Alignment Tool (COBALT; <http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi>), and a phylogenetic tree was constructed by using National Center for Biotechnology Information's Phylo TreeView. Values describe the levels of similarity and identity among 4 MiT family members.



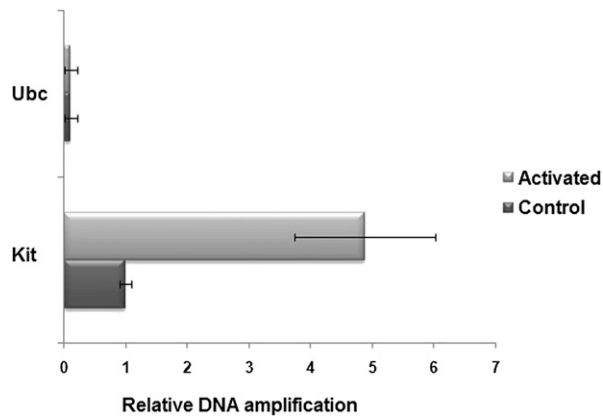


FIG E3. TFE3 directly associates with c-KIT promoter. ChIP of BMMC lysate from WT BMDCs with TFE3 antibody. The lysate of resting and 4-hour-activated cells was extracted and subjected to ChIP. The extracted DNA was quantified and used as a template for real-time PCR with specific primers to detect Kit promoter. Primers to detect ubiquitin C DNA were used as negative controls. Results represent means $\pm$ SEs ($n = 3$, $P \leq .05$).