TRAF6 Specifically Contributes to FcεRI-mediated Cytokine Production but Not Mast Cell Degranulation

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TRAF6 (tumor necrosis factor-associated factor 6) is an essential adaptor downstream from the tumor necrosis factor (TNF) receptor and Toll-like receptor superfamily members. This molecule is critical for dendritic cell maturation and T cell homeostasis. Here we show that TRAF6 is important in high affinity IgE receptor, FcεRI-mediated mast cell activation. In contrast to dendritic cells and T cells, TRAF6-deficient mast cells matured normally and showed normal IgE-dependent degranulation. Importantly, TRAF6-deficient mast cells showed impaired production of cytokine interleukin-6, CCL-9, interleukin-13, and TNF following FcεRI aggregation. Chromatin immunoprecipitation assay showed decreased NF-κB p65 binding to CCL-9 and TNF promoters in TRAF6-deficient mast cells. Antigen and IgE-induced IxB phosphorylation and NF-κB p65 translocation to the nucleus were diminished in TRAF6-deficient mast cells. NF-κB luciferase activity in response to antigen and IgE stimulation was severely impaired in TRAF6-deficient mast cells. In addition, antigen and IgE-induced phosphorylation of mitogen-activated protein kinase p38 and JNK, but not ERK1/2, was significantly reduced in TRAF6-deficient mast cells. These results identify TRAF6 as an important signal transducer in FcεRI-mediated signaling in mast cells. Our findings implicate TRAF6 as a new adaptor/regulator molecule for allergen-mediated inflammation in allergy.

FcεRI-mediated allergic inflammation is initiated by mast cells through secreting inflammatory cytokines, lipid mediators, and preformed mediators from the granules (1, 2). As a member of the immune receptor superfamily, the FcεRI consists of IgE-binding α subunit and signal-transducing β and γ subunits (3). The early molecular events mediated by FcεRI have been well characterized. Upon cross-linking of the FcεRI, Lyn which is an Src family protein-tyrosine kinase, is activated and phosphorylates the cytoplasmic domains of the β and γ subunits leading to the recruitment of Syk. Syk is then activated and phosphorylates linker for activation of T cells (LAT) (4). LAT functions as a scaffold and assembles a complex of signaling proteins containing Grb2, Gads, SLP-76, and phospholipase Cγ (PLCγ). FcεRI-mediated signals branch out at LAT into several pathways leading to the release of three major categories of mast cell mediators, including preformed mediators (degranulation), lipid mediators, and cytokines/chemokines (4, 5). Downstream molecular events associated with the activation of these three category mediators have been documented. Degranulation follows the activation of PKC and calcium mobilization (6). Cytokine and chemokine production follows the activation of transcription factor NF-κB, and MAP kinases p38 and JNK, whereas lipid mediator production follows the activation of ERK1/2 pathway (6). However, intermediate signaling molecules/adaptor proteins responsible for the secretion of a specific category of mast cell mediators remain incompletely defined.

Tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) are a family of proteins that were identified initially as signaling adaptors of the TNFR superfamily members (7). To date, seven members of the TRAF family have been identified (8–10). Among TRAF family members, TRAF6 exhibits the unique property in that its TRAF-C domain interacts with a peptide motif distinct from that recognized by other TRAF members (11). More recently, TRAF6 has been shown to be involved in signaling by Toll/IL-1 receptor family members (12–14), IL-17 receptor (15), IL-25 receptor (16), X-linked ectodysplasin-A2 receptor (17), and T cell receptor (TCR) (18, 19) in addition to TNFR superfamily members. The biological role of TRAF6 appears to be cell type-specific. TRAF6 is a T cell-intrinsic negative regulator required for the maintenance of immune homeostasis (19). In dendritic cells, TRAF6 is a critical positive regulator for dendritic cells maturation and development (20). TRAF6 can either directly associate with cell surface receptors or function as an adaptor molecule in the intermediate

3 The abbreviations used are: LAT, linker for activation of T cells; TNF, tumor necrosis factor; TRAF, TNF receptor; IL, interleukin; MAP, mitogen-activated protein; MAPK, MAP kinase; JNK, c-Jun NH2-terminal kinase; TNP, trinitrophenyl; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PLC, phospholipase C; PKC, protein kinase C; ELISA, enzyme-linked immunosorbent assay; Ab, antibody; TRAF, tumor necrosis factor-associated factor; TCR, T cell receptor; ChIP, chromatin immunoprecipitation; SCF, stem cell factor; ERK, extracellular signal-regulated kinase; TLR, Toll-like receptor.

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level of a signaling cascade. TRAF6 directly associates with surface receptors such as CD40 or receptor activator of NF-κB (21), IL-17 receptor (15), or IL-25 receptor (16) and functions at the top of a signaling cascade. In TLR and TCR signaling, TRAF6 functions at the intermediate level through interaction with other signaling molecules in the cytoplasm. The pathway of TLR-MyD88-IRAK4-TRAF6-TAK/TAB-1-MyD88-TRAF6-TAK/TAB-1 regulates TLR-mediated signaling, whether it functions in FcεRI-mediated signaling in mast cells has not been explored. In this study, we report that TRAF6 is an important positive regulator for FcεRI-mediated signaling in mast cells. TRAF6 plays a critical role in TLR-mediated host defense against microbial infection (22), whereas the pathway of TCR-PKC-Bcl10/molecule LAT or MAP kinase ERK1/2 and subsequent degranulation of mast cells. Thus, TRAF6 segregates from other signaling molecules in the cytoplasm. The pathway of TCR-mediated downstream signaling leading to activation of NF-κB and MAP kinase p38 and JNK and subsequent cytokine IL-6, CCL-9, IL-13, and TNF production. However, TRAF6 is not required for mast cell maturation, nor is it needed for FcεRI-mediated phosphorylation of proximal signaling molecule LAT or MAP kinase ERK1/2 and subsequent degranulation or leukotriene production. Thus, TRAF6 segregates FcεRI-induced cytokine production from mast cell degranulation and leukotriene production and represents a novel mechanism in FcεRI-mediated mast cell activation.

**MATERIALS AND METHODS**

**Antibodies**—Antibodies to phospho-JNK (Thr-183/Tyr-185), JNK, phospho-p38 MAPK (Thr-180/Tyr-182), phospho-p44/42 MAPK (ERK1/2), phospho-p38 MAPK, phospho-c-Jun (Ser-63/72), phospho-1KB-α (Ser 32), and 1KB-α were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies to NF-κB p65, p38 MAPK, proliferating cell nuclear antigen, FcεRIα (H-274), and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated rat anti-mouse CD117 (c-kit) monoclonal Ab (IgG2a, CL8936F), and FITC-conjugated goat anti-rabbit Alexa 549-conjugated IgG (ab’), was purchased from Invivogen (San Diego, CA).

**Mast Cells**—TRAF6-deficient mice were generated as described previously (23). The mouse liver-derived mast cells were obtained by culturing liver cells from the newborn mice (1–2 days) of TRAF6-/- colony. Briefly, liver tissue was removed to a sterile environment and was ground to produce a single cell suspension in RPMI 1640 medium. Cells were collected, centrifuged at 500 × g for 5 min at 4 °C, and resuspended to a density of 0.5 × 10^6^ cells/ml in complete medium (RPMI 1640 medium containing 10% FBS, 10% W/EHI-3B conditioned medium, 30 ng/ml stem cell factor, 50 units/ml each of penicillin and streptomycin, 50 μM 2-mercaptoethanol, and 200 nm prostaglandin E_2_. An aliquot of cells from each mouse was used for genotyping. Nonadherent cells were resuspended in complete medium twice per week and transferred to a fresh flask once per week. After 5–6 weeks, mast cell purity of >98% was achieved as assessed by toluidine blue staining of fixed cytospin preparations. Mast cells were passively sensitized with IgE from TIB-141 cells (ATCC number TIB-141) (24) and then activated by the addition of 10 ng/ml TNP-BSA (Biosearch Technologies, Inc., Novato, CA) or 1 μM calcium ionophore A23187 (Sigma).

**Western Blotting**—Cells were lysed in RIPA buffer for analysis of whole cell lysates. Samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk, membranes were incubated with primary antibodies and secondary antibodies and then detected by enhanced chemiluminescence detection reagent (Amersham Biosciences).

**Mast Cell Degranulation**—Mast cell degranulation was determined by measuring β-hexosaminidase activities.

**Real Time Quantitative PCR**—Total RNA was isolated using TRizol reagent (Invitrogen). Reverse transcription and quantitative PCR were performed as described previously (25). Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous reference. Data were analyzed using relative standard curve method according to the manufacturer’s protocol.

**Luciferase Assay for NF-κB Activities**—Liver mast cells (4 × 10^6^) were co-transfected with pNF-κB-Luc plasmid (Stratagene, La Jolla, CA) and RL-TK plasmid (Promega, Madison, WI) using Amaxa mouse T cell nucleofector kit (VPA-1006) in the Amaxa nucleofector device (program U-023, Gaithersburg, MD). After transfection, mast cells were allowed to recover for 24 h. Cells were then sensitized with anti-TNP IgE and challenged with TNP-BSA for 5 h. Firefly and Renilla activities were sequentially quantified using a dual-luciferase reporter assay system (Promega) in a Veritas microplate Luminometer (Turner Biosystems).

**Chromatin Immunoprecipitation (ChIP)**—ChIP assays were performed by using the ChIP-IT kit (catalog number 53009, Active Motif), according to the instructions of the manufacturer. In brief, mast cells (1 × 10^7^ cells/condition) were left unstimulated or stimulated for 20 min, 1 h, or 2 h with 10 ng/ml TNP-BSA. Cells were then fixed for 5 min at room temperature with 1% formaldehyde. To shear genomic DNA, the nuclei were sheared with 1% formaldehyde. Firefly and Renilla activities were then detected by enhanced chemiluminescence detection reagent (Promega) in a Veritas microplate Luminometer (Turner Biosystems).

**Total RNA Isolation**—Total RNA was isolated from liver mast cells using TRIzol reagent (Invitrogen). Reverse transcription and quantitative PCR was performed as described previously (25). Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous reference. Data were analyzed using relative standard curve method according to the manufacturer’s protocol.

**Immunofluorescence Study**—Staining was carried out as described previously (25). Mast cells were fixed, permeabilized, and stained with anti-TRAF6 Ab and followed by Alexa 594-goat anti-rabbit IgG as a secondary Ab. Cell nucleus were visualized with 4’,6-diamidino-2-phenylindole staining.

**Intracellular Calcium**—Mast cells were incubated for 30 min with 2 μM Fura-2 AM. After washing, mast cells were resus-
pended in phosphate buffer with 1.5 mM CaCl$_2$ at a concentration of 1 $\times$ 10$^6$ cells/ml. Fluorescence was measured by placing 2 ml of the mast cell suspension in a 37 °C thermostated quartz cuvette with magnetic stirring in a RF-1501 spectrofluorophotometer (Shimadzu Co., Tokyo, Japan).

Detection of Cytokine and Leukotriene C4 Production—IL-6, CCL-9, IL-13, TNF, and leukotriene C4 were measured by ELISA according to the manufacturer’s instruction.

Cytokine Protein Array—Mast cells were treated with 10 ng/ml of TNP-BSA for 6 h. Supernatants were collected and analyzed with the RayBio™ Mouse Inflammation Antibody Array I (catalog no. M0329801, Raybiotech, Norcross, GA), which can detect 40 different cytokines. The assay was performed in accordance with the manufacturer’s instructions.

RESULTS

Mast Cells Mature Normally in Vitro by Culturing Cells from Mouse Neonatal Liver in the Absence of TRAF6—TRAF6 has been shown to be essential for dendritic cell maturation and development (20) and T cell homeostasis (19). We found that mast cells also express TRAF6 (Fig. 1A). Immunofluorescence staining indicated that resting mouse bone marrow-derived

FIGURE 1. TRAF6 is not required for mast cell maturation. A, mouse bone marrow-derived mast cells were sensitized with anti-TNP IgE and stimulated with TNP-BSA (10 ng/ml) for 10 or 30 min. Cells were then fixed, permeabilized, and stained with anti-TRAF6 antibody and followed with Alexa Fluor® 594-conjugated goat anti-rabbit IgG (Fab′)2. Fluorescence-labeled mast cells were cyto-centrifuged onto slides. Slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Cells were examined using fluorescence microscope (Nikon ECLIPSE E600, Japan). Original magnification: ×40. & wild type, TRAF6 mutant, or heterozygotes were identified by PCR using DNA from liver cells. C, after 4 weeks of culture, liver-derived mast cells were examined by toluidine blue staining. Original magnification = ×100. KO, knock-out. D, liver-derived mast cells were sensitized with anti-TNP IgE, stained with FITC-conjugated anti-IgE, and examined by flow cytometry. E, liver-derived mast cells were stained with FITC-conjugated anti-c-kit antibody and examined by flow cytometry. F, liver-derived mast cells were stimulated with 1 μM calcium ionophore A23187 (A23) for 20 min or left untreated (NT). Release of β-hexosaminidase was determined.
mast cells express TRAF6, which appears to be in a punctated pattern. Stimulation with TNP-BSA appears to induce TRAF6 distribution more evenly in the cytoplasm (Fig. 1A). These data suggest that TRAF6 may be involved in FcεRI-induced mast cell activation. Accordingly, we decided to determine whether TRAF6 plays a role in mast cell development and IgE-dependent activation.

First, we tested if TRAF6 is required for mast cell development. Because TRAF6−/− mice die prematurely and show severe osteopetrosis (23), it is not feasible to use the traditional approach of culturing mast cells from bone marrow cells. Instead, livers from the newborn mice in the TRAF6+/− breeding colony were used to culture mast cells. TRAF6−/−, wild type, or heterozygote mice were identified by genotyping (Fig. 1B). Each individual experiment was performed using mast cells derived from the same litter animals. Liver cells were cultured in SCF and IL-3 containing media for 4–8 weeks and examined by metachromatic staining and flow cytometry. No morphological difference can be observed between TRAF6−/− and wild type mast cells by toluidine blue staining (Fig. 1C). TRAF6−/− and wild type mast cells express similar levels of IgE receptor and c-kit (Fig. 1D and E). To test mast cell function, calcium ionophore A23187 was used to induce mast cell degranulation. Similar levels of β-hexosaminidase release were seen in TRAF6-deficient and wild type mast cells following A23187 stimulation (Fig. 1F), suggesting that mast cell degranulation machinery is intact in TRAF6-deficient cells. Thus, TRAF6 deficiency does not affect mast cell maturation and degranulation machinery.

TRAF6 Deficiency Leads to Impaired IgE-dependent IL-6, CCL-9, IL-13, and TNF Production—In TLR- or CD40-mediated signaling events, TRAF6 plays an essential role in NF-κB-regulated gene expression. To examine whether TRAF6 plays a role in FcεRI aggregation-mediated mast cell activation, TRAF6-deficient and wild type mast cells were sensitized with IgE and stimulated with TNP-BSA for 6 h. Cell-free supernatants were analyzed by protein array assay. Among the 40 cytokines and chemokines tested, IL-6, CCL-9, and TNF showed visible diminished expression in TRAF6−/− cells following TNP-BSA stimulation (supplemental Fig. 1).

We performed ELISA and real time quantitative PCR to further determine IL-6 and CCL-9 expression. TNP-BSA-induced IL-6 production at the protein level was reduced in TRAF6−/−
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deficient mast cells compared with wild type mast cells (Fig. 2A). The impaired production of IL-6 was persistent throughout the time course examined (3–24 h). TNP-induced IL-6 mRNA expression, which peaked at 1 h, was reduced by 44% in TRAF6-deficient mast cells (Fig. 2, B and C). In contrast, liver-derived TRAF6+/− mast cells produced similar levels of IL-6 in response to TNP-BSA stimulation as compared with wild type mast cells (data not shown).

Similarly, TNP-BSA-induced CCL-9 expression at protein and mRNA levels was reduced in TRAF6-deficient cells (Fig. 2, D, E, and F). In addition, decreased IL-13 and TNF production was confirmed by ELISA (Fig. 2, G and H). These results indicated that TRAF6 plays a positive role in FceRI-induced signaling and contributes to FceRI-mediated production of IL-6, CCL-9, IL-13, and TNF.

It is important to note that unlike the complete inhibition of TLR-induced IL-6 and TNF production in TRAF6-deficient macrophages (26), TRAF6 deficiency only partially blocked FceRI-induced IL-6, CCL-9, TNF, and IL-13 production. These data suggested a differential role of TRAF6 in TLR- and FceRI-mediated signaling.

To examine whether TRAF6 is required for IgE-dependent lipid mediator production, TRAF6-deficient mast cells and wild type cells from the same litter were stimulated by TNP-BSA for 20 min. Cell supernatants were used to examine LTC4 production. No difference in LTC4 production was observed between TRAF6-deficient and wild type mast cells (Fig. 2I). Thus, TRAF6 selectively affects IgE-dependent cytokine IL-6, CCL-9, IL-13, and TNF production but not leukotriene production.

**TRAF6 Deficiency Leads to Impaired IgE-dependent IkB-NF-κB Pathway Activation—**NF-κB plays a central role in the regulation of IgE-dependent mast cell cytokine production. To examine whether TRAF6 is required for IgE-dependent NF-κB pathway activation, TRAF6-deficient and wild type mast cells after sensitization with IgE were transfected with NF-κB luciferase plasmid and stimulated with TNP-BSA. Significant TNP-induced NF-κB activation was seen in wild type mast cells as expected. However, TNP-induced NF-κB activity was severely impaired in TRAF6-deficient mast cells (Fig. 3A).

To confirm a role for TRAF6 in NF-κB activation, nuclei from mast cells following stimulation by TNP-BSA for various times were analyzed by ChIP assay using anti-NF-κB p65 Ab. Immunoprecipitates were subjected to PCR analysis using primers targeting the promoter regions of CCL-9 andTNF genes. Reduced PCR products for CCL-9 and TNF promoters were seen in TRAF6-deficient cells compared with wild type cells (Fig. 3B). Western blotting using nuclear proteins was performed to further confirm NF-κB p65 activation. TNP-BSA stimulation induced nuclear translocation of NF-κB p65 in wild type mast cells. TRAF6-deficient mast cells showed impaired p65 nuclear translocation (Fig. 3C).

We further determined IkB phosphorylation and degradation. Rapid and significant IkB phosphorylation was observed in wild type mast cells following TNP-BSA stimulation. Phosphorylation was accompanied by degradation of total IkB (5–20 min) and was followed by regeneration of IkB protein (1–6 h) in these cells (Fig. 3D). In contrast, TRAF6-deficient mast cells showed severely impaired IkB phosphorylation. Compared

![FIGURE 3](image)

TRAF6 deficiency leads to impaired IgE-dependent IkB-NF-κB pathway activation. A, liver-derived mast cells were transfected with 3 μg of pNF-κB-Luc plasmid (Firefly) and 1 μg pRL-TK plasmid (Renilla). Cells were then sensitized with IgE and stimulated with TNP-BSA for 5 h. Firefly and Renilla activities were quantified. n = 3 independent experiments. B, liver-derived mast cells after IgE sensitization were either left unstimulated (NT) or stimulated with TNP-BSA (10 ng/ml) for 20, 60, or 120 min. Nuclei were extracted and subjected to enzymatic digestion to shear genomic DNA. Sheared chromatin was immunoprecipitated with anti-NF-κB p65 Ab or control IgG. Then 0.4% of the input DNA (without immunoprecipitation) and 5% of the precipitated DNA were used as templates for each PCR analysis using primers targeting CCL-9 and TNF promoters. PCR products were stained by ethidium bromide. M, DNA marker. C, liver-derived mast cells were sensitized with IgE and stimulated with TNP-BSA (10 ng/ml) for various times or left untreated (NT). Nuclear proteins were isolated using a nuclear extraction kit (Active Motif Inc., Carlsbad, CA), according to the manufacturer’s protocol. Nuclear proteins were analyzed by Western blotting for phosphor-IkBα, total IkBα, and actin.
TRAF6 Deficiency Leads to Impaired IgE-dependent Phosphorylation of JNK and p38 but Not ERK

Liver-derived mast cells were sensitized with anti-TNP IgE and stimulated with TNP-BSA (10 ng/ml) for various times. Whole cell lysates were analyzed by Western blotting for phospho-JNK and total JNK (Fig. 4A), phospho-p38 and total p38 (C), phospho-ERK1/2 and total ERK1/2 (E), and phospho-c-Jun and total c-Jun (G). Densitometry analysis of phospho-JNK(1 and 2)/JNK ratio (B), phospho-p38/total p38 ratio (D), phospho-ERK/total ERK ratio (F), and phospho-Jun/total c-Jun ratio (H) was performed based on three separate experiments.

The effect of TRAF6 on JNK phosphorylation prompted us to examine Jun family members. TNP-induced phosphorylation of c-Jun was reduced in TRAF6-deficient mast cells (Fig. 4, C and D). However, TNP-induced expression of c-Fos, c-Jun, and JunB at the mRNA level was comparable between wild type and TRAF6-deficient mast cells (supplemental Fig. 3). These results suggest that TRAF6 may be selectively involved in JNK phosphorylation but not gene expression of these family members.

Effects of TRAF6 Deficiency on IgE-mediated Proximal Signaling Events—TRAF6 has been shown to be an adapter that can directly bind to a cell surface receptor, such as CD40 (12, 14), IL-17 (15), or IL-25 (16), in addition to functioning at a downstream level. No association between TRAF6 and FceRI or Lyn was observed in our co-immunoprecipitation experiment (data not shown). To test if FceRI-induced proximal signaling events are affected by TRAF6 deficiency, LAT phosphorylation was examined. Our results indicated that similar levels of LAT phosphorylation were observed in TRAF6-deficient and wild type mast cells (Fig. 5, A and B). Thus, it is likely that TRAF6 regulates FceRI-induced signaling downstream of LAT.

TRAF6 Deficiency Has No Effects on IgE-dependent Calcium Mobilization and Degranulation—To examine whether TRAF6 regulates calcium mobilization, mast cells were loaded with Fura-2/AM and stimulated with TNP-BSA. TNP-mediated role in IgE-dependent MAP kinase activation, TRAF6-deficient and wild type mast cells were sensitized with IgE and stimulated with TNP-BSA for various times. Total cell lysates were analyzed by Western blotting for phospho-JNK, phospho-p38, phospho-ERK, and their respective total proteins. A severe defect of TNP-BSA-induced JNK2 phosphorylation (Fig. 4A, top band of JNK2) was observed in TRAF6-deficient mast cells, suggesting a critical role of TRAF6 in IgE-dependent JNK activation (Fig. 4, A and B). Similarly, a significant reduction in TNP-BSA-induced p38 phosphorylation was found in TRAF6-deficient mast cells (Fig. 4, C and D). In contrast, similar levels of TNP-BSA-induced ERK phosphorylation were seen in TRAF6-deficient and wild type mast cells (Fig. 4, E and F), suggesting a lack of effect of TRAF6 in IgE-dependent activation of the ERK pathway. ERK pathway is responsible for leukotriene production. Thus, the lack of effect of TRAF6 on ERK1/2 activation is consistent with the result showing no effect of TRAF6 on leukotriene C4 production. Thus, TRAF6 is specifically required for...
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**FIGURE 5.** TRAF6 deficiency has no effect on the proximal signaling molecule LAT phosphorylation, calcium mobilization, and mast cell degranulation. After sensitization with anti-TNP IgE, liver-derived mast cells were stimulated with TNP-BSA (10 ng/ml) for various times. A, whole cell lysates were analyzed by Western blotting for phospho-LAT and total LAT. B, densitometry analysis of phospho-LAT/total LAT ratio was performed based on three separate experiments. C, intracellular calcium level was measured using Fura-2 AM as a probe. D, mast cell degranulation was determined by measuring β-hexosaminidase release after stimulation with TNP-BSA for 20 min. E, TRAF6 represents a novel signal transducer in FcεRI-mediated signaling. TRAF6 contributes to IgE-dependent ERK1/2 pathway activation and MAP kinase JNK and p38 pathway activation and subsequent cytokine production, but not mast cell degranulation. We also exclude a role for TRAF6 in IgE-dependent ERK1/2 pathway activation and its subsequent leukotriene production.

**DISCUSSION**

In this study we established a new approach to generate mature TRAF6-deficient mast cells. TRAF6-deficient mice show severe osteopetrosis and die within days after birth (23). Thus, it is not feasible to culture mast cells from bone marrow cells or to obtain sufficient tissue mast cells. Accordingly, we used liver cells from the newborn mice in the TRAF6+/− breeding colony and cultured these cells in the presence of SCF and IL-3 for differentiation into mast cells. This allows us to obtain TRAF6−/−, TRAF6+/−, and TRAF6+/* mast cells from the same litter of mice. By using these cells, we provide evidence that TRAF6 has a novel role in FcεRI-mediated signaling in mast cells. TRAF6 deficiency selectively affects FcεRI-induced activation of NF-κB, MAP kinase p38, and JNK but has no effect on ERK or proximal molecule LAT phosphorylation, leading to the selective regulation of cytokine production but not degranulation or lipid mediator LTC4 production. Our results suggest a model of TRAF6 in FcεRI-mediated signaling in mast cells (Fig. 5E). TRAF6 selectively functions downstream of LAT and upstream of NF-κB, MAP kinase p38, and JNK pathways that are responsible for gene expression of cytokines. TRAF6 is not involved in FcεRI-mediated pathways leading to degranulation or lipid mediator production.

FcεRI aggregation initiates Lyn and Syk phosphorylation leading to LAT activation. LAT organizes a complex of signaling proteins containing Grb2, Gads, SLP-76, and phospholipase Cγ (PLCγ) (5). Immunoprecipitation study showed no association between TRAF6 and FcεRI γ chain (data not shown). In addition, FcεRI-induced LAT phosphorylation was not affected in TRAF6-deficient cells. These results suggested that TRAF likely functions downstream of LAT.

Signals from FcεRI branch out at LAT into several pathways (5). Recruitment of Grb2 to LAT mediates activation of the Ras-Raf-MEK pathway leading to MAP kinase ERK1/2 phosphorylation and activation, which plays a primary role in IgE-dependent leukotriene production. No effect of TRAF6 deficiency on ERK1/2 phosphorylation and LTC4 production suggests that TRAF6 is not involved in this pathway activation. Although a role of TRAF6 in mediating CD40- or TLR-induced ERK activation has been shown in macrophages (27), our result excluded a role of TRAF6 in FcεRI-induced ERK activation in mast cells. Thus, TRAF6 has a distinct role in FcεRI-mediated signaling in mast cells.

Recruitment of Gads and SLP-76 to LAT is responsible for activation of Rac1 and Cdc42, leading to subsequent activation of MAP kinase JNK pathway MEKK1-SEK1/MKK7-JNK in mast cells (28, 29). TRAF6 has been shown to directly associate with the MAP3K, MEKK1 (30), and regulates JNK activity. TRAF6 deficiency dramatically blocked FcεRI-induced JNK phosphorylation. Thus, it is likely that TRAF6 mediates FcεRI-
induced JNK activation through TRAF6-MEKK1-SEK1/MKK7-JNK pathway. FcεRI aggregation induces MAP kinase p38 activation in mast cells through an unknown mechanism. However, TAK1-MKK6-p38 pathway has been well described in other immune effector cells (31). TRAF6 deficiency significantly reduced FcεRI-induced p38 phosphorylation. Given that a direct association between TRAF6 and TAK1 has been reported previously (32), it is possible that TRAF6 mediates FcεRI-induced p38 activation through the TRAF6-TAK1-MKK6-p38 pathway.

Recruitment of PLCγ to LAT leads to the subsequent activation of NF-κB, which is a master transcription factor that controls the expression of proinflammatory gene products (33). Binding of PLCγ to LAT induces generation of inositol 1,4,5-triophosphate and diacylglycerol, which are responsible for inducing calcium influx and protein kinase C (PKC) activation, respectively. We found that TRAF6 deficiency showed no effect on TNF-induced calcium mobilization, suggesting that TRAF6 functions downstream of calcium.

In TCR-mediated signaling cascade, TRAF6 has been shown to be associated with Malt1/Bcl10 and to relay signals from TCR to IkB-NF-κB in T lymphocytes (18). In mast cells, Malt1 and Bcl10 form a complex and link PKC to NF-κB activation from TNP-induced calcium mobilization, suggesting that TRAF6 is required for IgE-mediated cytokine production by mast cells.

It is interesting to note that TRAF6 deficiency had no effect on mast cell development from liver cells under the culture condition containing IL-3 and SCF. This result suggests that TRAF6 is not required for IL-3- or SCF-mediated signaling in mast cells, although TRAF6 is needed for signaling several cytokine receptors in other cells (8). The lack of effect of TRAF6 in mast cell development is in vast contrast to the essential role of TRAF6 for the development of dendritic cells (20) and plasma cells (35). These results support the concept that a role of TRAF6 in signal transduction is cell type-specific.

Collectively, our results suggest that TRAF6 segregates FcεRI-induced cytokine production from mast cell degranulation and leukotriene production. TRAF6 represents a novel mechanism in mediating FcεRI-induced IkB/NF-κB, MAP kinase p38, and JNK but not ERK1/2 activation. These results implicate TRAF6 as a regulator in mast cell-mediated allergy inflammation.

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*TRAF6 in IgE-dependent Mast Cell Activation*