

IgE production in CD40/CD40L cross-talk of B and mast cells and mediator release via TGase 2 in mouse allergic asthma



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ABSTRACT

TGase 2 is over-expressed in a variety of inflammatory diseases including allergic asthma. This study aimed to investigate the role of TGase 2 on IgE production and signaling pathways in mast cell activation related to OVA-induced allergic asthma. Bone marrow-derived mast cells (BMMCs) isolated from WT or TGase 2^{-/-} mice were activated with Ag/Ab (refer to act-WT-BMMCs and act-KO-BMMCs, respectively). B cells isolated from splenocytes were activated with anti-mouse IgM (act-B cells), and B cells were co-cultured with BMMCs. WT and TGase 2^{-/-} mice were sensitized and challenged with OVA adsorbed in alum hydroxide. Intracellular Ca²⁺ ([Ca²⁺]_i) levels were determined by fluorescence intensity; IgE, mediators and TGase 2 activity by ELISA; the CD138 expression by FACS analyzer; cell surface markers and signal molecules by Western blot; NF-κB by EMSA; co-localization of mast cells and B cells by immunohistochemistry; FcεRI-mediated mast cell activation by PCA test; expression of cytokines, MMPs, TIMPs, TLR2 and FcεRI by RT-PCR. In vitro, act-KO-BMMCs reduced the [Ca²⁺]_i levels, NF-κB activity, expression of CD40/CD40L, plasma cells, total IgE levels and TGase 2 activity in act-B cells co-cultured with act-BMMCs, expression of inflammatory cytokines and MMPs2/9, release of mediators (TNF-α, LTs and cytokines), and activities of signal molecules (PKCs, MAP kinases, I-κB and PLA2), which were all increased in act-WT-BMMCs. TGase 2 siRNA transfected/activated-BMMCs reduced all responses as same as those in act-KO-BMMCs. In allergic asthma model, TGase 2^{-/-} mice protected against PCA reaction, OVA-specific IgE production and AHR, and they reduced co-localization of mast cells and B cells or IgE in lung tissues, expression and co-localization of surface molecules in mast cells (c-kit and CD40L) and B cells (CD23 and CD40), inflammatory cells including mast cells, goblet cells, amounts of collagen and mediator release in BAL fluid and/or lung tissues, which were all increased in WT mice. TLR expression in TGase 2^{-/-} mice did not differ from those in WT mice. Our data suggest that TGase 2 expression and Ca²⁺ influx required by bidirectional events in mast cell activation facilitate IgE production in B cells via up-regulating mast cell CD40L expression, and induce the expression of numerous signaling molecules associated with airway inflammation and remodeling in allergic asthma.

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1. Introduction

Mast cells, which are well-known as major effector cells for allergic reactions, are activated by cross-linking of antigen-specific IgE bound to the high-affinity receptor (FcεRI) on their membranes. Activated mast cells secrete a variety of mediators [1,2], which contribute to airway inflammation and remodeling in allergic asthma [3]. Mast cells as well

as activated T cells express costimulatory molecules (CD40L), which is natural ligand for CD40 and a member of TNF-α family [4]. CD40, which is a type I transmembrane protein belonging to the TNFR superfamily, is expressed in the various cell types such as B cells, dendritic cells, macrophages, endothelial cells, astrocytes and mast cells [4,5]. Mast cell expressing CD40L can trigger IgE synthesis [6,7] or IgA synthesis [8] through B cell Ig class switching via CD40/CD40L interaction and soluble factors such as IL-4, IL-13 or IL-6.

TGase 2, a cross-linking enzyme with diverse function, contributes to induction of experimental allergic encephalomyelitis (EAE) [9] and drug resistance in various cancer cells [10]. TGase 2 gene ablation protects against renal ischemia injury by blocking NF-κB [11]. Epithelial TGase 2 is a critical inducer of pulmonary inflammation in bleomycin-treated mice [12].

TGase 2, which activates NF-κB via two different pathways: IKK-independent and IKK-dependent pathways, activates LPS-stimulated

Abbreviations: AHR, Airway hyperresponsiveness; BAL, Bronchoalveolar lavage; BMMCs, Bone marrow-derived mast cells; MCh, Methacholine; MMPs, Matrix metalloproteinases; PAS, Periodic acid-Schiff; PLA2, Phospholipase A2; PCA, Passive cutaneous anaphylaxis; TLR, Toll like receptor; TGase 2, Transglutaminase 2; TGase 2^{-/-}, TGase 2 knock-out; TIMPs, Tissue inhibitor metalloproteinases; WT, wild type.

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inflammatory responses [13] and regulates maturation and responsiveness of dendritic cells to LPS treatment [14] and TNF- α -mediated NF- κ B activation [15]. Recently, TGase 2 was found to serve as a key initiator of airway inflammatory asthma pathogenesis [16,17]. Our laboratory has also reported that a TGase 2 inhibitor attenuates allergic responses by regulating NF- κ B/TGase 2 activity in OVA-induced allergic asthma in mice [3]. However, a role of TGase 2 on IgE production in interaction of B cells and mast cells via CD40/CD40L, and on signaling pathways in mast cell activation related to Ag-induced allergic asthma is not clarified yet.

Based on the information presented above that mast cells are major effector cells in allergic asthma and associated with B cell Ig class switching, and TGase 2 induces Ag-induced airway inflammatory responses, we hypothesized that TGase 2 expressed in mast cell activation may facilitate IgE production in B cells via CD40/CD40L, and may cause allergic asthma responses. We observed that TGase 2 expression and Ca^{2+} influx required by bidirectional events in mast cell activation had an important role in IgE production in B cells via CD40/CD40L interaction, and in airway inflammation and remodeling of OVA-specific allergic asthma.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice, 8 wk old, were obtained from ORIENT BIO (Seongnam Co., Osan, Korea), and maintained in specific pathogen-free conditions before sacrifice. All animals were housed in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All protocols were approved by the institutional review board and conducted in the Laboratory Animal Research Center of Sungkyunkwan University (Suwon, Korea).

2.2. Targeting of mouse TGase2 and genotyping

TGase 2^{-/-} mice (female) were generated by homologous recombination as previously described [18] and were back-crossed to C57BL/6 mice for more than ten generations [11] (see Supplementary data).

2.3. Culture and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells flushed from femurs and tibias of WT and TGase 2^{-/-} mice were cultured for 5 wk in RPMI-1640 containing IL-3 [4]. The purity for BMMCs using May–Grünwald–Giemsa staining was more than 95% of total cells (see Supplementary data).

Both BMMCs (1×10^6 cells) were sensitized with anti-DNP IgE Ab (0.1 $\mu\text{g}/\text{mL}$) overnight at 37 °C, and then challenged with 10 ng/mL DNP-HSA (Sigma-Aldrich, St. Louis) (refer to act-BMMCs) for period indicated at 37 °C in Tyrode's buffer [4]. Ca^{2+} inhibitor (100 μM 2-APB), and R2 or scrambled (S2) peptide (200 $\mu\text{g}/\text{mL}$ TGase 2 inhibitor and positive control for R2, respectively) were added 10 min and 30 min, respectively, before DNP-HSA stimulation. To investigate signaling cascades between Ca^{2+} levels and TGase 2, Ca^{2+} blocker in bone marrow cell culture media was treated at 1, 2, 3 or 4 wk, respectively.

2.4. Western blot analysis

Immunoblotting was performed for each protein extract from homogenized BMMCs, BAL cells (1×10^6 cells/50 μL) or lung tissues (50 mg/500 μL) using a previously described method [3] (see Supplementary data).

2.5. Measurement of intracellular Ca^{2+} [Ca^{2+}]_i level

The [Ca^{2+}]_i levels in act-BMMCs (3×10^6 cells) were quantified with fluorescence intensity using a LSM 510 laser scanning microscope

(Carl Zeiss, Oberkochen, Germany) [4]. Fluorescence intensity for control ($F = 0$) was adjusted as 1 (see Supplementary data).

2.6. TGase 2 activity assay

The TGase 2 activity was determined using the TG-Covtest kit (Covalab, Villeurbanne, France) [19]. TGase 2 activity was calculated using standard curves generated with specific TGase 2 standards, and expressed in $\mu\text{U}/\text{mg}$ protein (see Supplementary data).

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from BMMCs, BAL cells (3×10^6 cells) or lung tissues (50 mg/500 μL), and then EMSA were performed using a previously described method [3] (see Supplementary data).

2.8. Isolation and activation of B cells or co-culture of B cells and mast cells

B cells (1×10^6 cells) isolated using the MagCelect mouse B cell isolation kit (R&D Systems, Inc., Minneapolis, purity of more than 94%) were incubated with 0.5 $\mu\text{g}/\text{mL}$ anti-mouse IgM Ab (refer to act-B cells) for 48 h [8]. The act-B cells (1×10^6 cells) were added on non-act-BMMCs (BMMCs alone without Ag/Ab reaction) or act-BMMCs (1×10^6 cells) for 5 h [1], and co-cultured for 72 h. The optimal incubation time and ratio (1:1) for co-cultured of both cells was yielded in preliminary experiments. IgE secreted in culture media was determined by ELISA method [20].

To assess formation of plasma cells, B cells (1×10^6 cells) were incubated with phycoerythrin-conjugated anti-mouse CD138 (BD Bioscience, San Jose, CA) for 1 h at 4 °C. The CD138 expression was measured by FACS analyzer (Becton and Dickinson, San Jose) [20] (see Supplementary data).

2.9. Measurement of IgE level, cytokines, and MMPs by ELISA

IgE amounts in the supernatants (200 μL) isolated from act-B cells with or without act-BMMCs or sera were analyzed using ELISA [3] (see Supplementary data).

Amounts of cytokines or MMP2/9 were determined using an ELISA kit (BD Biosciences). The lowest detection limit for IL-4, IL-5, IL-6, IL-8 and TNF- α was better than 31.3 pg/mL, and for IL-13, RANTES, TGF- β was 15.6 pg/mL, and for MMP2/9 was 0.078 pg/mL (see Supplementary data).

2.10. TGase 2 small interfering RNA (siRNA) transfection

Transfection for TGase 2 was carried out according to the manufacture's protocol (Santa Cruz Biotech, Santa Cruz). TGase 2 siRNA transfected BMMCs were used in CD40/CD40L, [Ca^{2+}]_i level and TGase 2 activity, measurement of IgE level [4] (see Supplementary data).

2.11. Sensitization, challenge, and experimental protocol

C57BL/6 mice were divided into six groups (eight mice/group): NC (negative control) or WT, WT mice sensitized and nebulized with PBS and OVA, respectively; Con or TGase 2^{-/-}, TGase 2^{-/-} mice sensitized and nebulized with PBS and OVA, respectively. R2 or S2 peptide (20 mg/kg), WT-mice sensitized and nebulized with OVA pretreated with R2 and S2 peptides once a day 10 min before OVA challenge for 3 days. The mice were sensitized with 20 $\mu\text{g}/200 \mu\text{L}$ OVA adsorbed in 1 mg/50 μL aluminum hydroxide gel adjuvant delivered by i.p. injection on days 1 and 15. Mice were then challenged with 5% OVA in PBS to the nose for 30 min once a day for 3 days (from day 21 to day 23) using a nebulizer (Mega Medical, Seoul, Korea). The NC (negative

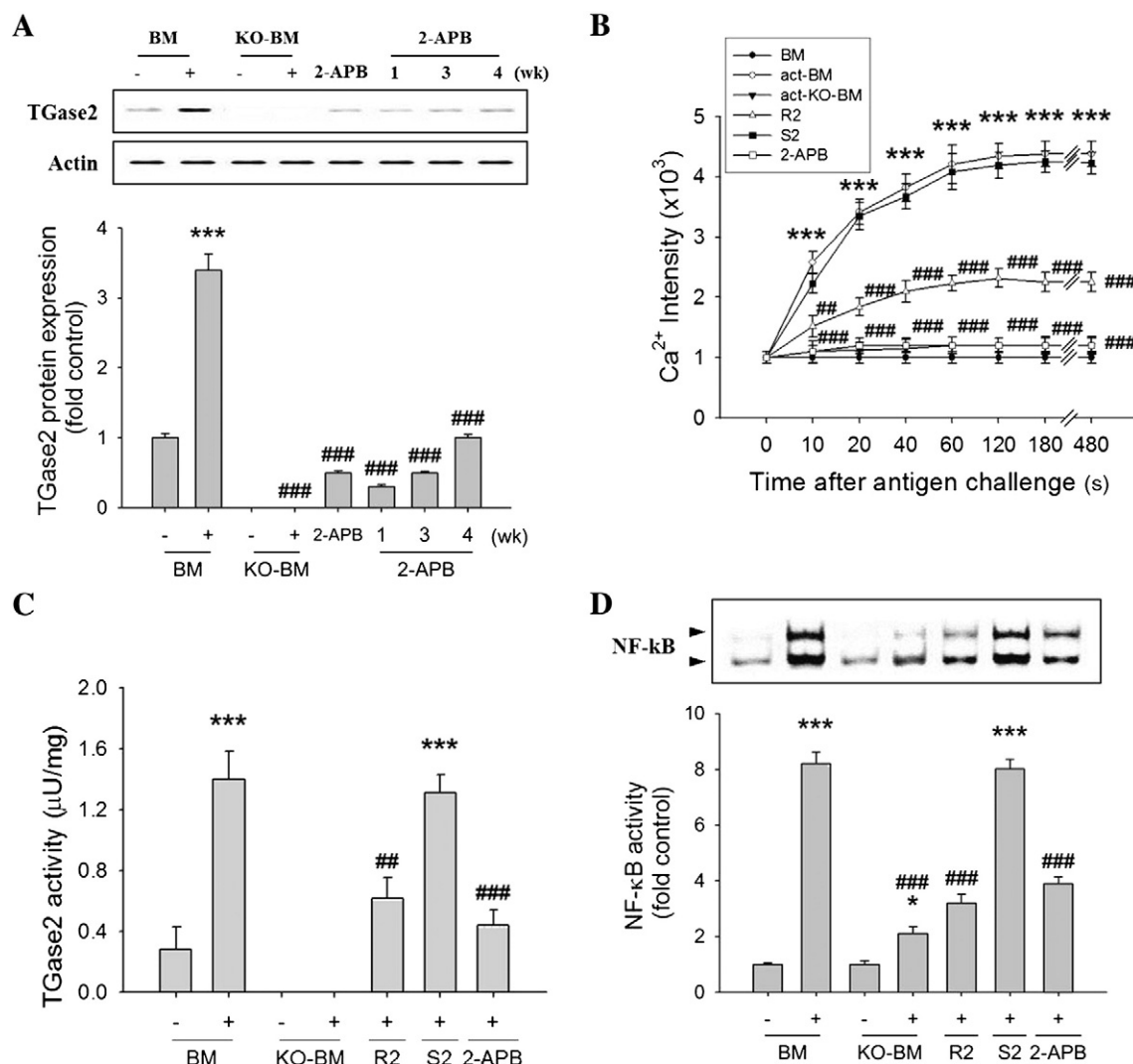


Fig. 1. Effects of TGase 2^{-/-} or inhibitors on the expression of TGase 2, [Ca²⁺]_i level, activities of TGase 2 and NF-κB in act-BMMCs. BMMCs (1 × 10⁶ cells) from WT or TGase 2^{-/-} mice were activated with Ag/Ab reaction (act-WT-BMMCs or act-KO-BMMCs) for the indicated periods (2 h for TGase 2; 1 h for TGase 2 activity and 5 h for NF-κB activity) as described in [Materials and methods](#). Ca²⁺ blocker (100 μM 2-APB), TGase 2 inhibitor (R2 peptide, 200 μg/mL) or scrambled (S2) peptide (200 μg/mL) was pretreated for 10 min, 30 min and 30 min, respectively, before Ag stimulation. BM or KO-BM, BMMCs isolated and cultured from WT and TGase 2^{-/-} mice, respectively. — or +, non-act-BMMCs and act-BMMCs, respectively. TGase 2 expression or activities of NF-κB were determined in protein and nuclear extract isolated from BMMCs using Western blot and EMSA, respectively. Fluo-3-AM (5 μM) was added to BMMCs and incubated for 30 min. The [Ca²⁺]_i level was analyzed by confocal microscopy. TGase 2 activity was determined by TG Covtest kit. (A) Bands and histogram for TGase 2 expression in BMMCs or in BMMCs cultured after adding 2-APB at 1, 3 and 4 (wk). Numbers indicate wk adding the Ca²⁺ blocker into bone marrow cell culture media. (B) Time courses of [Ca²⁺]_i level in after inhibitor treatment. (C) and (D) Activities of TGase 2 and NF-κB in both BMMCs or after inhibitor treatment. The histogram from the densitometry analysis shows the mean ± SEM (n = 4) obtained from the ratio of each band density versus those of control and actin or total protein from four independent experiments. *, P < 0.05; ***, P < 0.001 versus the non-act-WT-BMMCs or non-act-KO-BMMCs. ##, P < 0.01; ###, P < 0.001 versus the act-WT-BMMCs. Data in the [Ca²⁺]_i level or TGase 2 activity are expressed as the mean ± SEM (n = 8).

control) or Con mice (TGase 2^{-/-} alone) were sensitized and nebulized by PBS. All mice were sacrificed on day 24.

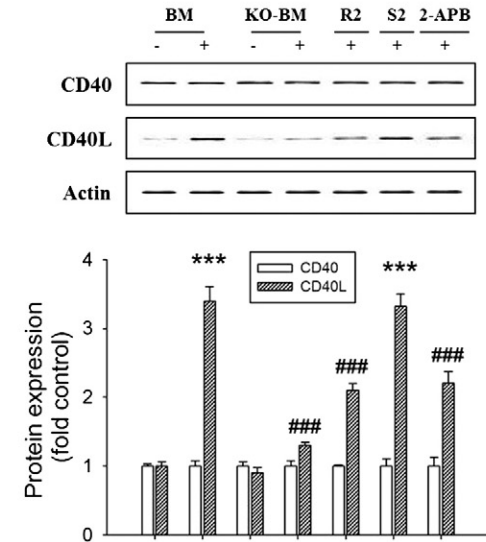
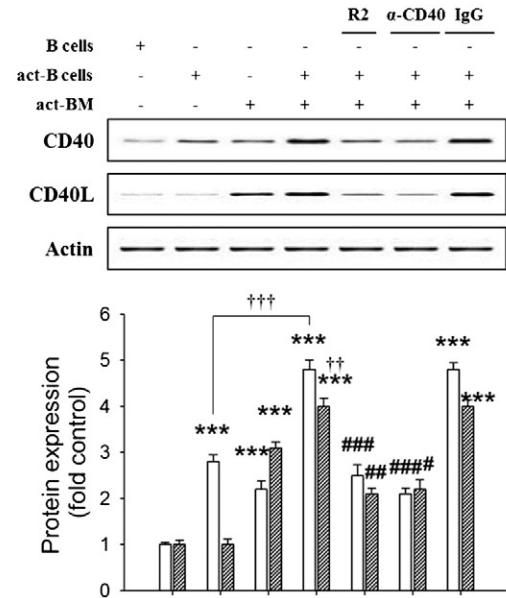
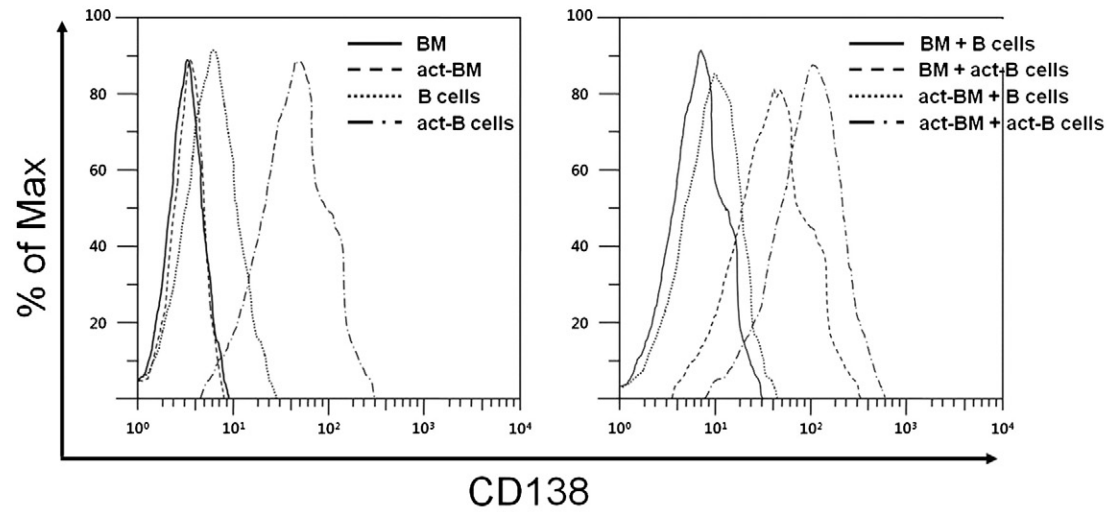
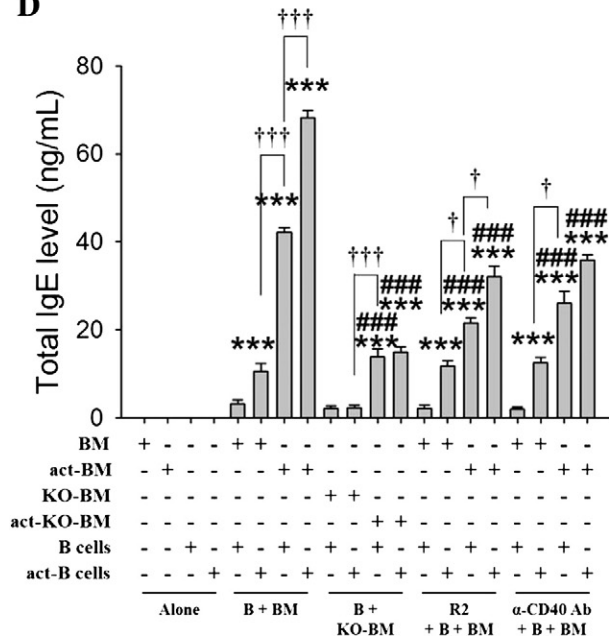
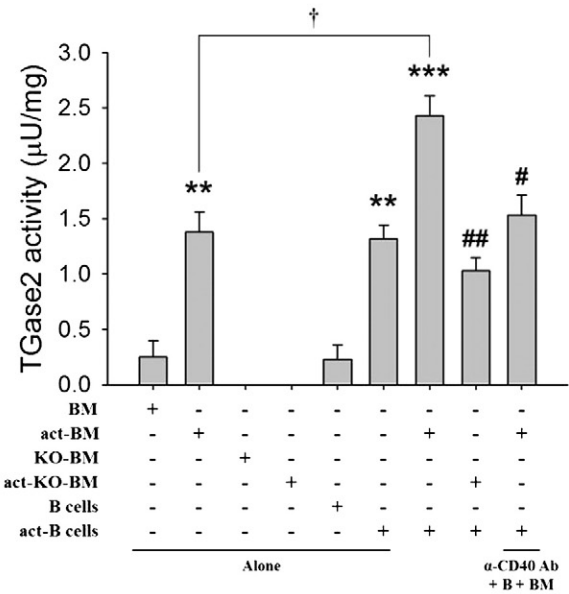
R2 or S2 peptide (20 mg/kg) was dissolved in PBS and given by i.p. injection once a daily 10 min before OVA challenge for 3 days (from days 21 to days 23). All the data for pretreatment with R2 or S2 peptide alone in non-sensitized and non-challenged mice are not shown because these treatments were no effects. The

concentration for R2 or S2 peptide (20 mg/kg) is the highest dose used in the previous experiment.

2.12. Bronchoalveolar lavage (BAL) fluid

BAL fluid was collected, stained and counted using previously described [Materials and methods](#) [3]. Infiltrated inflammatory cells

Fig. 2. Effects of TGase 2^{-/-} or inhibitors on the expression of CD40/CD40L, plasma cells or IgE production in act-B cells with act-BMMCs. Experimental details for activation of BMMCs, inhibitor treatment and definition of all symbols used was described in [Fig. 1](#) legend. B cells (1 × 10⁶ cells) isolated from spleen of WT mice were activated with 0.5 μg/mL anti-mouse IgM Ab for 48 h, and co-cultured with act-BMMCs for 72 h. Anti-CD40 Ab (300 ng/mL) was pretreated for 1 h before B cell activation. Expression of CD40/CD40L was determined in protein extract isolated from BMMCs or B cells using Western blot. Total IgE production was determined by ELISA, plasma cells by FACS analyzer, and TGase 2 activity by TG-Covtest kit. — or +, non-act-BMMCs or non-act-B cells and act-BMMCs or act-B cells, respectively. (A) Expression of CD40/CD40L in both BMMCs or inhibitor pretreatment. (B) Expression of CD40/CD40L in B cells co-cultured with BMMCs or inhibitor pretreatment. (C) Percentage of positive plasma cells in B cells with BMMCs. (D) Total IgE production in B cells with BMMCs. (E) TGase 2 activity in B cells with BMMCs. The histogram from the densitometry analysis was obtained as described in [Fig. 1](#) legend (n = 4). **, P < 0.01; ***, P < 0.001 versus the non-act-WT-BMMCs or non-act-KO-BMMCs. #, P < 0.05; ##, P < 0.01; ###, P < 0.001 versus the act-WT-BMMCs or act-B cells with act-BMMCs. †, P < 0.05; ††, P < 0.01; †††, P < 0.001 versus act-B cells, act-BMMCs or act-B cells with non-act-BMMCs.

A**B****C****D****E**

stained with Diff-Quick or mast cells stained with May–Grünwald–Giemsa were quantified under light microscopy (see Supplementary data).

2.13. Lung tissue histology

Lungs tissue sections from the mice embedded in paraffin (3 μ m) were stained with H&E to observe the general morphology, PAS for goblet cells [3], and Masson's trichrome for collagen deposition [21]. The amount of collagen was quantified by a Sircol collagen assay [21] (see Supplementary data).

2.14. Measurement of airway hyperresponsiveness (AHR)

AHR was assessed by measuring changes in the airway resistance (R_L , cmH₂O/mL/s) after mice were treated with MCh using the Flexivent system (SCIREQ, Montreal, Quebec, Canada). Provocative concentration (PC₁₀₀) was calculated by the dose of MCh which induced a 100% increase in R_L [3] (see Supplementary data).

2.15. Passive cutaneous anaphylaxis (PCA) analysis

The PCA analysis was performed using a previously described method [22] (see Supplementary data).

2.16. Immunostaining and immunofluorescence

Lung sections (3 μ m) incubated for overnight with a primary Abs against c-kit, CD23, CD40 and CD40L (1:100, Santa Cruz Biotechnology, Santa Cruz) were incubated with HRP-conjugated goat anti-mouse or HRP-conjugated goat anti-rabbit (1:1000, Zymed Laboratory Inc. San Francisco, CA) for 1 h, and followed by staining with 3-amino-9-ethylcarbazole (AEC, Dako, Glostrup, Denmark). Slides were counterstained with hematoxylin and finally mounted using aqueous mounting medium (Thermo Shandon, Pittsburgh), and then examined under a light microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany) [4].

The deparaffinized and blocked lung tissue sections (slides) were incubated with the Abs against c-kit and CD23 (1:25) or c-kit and IgE (1:50) in blocking solution for 5 h. The slides were then treated with the corresponding FITC- or TEXAS-conjugated anti-IgG (Zymed Laboratory Inc. San Francisco) for 1 h in room temperature. Washing steps between each stage were performed for 1–2 min. After washing, the slides were mounted using aqueous mounting medium (Thermo Shandon, Pittsburgh), and examined by confocal microscope (Carl Zeiss, Oberkochen, Germany) [4].

2.17. Measurement of phospholipase A2 (PLA2) activity

PLA2 activity was measured in BAL fluid and cells, lung tissues or BMMCs (1 $\times$ 10⁶ cells) by the technique previously described [3] (see Supplementary data).

2.18. Immunoassay for leukotrienes (LTs)

The LTs contents in supernatants isolated from act-BMMCs, BAL fluid or supernatants obtained from lung tissue homogenates were determined using an enzyme immunoassay kit (Santa Cruz Biotechnology, Inc., Santa Cruz). The results are expressed in pg/mL (see Supplementary data).

2.19. Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from BMMCs (1 $\times$ 10⁶ cells), BAL cells (1 $\times$ 10⁶ cells) or lung tissues (50 mg/500 μ L) using Trizol reagent (Invitrogen Life Technologies, Carlsbad). RT-PCR was performed in a final volume of 50 μ L using an amfiRivert One-step RT-PCR kit (GenDEPOT, Barker) in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR was performed for 35 cycles [3] (see Supplementary data).

2.20. Statistical analysis

Unless otherwise noted, experimental data are presented as the mean $\pm$ SEM (n = 8). Multiple group comparisons were performed using a one-way ANOVA followed by Scheffe's post hoc test using SPSS (SPSS Inc., Chicago). P values < 0.05 were regarded as significant. Densitometry analyses of western blot, PCR assay, and EMSA were performed with Quantity One version 4.6.3 (Bio-Rad, Hercules). Histograms for densitometry analysis represent the mean $\pm$ SEM (n = 4) obtained from four independent experiments.

3. Results

3.1. Maturity of bone marrow-derived mast cells (BMMCs)

The maturity in knockout (KO)-BMMCs showed similar results to that in all wild type (WT)-BMMCs. However, amounts of histamine released decreased in KO-BMMCs activated with DNP-HSA/anti-DNP IgE Ab (refer to act-KO-BMMCs) versus WT-BMMCs activated with Ag/Ab reaction (refer to act-WT-BMMCs) (Supplement Fig. 1A–D). This implies that histamine release may depend on TGase 2, but its storage does not affect it.

Non-act-WT-BMMCs originally contained TGase 2, and act-WT-BMMCs showed two peaks (2 min and 2 h) in expression of TGase 2 protein and mRNA (Supplement Fig. 1E) versus that in non-act-WT-BMMCs (BMMCs without Ag/Ab reaction), whereas non-act- or act-KO-BMMCs did not express it (Fig. 1A).

3.2. Effects of TGase 2^{-/-} on the intracellular calcium ([Ca²⁺]_i) level in BMMCs

As increment of [Ca²⁺]_i levels are necessary for the release of mediators in mast cell activation [4,21], we examined the [Ca²⁺]_i levels. Act-WT-BMMCs initiated to increase [Ca²⁺]_i levels at 2–5 s (Fig. 1B), reached plateau at 2 min (from 1.0 $\pm$ 0.00 to 4.38 $\pm$ 0.21 OD) and prolonged for 8 min, after that, it decreased, whereas act-KO-BMMCs showed the [Ca²⁺]_i levels similar to those in non-act-WT-BMMCs.

Fig. 3. Effects of TGase 2^{-/-} or inhibitors on the numbers of mast cells, serum IgE level, PCA test, interaction of mast cells and B cells in lung tissues of OVA-challenged allergic asthma in mice or surface markers in B cells and BMMCs. Experimental details for the sensitization of WT or TGase 2^{-/-} mice, PCA test, definition of all symbols used, expression of surface markers, IHC and double staining, and activation of BMMCs have been described in [Materials and methods](#) and [Fig. 1](#) legend. (A) Mast cell population and serum OVA-specific IgE level. Histogram for numbers of mast cells were quantified by cell counts in 200 $\times$ 200 μ m areas under a microscopy (5 areas/each slide $\times$ 8 mice/each group = 40 areas), and IgE by ELISA. (B) PCA test by TGase 2^{-/-} and inhibitor pretreatment. Values above box showed dye intensity extravasated in responses to Ag. (C) Histogram for protein expression of CD72 and CD23 in lung tissues, both BMMCs and B cells. (D) Histogram for each surface marker expression in lung tissues. The histogram from the densitometry analysis was obtained as described in [Fig. 1](#) (n = 4). Band images for Fig. 3C and D were shown in Supplement Fig. 3G and H. (E) Expression of surface markers c-kit/CD40L (mast cells) and CD23/CD40 (B cells) in lung tissues. (F) Co-localization (yellow) of c-kit (green) and CD23 (red) in lung tissues. (G) Co-localization (yellow) of c-kit (green) and IgE (red) in lung tissues. Scale bar in Con (NC) indicated 100 μ m, and magnification in Fig. 3E was $\times$ 200, it in Fig. 3F and G was $\times$ 400. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus the NC or Con mice, non-act-WT-BMMCs or non-act-KO-BMMCs. ##, P < 0.01; ###, P < 0.001 versus the WT mice or act-WT-BMMCs. Data shown are expressed as the mean $\pm$ SEM (n = 8).

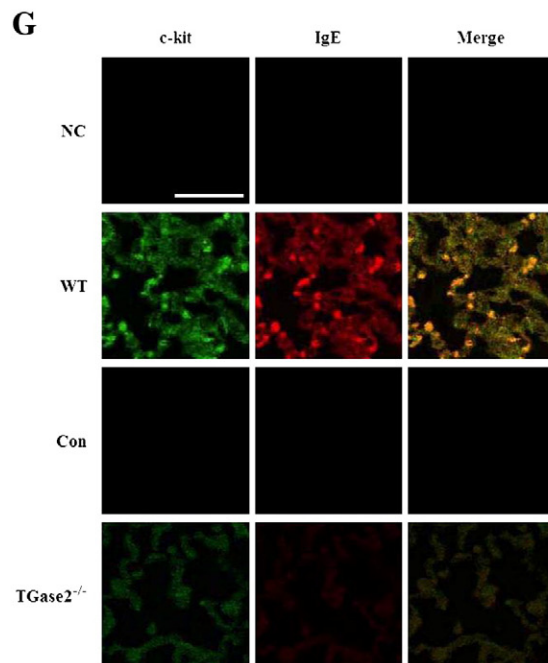
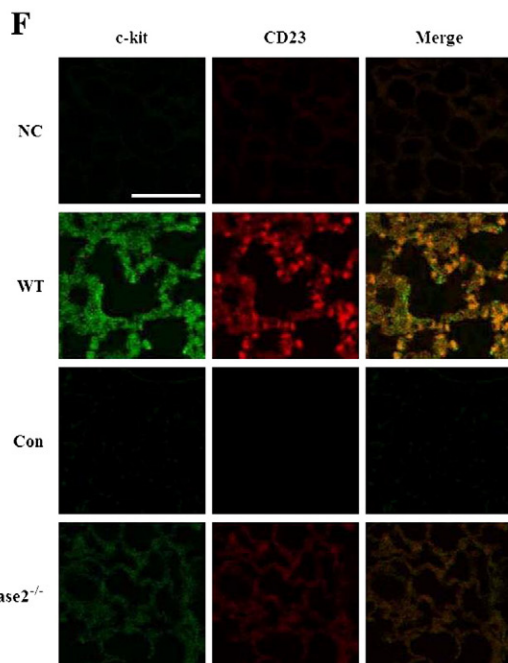
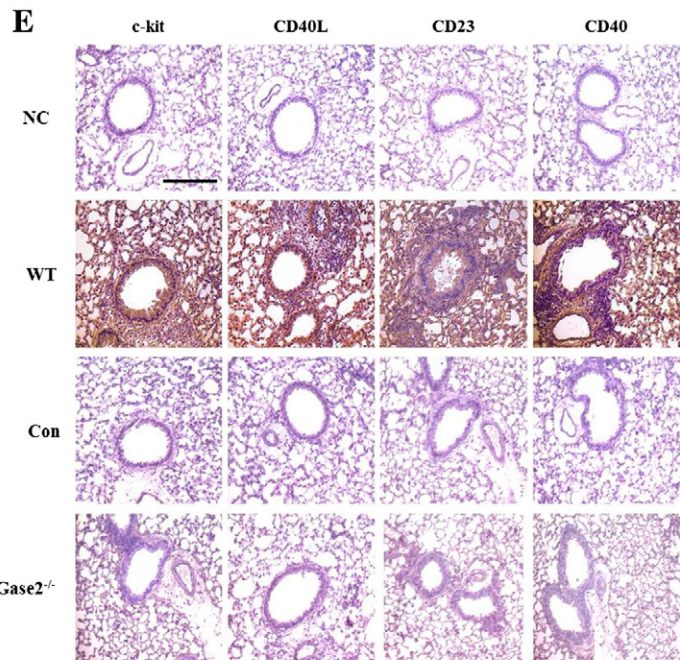
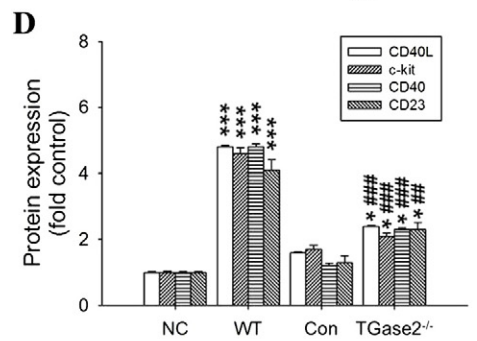
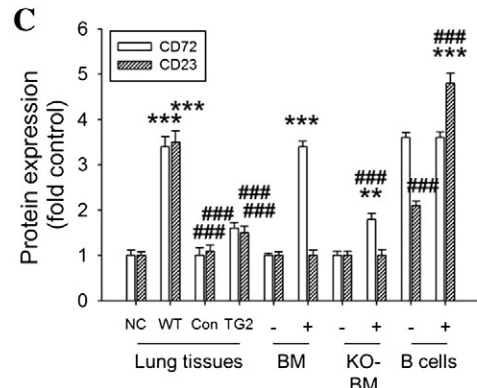
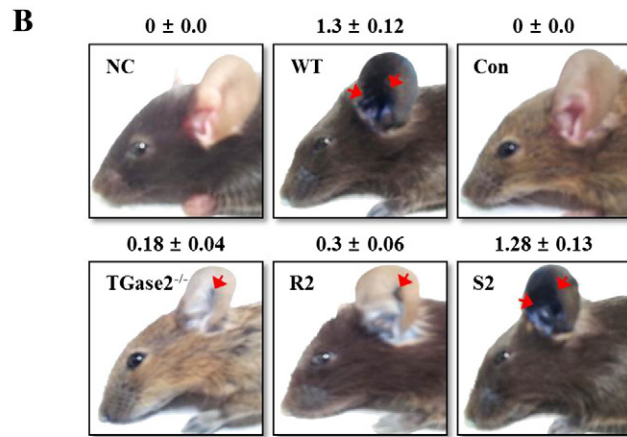
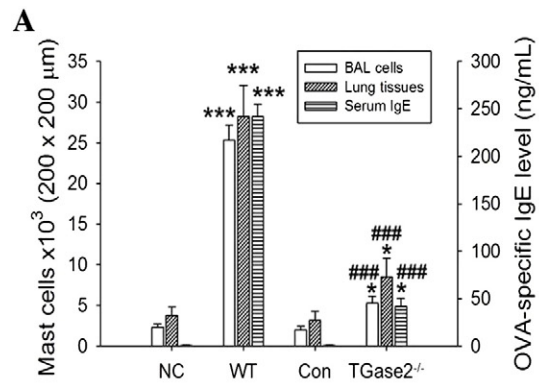


Table 1Effects of TGase 2 on the release of mediators in activated-BMMCs or in BAL fluid and lung tissues of OVA-challenged allergic asthma in mice^a.

Groups	TNF- α (pg/mL)		Leukotrienes (pg/mL)		
	BMMCs	Lung tissues	BMMCs	BAL fluid	Lung tissues
NC	033 $\pm$ 2.4	0650 $\pm$ 21.6	0114 $\pm$ 2.5	009 $\pm$ 2.3	024 $\pm$ 2.5
WT	584 $\pm$ 32.9***	1653 $\pm$ 32.9***	4026 $\pm$ 41.3***	290 $\pm$ 23.6***	663 $\pm$ 41.3***
Con	034 $\pm$ 3.1	0646 $\pm$ 28.1	0110 $\pm$ 5.2***	010 $\pm$ 1.6	020 $\pm$ 2.2
TGase 2 ^{-/-}	094 $\pm$ 32.9***,+++	0767 $\pm$ 23.2***,+++	0255 $\pm$ 25.0***,+++	044 $\pm$ 8.3***,+++	101 $\pm$ 25.5***,+++
R2	152 $\pm$ 32.9***,+++	0823 $\pm$ 22.5***,+++	0521 $\pm$ 25.0***,+++	128 $\pm$ 9.2***,+++	223 $\pm$ 21.2***,+++
S2	596 $\pm$ 31.5***	1658 $\pm$ 31.8***	4039 $\pm$ 40.2***	296 $\pm$ 24.7***	672 $\pm$ 35.2***

^a Activation of BMMCs (1×10^6 cells) from WT or TGase 2^{-/-} mice, experimental details for the sensitization of WT and TGase 2^{-/-} mice and all symbol used were described in Figs. 1 and 3 legend. Amounts of TNF- α and LTs from act-BMMCs, and BAL fluid and supernatants obtained from lung homogenates (50 mg) of allergic asthma were determined by ELISA. Data are expressed as the mean $\pm$ SEM (n = 8).

* $P < 0.05$.

** $P < 0.001$.

*** $P < 0.001$ vs. the NC-BMMCs or Con-BMMCs, and NC mice or Con mice.

+++ $P < 0.001$ vs. act-WT-BMMCs or WT mice.

TGase 2 inhibitor (200 μ g/mL R2 peptide) inhibited the $[Ca^{2+}]_i$ levels by 49% (to 2.25 ± 0.16 OD). Scrambled (S2) peptide as control of R2 peptide did not affect it. Ca^{2+} blocker (100 μ M 2-APB) pretreatment inhibited TGase 2 expression (Fig. 1B). Hereafter, we did not describe the data for the pretreatment of R2 and S2 peptides or Ca^{2+} blocker due to showing similar results to all responses and the previous report [3].

To confirm whether TGase 2 is Ca^{2+} -dependent enzyme, we observed that Ca^{2+} blocker treated in culture media at individual wk reduced the expression of TGase 2 (Fig. 1A right column).

TGase 2 catalytic enzyme activity (from 0.3 ± 0.08 to 1.4 ± 0.12 μ U/mg) or TGase 2-mediated NF- κ B activity was increased in act-WT-BMMCs versus non-act-WT-BMMCs, whereas they in act-KO-BMMCs was not detected and reduced, respectively (Fig. 1C and D).

3.3. Effects of TGase 2^{-/-} on the CD40/CD40L expression in BMMCs

We previously reported that TGase 2 inhibitor reduced serum OVA-specific IgE level [3]. CD40 and CD40L, which are related to IgE class switching, are expressed on diverse cell types including mast cells to regulate immune responses [3,4]. Therefore, we observed that CD40 was originally expressed in both non-act-WT- and non-act-KO-BMMCs, but not enhanced in both act-WT- and act-KO-BMMCs (Fig. 2A). The CD40L expression was enhanced in act-WT-BMMCs versus non-act-WT-BMMCs, whereas it was weakly expressed in act-KO-BMMCs versus act-WT-BMMCs.

To investigate the relationship between mast cells and B cells in IgE production, both cells were co-cultured by 1:1 optimal ratio obtained from preliminary experiments (data not shown). Expression of CD40 was increased in B cells activated with anti-IgM Ab (act-B cells) versus non-act-B cells, and enhanced in act-B cells co-cultured with act-BMMCs versus each act-cells (Supplement Fig. 1F and Fig. 2B). CD40L expression was enhanced in act-B cells with act-BMMCs versus act-B cells. Anti-CD40 Ab inhibited CD40 and CD40L expression in act-B cells with act-BMMCs, but IgG Ab as positive control did not inhibit CD40/CD40L expression. These surface markers (CD40 and CD40L) on B and mast cells were confirmed by flow cytometry (Supplement Fig. 2A and B).

3.4. Effects of TGase 2^{-/-} on the IgE production in interaction of B cells with mast cells

Percentage of IgE-secreting plasma cells (CD138) was enhanced (right shift) (Fig. 2C), and CD138 protein expression was enhanced in act-B cells co-cultured with act-BMMCs versus individual act-cell (Supplement Fig. 3A). Total IgE production was increased in non-act-B

cells co-cultured with act-BMMCs (42 ± 1.0 ng/mL), and it was enhanced in act-B cells with act-WT-BMMCs (68 ± 1.5 ng/mL) versus non-act-B cells or act-B cells with non-act-BMMCs (11 ± 1.8 ng/mL), whereas it was reduced by 67% and 78%, respectively, in non-act-B cells with act-KO-BMMCs (from 42 ± 1.0 to 14 ± 0.8 ng/mL) or act-B cells with act-KO-BMMCs (from 68 ± 1.5 to 15 ± 1.2 ng/mL) (Fig. 2D).

Act-WT-B cells with act-WT-BMMCs enhanced TGase 2 activity (2.4 ± 0.18 μ U/mg) versus that in act-B cells with act-KO-BMMCs (1.0 ± 0.12 μ U/mg) or act-B cells (1.3 ± 0.12 μ U/mg) (Fig. 2E). Anti-CD40 Ab (1.5 ± 0.18 μ U/mg) reduced TGase 2 activity by 38% in act-B cells with act-BMMCs.

As KO-mice shows deficiency in all cell types, we needed to confirm the relationship of mast cells and TGase 2 in co-cultures using TGase 2 transfected cells. Thus, we found that act-B cells with TGase 2 transfected-WT-BMMCs reduced expression of all surface markers (CD40L, CD72 and CD23), $[Ca^{2+}]_i$ level, TGase 2 activity and total IgE level, but not affected CD40 expression (Supplement Fig. 3B-F).

3.5. Effect of TGase 2^{-/-} on inflammatory cell recruitment and histopathologic changes

Next, we investigated whether events for BMMCs in vitro occur in vivo. We first confirmed that TGase 2^{-/-} mice completely lacked TGase 2 expression versus that increased in BAL cells and lung tissues of WT mice (Supplement Fig. 4A).

Reduction of infiltration of inflammatory cells in the peribronchial and perivascular areas, the goblet cell hyperplasia (pink dots) containing mucus (the percentage of PAS-positive cells/total cells) and individual inflammatory cells in BAL fluid were found in TGase 2^{-/-} mice versus those in WT mice (Supplement Fig. 4B, C and E).

TGase 2^{-/-} mice showed very low levels in collagen deposition in lung tissues, and the reduction of collagen amounts by 71% in lung tissues (from 18.0 ± 1.15 to 5.3 ± 0.24 μ g/mL) versus WT mice (Supplement Fig. 4D and F).

Serum OVA-specific IgE levels in TGase 2^{-/-} mice were reduced by 82.6% (from 242 ± 12.9 to 42 ± 8.2 ng/mL) versus WT mice (from 1 ± 0.5 to 242 ± 12.9 ng/mL) (Fig. 3A right axis).

3.6. Effect of TGase 2^{-/-} on AHR to MCh

The airway resistance (R_L) in WT mice was increased by MCh administration whereas TGase 2^{-/-} mice showed similar R_L to that in NC mice. The WT mice (from 28.8 ± 0.84 to 4.2 ± 1.84 mg/mL) remarkably decreased the concentration of MCh which induced a 100% increase in respiratory system (PC_{100}). TGase 2^{-/-} mice (to 27.5 ± 1.84 mg/mL) recovered PC_{100} similar to that in NC or Con mice (27.6 ± 2.84 mg/mL) (Supplement Fig. 4G and H).

3.7. Relationship of mast cells and TGase 2 in allergic asthma model

As the release of mediators [23], IgE production and changes of AHR were remarkably reduced in act-KO-BMMCs or TGase 2^{-/-} mice versus act-WT-BMMCs and WT mice, respectively, we first observed that the numbers of mast cells in WT mice were increased in BAL cells (from 2.3 ± 0.41 cells to 25.3 ± 1.83 cells) and lung tissues (from 3.8 ± 1.00 cells to 28.2 ± 3.82 cells), whereas TGase 2^{-/-} mice reduced them in BAL cells (to 5.3 ± 0.84 cells) and lung tissues (to 8.5 ± 2.32 cells) versus WT mice (Fig. 3A left axis).

Next, we focused on the relationship of mast cells and TGase 2 using PCA test caused via FcεRI-derived mast cell activation. TGase

2^{-/-} mice almost suppressed the mast cell-mediated PCA reaction versus that in WT mice (Fig. 3B).

B cell surface markers CD72 and CD23 expression, which are expressed in various cell types such as endothelial cells, dendritic cells and mast cells, were increased in lung tissues of WT mice, whereas these markers were reduced in TGase 2^{-/-} mice (Fig. 3C left column; see band images in Supplement Fig. 3G). However, CD72, which is known as a typical B cell surface marker and was expressed in human mast cells [24], was increased in act-BMMCs, but not enhanced in act-B cells (Fig. 3C right column). And, act-B cells enhanced CD23 expression versus non-act-B cells, but not act-BMMCs. These surface markers were reduced in lung tissues of

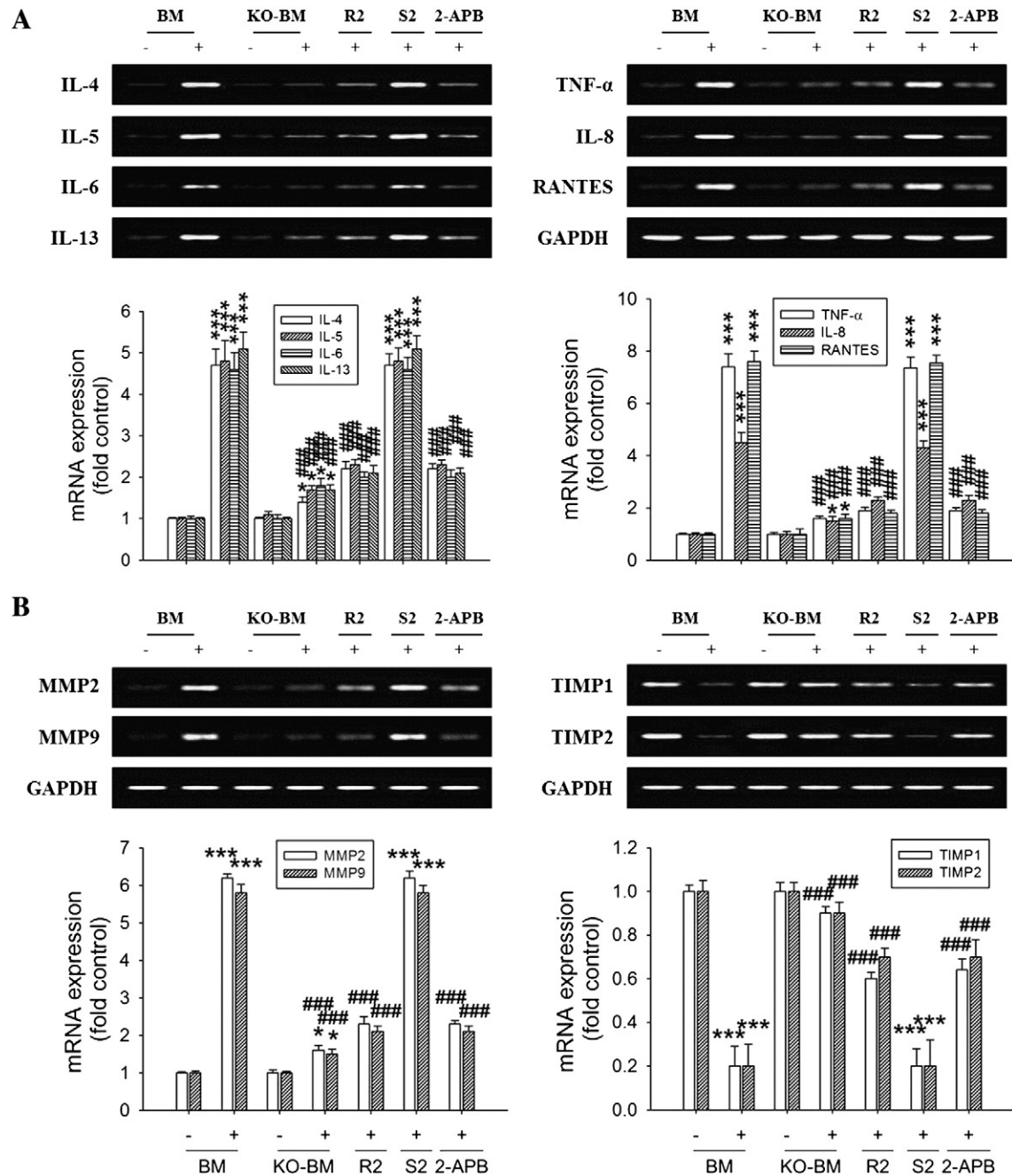


Fig. 4. Effects of TGase 2^{-/-} or inhibitor on expression of cytokine, MMPs and TIMPs mRNA in act-BMMCs. Experimental details for activation of BMMCs for 5 h, inhibitor treatment and definition of all symbols used were described in Fig. 1 legend. Expression of cytokines and MMPs/TIMPs mRNA were determined mRNA extracts isolated from BMMCs using RT-PCR. (A) and (B) Band images and corresponding histogram for mRNA expression of cytokines/chemokines and MMPs/TIMPs, respectively. The histogram from the densitometry analysis was obtained as described in Fig. 1 legend (n = 4). *, P < 0.05; ***, P < 0.001 versus the non-act-WT-BMMCs or non-act-KO-BMMCs. ###, P < 0.001 versus the act-WT-BMMCs.

TGase 2^{-/-} mice versus those in WT mice (Fig. 3C). Thus, we chose CD23 as B cell markers. Mast cell surface marker CD40L and the other marker c-kit expression were increased in lung tissues of WT mice, but TGase 2^{-/-} mice showed lower level expression of in these molecules (Fig. 3D; see band images in Supplement Fig. 3H). All surface markers on B and mast cells in vitro and in vivo were confirmed by flow cytometry (Supplement Fig. 2C and D).

The expression of CD23 and CD40 were reduced in lung tissues of TGase 2^{-/-} mice versus those in WT mice (Fig. 3E). However, the changes of lung tissue morphology in TGase 2^{-/-} mice were not observed compared to those in WT mice. In double labeling with c-kit (green) for mast cells and CD23 (red) for B cells, both cells (merge) were co-localized in WT mice (yellow), but not in lung tissues of KO mice (Fig. 3F). IgE-secreting plasma cells were selectively localized in adjacent areas populated by mast cells in WT mice because the secreted IgE may bind to mast cell receptor FcεRI, but not TGase 2^{-/-} mice (Fig. 3G).

3.8. Effects of TGase 2^{-/-} on expression or activity of signaling molecules in vitro and in vivo

In signaling molecules, phosphorylation of PKC isoforms or MAPKs reached a maximum at 3 min and 5 min, respectively, in act-BMMCs. The phosphorylation of PKCs was not inhibited by MAPK inhibitor (PD98059), but phosphorylation of ERK was inhibited by specific PKC inhibitor (Gö6976). The act-KO-BMMCs weakly increased the activities of PKCs and MAPKs versus non-act-KO-BMMCs (Supplement Fig. 5A–D).

And, act-WT-BMMCs decreased expression of I-κB, and increased activity of p-I-κB and expression and activity of PLA2 (Supplement Fig. 5E–H), followed by secretion of the granular and secreted TNF-α (total TNF-α amounts) and newly synthesized LTs (Table 1) versus non-act-WT-BMMCs, whereas act-KO-BMMCs weakly increased p-I-κB and PLA2 and mediator release versus non-act-KO-BMMCs. We also observed the same results as those in vitro for all signaling molecules (data not shown) and mediator release in vivo.

3.9. Effects of TGase 2^{-/-} on the expression of cytokines and MMPs

Expression of cytokines [23,25] and MMPs [26,27] is up-regulated in mast cells via a NF-κB. Therefore, we observed that act-WT-BMMCs or lung tissues had increased expression of cytokine mRNA and protein (IL-4, -5, -6, -8, -13, TNF-α and RANTES) and of constitutive MMP2 and inducible MMP9 mRNA and protein versus non-act-WT-BMMCs, but act-KO-BMMCs or TGase 2^{-/-} mice reduced all mRNA and protein expression of cytokines and MMPs (Fig. 4 and Table 2).

3.10. Effect of TGase 2^{-/-} on expression of TLR mRNA and FcεRIα mRNA

Mast cells play a role as a sentinel in host defense against bacterial, viral and fungal pathogens, and can be selectively activated by pathogens via various Toll-like receptors (TLRs) [28]. TLR2 or FcεRI mRNA expression was enhanced in WT-BMMCs or KO-BMMCs activated with Ag/Ab reaction or lysophosphatidic acid (LPA, Sigma-Aldrich, St Louis, MO). The expression of TLR2 and FcεRI mRNA did not show any differences between WT and KO mice, but TNF-α mRNA expression as mediator secreted differed from both BMMCs or both OVA-challenged mice (Fig. 5).

4. Discussion

This study demonstrates that Ca²⁺-dependent TGase 2 expressed in mast cells enhances IgE production in B cells via up-regulating CD40/CD40L expression, as supported by PCA test which is related to FcεRI-mediated mast cell activation. And, the data also demonstrate that Ca²⁺/TGase 2 may induce the downstream signals of PKCs activities to release the granular histamine and TNF-α (total TNF-α) and newly synthesized LTs, and induce MAPKs/I-κB-dependent NF-κB activity to express inflammatory cytokines and various molecules related to the remodeling, whereas KO-BMMCs and TGase 2^{-/-} mice protect against all events. Thus, it can be inferred that Ca²⁺/TGase 2, surface molecules, and mediators including Th2-mediated cytokines expressed/released in mast cell activation may induce IgE production, changes of AHR to MCh, airway inflammation and tissue remodeling in OVA-induced allergic asthma responses.

The [Ca²⁺]_i level generally reaches a plateau at 10–15 s in a single mast cell activation [29]. However, in this study, the reason that the [Ca²⁺]_i level is gradually increased can be due to the measurement of total [Ca²⁺]_i level in total BMMCs used. Our data, by showing that TGase 2 expression requires cytosolic Ca²⁺, and the Ca²⁺ influx requires TGase 2 expression (Fig. 1A and B), can be inferred that TGase 2 existed originally in mast cells may initially induce Ca²⁺ influx in mast cell activation, and the increase of [Ca²⁺]_i level may induce TGase 2 expression. However, further study needs to confirm it.

Another member of TNF family, the CD40L (CD154) is expressed in BMMCs [4]. The CD40L expressing mast cells trigger IgE synthesis through B cell Ig isotype class switching in the presence of IL-4 and IL-13 [7,30]. However, there are reports that cultured basophils induce human IgE synthesis in B cell activation, but mast cells co-cultured with B cells do not synthesize it [31] or produce IgA [8]. However, our data suggest that Ca²⁺-TGase 2 expressed in mast cell activation may induce IgE production through B cell Ig isotype class switching via up-regulating CD40L expression in mast cells and cytokines (IL-4 and IL-13), which are secreted in various cells. And, the reason that

Table 2
Effects of TGase 2 on cytokines or MMPs levels in act-BMMCs or lung of OVA-challenged allergic asthma.^a

ILs	BMMCs (pg/mL)				Lung tissues (pg/mL)			
	NC	WT	Con	TGase2 ^{-/-}	NC	WT	Con	TGase2 ^{-/-}
IL-4	018 ± 2.6	426 ± 12.2***	016 ± 2.1	068 ± 4.2+++	102 ± 4.9	0886 ± 7.2***	101 ± 3.8	156 ± 9.9+++
IL-5	030 ± 3.2	146 ± 11.8***	031 ± 2.2	024 ± 8.8+++	370 ± 8.4	0714 ± 14.8***	375 ± 4.6	394 ± 8.2+++
IL-6	042 ± 3.2	542 ± 13.2***	040 ± 3.2	090 ± 5.2+++	208 ± 2.9	1284 ± 62.6***	210 ± 3.2	386 ± 14.2+++
IL-8	031 ± 8.2	528 ± 33.5***	029 ± 6.2	092 ± 14.2+++	182 ± 8.9	1634 ± 52.6***	186 ± 15.2	298 ± 22.2+++
IL-13	028 ± 2.2	659 ± 8.2***	030 ± 2.2	082 ± 3.2+++	664 ± 7.8	1727 ± 9.6***	668 ± 5.8	709 ± 3.2+++
TGF-β	082 ± 3.2	483 ± 5.4***	079 ± 2.2	112 ± 5.4+++	152 ± 5.8	1642 ± 72.4***	146 ± 7.8	192 ± 8.4+++
RANTES	004 ± 1.2	34 ± 5.4***	004 ± 1.4	016 ± 8.1+++	082 ± 5.8	0534 ± 68.4***	180 ± 5.2	121 ± 6.2+++
MMP2	038 ± 0.5	209 ± 6.2***	038 ± 0.2	054 ± 6.2+++	064 ± 0.5	0548 ± 6.2***	064 ± 0.6	089 ± 3.2+++
MMP9	0.4 ± 0.43	01.9 ± 0.68***	0.3 ± 0.23	0.6 ± 0.34+++	001 ± 0.9	0034 ± 4.8***	001 ± 0.6	006 ± 2.1+++

^a Activation of BMMCs (1 × 10⁶ cells) from WT or TGase 2^{-/-} mice, experimental details for the sensitization of WT and TGase 2^{-/-} mice and all symbols used were described in Figs. 1 and 3 legend and methods. Individual cytokine and MMP 2/9 levels in BMMCs or lung tissues were determined by ELISA. Data are expressed as the mean ± SEM (n = 8).

*** P < 0.001 vs. the NC-BMMCs or NC mice.

+++ P < 0.001 vs. the act-WT-BMMCs or WT mice.

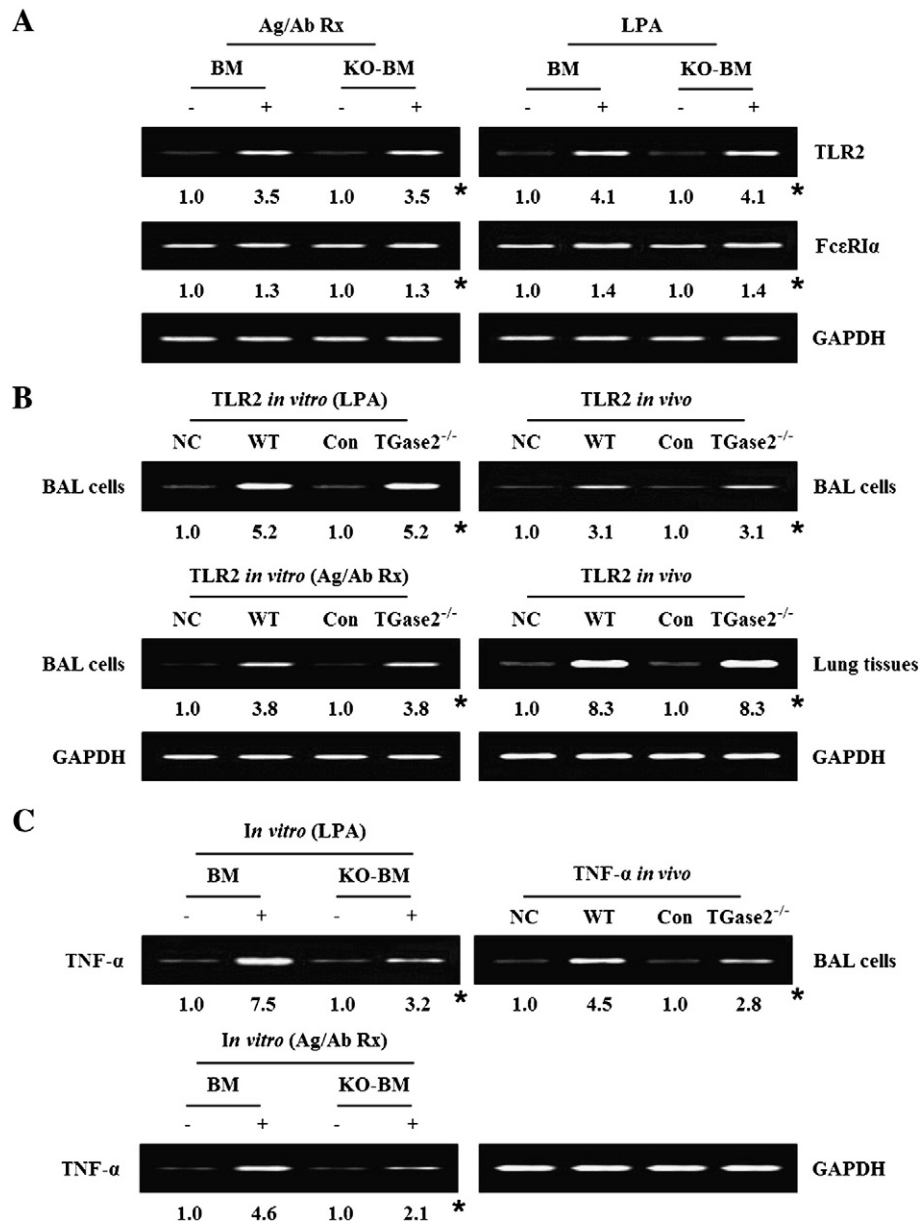


Fig. 5. Effects of TGase 2^{-/-} on the expression of TLR2 or FcεRI mRNA in BMMCs, BAL cells activated with stimuli or in BAL cells and lung tissues of OVA-challenged allergic asthma in mice. Activation of BMMCs, experimental details for the sensitization of WT or TGase 2^{-/-} mice, and definition of all symbols used have been described in Figs. 1 and 3 legends. Expression of TLR2, FcεRI and TNF-α mRNA was determined or mRNA extracts isolated from BAL cells of OVA-challenged mice or BMMCs (BM or KO-BM) using RT-PCR. (A) Expression of TLR2 or FcεRI mRNA in BMMCs activated with Ag/Ab reaction (left panel) or LPA (right panel). (B) Expression of TLR2 mRNA in BAL cells (from normal mice) activated with LPA or Ag/Ab reaction (left panel) or lung tissues of OVA-challenged mice (right panel). (C) Expression of TNF-α mRNA in act-BMMCs with LPA or Ag/Ab reaction (left panel) or OVA-challenged BAL cells (right panel). *, Numbers below bands are mean (n = 4) obtained from the ratio of band density of each group versus those of control and actin or GAPDH mRNA. NC, negative control. Rx, BMMCs activated with DNP-HSA/anti-DNP IgE Ab (act-BMMCs). Con, Control for TGase 2^{-/-}. LPA, LPA-stimulated BMMCs or BAL cells isolated from non-OVA-challenged WT or TGase 2^{-/-} mice.

anti-CD40 Ab inhibits expression of CD40L as well as CD40 in act-B cells with act-BMMCs can be inferred that CD40L in the absence of CD40 by anti-CD40 Ab may be degraded in proper time or anti-CD40 Ab may have cross-inhibitory response to CD40L molecule. However, further study needs to confirm.

NF-κB is degraded and translocated from the I-κB complex via I-κB phosphorylation, and activated in the nucleus. It increases the expression of multiple inflammatory genes in mast cells [32] and allergic asthma [3]. Thus, the expression of inflammatory cytokines [23,25,33] and MMPs [26] are up-regulated in mast cell activation and allergic asthma following NF-κB activation. TGase 2 also contributes to the pathogenesis of inflammation through TGase 2-mediated NF-κB activation [15].

TGase 2, which is over-expressed by inflammatory cytokines following I-KK-dependent NF-κB activation [15], has been identified as a novel mediator of asthma pathogenesis [3,16,17]. Therefore, our data suggest that expression of cell surface molecules (CD40/CD40L), various molecules related to tissue remodeling (MMPs and collagen) and more TGase 2 may be induced by TGase 2 (originally existed in cells)-mediated NF-κB activation in mast cell activation, and also suggest that TGase 2 may be expressed in downstream of NF-κB activation (TGase 2 expressed at 2 h after Ag/Ab reaction). This relationship between mast cells and TGase 2 is supported by the data that TGase 2^{-/-} protected against PCA caused by FcεRI-mediated mast cell activation, and that it also reduced the recruitment of airway inflammatory

cells and tissue remodeling via reducing cytokines/chemokines, AHR, goblet cells, and various remodeling molecules, such as imbalance of MMP2/9 and TIMP1/2, and collagen, via I- κ B-dependent NF- κ B.

TGase 2 increases PLA2 catalytic activity by forming intramolecular linkages that facilitate PLA2 dimerization [3]. The PLA2 activation induces LTs releases in mast cells [34], and PLA2-cycLTs axis induces airway inflammation and structural remodeling in asthma [1]. Our data are consistent with the data reported by other laboratories. However, it may be difficult to convince that the phosphorylation of cPLA2 and Ca^{2+} influx are associated with LTs releases because they are non-specific responses, but there are many reports that cPLA2 are controlled by phosphorylation and Ca^{2+} influx. And, PLA2 activity was additionally confirmed to cover non-specific responses in this study.

Mast cells play an important role in host defense against pathogens via TLRs [28]. TGase 2 participates in the host defense using NF- κ B activation. Recently, it was reported that TGase 2 reduced *Drosophila* larvae show increased mortality in septic injury and are more susceptible to a natural infection involving entomopathogenic nematodes and their symbiotic bacteria [35]. However, our data can be inferred that host defense system in TGase 2^{-/-} mice may be enhanced due to the increment of TLR2 expression in act-KO-BMMCs and act-KO-BAL cells from TGase 2^{-/-} mice as same as those in act-WT-BMMCs or act-BAL cells (Fig. 5), although NF- κ B-reduced activity in TGase 2^{-/-} mice may anticipate the reduction of host defense. And, it is also supported by recent report that epithelial TGase 2 is needed for pulmonary inflammation and fibrosis in bleomycin-treated mice [12]. However, TGase 2, which is widely distributed in various tissue cells such as macrophages, endothelium and epithelium, may play a role of NF- κ B activation in these tissue cells [17] as well as mast cells shown in this study. Thus, effect of TGase 2 deficiency on these cells, not limited to the mast cells, needs to investigate further.

TGase 2 does not seem to control all aspects of signaling pathways in mast cells which are activated through a variety of signaling pathways in upstream of TGase 2, although TGase 2 is controlled through Fc ϵ RI pathways by Ag/Ab reaction in this study. However, it needs further study. And, although TGase 2 expressed/activated from mast cell activation contributes to allergic asthma in mice, TGase 2 expressed by mast cells is not the sole exclusive mechanism in eliciting allergic asthma. And, Ca^{2+} /TGase 2 expressed in BMMCs does not affect histamine storage in granules, but it affects histamine release by Ag/Ab reaction.

In conclusion, although TGase 2 has been detected in a variety of cell types, our report is the first to demonstrate the TGase 2 expression in mast cells. Our data suggest that TGase 2 expressed during mast cell activation enhances IgE production in the B cells by up-regulating CD40L expression. TGase 2 may contribute to the development of allergic airway inflammation by activating several signaling molecules associated with inflammatory and lung tissue remodeling pathways in mice. This is accomplished by observing that OVA-challenged allergic asthma is induced through mediators such as TNF- α , LTs and cytokines produced in mast cell activation via Ca^{2+} -TGase 2 at up-stream of PKCs/MAPKs/I- κ B/NF- κ B and PLA2 activation (Fig. 6). Thus, TGase 2 inhibitors may be developed as a potential therapeutic agent for treating airway inflammation and remodeling caused with mast cell activation in patients with allergic asthma.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cellsig.2013.03.010>.

Conflict of interest statement

The authors have no financial conflicts of interest.

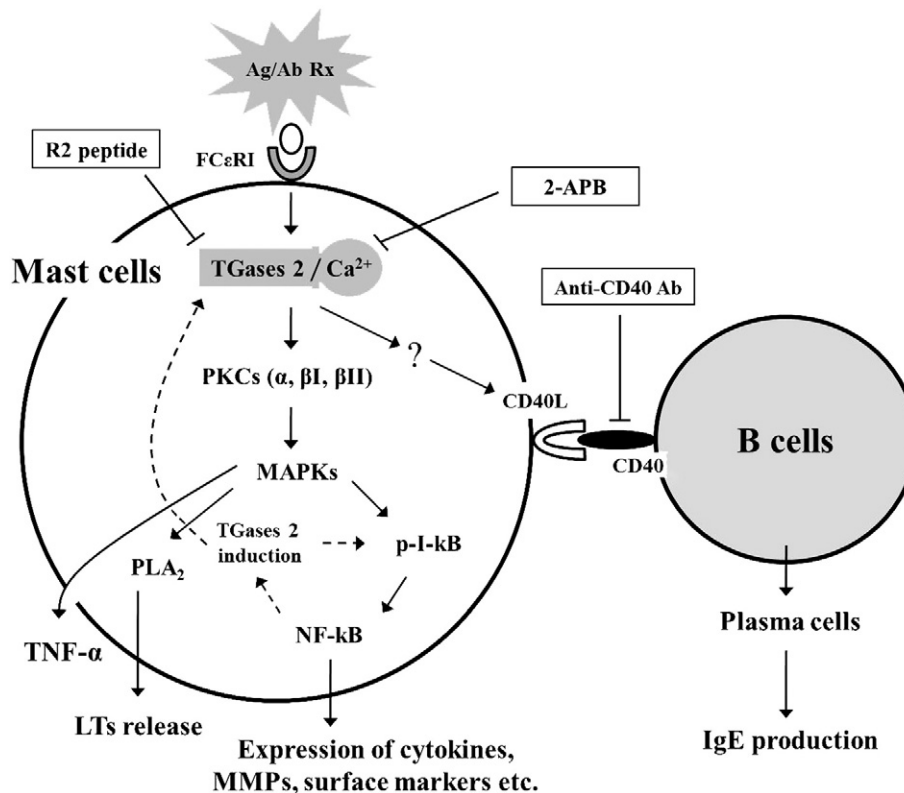


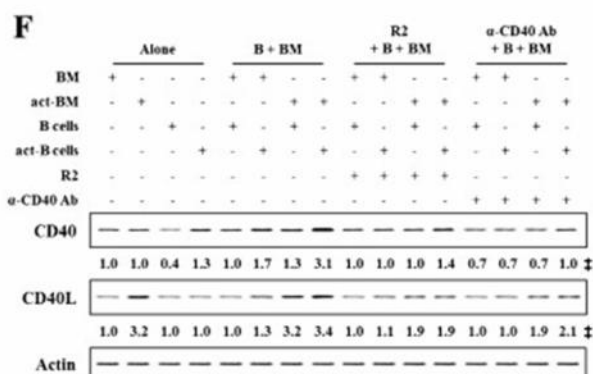
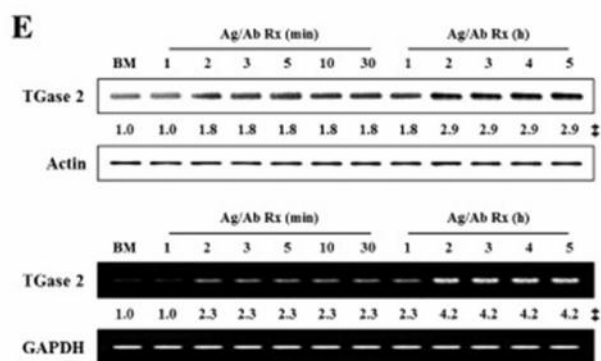
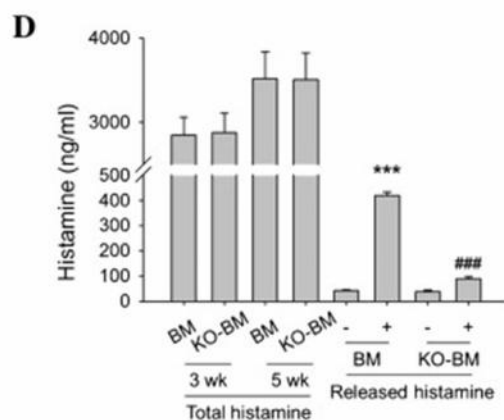
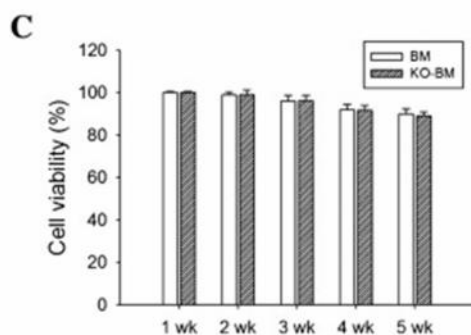
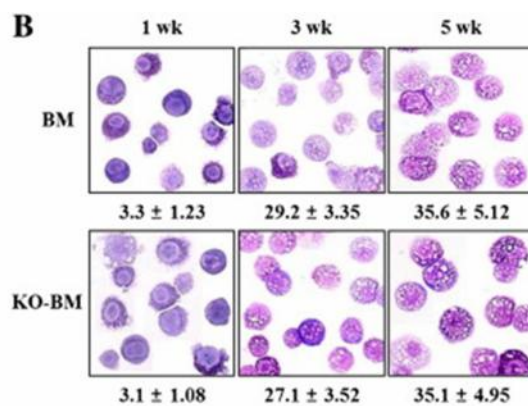
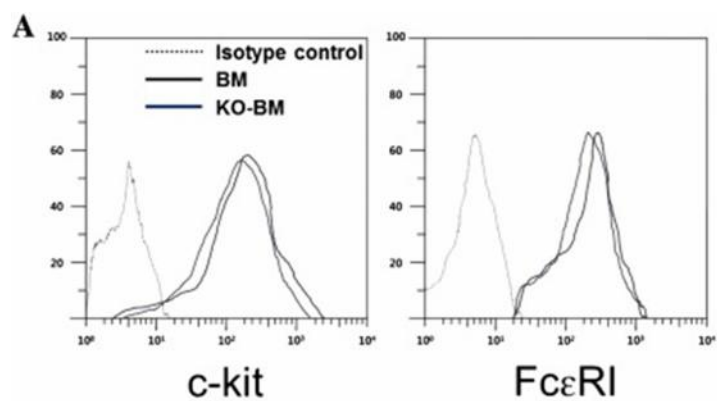
Fig. 6. A schematic diagram for signaling pathways in mast cells activated with Ag/Ab reaction in vivo and in vitro. The data suggest that mast cells activated with Ag/Ab reaction express TGase 2 that it with Ca^{2+} influx are bidirectional requisition, and the expressed TGase 2 induces mast cell surface marker CD40L, although we could not confirm if TGase 2 directly expresses CD40L or expresses it via any molecules. Thus, TGase 2 expressed/activated in mast cells may enhance IgE production through B cell Ig isotype class switching via interaction of B cell surface marker CD40, which is inhibited by anti-CD40 Ab. And, our data also suggest that Ca^{2+} level/TGase 2 produces mediators, such as TNF- α , LTs and inflammatory cytokines/chemokines, through PKCs/MAPKs/I- κ B/NF- κ B or PLA2 activation in vitro and in vivo. It can be also inferred that NF- κ B activation may express TGase 2, but further study needs to confirm. $\rightarrow$ or the dotted arrow indicates blocking action by inhibitors and a response that was not determined in this study, respectively.

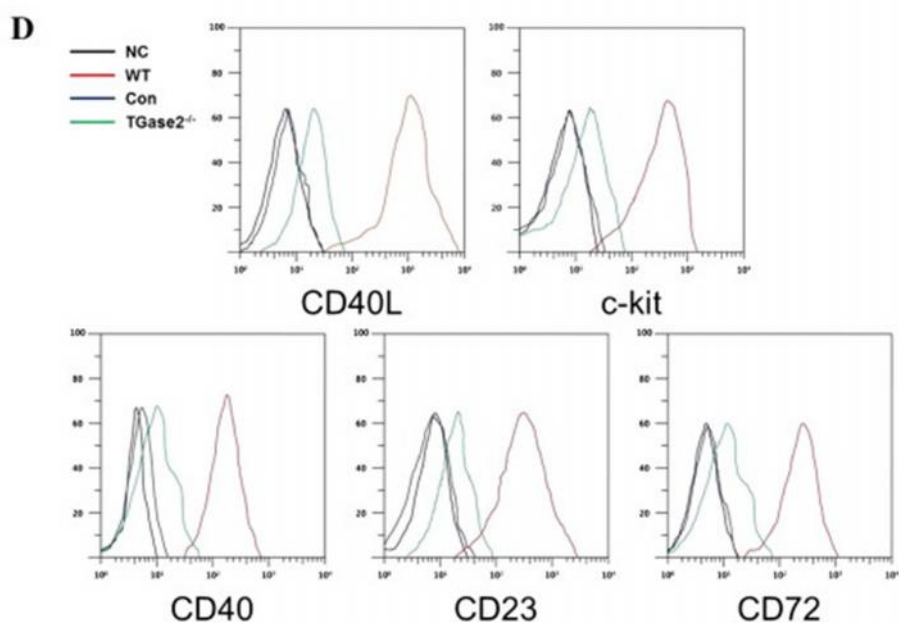
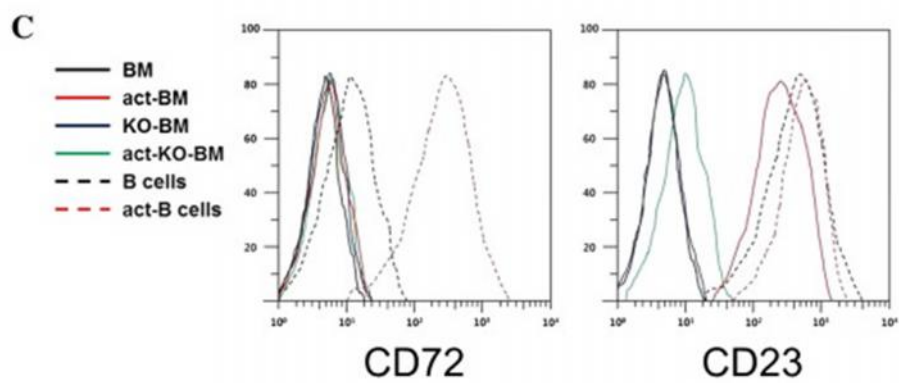
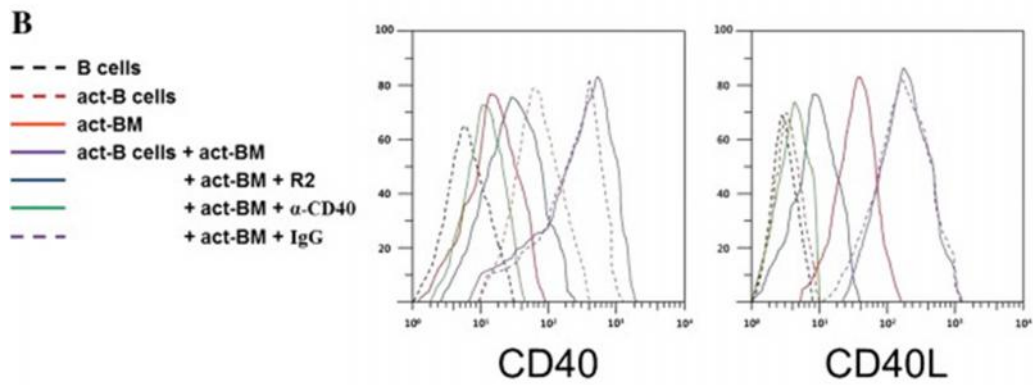
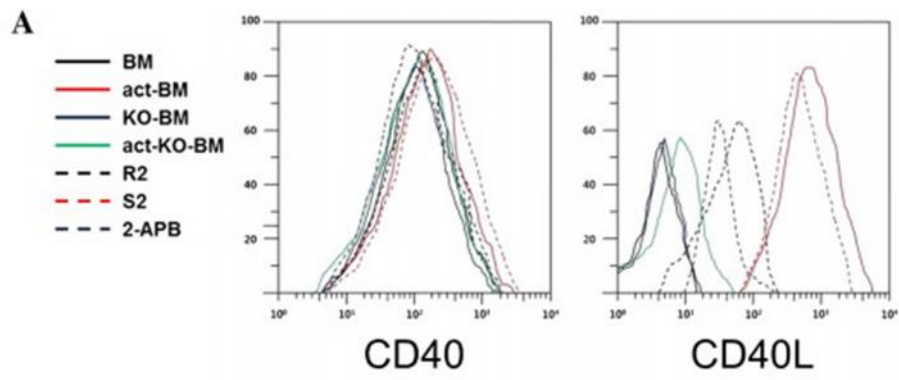
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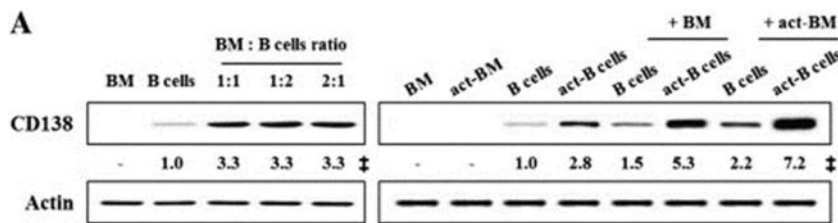
References

- [1] C. Jin, C.P. Shelburne, G. Li, E.N. Potts, K.J. Riebe, G.D. Sempowski, W.M. Foster, S.N. Abraham, *Journal of Clinical Investigation* 121 (2011) 941–955.
- [2] L.C. Wu, *Journal of Biological Chemistry* 286 (2011) 32891–32897.
- [3] D.Y. Kim, B.S. Park, G.U. Hong, B.J. Lee, J.W. Park, S.Y. Kim, J.Y. Ro, *British Journal of Pharmacology* 162 (2011) 210–225.
- [4] D.Y. Kim, D. Jeoung, J.Y. Ro, *Journal of Immunology* 185 (2010) 273–283.
- [5] S.T. Galli, S. Nakae, M. Tsai, *Nature Immunology* 6 (2005) 135–145.
- [6] G.A. Bishop, *Journal of Immunology* 188 (2012) 4127–4129.
- [7] J. Pene, F. Guihot, I. Cognet, A. Guay-Giroux, J.Y. Bonnefoy, G.C. Elson, H. Yssel, J.F. Gauchat, *Methods in Molecular Biology* 315 (2006) 319–329.
- [8] S. Merluzzi, B. Frossi, G. Gri, S. Parusso, C. Tripodo, C. Pucillo, *Blood* 115 (2010) 2810–2817.
- [9] K. Oh, H.B. Park, M.W. Seo, O.J. Byoun, D.S. Lee, *Clinical Immunology* 145 (2012) 122–132.
- [10] K.S. Park, H.K. Kim, J.H. Lee, Y.B. Choi, S.Y. Park, S.H. Yang, S.Y. Kim, K.M. Hong, *Journal of Cancer Research and Clinical Oncology* 136 (2010) 493–502.
- [11] D.S. Kim, B. Kim, H. Tahk, D.H. Kim, E.R. Ahn, C. Choi, Y. Jeon, S.Y. Park, H. Lee, S.H. Oh, S.Y. Kim, *Biochemical and Biophysical Research Communications* 403 (2010) 479–484.
- [12] K. Oh, H.B. Park, O.J. Byoun, D.M. Shin, E.M. Jeong, Y.W. Kim, Y.S. Kim, G. Melino, I.G. Kim, D.S. Lee, *The Journal of Experimental Medicine* 208 (2011) 1707–1719.
- [13] K.S. Ghanta, S.B. Pakala, S.D. Reddy, D.Q. Li, R. Kumar, *Journal of Biological Chemistry* 286 (2011) 7132–7138.
- [14] I. Matic, A. Sacchi, A. Rinaldi, G. Melino, C. Khosla, L. Falasca, M. Piacentini, *Journal of Leukocyte Biology* 88 (2010) 181–188.
- [15] K.S. Park, D.S. Kim, C. Ko, S.J. Lee, S.H. Oh, S.Y. Kim, *Frontiers in Bioscience* 3 (2011) 341–354.
- [16] T.S. Hallstrand, M.M. Wurfel, Y. Lai, Z. Ni, M.H. Gelb, W.A. Altemeier, R.P. Beyer, M.L. Aitken, W.R. Henderson, *PLoS One* 5 (2010) e8583.
- [17] Y. Kim, S. Eom, K. Kim, Y.S. Lee, J. Choe, J.H. Hahn, H. Lee, Y.M. Kim, K.S. Ha, J.Y. Ro, D. Jeoung, *Molecular Immunology* 47 (2010) 1010–1022.
- [18] H. Lee, D.J. Lee, S.P. Oh, H.D. Park, H.H. Nam, J.M. Kim, D.S. Lim, *Molecular and Cell Biology* 26 (2006) 5373–5381.
- [19] M.E. van Strien, J.J. Breve, S. Fratantoni, M.W. Schreurs, J.G. Bol, C.A. Jongenelen, B. Drukarch, A.M. van Dam, *PLoS One* 6 (2011) e25037.
- [20] D.P. Calado, B. Zhang, L. Srinivasan, Y. Sasaki, J. Seagal, C. Unitt, S. Rodig, J. Kutok, A. Tarakhovsky, M. Schmidt-Suppran, K. Rajewsky, *Cancer Cell* 18 (2010) 580–589.
- [21] T.A. Doherty, P. Soroosh, N. Khorram, S. Fukuyama, P. Rosenthal, J.Y. Cho, P.S. Norris, H. Choi, S. Scheu, K. Pfeffer, B.L. Zuraw, C.F. Ware, D.H. Broide, M. Croft, *Nature Medicine* 17 (2011) 596–603.
- [22] Y. Kim, K. Kim, D. Park, E. Lee, H. Lee, Y.S. Lee, J. Choe, D. Jeoung, *Journal of Biological Chemistry* 287 (2012) 25844–25859.
- [23] W. Ellmeier, A. Abramova, A. Schebesta, *FEBS Journal* 278 (2011) 990–1000.
- [24] T.R. Kataoka, A. Kumanogoh, G. Bandara, D.D. Metcalfe, A.M. Gilfillan, *Journal of Immunology* 184 (2010) 2468–2475.
- [25] J.S. Dahlin, M.A. Ivarsson, B. Heyman, J. Hallgren, *PLoS One* 6 (2011) e20261.
- [26] H. Maxova, L. Bacakova, V. Lisa, J. Novotna, H. Tomasava, M. Vizek, J. Herget, *Physiological Reviews* 59 (2010) 711–719.
- [27] Z. Zietkowski, R. Skiepkio, M.M. Tomasiak-Lozowska, B. Mroczko, M. Szmitkowski, A. Bondzenta-Lukaszyk, *Respiration* 80 (2010) 463–471.
- [28] A.R. Rodriguez, J.J. Yu, M.N. Guentzel, C.S. Navara, K.E. Klose, T.G. Forsthuber, J.P. Chambers, M.T. Berton, B.P. Arulanandam, *Journal of Immunology* 188 (2012) 5604–5611.
- [29] J.Y. Ro, B.C. Lee, J.Y. Kim, Y.J. Chung, M.H. Chung, S.K. Lee, T.H. Jo, K.H. Kim, Y.I. Park, *Journal of Pharmacology and Experimental Therapeutics* 292 (2000) 114–121.
- [30] L.K. Poulsen, L. Hummelshoj, *Annals of Medicine* 39 (2007) 440–456.
- [31] Y. Yanagihara, K. Kajiwar, Y. Basaki, K. Ikizawa, M. Ebisawa, C. Ra, *Clinical and Experimental Immunology* 111 (1998) 136–143.
- [32] M.H. Hong, J.H. Kim, S.H. Na, H. Bae, Y.C. Shin, S.H. Kim, S.G. Ko, *Bioscience, Biotechnology, and Biochemistry* 74 (2010) 1152–1156.
- [33] A.J. MacNeil, Y.J. Yang, T.J. Lim, *Journal of Immunology* 187 (2011) 3374–3382.
- [34] S.G. Payne, C.A. Oskeritzian, R. Griffiths, P. Subramanian, S.E. Barbour, C.E. Chalfant, S. Milstien, S. Spiegel, *Blood* 109 (2007) 1077–1085.
- [35] Z. Wang, C. Wilhelmsson, P. Hyrsi, T.G. Loof, P. Dobes, M. Klupp, O. Loseva, M. Mörgelin, J. Iklé, R.M. Cripps, H. Herwald, U. Theopold, *PLoS Pathogens* 6 (2010) e1000763.

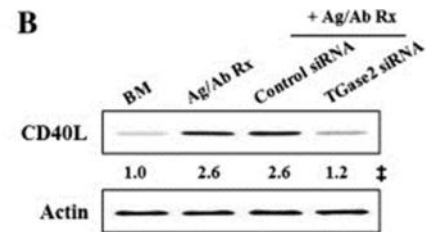




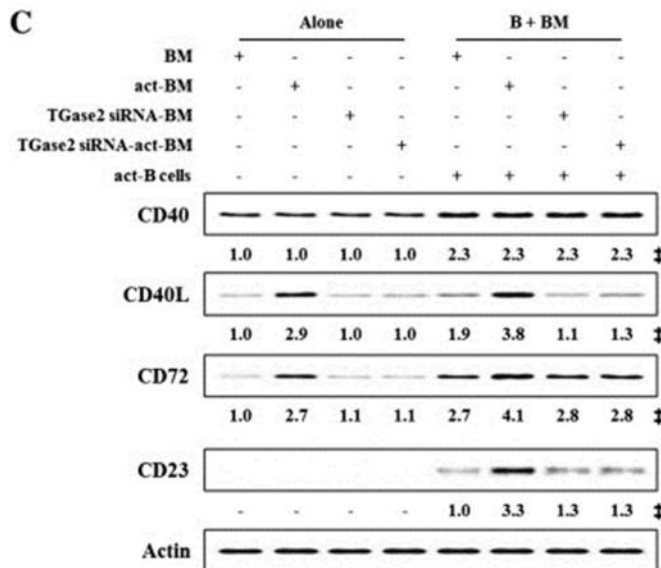
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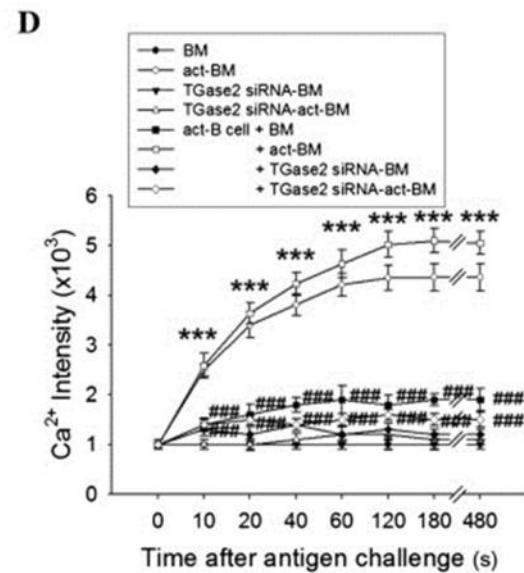
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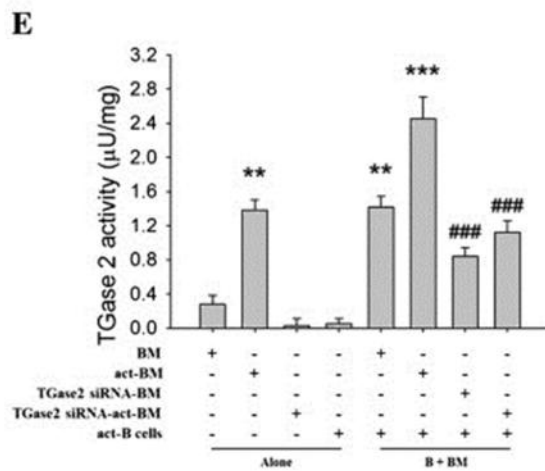
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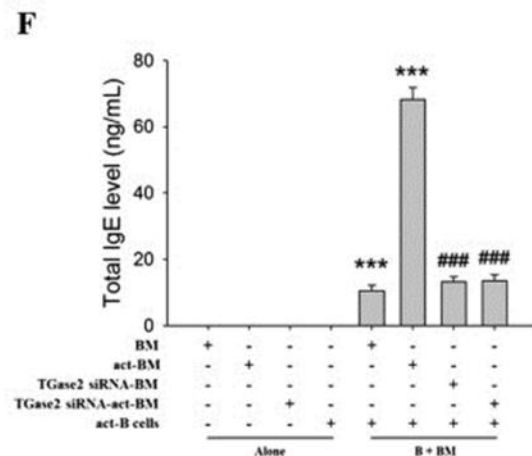
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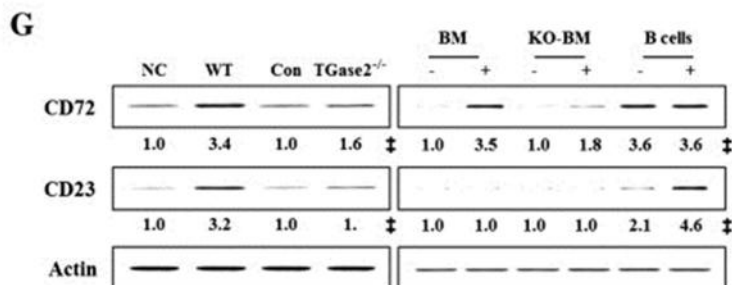
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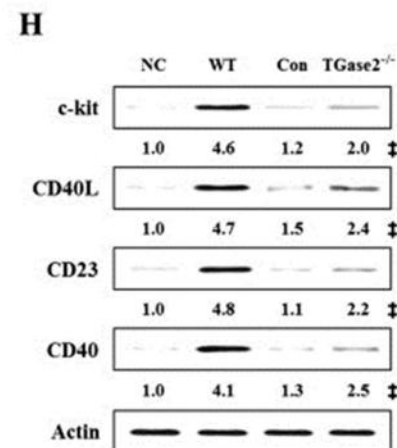
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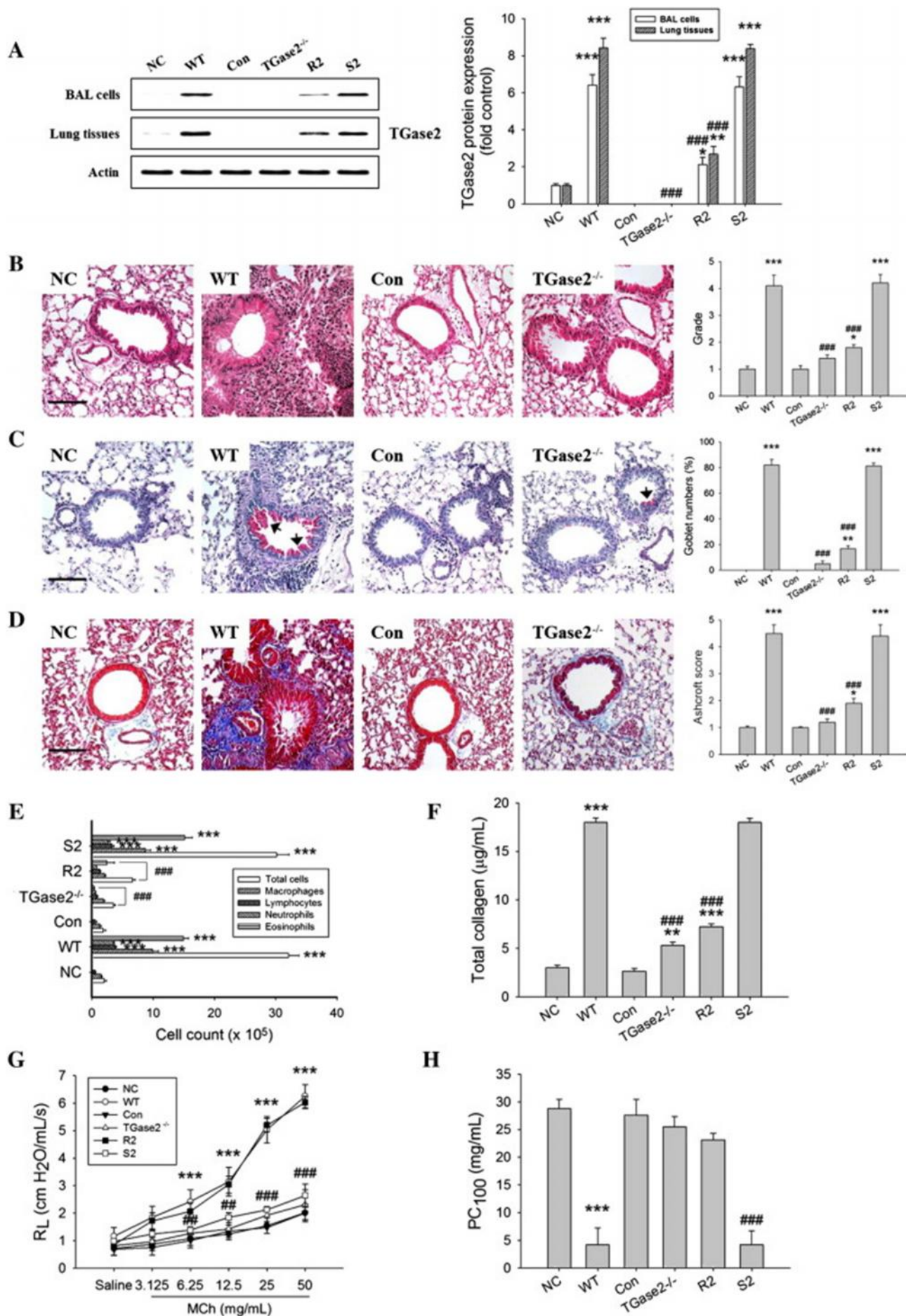


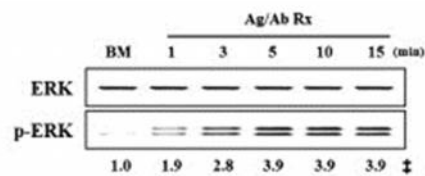
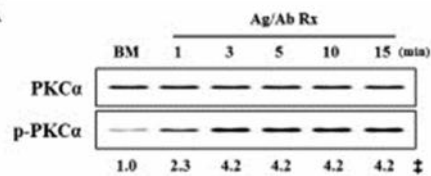
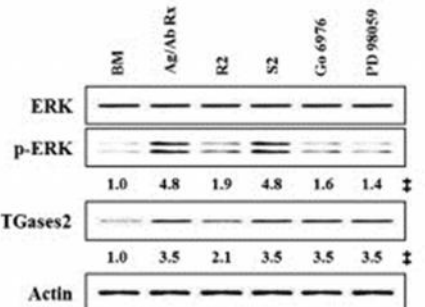
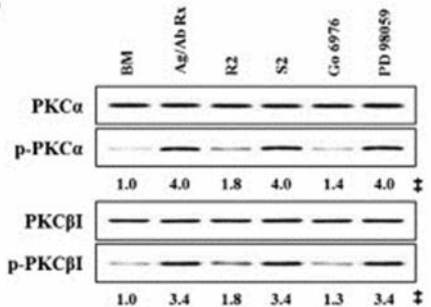
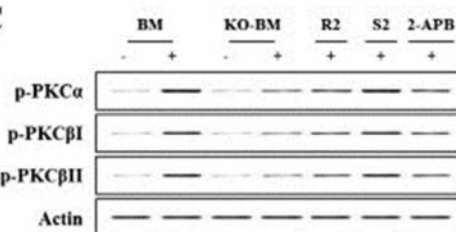
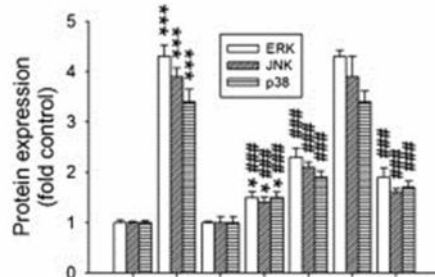
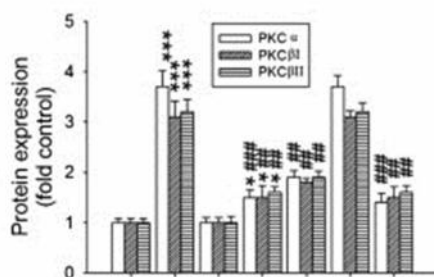
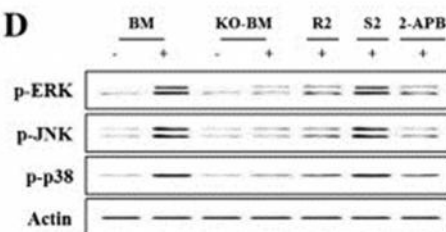
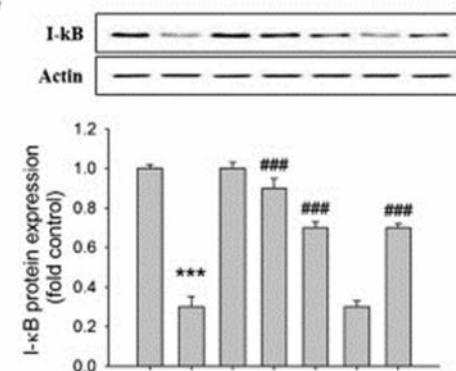
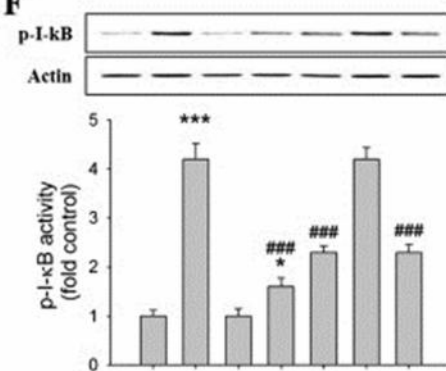
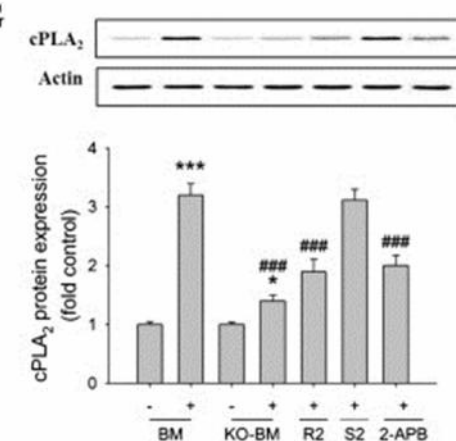
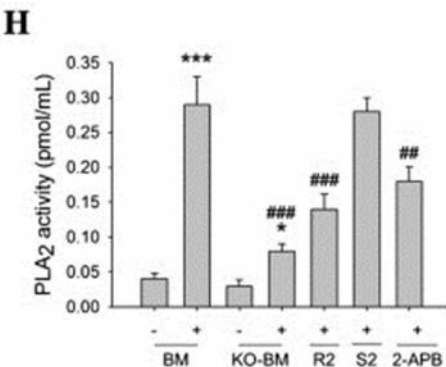
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Supplementary data

IgE production in CD40/CD40L cross-talk of B and mast cells and mediator release via TGase 2 in mouse allergic asthma

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Key words: allergic asthma, B cells, IgE, mast cells, transglutaminase 2 (TGase 2), mediators, cell surface molecules

Abbreviations: AHR, Airway hyperresponsiveness; BAL, Bronchoalveolar lavage; BMMCs, Bone marrow-derived mast cells; MCh, Methacholine; MMPs, Matrix metalloproteinases; PAS, Periodic acid-Schiff; PLA2, Phospholipase A2; PCA, Passive cutaneous anaphylaxis;

TLR, Toll like receptor; TGase 2, Transglutaminase 2; TGase 2^{-/-}, TGase 2 knock-out; TIMPs, Tissue inhibitor metalloproteinases; WT, wild type.

Materials and methods

Targeting of mouse TGase2 and genotyping

A 14,327-base pair genomic DNA fragment (from the *EcoRV* site to the *XhoI* site) was retrieved from a BAC clone (bMQ-272N7, Sanger Institute) and placed into the *SalI-NotI* site of the pBluescript phagemid system in accordance with a previously reported procedure (29). A neomycin-resistance cassette restricted from PL451 (provided by N Copeland) was inserted within the *EcoRV-SmaI* site, leading to disruption of exons 5 and 6 of mouse TGase 2. The diphtheria toxin A chain cassette (DT-A) was employed as a negative selection marker. The targeting vector was linearized and used for gene targeting in the E14Tg2A embryonic stem (ES) cell line (129OlaHsd-derived, Baygenomics). Electroporation of mouse ES cells in culture was performed as previously describe (18). Homologous recombination was confirmed by Southern blot analysis of genomic DNA prepared from neomycin-resistant ES cell clones using external probes (11).

The 5'-external probe (760 bp) was obtained by PCR using two primers, 5'-TCT GAG CTC TTC GCT CAT GGG-3' and 5'-GAA TTC CTG GCA GGC AGA GCA-3'. Two of the 12 correctly targeted TGase 2^{+/-} ES cell clones were subsequently injected into C57BL/6 blastocysts, resulting in germ line-transmitting chimeric mice.

Mouse genotyping

Genomic DNA extracted from tail biopsies taken 3 wk postnatally was screened for the presence of wild-type and mutant alleles by PCR. To detect the wild-type and mutant TGase 2 alleles, amplification reactions were performed with the following primers: 5'-CTT TCT AGT CTC CAG GAC ACA C-3' and 5'-GAC TGT CTT CCT CTG TCT CCT G-3' for PCR-1, and 5'-CTT TCT AGT CTC CAG GAC ACA C-3 and 5'-GGA TGA TCT GGA CGA AGA GC-3' for PCR-2. WT and mutant alleles yielded products of 465 bp and 427 bp, respectively

(11).

Culture and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells were flushed from femurs and tibias of WT and TGase^{-/-} mice. RBC was lysed using 0.1 M NH₄Cl and remaining cells were washed, resuspended, and cultured for 5 wk in RPMI-1640 supplemented with 10 % heat-inactivated FBS and 50 % WEHI-3B conditioned media which contains IL-3. BMMCs (1×10^6 cells) were confirmed by May-Grünwald-Giemsa staining [4]. The purity for BMMCs was more than 95 % of total cells.

Both BMMCs (1×10^6 cells) were sensitized with anti-DNP IgE Ab (0.1 µg/mL) overnight at 37 °C, washed twice, and then challenged with 10 ng/mL DNP-HSA (Sigma-Aldrich, St. Louis) (refer to act-BMMCs) for period indicated at 37 °C in Tyrode's buffer [4]. Ca²⁺ inhibitor (100 µM 2-APB), and R2 or scrambled (S2) peptide (200 µg/mL TGase 2 inhibitor and positive control for R2, respectively) were added 10 min and 30 min, respectively, before DNP-HSA stimulation. To investigate signaling cascades between Ca²⁺ levels and TGase 2, Ca²⁺ blocker in bone marrow cell culture media was treated at 1, 2, 3 or 4 wk, respectively. Optimal time and concentration were first determined in preliminary experiments.

Western blot analysis

BMMCs, BAL cells (1×10^6 cells) or lung tissues (50 mg/500 µL) were suspended in a low-salt lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSE, 2.0 µg/mL aprotinin, 2.0 µg/mL leupeptin] and allowed to swell on ice for 30 min. The cells or tissues were then homogenized using a Polytron (Kinematica, Littau, Switzerland). Supernatants obtained after centrifugation (20 µg) were subjected to 10 %

SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were washed with PBS containing 0.1 % Tween 20 (PBST), and then blocked for 1 h in 5 % skim milk in PBST. After the membranes were washed with PBST, they were incubated for 60 min at room temperature with primary Abs. Membranes were washed with PBST and treated with HRP-conjugated goat anti-mouse or HRP-conjugated rabbit anti-goat IgG (diluted to 1:5,000 ~ 1:10,000) (Bethyl Laboratories, Montgomery, TX) in PBST for 60 min. After washing, the protein bands were visualized by ECL using a chemiluminometer (Amersham Biosciences) [3].

Measurement of intracellular Ca^{2+} [Ca^{2+}]_i level

BMMCs (3×10^6 cells) were incubated for 30 min after adding Fluo-3 AM (5 μ M) and placed on a glass slide treated with poly-L-lysine. The [Ca^{2+}]_i levels in act-BMMCs were quantified with fluorescence intensity using a LSM 510 laser scanning microscope (Carl Zeiss, Oberkochen, Germany) [4]. Fluorescence intensity for control (F = 0) was adjusted as 1.

TGase 2 activity assay

The TGase 2 activity in the supernatants isolated from act-BMMCs, act-B cells with anti-IgM Ab (1×10^6 cells) for 1 h (optimal time yielded by preliminary experiment) or lung tissue homogenates (50 mg/500 μ L) were determined using the TG-Covtest kit (Covalab, Villeurbanne, France) following the manufacturer's instructions [19]. TGase 2 activity was calculated using standard curves generated with specific TGase 2 standards, and expressed in μ U/mg protein. All assays were done in triplicate.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from BMMCs (3×10^6 cells), BAL cells (3×10^6 cells) or lung tissues (50 mg/500 μ L). Cells or brain tissues were washed twice with ice-cold PBS, and resuspended in 1mL ice-cold buffer A [10 mM Hepes/KOH (pH 7.9), 10 mM KCl, 1.5 mM MgCl_2 , 0.5 mM DTT, 0.2 mM PMSF, 1 μ g/mL leupeptin and 1 μ g/mL aprotinin]. After incubation on ice for 15 min, the cells were lysed by adding 10 % Nonidet P40 (10 μ L) and immediately vortexed for 10 s. Nuclei were harvested by centrifugation at 20,000 $\times$ g for 1 min and resuspended in 40 μ L ice-cold buffer C [20 mM Hepes/KOH (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, 25 % glycerol, 0.2 mM PMSF, 1 μ g/mL leupeptin and 1 μ g/mL aprotinin]. After incubation at 4°C for 20 min on a shaking platform, the nuclei were clarified by centrifugation at 15,000 $\times$ g for 10 min. The supernatant (nuclear extract) was then transferred to a new tube, and quantified using Bradford's method. Nuclear extract was stored at -70°C until required [3].

Ten microliter of a mixture of NF- κ B or AP-1 oligonucleotide, T4 polynucleotide kinase 10 X buffer, [α - ^{32} P]ATP (10 μ Ci; 3,000 Ci/mmol), nuclear-free water, and T4 polynucleotide kinase (5 ~ 10 U/ μ L) were incubated at 37 °C for 30 min. After adding 89 μ L Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], unincorporated nucleotides were removed from the DNA probe by chromatography through a G-25 spin column. The nuclear extract and gel shift binding 5 X buffer [20 % glycerol, 5 mM MgCl_2 , 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50mM Tris-HCl (pH 7.5), 0.25 mg/mL poly (dI-dC)] were incubated at room temperature for 10 min, and then 20-30 fmol of ^{32} P-labeled NF- κ B or AP-1 oligonucleotide was added, and incubated at room temperature for 20 min. After stopping the reaction, 1 μ L of 10 X gel loading buffer was added to each reaction. Reaction mixtures were electrophoresed on 6 % polyacrylamide gels, and the gels were analyzed using FLA-2000 (FUJIFILM, Japan).

Isolation and activation of B cells or co-culture of B cells and mast cells

B cells were isolated from spleens of C57BL/6 mice using the MagCelect mouse B cell isolation kit (R&D Systems, Inc, Minneapolis) according to the manufacturer's protocol. The purity of B cells was more than 94% of total cells.

BMMCs (1×10^6 cells) were activated with Ag/Ab for 5 h as presented above [1]. B cells (1×10^6 cells) were incubated with 0.5 $\mu\text{g/mL}$ anti-mouse IgM Ab (refer to act-B cells) (Jackson ImmunoResearch Laboratories, West Grove) for 48 h [8]. The act-B cells (1×10^6 cells) were added on non-act-BMMCs (BMMCs alone without Ag/Ab reaction) or act-BMMCs (1×10^6 cells), and co-cultured for 72 h. The optimal incubation time and ratio (1:1) for co-cultured of both cells was yielded in preliminary experiments. IgE secreted in culture media was determined by ELISA method [20].

To assess formation of plasma cells, B cells (1×10^6 cells) were first washed twice in FACS buffer (PBS containing 5 % FBS, 0.1 % sodium azide). The cells were incubated with phycoerythrin-conjugated anti-mouse CD138 (BD Bioscience, San Jose, CA) for 1 h at 4 °C. The CD138 expression was measured by FACS analyzer (Becton and Dickinson, San Jose) [20].

Measurement of IgE level, cytokines, and MMPs by ELISA

The supernatants (200 μL) were isolated from act-B cells with or without act-BMMCs. To obtain serum, blood samples collected by cardiac puncture immediately after anesthesia were centrifuged at 550 x g for 30 min after clotting at room temperature. IgE amounts in the supernatants or sera were analyzed using ELISA [3]. Briefly, for total IgE or OVA-specific IgE in serum, microplates were coated with purified rat anti-mouse IgE, and then treated with supernatants or mouse sera followed by total IgE (Bethy Laboratories, Montgomery, TX) or

biotinylated OVA which is prepared with a peroxidase-conjugated goat anti-biotin Ab, respectively (MD Bioproducts, St. Paul), which was used as secondary Ab. Mouse IgE (Bethyl Laboratories) was used for calibrating the assay. Reactions were read using an ELISA plate reader at 450 nm.

Amounts of cytokines or MMP2/9 in the supernatants from act-BMMCs and obtained from lung tissues (20,000 x g, 10 min) were determined using an ELISA kit (BD Biosciences) according to manufacturer's methods. Standard curves were generated using different concentrations of each of the recombinant cytokines or MMPs. The lowest detection limit for IL-4, IL-5, IL-6, IL-8 and TNF- α was better than 31.3 pg/mL, and for IL-13, RANTES, TGF- β was 15.6 pg/mL, and for MMP2/9 was 0.078 pg/mL.

TGase 2 small interfering RNA (siRNA) transfection

Specific oligonucleotide for siRNA-TGase 2 (5'-AAGGGCGAACCACCTGAACAA-3') was purchased from Invitrogen (Invitrogen Life Technologies, Carlsbad). To control general toxicity of siRNA, a negative oligonucleotide control sequence, which has not homologous to any human mRNA (5'-AATTCTCCGAACGTGTCACGT-3') (Invitrogen Life Technologies, Carlsbad), was used. Transfection was carried out according to the manufacture's protocol (Santa Cruz Biotech, Santa Cruz). TGase 2 siRNA transfected BMMCs were used in CD40/CD40L, $[Ca^{2+}]_i$ level and TGase 2 activity, measurement of IgE level [4]. Negative control was performed by adding the PBS.

Bronchoalveolar lavage (BAL) fluid

Immediately after blood collection, thoracic cavities were carefully opened. Tracheas were exposed, and BAL fluid was collected by cannulating the upper part of the trachea, by lavaging twice with 1 mL and then 0.8 mL of PBS (85-90 % of the volume inputted was

recovered). Lavaged sample from each mouse were kept on ice until required. BAL fluid was centrifuged at 400 x g for 5 min at 4 °C. After centrifugation, lavage supernatants were removed and kept, and then pellets were resuspended in 100 µl PBS, and total viable cell numbers were counted by trypan blue exclusion using a hemacytometer. BAL cells were adjusted to a concentration of 4×10^5 cells/mL of PBS. For cytospin preparations, cells were centrifuged at 400 x g for 3 min using a Cytospin III (Shandon, Pittsburg), and the cells were stained with Diff-Quik (International Reagents Corp., Tokyo, Japan) [3]. Mast cells were stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany), and quantified by cell counts in 200 x 200 µm areas (5 areas/each slide x 8 mice/each group = 40 areas. Differential cell counting was performed using standard morphological criteria under a light microscopy. The remnant BAL cells were kept at -70 °C to be determined for signaling molecules, cytokines, NF-κB activity etc.

Lung tissue histology

Lung tissues were perfused with 5 mL PBS via the right ventricle for elimination the blood in lung tissues of mice. Left lung tissues were removed from the chest cavity, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 3 µm. Tissue sections were subjected to H&E staining to investigate changes in the general histology of lung tissues, Periodic acid-Schiff (PAS) staining to investigate goblet cell hyperplasia in the bronchial epithelium [3] and Masson's trichrome (Sigma-Aldrich, St. Louis) staining to investigate collagen deposition [21]. Histological changes for H&E were scored in terms of the extent of infiltration of inflammatory cells into lung tissues, which were 1, none; 2, slight; 3, mild; 4, moderate; 5, severe. Goblet cell hyperplasia was quantified as the percentage of PAS-positive cells/total cells in 5 sites of 200 µm x 200 µm areas (5 sites/each slides x 8 mice/group = 40 sites) under a microscopy. The grading data are presented in histograms. Histopathologic

evaluation of pulmonary fibrosis was performed using Ashcroft scoring method [21]. Mast cells were stained with May-Grünwald-Giemsa (Merk, Darmstadt, Germany) [4].

Collagen levels in lung tissues were assessed by the Sircol collagen assay (Biocolor Ltd., Carrickfergus, UK), according to the manufacturer's instructions. Absolute collagen content was calculated by comparing the sample values to a pertinent standard curve [21].

Measurement of airway hyperresponsiveness (AHR)

AHR was assessed by measuring changes in the airway resistance (R_L , cmH₂O/mL/s) after mice were treated with MCh using the Flexivent system (SCIREQ, Montreal, Quebec, Canada). Anesthetized (pentobarbital sodium 70 ~ 90 mg/kg, i.p. injection) and tracheostomized (18G cannula) mice were mechanically ventilated (160 breaths/min, tidal volume of 10 mL/kg, positive end expiratory pressure of 3 cmH₂O). Increasing concentrations (3 ~ 25 mg/mL) of MCh aerosol were administered by nebulization (3 ~ 25 mg/mL). The R_L was measured at 60 s and 120 s, respectively, after the administration of MCh [3]. Provocative concentration (PC_{100}) was calculated by the dose of MCh which induced a 100% increase in R_L .

Passive cutaneous anaphylaxis (PCA) analysis

In four groups (ten mice/group), an anti-DNP-specific IgE Ab (0.5 µg) was injected intradermally into one ear of each mouse. After 24 h, the mice were challenged with an i.v. injection of DNP-HSA (200 µg; Ag) in 200 µL of PBS containing 3 % Evans blue. Two h after Ag challenge, the mice were euthanized and the treated ear was excised to measure the intensity of dye that had extravasated in response to Ag. The dye was extracted for measuring the dye absorbance at 620 nm [22].

Immunostaining and Immunofluorescence

Lung tissue sections (slides) were deparaffinized with xylene and washed in ethanol. Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide in methanol for 5 min. Slides were treated with citrate buffer using microwave to antigen retrieval, and then blocked with 1% BSA in PBS for 1 h. Slides were incubated for overnight with a primary Abs against c-kit, CD23, CD40 and CD40L (1:100, Santa Cruz Biotechnology, Santa Cruz), and incubated with HRP-conjugated goat anti-mouse or HRP-conjugated goat anti-rabbit (1:1000, Zymed Laboratory Inc. San Francisco, CA) for 1 h, and then washed again with PBS followed by staining with 3-amino-9-ethylcarbazole (AEC, Dako, Glostrup, Denmark). Slides were counterstained with hematoxylin and finally mounted using aqueous mounting medium (Thermo Shandon, Pittsburgh), and then examined under a light microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany) [4].

The deparaffinized and blocked lung tissue sections (slides) were incubated with the Abs against c-kit and CD23 (1:25) or c-kit and IgE (1:50) in blocking solution for 5 h. The slides were then treated with the corresponding FITC- or TEXAS-conjugated anti-IgG (Zymed Laboratory Inc. San Francisco) for 1 h in room temperature. Washing steps between each stage were performed for 1-2 min. After washing, the slides were mounted using aqueous mounting medium (Thermo Shandon, Pittsburgh), and examined by confocal microscope (Carl Zeiss, Oberkochen, Germany) [4].

Measurement of Phospholipase A2 (PLA₂) activity

PLA₂ activity was measured in 96-ELISA well plates by the technique previously described [3]. The standard assay mixture contained 200 μ l of buffer (10 mM Tris-HCl, 10 mM CaCl₂ and 100 mM NaCl, pH 8.0), 20 μ L of substrate (4N3OBA), 20 μ L of water and 20 μ L of samples (supernatant from act-BMMCs, BAL fluid or lung tissue homogenates) in a final

volume of 260 μ L. The mixture was incubated for up to 40 min at 37 °C, and read at absorbance (425 nm) in 10 min intervals using a Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Enzyme activity, expressed as initial velocity of the reaction (V_o), was calculated based on the increase in absorbance after 20 min. All assays were done in triplicate.

Immunoassay for leukotrienes (LTs)

The LTs contents in supernatants from act-BMMCs, BAL fluid or supernatants obtained from lung tissue homogenates were determined using an enzyme immunoassay kit (Santa Cruz Biotechnology, Inc., Santa Cruz). The concentrations of LTs were then calculated using standard curves generated with specific LTs standards and analyzed using analysis tools on the Cayman Chemical website (<http://www.caymanchem.com/app/template/analysis%2CEIA.vm/a/z>). The results are expressed in pg/mL (1×10^6 cells).

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from BMMCs (1×10^6 cells), BAL cells (1×10^6 cells) or lung tissues (50 mg/500 μ L) using Trizol reagent (Invitrogen Life Technologies, Carlsbad). RT-PCR was performed in a final volume of 50 μ L using an amfiRivert One-step RT-PCR kit (GenDEPOT, Barker) in an automated thermal cycler (BIOER Technology, Hangzhou, China). PCR was performed for 35 cycles [3].

The primer sequences used were as follows: IL-4 sense, 5-TCG GCA TTT TGA ACG AGG TC-3; IL-4 anti-sense, 5-GAA AAG CCC GAA AGA GTC TC-3; IL-5 sense, 5-ATG GAG ATT CCC ATG AGC AC-3; IL-5 anti-sense, 5-GTC TCT CCT CGC CAC ACT TC-3; IL-6 sense, 5-TGG AGT CAC AGA AGG AGT GGC TAA G-3-3; IL-6 anti-sense, 5-TCT GAC CAC AGT GAG GAA TGT CCA C-3; IL-13 sense, 5-CAG CTC CCT GGT TCT CTC AC-

3; IL-13 anti-sense, 5-CCA CAC TCC ATA CCA TGC TG-3; TNF- α sense, 5-TTA TCT CTC AGC TCC ACG CC-3; TNF- α anti-sense, 5-TGC GCA CTG AAA GCA TGA TC-3; IL-8 sense, 5-CAA ACC TTT CCC CAA AT-3; IL-8 anti-sense, 5-ATT GCA TCT GGC AAC CCT AC-3; RANTES sense, 5-GAT GGA CAT AGA GGA CAC AAC T-3; RANTES anti-sense, 5-TGG GAC GGC AGA TCT GAG GG-3; MMP2 sense, 5-CAC ACC AGG TGA ACC ATG TG-3; MMP2 anti-sense, 5-AGG GCT GCA TTG CAA ATA TC-3; MMP9 sense, 5-GTA TGG TCG TGG CTC TAA GC-3; MMP9 anti-sense, 5-AAA ACC CTC TTG GTC TGC GG-3; TIMP1 sense, 5-CAC CAC CTT ATA CCA GCG TT-3; TIMP1 anti-sense, 5-GTC ACT CTC CAG TTT GCA AG-3; TIMP2 sense, 5-CCA GGT CCT TTT CAT CCT GA-3; TIMP2 anti-sense, 5-TCC ATT CGC TGA AGT CTG TG-3; TLR2 sense, 5- CTT CCT GGT TCC CTG CTC GTT CTC-3; TLR2 anti-sense, 5- CAA GAA CAA AGA AAA TGA GTC AAG-3; Fc ϵ RI sense, 5-CTG TTC TTC GCT TCC AGA TGG GCT-3; Fc ϵ RI anti-sense, 5-TAC AGT GTT GAG GGG CTG AG-3; GAPDH sense, 5-AAC TTT GGC ATT GTG GAA GG-3; GAPDH anti-sense, 5-GTG AAG GTC GGT AAC GG-3.

Supplement Fig. 1. Effects of TGase 2^{-/-} on the surface molecules related to the mast cell maturity, cell viability, the expression of TGase 2 mRNA in BMMCs or surface markers of B cells with BMMCs. Bone marrow cells from femurs and tibias of WT and TGase 2^{-/-} mice were cultured for 5 wk as described in Materials and methods. Both BMMCs (1 x 10⁶ cells) were activated with Ag/Ab reaction (act-BMMCs) for 1 h or 5 h as described in Materials and methods. In order to confirm the differences between both BMMCs (BM and KO-BM), surface markers (c-kit and FcεRIα) were determined by FACS analyzer, morphology of BMMCs by May-Grünwald-Giemsa staining, granule formation by microscopy for every wk, cell viability by MTT assay, amounts of total and release histamine by ELISA, respectively. Anti-CD40 Ab (300 ng/mL) was pretreated for 1 h before Ag stimulation. BM or KO-BM, BMMCs isolated and cultured from WT and TGase 2^{-/-} mice, respectively. – or +, non-act-BMMCs and act-BMMCs, respectively. (A) Expression of c-kit and FcεRIα in BMMCs. (B) Morphology of BMMCs under a microscopy (magnification: x 400). Values below figure indicate the numbers of granules in single BMMC, which were quantified by all granules counted in single cell (mean ± SEM for granule counts/single cell x 20 BMMCs under x1000 magnification). (C) Cell viability of BMMCs. (D) Total histamine (1 x 10⁶ cells) and histamine released by Ag/Ab reaction in BMMCs. (E) Time course of expression of TGase 2 protein or mRNA, respectively, in BMMCs activated with Ag/Ab reaction. (F) Expression of CD40 and CD40L in B cells co-cultured with BMMCs after R2 or α-CD40 Ab pretreatment. wk, week; Rx, reaction; R2, R2 peptide; act-BM, act-BMMCs; B, B cells; act-B, act-B cells. ‡, Numbers below bands are mean obtained from the ratio of band density of each group versus those of control and actin or GAPDH mRNA from four independent experiments (n = 4). ***, *P* < 0.001 versus the non-act-WT-BMMCs. ###, *P* < 0.001 versus the act-WT-BMMCs.

Supplement Fig. 2. Effects of TGase 2^{-/-} or inhibitors on the expression of various surface

markers in B cells co-cultured with BMMCs or in lung tissues from OVA-challenged allergic asthma in mice. Activation of BMMCs, experimental details for the sensitization of WT or TGase 2^{-/-} mice have been described in Materials and methods. Ca²⁺ blocker (100 μM 2-APB), R2 or S2 peptide (200 μg/ml) was pretreated for 10 min, 30 and 30 min, respectively, before Ag stimulation. The symbols in mouse model; **NC** (negative control) or **WT**, WT mice sensitized and nebulized with PBS and OVA, respectively; **Con** or **TGase 2^{-/-}**, TGase 2^{-/-} mice with sensitized and nebulized with PBS and OVA, respectively. Lung tissues (50 mg) were homogenized, and then various surface markers were determined by flow cytometry. Protein expression for flow cytometry was shown in Fig. 2A, B and Fig. 3C, D. BM or act-BM, WT-BMMCs or WT-BMMCs activated without and with Ag/Ab reaction, respectively. KO-BM or act-KO-BMMCs, KO-BMMCs or KO-BMMCs without and with Ag/Ab reaction, respectively. **(A)** Expression of CD40 and CD40L in both BMMCs and after inhibitors. **(B)** Expression of CD40 and CD40L in B cell with BMMCs after R2 peptide, anti-CD40 Ab or IgG Ab (as a positive control) pretreatment. **(C)** and **(D)** Expression of surface markers c-kit/CD40L in mast cells and CD40/CD23/CD72 in B cells with both BMMCs (C) or in lung tissues in OVA-challenged mice (D), respectively. Data shown are representative of four independent experiments (n = 4).

Supplement Fig. 3. Effects of TGase 2^{-/-} on expression of CD138, CD40/CD40L, CD72, CD23, [Ca²⁺]_i level, TGase 2 activity, IgE production in B cells with BMMCs or with TGase 2 siRNA-BMMCs. Experimental details for activation of BMMCs, and definition of all symbols used were described in Supplement Fig. 1 legend. Anti-CD40 Ab (300 ng/mL) was pretreated for 1 h before Ag stimulation. TGase 2 siRNA transfection was performed as described in Materials and methods. Expression of CD138 or surface markers was determined in protein extract isolated from BMMCs or B cells with BMMCs using western

blot. The $[Ca^{2+}]_i$ level was analyzed by confocal microscopy. TGase 2 activity was determined by TG Covtest kit, IgE production by ELISA. – or +, non-act-BMMCs and act-BMMCs, respectively. (A) Expression of plasma cells in the ratio for each cell co-culture (left column) and in B cells co-cultured with BMMCs (right column). (B) Expression of CD40L in TGases2 siRNA transfected-BMMCs. (C) Expression of CD40, CD40L, CD72 and CD23 in B cells co-cultured with TGase 2 siRNA-BMMCs. (D) Time courses of $[Ca^{2+}]_i$ level in B cells with TGase 2 siRNA-BMMCs. (E) TGase 2 activity in B cells with TGase 2 siRNA-BMMCs. (F) Total IgE production in B cells with TGase 2 siRNA-BMMCs. (G) Expression of CD72 and CD23 in lung tissues (left column) and in act-B cell with act-BMMCs (right column) as band images for histogram shown in Fig. 3C. (H) Expression of surface markers in mast cells (CD40L and c-kit) and B cells (CD23 and CD40) in lung tissues as band images for histogram shown in Fig. 3D. ‡, Numbers below bands are mean values (n = 4) as described in Supplement Fig. 1 legend. **, $P < 0.01$; ***, $P < 0.001$ versus the non-act-WT-BMMCs. ###, $P < 0.001$ versus act-B cells with act BMMCs. Data in the $[Ca^{2+}]_i$ level, TGase 2 activity or total IgE production are expressed as the mean $\pm$ SEM (n = 8).

Supplement Fig. 4. Effects of TGase 2^{-/-} or inhibitors on the TGase 2 expression, the recruitment of inflammatory cells, collagen amounts and AHR in BAL cells or lung tissues of OVA-challenged allergic asthma in mice. Experimental details for the sensitization of WT or TGase 2^{-/-} mice, pretreatment of R2 and S2 peptides (20 mg/kg) and all symbols used were described in Materials and methods and Fig. 3 legend (symbols for mouse model). (A) Expression of TGase 2 in BAL cells and lung tissues. Expression of TGase 2 was determined in protein extracts isolated from BAL cells and lung tissues using Western blot. (B) Lung tissues stained with H&E (magnification: x200). Histological appearances were scored as described in Materials and methods (Histogram). (C) Lung tissues stained with PAS

(magnification: x200). Numbers of goblet cell hyperplasia were quantified as the percentage of PAS-positive cells / total cells in 5 sites of 200 μm $\times$ 200 μm areas (5 sites/each $\times$ 8 mice/group = 40 sites). Arrow (pink color) in Fig. 1C (WT or TGase 2^{-/-}) indicates goblet cells. (D) Collagen deposition stained with Masson's trichrome. Histological appearances were scored for the presence of peribronchial and perivascular inflammation (blue color), and these scores were added together to give a total lung collagen deposition score. (E) Numbers of a variety of BAL cells counted by standard morphological criteria. (F) Collagen amounts in lung tissues of OVA-challenged mice by Sircol assay. (G) R_L values. (H) PC₁₀₀ (the dose of MCh which induces a 100% increase in the R_L values). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the NC or Con mice. ##, $P < 0.01$; ###, $P < 0.001$ versus the WT mice. Data shown are expressed as the mean $\pm$ SEM (n = 8).

Supplement Fig. 5. Effects of TGase 2^{-/-} inhibitors on the PKCs, MAPKs, I- κ B or PLA2 activity in BMMCs activated with Ag/Ab reaction. BMMCs (1 $\times$ 10⁶ cells) from WT or TGase 2^{-/-} mice were activated with Ag/Ab reaction (act-BMMCs) for the indicated periods (3 min for PKCs; 5 min MAPKs; 10 min for p-I- κ B and PLA2 activity) as described in Materials and methods. Ca²⁺ blocker (100 $\mu\text{g/mL}$ 2-APB), TGase 2 inhibitor (R2 peptide, 200 $\mu\text{g/mL}$) or scrambled (S2) peptide (200 $\mu\text{g/mL}$) was pretreated for 10 min and 30 min, respectively, before Ag stimulation. BM or KO-BM, BMMCs isolated and cultured from WT mice and TGase 2^{-/-} mice, respectively. – or +, non-act-BMMCs and act-BMMCs, respectively. Expression of PKCs, MAPKs, I- κ B, p-I- κ B and cPLA2 was determined in protein extracts isolated from BMMCs using Western blot. PLA2 activity was determined as described in Materials and methods. (A) Time courses for activities of PKC α and ERK. (B) Activities of PKCs and MAPKs by individual inhibitor pretreatment. ‡, Numbers below

bands are mean values ($n = 4$) as described in Supplement Fig. 1 legend. (C) and (D) Band images and histogram for activities of PKCs and MAPKs by TGase 2 inhibitor and Ca^{2+} blocker. (E) and (F) Band images and histogram for I- κ B protein expression and p-I-KB activity. (G) and (H) Band images and histogram for PLA2 protein expression and activity. The histogram from the densitometry analysis shows the mean $\pm$ SEM ($n = 4$) obtained from the ratio of each band density versus those of control and loading control (band image not shown) from four independent experiments. *, $P < 0.05$; ***, $P < 0.001$ versus the non-act-WT-BMMCs or non-act-KO-BMMCs. ###, $P < 0.001$ versus the act-WT-BMMCs.