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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 Ca^{2+} ($[Ca^{2+}]_i$) 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 $[Ca^{2+}]$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 Ca^{2+} 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.

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-κB 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-κB/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$^{-/-}$ 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 μg/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 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 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 μU/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 MagCellect mouse B cell isolation kit (R&D Systems, Inc., Minneapolis, purity of more than 94%) were incubated with 0.5 μg/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 μg/200 μL 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