Thrombomodulin inhibits the activation of eosinophils and mast cells

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A B S T R A C T

Eosinophils and mast cells play critical roles in the pathogenesis of bronchial asthma. Activation of both cells leads to the release of pro-inflammatory mediators in the airway of asthmatic patients. Recently, we have shown that inhaled thrombomodulin inhibits allergic bronchial asthma in a mouse model. In the present study, we hypothesize that thrombomodulin can inhibit the activation of eosinophils and mast cells. The effect of thrombomodulin on the activation and release of inflammatory mediators from eosinophils and mast cells was evaluated. Thrombomodulin inhibited the eotaxin-induced chemotaxis, upregulation of CD11b and degranulation of eosinophils. Treatment with thrombomodulin also significantly suppressed the degranulation and synthesis of inflammatory cytokines and chemokines in eosinophils and mast cells. Mice treated with a low-dose of inhaled thrombomodulin have decreased number of eosinophils and activated mast cells and Th2 cytokines in the lungs compared to untreated mice. The results of this study suggest that thrombomodulin may modulate allergic responses by inhibiting the activation of both eosinophils and mast cells.

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1. Introduction

Eosinophils and mast cells are the key mediators of allergic inflammatory responses in the airways of asthmatic patients [1,2]. A large body of evidence gained from clinical and experimental studies has shown that excessive infiltration and secreted products of both cells in the airways are associated with disease severity and enhanced airway hyperresponsiveness [1–4]. Eosinophils can be activated by several ligands of CCR3 including eotaxin, which is secreted by airway epithelial cells [4]. Activated eosinophils secrete proinflammatory factors including cytokines (IL-13, IL-5, osteopontin), chemokines (CCL11 or eotaxin, CCL22), leukotrienes, matrix metalloproteinases and granule molecules such as eosinophil peroxidase (EPO), eosinophil cationic protein, major basic protein and eosinophil-derived neurotoxin [1,4]. Mast cells are activated by antigen-mediated cross-linking of immunoglobulin E (IgE)-bound Fcε receptors (FcεR) leading to the release of inflammatory mediators including histamine, serotonin, proteoglycans, lipid mediators (prostaglandins, leukotrienes), Th2 cytokines (IL-4, IL-5) and chemokines (CCL2, CCL5) [2,3].

Thrombomodulin (TM) is a cell membrane-bound protein also known as CD141 or blood dendritic cell antigen (BDCA)-3 [5,6]. When TM binds thrombin, a critical pro-coagulant protease of the clotting system, thrombin is converted to an anticoagulant and anti-inflammatory factor. TM-bound thrombin is unable to cleave fibrinogen, protease-activated receptor (PAR)-1, and other substrates of the coagulation cascade, but it is able to cleave protein C to activated protein C, an anticoagulant and antithrombin factor and thrombin-activatable fibrinolysis inhibitor (TAI) to activated TAI, an anti-inflammatory and anti-thrombotic factor [5–7]. In addition, TM can exhibit direct anti-inflammatory properties by inhibiting the pro-inflammatory activity of high-mobility group protein B-1 (HMGB-1), and recently, we discovered that soluble and membrane-bound TM is protective in allergic diseases by modulating the pro-inflammatory activity of dendritic cells (DCs) [8,9].

In the current study we hypothesized that TM may also exert protective effect in allergic conditions by modulating the activation of eosinophils and mast cells.
2. Materials and methods

2.1. Reagents

Soluble recombinant human (rh) TM (ART123; rhTM) was supplied by Asahi Kasei Corporation (Tokyo, Japan) and the concentration of endotoxin was below the detection limit. The rhTM was the same as that approved for use in the clinic in Japan and therefore contains no endotoxins.

2.2. Cell culture

EoL-1 cells (RIKEN Cell Bank, Tsukuba, Japan) is an established human eosinophilic leukemia cell line with cytological features of myeloblasts under normal culture conditions but differentiate phenotypically and functionally into eosinophils after treatment with butyric acid [10]; the cells were cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and l-glutamine. Differentiation of EoL-1 cells was done by culturing with 0.5 mM butyric acid for 7 days. Cell number readjustment to 5 × 10^5/ml was performed every 3 days. Bone marrow-derived mast cells (BMMCs) were obtained by a long-term culture (>3 weeks) of bone marrow cells from BALB/c mice in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and 5 ng/ml murine IL-3. Recovered cells were >90% FceRI+ and c-Kit+. Human eosinophils were isolated and cultured as previously described [11]. All cells were cultured at 37 °C with 5% CO2 in a humidified atmosphere. The Committee for Animal Investigation of Mie University approved the experimental protocol. Informed consent was obtained from all healthy volunteers and the protocol was approved by the Ethic Board of Mie University for Clinical Investigation.

2.3. Cell stimulation

EoL-1 cells and primary human eosinophils were starved in serum-free media containing 0.2% bovine serum albumin (BSA) for 2 h, pretreated with 200 nM rhTM for 30 min and stimulated with 100 ng/ml of eotaxin. BMMCs (1 × 10^5 cells/200 μl) were sensitized by incubating with 1 μg/ml trinitrophenyl (TNP)-specific IgE (TNP-IgE) for 18 h. Cells were starved in serum-free media containing 0.2% BSA for 2 h, pretreated with 200 nM TM for 30 min and stimulated with 10 ng/ml of TNP-conjugated ovalbumin (TNP-OVA).

2.4. RNA extraction and RT-PCR

Total RNA was extracted using TriZol following the instructions of the manufacturer. The extracted RNA was dissolved in diethylpyrocarbonate water and its concentration was measured by spectrophotometer. One microgram of total RNA was denatured at 65 °C for 10 min, and then reverse-transcribed using the superscript II RT enzyme and Oligo (dT) primer (Life Technologies) in a volume of 15 μl according to the manufacturer’s protocol. The cDNA was amplified by PCR and the conditions were as follows: 94 °C, 1 min; 60 °C, 1 min and 72 °C, 1 min for 28–35 cycles. The sequences of the primers are described in Table 1. PCR products were electrophoresed on a 1.5 % agarose gel and then stained with ethidium bromide solution.

2.5. Eosinophil degranulation assay

Eosinophil peroxidase (EPO) release was measured in the supernatants by the o-phenylenediamine (OPD) method. Ninety microliter of cells (5 × 10^5 cells/ml in RPMI-1640 medium without phenol red) stimulated with or without eotaxin (100 ng/ml), in the absence or in the presence of 200 nM of rhTM, were incubated for 2 h at 37 °C and 5% CO2. The calcium ionophore A23187 (5 μM, 30 min) was used as a positive control. At the end of the incubation period the reaction was stopped by cooling on ice for 3 min. The samples were centrifuged at 250 × g for 15 min at 4 °C and the cells discarded. Aliquots of the supernatants (50 μl; triplicate) were dispensed into each well of a microplate, and 50 μl of peroxidase substrate (1 mM H2O2, 1 mM OPD and 0.1% Triton X-100 in Tris buffer, pH 8.0) was added to each well. After measurement of the optical density at 490 nm, the concentration of EPO was calculated from standard curve prepared using known concentrations of peroxidase.

2.6. Migration assay

The migration of the EoL-1 cells was assayed using Transwell inserts (pore size, 5 μm) and 24-well culture plates (Corning Costar, Cambridge, MA) in the presence or absence of rhTM.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
<th>BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>GAPDH</td>
<td>CCACCATGCCAATATTCATGACA</td>
<td>TCTAGACGGCAGTCAGGTCACC</td>
</tr>
<tr>
<td>IL-4</td>
<td>CCAAATCTCTCTCCCTGTG</td>
<td>TCTGTTACGGTCAACTCGGT</td>
<td>150 bp</td>
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<tr>
<td>IL-5</td>
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<tr>
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<td>GAGTGGATCATGCTGGCAGC</td>
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<tr>
<td>IL-13</td>
<td>TGCAATGCGACCAGTGATATG</td>
<td>GCAGGCCTTCTACTGAC</td>
<td>212 bp</td>
</tr>
<tr>
<td>TNFα</td>
<td>GAAAACATGATCCGGAGCGT</td>
<td>TTGGTCTGCTAGAGA</td>
<td>489 bp</td>
</tr>
<tr>
<td>Mouse</td>
<td>GAPDH</td>
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<td>GAGGGGCCATACCCAGCTTCTG</td>
</tr>
<tr>
<td>IL-4</td>
<td>ATGGGCCGCTCCACCCAGTACT</td>
<td>GCCCTTGAAGGTCCACCCAGTAC</td>
<td>399 bp</td>
</tr>
<tr>
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<td>CCAGACTCTCTTCTTGGG</td>
<td>201 bp</td>
</tr>
<tr>
<td>IL-13</td>
<td>GACCCAGAGGATCCGGGACGT</td>
<td>CACAGAACTTCTGGG</td>
<td>214 bp</td>
</tr>
<tr>
<td>CCL2</td>
<td>ATGGGACGAGCTGTCTCAGTCTCTC</td>
<td>ACTGTTCAGTCGTTCACTGTC</td>
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<tr>
<td>TNFα</td>
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<td>CTGCTTACCTGTGGATTG</td>
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<tr>
<td>Cma1</td>
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<td>AGAAGCAGCCTTCTATAGA</td>
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<tr>
<td>Cpa3</td>
<td>CTTCAATGGCGACTGCTTAC</td>
<td>AAGTGTGCGAGCCTTGTC</td>
<td>135 bp</td>
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</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Cpa3, mast cell carboxypeptidase A; TNFα, tumor necrosis factorα; Cma, mast cell protease-1; IL-4, interleukin-4; CCL2, chemokine (C–C motif) ligand 2.
(200 nM). Briefly, 1 × 10⁶ cells suspended in 0.1 ml RPMI-1640 medium containing 0.1% BSA were transferred to the Transwell insert (the upper compartment). Eotaxin (100 ng/ml) was added to 0.6 ml RPMI-1640 medium containing 0.1% BSA in the well of the culture plate (the lower compartment). After incubation at 37 °C for 2 h in an atmosphere of 95%-air and 5% CO₂, the number of cells that had migrated from the upper to the lower compartment was counted using a hemocytometer.

2.7. Mast cell degranulation assay

Degranulation of BMMCs was assessed by measuring the release of β-glucosaminidase activity. To examine the effects of rhTM on IgE-bound BMMCs, cells were sensitized for 18 h with mouse TNP-IgE (1 μg/ml), washed with Tyrode’s buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% BSA) to remove unbound antibody, and then 1 × 10⁵ cells were plated in 96-well plates, pre-treated with 200 nM rhTM for 30 min, and then TNP-OVA was added to cross-link IgE. Fifty-μl aliquot of the supernatant from each sample was placed into each well of another 96-well plate together with 100 μl of 2.5 mM p-nitrophenyl-N-acetyl β-glucosaminide solubilized in 0.04 M citrate buffer (pH 4.5). After incubation at 37 °C for 90 min, the reactions were terminated by adding 50 μl of 0.4 M glycine (pH 10.7). The colored products were measured using a multiwell plate reader at 405 nm. The activity of β-glucosaminidase was expressed as the percentage of β-glucosaminidase release by cells after freeze–thaw treatment.

2.8. Mast cell activation assay

TNP-IgE–sensitized BMMCs were pre-treated with 200 nM TM and stimulated with TNP-OVA for 4 h at 37 °C and then culture supernatants were collected for protein measurements.

2.9. Experimental animals

The Mie University Committee on Animal Investigation approved the experimental protocols. Female Balb/c mice (8-week-old) weighing 18–20 g were purchased from Nihon SLC (Hamamatsu, Shizuoka, Japan). The experimental mice were housed in the animal facility of Mie University where they were maintained on a constant 12-h light/12-h dark cycle in a temperature-controlled and humidity-controlled room. They were given food and water ad libitum.

2.10. Animal model of bronchial asthma

Ten microgram of aluminum (Pierce Biotechnology Inc., Rockford, IL)–precipitated ovalbumin (Sigma, St. Louis, MO, USA) was administered to female Balb/c mice by intraperitoneal injection on days 0 and 14. Control animals received ip injection of normal saline following the same schedule. Bronchial asthma was induced in sensitized mice by exposure to aerosolized 2% ovalbumin in a chamber (MIPS, Osaka, Japan) on days 23, 23, 24 and 25; airway hyperresponsiveness was measured on day 26, re-challenged with aerosolized 2% ovalbumin before sacrifice by intraperitoneal overdose injection of pentobarbital. Mice were treated with 0.03 mg/kg of rhTM for 20 min on days 22, 23, 24, 25 and 26 immediately before challenge with ovalbumin by inhalation.

2.11. Biochemical and histological evaluation

The mice were euthanized by intraperitoneal injection of pentobarbital overdose and samples for biochemical and histologic examinations were taken. Bronchoalveolar lavage fluid (BALF) was obtained as previously described, centrifuged (1000 × g, 10 min, 4 °C) and the cell-free supernatant was stored at −80 °C until analysis [9]. The total cell number in BALF was counted using a nuclocounter from ChemoMetec (Allerød, Denmark) and then pelleted cells were stained with May–Grunwald–Giemsa (Merck, Darmstadt, Germany) for differential cell counting. The immunoassay kits for measuring interleukin (IL)-5 and IL-4 were from BD Biosciences Pharmingen, and TNF-α, CCL2 and IL-13 was from R&D Systems. The lungs were incised, fixed in formalin, embedded in paraffin, and prepared for staining with May–Grunwald–Giemsa and toluidine blue by standard methods.

2.12. Western blotting

BMMCs were cultured and activated by IgE cross-linking in the presence or absence of rhTM, and then the expression of mast cell protease-1 and mast cell carboxypeptidase A were measured in the cell supernatant by Western blot analysis using rat anti-mouse mast cell protease-1 (R&D, Minneapolis, MN), rabbit anti-mouse mast cell carboxypeptidase A (Gen Way Biotech, San Diego, CA) as first antibody and goat anti-rat (R&D) or anti-rabbit (BIO-RAD, Hercules, CA) antibody coupled to horseradish peroxidase as second antibody.

2.13. Statistical analysis

Data are expressed as means ± S.D. unless otherwise specified. Statistical evaluation was performed using analysis of variance (ANOVA) for multiple comparisons with post hoc analysis using Tukey’s test. Unpaired Student’s t-test was performed when the distribution of the samples was normal. A value of P < 0.05 was taken as significant.

3. Results

3.1. rhTM inhibits activation, degranulation and migration of EoL-1 cells

Differentiated EoL-1 cells were pre-treated with rhTM before activation, degranulation or migration were induced by eotaxin. The surface expression of CD11b, a marker of eosinophil activation, the secretion of EPO, a protein contained in eosinophil granules, the Th2 cytokines IL-13 and IL-4, and the migration of EoL-1 were significantly stimulated by eotaxin but repressed in cells pre-treated with rhTM (Fig. 1A,B,C). The mRNA expression of IL-4, IL-13, TNFα and IL-6 were significantly increased by eotaxin but they were significantly reduced in cells pre-treated with rhTM (Fig. 1D).

3.2. rhTM inhibits expression of cytokines from primary human eosinophils

Primary human eosinophils were isolated, cultured and then stimulated with eotaxin in the presence or absence of rhTM. The concentration of IL-13 and TNFα in the cell supernatant (Fig. 2A) and the mRNA expression of IL-4 and TNFα enhanced by eotaxin were significantly inhibited by rhTM (Fig. 2B). No significant differences were observed in the protein level of IL-4 or mRNA expression of IL-13 between groups (data not shown).

3.3. rhTM inhibits degranulation and expression of leukotrienes and proteases from mouse mast cells

Mouse mast cells were activated by IgE cross-linking in the presence or absence of rhTM. The activity of hexosaminidase and the concentration of cysteinyl leukotrienes in the cell supernatant
was significantly decreased by rhTM (Fig. 3A). The mRNA expression and the protein content in cell supernatant of mast cell protease-1 and mast cell carboxypeptidase A induced by IgE cross-linking were significantly suppressed by rhTM (Fig. 3B and C).

3.4. rhTM inhibits cytokines expression from mouse mast cells

Mouse mast cells were prepared from mouse bone marrow stem cells, cultured and activated by IgE cross-linking in the presence or absence of rhTM. The cell culture supernatant concentration and the mRNA expression of IL-5 and TNFα were significantly increased by IgE cross-linking but they were significantly reduced in the presence of rhTM (Fig. 4A and B). The cell supernatant concentration of IL-13 and CCL2 tended to decrease but the mRNA expression of IL-13 and CCL2 was significantly suppressed in cells treated with rhTM compared to controls (Fig. 4A and B).

3.5. rhTM inhibits migration and activation of eosinophils and mast cells in vivo

Airway hyperresponsiveness, the bronchoalveolar lavage fluid concentration of IL-5 and the number of lung eosinophil were significantly suppressed in asthma mice receiving inhalation of rhTM compared to mouse controls (Fig. 5A). Giemsa staining (Fig. 5B and C) of the bronchoalveolar lavage fluid cells and the lungs revealed decreased infiltration of eosinophils, and toluidine blue staining (Fig. 5D) showed reduced infiltration and activation of mast cells in the peribronchial surrounding areas.

4. Discussion

This study showed that TM can inhibit the release and expression of pro-allergic factors from eosinophils and mast cells.

4.1. Coagulation factors in allergy

The coagulation system is involved in the pathogenesis of allergic diseases including bronchial asthma, urticaria and angioedema [12–14]. Thrombin, the key procoagulant enzyme, is protective at low concentration because it forms a complex with membrane-bound TM, thereby stimulating enhanced generation of activated protein C and activated thrombin-activatable fibrinolysis inhibitor, both with suppressive activity on inflammation and allergy [15]. In addition to these indirect beneficial effects, endogenous TM expressed by airways epithelial cells and dendritic cells can also directly inhibit allergic inflammation by inhibiting the pro-inflammatory activity of thrombin and HMGB1 [9]. The administration of rhTM by inhalation has been also shown to block bronchial asthma by inhibiting allergic inflammation, airway hyperresponsiveness and by increasing the number of tolerogenic dendritic cells, suggesting the potential therapeutic value of rhTM [9]. However, the direct effect of TM on eosinophils and mast cells, which play critical function in the allergic response, has not been so far appraised.
Fig. 2. rhTM inhibits expression of cytokines from primary human eosinophils. Primary human eosinophils were separated from blood of volunteers, cultured and the suppressive effect of rhTM was evaluated. The concentration of IL-13, TNFα (A) in the cell supernatants and the mRNA expression of IL-4 and TNFalpha (B) induced by eotaxin were suppressed by rhTM. Data are expressed as the mean ± S.D.

Fig. 3. rhTM inhibits degranulation and expression of leukotrienes and proteases from mouse mast cells. Bone marrow-derived mast cells were stimulated by IgE cross-linking in the presence or absence of rhTM. The activity of β-hexosaminidase, cysteinyl leukotriene level in the cell supernatants (A) and the mRNA expression of mast cell protease-1 (Cma1) and mast cell carboxypeptidase (Cpa3; B) were significantly suppressed by rhTM. The protein contents of Cma1 (B) and Cpa3 (C) in the cell supernatant, as analyzed by Western blotting, were also decreased in the presence of rhTM compared to controls. Data are expressed as the mean ± S.D.
Fig. 4. rhTM inhibits cytokines expression from mouse mast cells. Bone marrow mast cells were stimulated by IgE cross-linking in the presence or absence of rhTM. The concentration of IL-5, IL-13, TNFα and CCL2 (A) and the mRNA expression of IL-5, IL-13, TNFα and CCL2 (B) were suppressed by rhTM. Data are expressed as the mean ± S.D.

Fig. 5. rhTM inhibits eosinophils and mast cells in an experimental asthma model. Inhalation of low dose of rhTM decreases airway hyperresponsiveness, the number of infiltrating eosinophils in the lungs and the bronchoalveolar lavage fluid (BALF) levels of IL-5 compared to controls (A). Giemsa staining of BALF cells (B) and lung tissue (C) shows eosinophil infiltration and toluidine blue staining of lung tissue (D) disclosed mast cell activation that were decreased in mice treated with inhaled rhTM. n = 4 mice in each group. Error bars indicate S.E.M. Arrows indicate eosinophils or mast cells. Scale bars indicate 20 µm.
Here we showed that rhTM decreases the expression of CD11b, an activity marker, and the degree of migration in eosinophil-stimulated eosinophils, the secretion of cysteinyl leukotrienes in mast cells after IgE cross-linking, and the number of eosinophils and mast cells in the lung of mice sensitized and challenged with an allergen. Overall, these observations suggest the suppressive effect of rhTM on the allergic response mediated by both eosinophils and mast cells.

4.2. Effector cell activity and TM

During the allergic response, recognition of the allergen by antigen-presenting cells is followed by differentiation and activation of effector T cells releasing Th2 cytokines that, apart from activating B cells, stimulate eosinophils and mast cells that become malignant drivers of the immune response by releasing excessive amount of inflammatory mediators by degranulation or by de novo synthesis [1–4,16]. Eosinophils contain cytokines, chemokines and highly basic and cytotoxic proteins in their granules including eosinophil cationic protein, major basic protein and EPO that, if overexpressed, may cause tissue damage hence exacerbating inflammation [1]. Similarly, granules of mast cells also contain pro-inflammatory factors including histamine, chemokines and enzymes such as mast cell protease-1 and mast cell carboxypeptidase A that may cause tissue injury [1,2]. In the present study, the concentration of the granular components of eosinophils (EPO) and mast cells (hexoxaminidase and proteases) in the cell supernatants was significantly decreased by rhTM compared to controls, suggesting that rhTM can inhibit the degranulation of both cells.

Mast cells and eosinophils can produce and secrete Th2 (IL-4, IL-5, IL-6, IL-13), Th1 (TNFα) cytokines and chemokines (CCL2) that can further favor and perpetuate the inflammatory allergic response [16]. In the present study, we found that the protein and mRNA expression of IL-4, IL-13 and the mRNA expression of TNFα and IL-6 in eosinophils, and the protein and mRNA expression of IL-5, IL-13, TNFα and the mRNA expression of CCL2 in mast cells were significantly reduced by rhTM compared to controls, suggesting that rhTM can also inhibit inflammatory cytokines at transcription level.

4.3. In vivo relevance

The relevance of these findings was evaluated in in vivo conditions, using a mouse model of allergic bronchial asthma as previously described but using rhTM here at low-dose [9]. Consistent with our previous observation, airway hyperresponsiveness, the number of eosinophils, the number and activity of mast cells, and the lung concentration of IL-5 were significantly reduced in mice treated with rhTM compared to control mice [9]. The fact that rhTM significantly suppressed the secretion of IL-13 and TNFα, and the mRNA expression of IL-4 and TNFα in primary culture of eosinophils drawn from healthy controls suggests that findings of the present study may also be relevant to human diseases.

The cellular mechanism of this TM protective action remains unclear. A substrate of TM, HMGB-1, has been involved in the activation and release of pro-inflammatory mediators from eosinophils and mast cells [17–20]; thus, it is conceivable that the suppressive effect of TM is due to its inhibitory activity of HMGB1. In addition, TM may also act through an as yet unidentified receptor as previously suggested in dendritic cells [21].

5. Conclusion

The results of this study showed that TM can inhibit the activation and degranulation of both eosinophils and mast cells further supporting the protective role of TM against allergic inflammation.

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References

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