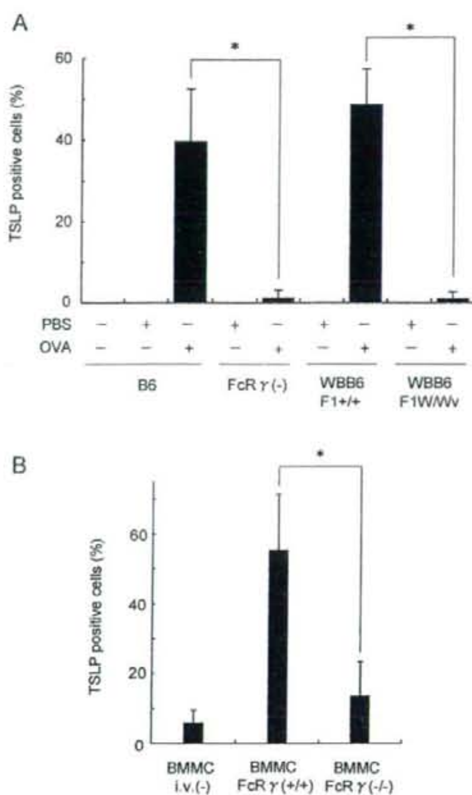

Figure 2. Deficiency of mast cells and FcγR abolishes TSLP expression in the nasal epithelium during allergic rhinitis. (A) A quantitative analysis of TSLP expression in the nasal epithelium during allergic rhinitis using C57BL/6, FcγR-deficient, WBB6F1-+/+, WBB6F1-W/W^v mice as described in the text. (B) A quantitative analysis of TSLP expression in the nasal epithelium during allergic rhinitis using C57BL/6-KitW-sh/W-sh mice reconstituted with wild-type BMMC (BMMC FcγR(+/+)) or with FcγR-deficient BMMC (BMMC FcγR(-/-)), as described in the text. BMMC i.v.(-): C57BL/6-KitW-sh/W-sh mice without the transfer of BMMC. Values represent the mean + SD of eight mice in each group. *$p<0.05$.

via FcεRI-mediated release of TNF-α) might play a critical role in the initial phase of allergic rhinitis through the induction of epithelial TSLP expression.

Conversely, TSLP has been shown to stimulate the production of high levels of Th2 cytokines by human mast cells synergistically with IL-1 and TNF-α [12]. Therefore, it is possible that there is an important interaction between epithelial cells and mast cells *via* TSLP and proinflammatory cytokines for the development of allergic rhinitis. Mast cells may induce TSLP expression in the nasal epithelium following the allergen challenge, and then epithelial-derived TSLP could stimulate mast cells to produce Th2 cytokines to further augment allergic inflammation.

Treatment of the mice with anti-TSLP neutralizing antibody inhibited the development of allergic rhinitis (Fig. 3). These results

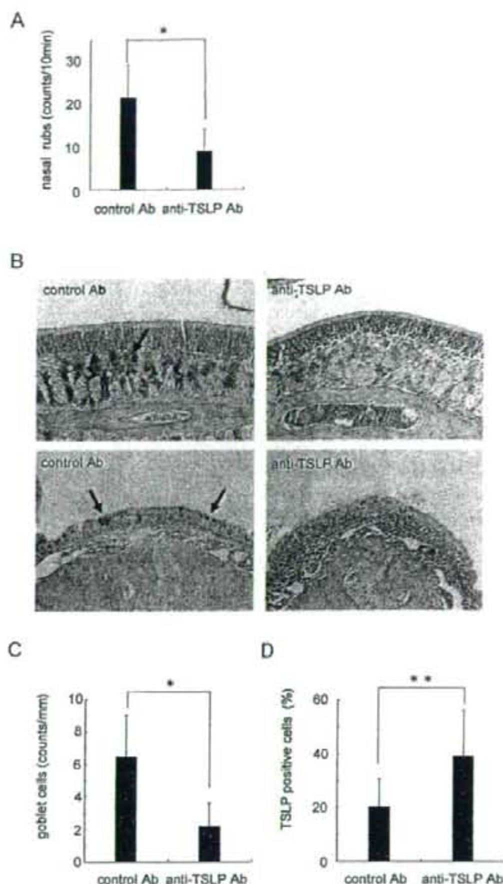

Figure 3. Anti-TSLP neutralizing antibody inhibits the development of allergic rhinitis. The OVA-sensitized mice were intranasally challenge with 100 μg OVA in 10 μL PBS twice per day for 1 week (total 14 time week). The mice were challenged intranasally with PBS in a simil manner for the negative control. During the OVA challenge, rat ant mouse TSLP neutralizing antibody or the isotype control rat IgG (15 mg/kg per mouse) was administered intraperitoneally every oth day. (A) Appearance of nasal symptom (nasal rubs). The frequency nasal rubs was counted for 10 min after the last intranasal challen with OVA. Control Ab: mice treated with control IgG antibody, anti-TSl Ab: mice treated with anti-TSLP antibody. (B) Representative P staining of the nasal mucosa obtained from C57BL/6 mice treated wi anti-TSLP antibody (anti-TSLP Ab) or control IgG (control Ab). Upp panels: submucosa; lower panels: epithelial area. Note the decreas number of PAS-positive cells in the nasal submucosa and epitheliu The arrows indicate the PAS-positive cells. (C) A quantitative analysis the epithelial PAS-positive cells. (D) A quantitative analysis of t epithelial TSLP-positive cells. Values represent the mean + SD of eig mice in each group. Both * and ** represent $p<0.05$.

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are consistent with the previous *in vivo* studies using mouse models of asthma and atopic dermatitis [5–7]. Thus, the current results also support the importance of TSLP in allergic inflammation. In addition, the findings that treatment with anti-TSLP neutralizing antibody up-regulated TSLP expression in the nasal epithelium (Fig. 3) may suggest the presence of negative feedback regulation in the epithelial TSLP expression.

Concluding remarks

In summary, this study provides significant evidence that mast cells play a critical role in TSLP expression in the nasal epithelium in allergic rhinitis and subsequent development of the disease. FcεRI, expressed on mast cells, is likely to be involved in the epithelial TSLP expression. To our knowledge, this is the first study suggesting TSLP to play a potential role in allergic rhinitis.

Materials and methods

Mice

Female 4–6 wk C57BL/6 mice, WBB6F1-+/+, and WBB6F1-W/W^{v} mice [18] were purchased from Japan SLC (Tokyo, Japan), FcγR-chain deficient mice (C57BL/6 background) and mast cell-deficient KitW-sh/W-sh mice on the C57BL/6 background were previously described [19, 23, 24], and were bred under specific pathogen-free conditions.

Allergic rhinitis model

An allergic rhinitis model was established as previously described with some modifications [26]. Briefly, the mice were actively immunized i.p. with 10 μg OVA (Sigma Aldrich, St. Louis, MO) in 4 mg aluminum hydroxide on days 1 and 7. Starting on day 14, they were challenged intranasally with 100 μg OVA in 10 μL PBS twice per day for 1 week (total 14 times/week). The mice were challenged intranasally with PBS in a similar manner for the negative control. For some experiments, rat anti-mouse TSLP neutralizing antibody or control rat IgG2a (15 mg/kg per mouse, R&D Inc., Minneapolis, MN) were administered intraperitoneally every other day, starting on day 14 until sacrifice. The dosage of the antibody (15 mg/kg) was based on previous experiments [27]. The animal experiments were approved by the Institutional Review Board of the University of Yamanashi.

Histological examination

At 12 h after the final nasal challenge, mice were killed with carbon dioxide. The heads were removed, fixed, and decalcified. Coronal nasal sections were visualized by staining with hematoxylin and eosin (H&E) or PAS/hematoxylin (to demonstrate goblet cells). For immunohistochemistry, the coronal nasal sections were deparaffinized and stained with anti-TSLP antibody or control IgG antibody (Santa Cruz Biotechnology Inc.) through the use of peroxidase-based Vectastain ABC kits with DAB substrate (Vector Laboratories, Burlingame, CA).

Nasal symptom

Nasal rubs were observed for 10 min after the last intranasal challenge using a video recorder and the frequency of the nasal rubs were counted by investigators who were blind to the treatment protocol.

Assessment and quantification of histological examination

The length of the positively TSLP-stained epithelial layer in the total nasal epithelial lining of the coronal sections was measured and expressed as percentage of the total length of the total nasal epithelial lining of the coronal sections (% of the total length). The number of PAS-positive cells in the total nasal epithelial lining of the coronal sections was counted microscopically in a blinded manner and it was expressed as the number per the total length of the total nasal epithelial lining of the coronal sections (number/mm). Two or four specimens of the TSLP- or PAS-stained coronal sections from one mouse were selected. The mean score was counted, and then the final mean scores were calculated from eight animals.

Reconstitution of KitW-sh/W-sh mice with BMMC

BMMC were generated from the femoral BM cells of mice and maintained in the presence of 10% pokeweed mitogen-stimulated spleen-conditioned medium as a source of mast cell growth factors as previously described [28]. Mast cell deficiency of KitW-sh/W-sh was reconstituted by the intravenous injection, 4 weeks after starting the culture, of 2×10^{6} BMMC derived from C57BL/6 or FcγR-chain deficient mice, as previously described [23, 24]. The mice were used for experiments 5 wk after the injection of BMMC. The reconstitution of the mast cells was confirmed by toluidine blue staining of the nasal mucosa.

Data analysis

Data are represented as the mean + SD. The statistical analysis was performed by unpaired Student's *t*-test. A value of $p<0.05$ was considered to be significant.

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Abbreviations: **BMMC:** bone marrow-derived mast cells · **FcεR:** Fc receptor ε chain · **FcγR:** Fc receptor γ chain · **PAS:** periodic acid-Sch **TSLP:** thymic stromal lymphopoietin

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Identification of epigallocatechin-3-*O*-gallate as an active constituent in tea extract that suppresses transcriptional up-regulations of the histamine H_1 receptor and interleukin-4 genes

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Accumulating evidence suggests the anti-allergic effect of green tea extract. Although histamine is a major chemical mediator in the pathogenesis of allergic rhinitis, the effect of green tea extract on histamine signaling was unknown. In this study, we investigated the effect of tea extract on the toluene-2, 4-diisocyanate (TDI)-induced up-regulations of the histamine H_1 receptor (H1R) and Th2 cytokines gene expressions in the nasal mucosa of TDI-sensitized allergy model rats. Pre-treatment with tea extract once daily for 3 weeks significantly suppressed the TDI-induced mRNA elevations of Th2 cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13, and tended to suppress H1R mRNA elevation induced by TDI. Further investigations revealed that the 80% ethanol eluate from a TOYOPEARL HW40EC column, in which epigallocatechin-3-*O*-gallate (EGCG) was a major constituent, inhibited the IgE-stimulated mRNA elevations of Th2 cytokines in RBL-2H3 cells. EGCG suppressed the elevations of IgE-stimulated IL-4 mRNA and phorbol-12-myristate-13-acetate (PMA)-induced H1R mRNA in a dose-dependent manner. In TDI-sensitized rats, pre-treatment with EGCG once daily for 3 weeks decreased the number of sneezes and suppressed the TDI-induced elevations of H1R and IL-4 mRNAs in the nasal mucosa. These findings suggest that EGCG alleviates nasal symptoms by inhibiting histamine signaling as well as IL-4 signaling by suppressing TDI-induced H1R and IL-4 gene up-regulations in TDI-sensitized rats.

Key words allergy, epigallocatechin-3-*O*-gallate, gene expression, histamine H_1 receptors, histamine signaling, Th2 cytokines.

Introduction

Pollinosis is a seasonal allergic rhinitis caused by hypersensitivity to tree or grass pollens and about 16% of the Japanese population suffer from it.[1] Histamine is a major chemical mediator of allergic rhinitis. Many studies have shown that activation of the histamine H_1 receptors (H1R) by histamine is responsible for the symptoms of allergic rhinitis including sneezing, watery rhinorrhea and nasal itching and that antihistamines are effective in controlling nasal hypersensitivity and symptoms in the eliciting/effector phase of nasal allergy.[2-5]

Th1/Th2 imbalance towards Th2 in the immune system results in the clinical expressions of nasal allergy and asthma.[6] Therefore, Th2 cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13 are other important mediators of allergic rhinitis.[7] Among them, IL-4 has a central role in IgE production by B cells and enhancement of the Th2 responses.[6]

Increasing experimental evidence suggests the existence and important role of the histamine-cytokine network in allergic inflammation, in which histamine influences the expressions and actions of several cytokines and some cytokines modulate the production and release of histamine.[2-4] Pre-treatment with IL-4 primes the release of histamine, prostaglandins, leukotrienes, and cytokines in response to FcεRI.[8,9] Histamine, on the other hand, modulates the releases of IL-4 and interferon-gamma (IFN-γ) from T cells[10] and induces the release of IL-5.[11]

Toluene 2, 4-diisocyanate (TDI) is an organic solvent that causes occupational asthma in 5-15% of all persons exposed to it.[12,13] Intranasal application of TDI caused neuropeptide-mediated release of histamine from mast cells in the nasal mucosa and led to the development of nasal allergy-like symptoms such as sneezing and watery rhinorrhea in TDI-sensitized guinea pigs and rats.[14-19] Although allergic rhinitis, defined as an IgE mediated disease, could be different from TDI-induced rhinitis, which is a non-IgE mediated disease,[20,21] nasal allergy-like symptoms induced by TDI are similar to those observed in allergic rhinitis patients. In addition to the symptoms, TDI-sensitized rats also display many of the characteristic features of allergic rhinitis in humans, including infiltration of eosinophils and mast cells,[22] increase in the levels of cytokines,[23-26] elevations of the H1R mRNA and protein levels,[18] and increases

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in the histidine decarboxylase (HDC) mRNA level, HDC activity and histamine content,[27] even though histamine release is triggered by neuropeptide, but not IgE in TDI-sensitized animals. The expressions of IL-4 and IL-5 mRNAs were also up-regulated in the nasal mucosa of TDI-sensitized rats after provocation with TDI.[28]

Tea is one of the most widely consumed beverages in the world. It has been reported that green tea extract has many pharmacological activities, including anti-oxidative, anti-carcinogenic, anti-hypertensive, and anti-hypercholesterolemic activities.[29-32] The anti-allergic activity of tea extract has also been reported.[33-38] Catechins, which are flavanols derived from green tea, are reported to be responsible for many of the activities of tea extract. Epigallocatechin-3-*O*-gallate (EGCG) is the major polyphenolic constituent in green tea extract and there is accumulating evidence suggesting its anti-allergic effect. EGCG inhibits tumor necrosis factor alpha (TNFα)-induced production of monocyte chemotactic protein-1 (MCP-1), which is a potent chemotactic cytokine.[39] It also inhibits IgE-stimulated and Ca^{2+} ionophore-induced histamine release by RBL-2H3 cells.[40,41] Recently, an *O*-methylated derivative of EGCG has been shown to be a more potent inhibitor of allergies than EGCG.[42] Tachibana *et al.* identified the 67 kD laminin receptor (67LR) as a specific cell-surface receptor for EGCG and *O*-methylated EGCG.[43,44] They also reported that the anti-allergic activity of these compounds was mediated by 67LR.[45] EGCG has been reported to inhibit histamine release through the 67LR-mediated reduction of myosin II right chain phosphorylation, which causes cytoskeletal rearrangement leading to histamine release.[46] On the other hand, there are reports that EGCG is the major causative agent of green tea-induced asthma.[47,48] Thus, the suppressive effect of EGCG on allergic diseases is not conclusive, and the molecular mechanism of the anti-allergic effect of EGCG requires study.

In the present study, we examined the effect of tea extract on the TDI-induced up-regulations of H1R and Th2 cytokines genes in the nasal mucosa of TDI-sensitized rats. Then, we fractionated the constituents of tea extract by column chromatography, and examined which fraction suppressed the mRNA levels of H1R and Th2 cytokines using cells. We identified the active compound and finally evaluated its suppressive effect on the TDI-induced elevations of H1R and Th2 cytokine mRNAs using TDI-sensitized rats.

Materials and Methods

TDI sensitization and provocation. Six-week-old male Brown Norway rats (SLC, Hamamatsu, Japan) were used. The rats were kept in a room maintained at 22 ± 1°C and 50% humidity under a 12-h light/dark cycle and divided into three groups of 4 rats each. Sensitization to TDI was achieved as described by Tanaka *et al.*[49] with slight modifications using the protocol shown in Fig. 1. Briefly, 10 µl of 10% TDI (Wako Chemical Co., Tokyo, Japan) dissolved in ethyl acetate was applied bilaterally to the nasal vestibule once daily for five consecutive days. This sensitization procedure was then repeated after a 2-day interval. Nine days after the second sensitization, 10 µl of 10% TDI solution was again applied to the nostrils to provoke nasal allergy-like behavior. Of the three groups, groups 2 and 3 were sensitized with TDI solution, while group 1 was treated with ethyl acetate and phosphate buffer as a control (Fig. 1). Tea extract (60 mg/rat) was administered orally once a day for 3 weeks before provocation with TDI (group 3). The number of sneezes during a 10-min after TDI provocation was counted. All animal experiments were approved by the Ethical Committee for Animal Studies of the School of Medicine, University of Tokushima, Japan.

Preparation and fractionation of tea extract. A total of 250 g of green tea [Yabukita (first flush green tea at Shizuoka, Japan), kindly provided by ITO EN, LTD., Tokyo, Japan] was mixed with 1.5 L of 30% ethanol and stood for

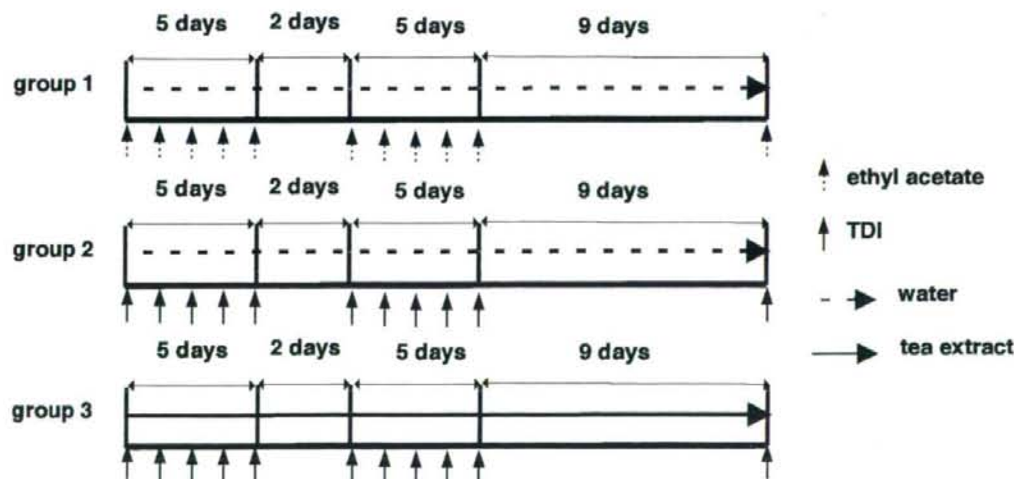

Figure 1. Preparation for nasal allergy model rats.

3 days at room temperature. This extraction was repeated three times. The mixture was centrifuged; the supernatant was filtered to remove insoluble materials, evaporated and designated as crude extract (80.89 g). The crude extract was then applied to TOYOPEARL HW40EC (Tosoh Co., Tokyo, Japan) column chromatography successively eluted with 100% water, 80% ethanol and acetone to separate catechins into gallated and non-gallated form.[50] The fractions eluted with both 80% ethanol and acetone were combined and designated as fraction 1. The flow-through fraction from the HW40EC column, which mainly contained non-gallated catechins and caffeine, was then applied on a DIAION HP20 (Mitsubishi Chemical Co., Tokyo, Japan) column.[50] Materials that bound to the column were eluted with 15% and then 80% ethanol (designated as fractions 3 and 4, respectively) to gain a polyphenol glycoside fraction. The flow-through fraction from the HP20 column was designated as fraction 2. Each fraction was further analyzed to identify its constituents including main catechins, caffeine, and polyphenol derivatives (summarized in Table 1). Pure epigallocatechin 3-*O*-gallate is provided by ITO EN, LTD.

Cells. HeLa cells were cultured at 37℃ under a humidified 5% CO_2/95% air atmosphere in MEM-alpha medium (Gibco Grand Island, NY, USA) containing 8% fetal calf serum (Sigma, MO, USA) and supplemented with 100 IU/ml penicillin (Sigma) and 50 μg/ml streptomycin (Sigma). Serum was removed for 24 h before treatment with tea extract. The cells were stimulated for 3 h with phorbol-12-myristate-13-acetate (PMA, 100 nM) or histamine (100 μM) and then harvested and their total RNA was extracted. RBL-2H3 cells were cultured in MEM-alpha containing 10% fetal calf serum and antibiotics and were treated with tea extract (100 μg/ml) for 6 h. Then, 100 ng/ml of monoclonal anti-dinitrophenyl (DNP) IgE (Sigma) was added and the cells were further incubated for 12 h. Then they were stimulated with 100 ng/ml of DNP-albumin (Sigma) for 1 h, and harvested and their total RNA was extracted.

Isolation of total RNA. Nasal mucosa was removed from the nasal septum. Samples were frozen in RNA*later*® (Applied Biosystems, Foster City, CA, USA) and stored at -80℃ until use. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacture's instructions. Samples were homogenized in a Polytron (Model PT-K; kinematica AG, Littau/Luzern, Switzerland) in 10 volumes of TRIzol reagent. The homogenates were mixed with chloroform and centrifuged at 15,000 rpm for 15 min at 4℃. RNA in the aqueous phase was precipitated with isopropanol. The precipitated RNA was washed with 70% ice-cold ethanol, air-dried and dissolved in 20 μl of diethylpyrocarbonate (DEPC)-treated water.

Cells cultured to ~70% confluency in 35 mm dishes were treated with tea extract. After the treatment, the cells were harvested with 700 μl of TRIzol reagent, mixed with 140 μl of chloroform and centrifuged at 15,000 rpm for 15 min at 4℃. The aqueous phase was collected and total RNA was prepared as described above. The purity and yield of total RNA were determined spectrophotometrically at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Table 1. Summary of separation of green tea extract using column chromatography

Fractions	Steps	Recovery (% of crude extract)	Major constituents**
	Crude extract*	100	
	HW40EC		
Fraction 1	80% Ethanol	40.56	EGCG, ECG
	Acetone	1.32	
	HP-20		
Fraction 2	Flow-through	40.39	Polysaccharides
Fraction 3	15% Ethanol	12.83	Caffeine, EGC, EC, +C
Fraction 4	80% Ethanol	4.90	Polyphenol glycosides

*Starting from 250 g of Yabukita green tea leaves, 80.89 g of crude extract was obtained.
**Catechins and caffeine were measured with HPLC[50] and polyphenol glycosides were detected changing the wavelength of this method to 340 nm.
Abbreviations; EGCG, epigallocatechin-3-*O*-gallate; ECG, epicatechin-3-*O*-gallate; EGC, epigallocatechin; +C, catechin

Real-time quantitative reverse transcription polymerase chain reaction. RNA samples were reverse-transcribed to cDNA in a reaction volume of 40 μl in first-strand buffer [250 mM Tris-HCl, pH 8.3, at room temperature containing 375 mM KCl, 15 mM $MgCl_2$, 0.8 mM concentrations of each deoxyribonucleoside triphosphate (dNTP), 40 μM oligo (dT) primers, 0.004 units of RNase inhibitor, and 8 units of reverse transcriptase (Superscript II, Invitrogen)]. Samples were incubated at 37℃ for 60 min. Then, 2 μl of 2 N NaOH was added and incubation was continued at 65℃ for 30 min. The reaction mixture was then neutralized by adding 12.8 μl of 1 M Tris-HCl, pH 8.0. The samples were then heated at 95℃ for 10 min and chilled to 4℃ for 5 min. TaqMan primers and probe were designed using Primer Express primer design software (Applied Biosystems). The sequences of primers and probes used in this study are summarized in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The primer and probe for GAPDH were from Applied Biosystems. The transcripts were used for a 40-cycle, 3-step polymerase chain reaction (PCR) with the GeneAmp 7300 Sequence Detection System (Applied Biosystems) in 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 200 μM dNTPs, 900 nM of each primer and 0.25 units of platinum Taq polymerase. The size and reaction specificity of amplicon were confirmed by agarose gel electrophoresis. PCR products were identified using a genetic analysis system (SEQ8000; Beckman Coulter Inc. Fullerton, USA). For determination of whether the amplification products were derived exclusively from the RNA, a reverse transcriptase (RT)-negative reaction was run in which the enzyme was replaced by an RNase-free sample.

Statistical analysis. Results are presented as means ± S.E.M. for at least three independent experiments, each carried out in triplicate. Statistical analyses were performed by Fisher's paired least-significant difference test or One-way

Table 2. Primer and probe sequences used for real-time RT-PCR

Primer/probe name		Sequence
human	Sense primer	5'-CAGAGGATCAGATGTTAGGTGATAGC-3'
H1R	Anti sense primer	5'-AGCGGAGCCTCTTCCAAGTAA-3'
mRNA	Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA
rat H1R	Sense primer	5'-TATGTGTCCGGGCTGCACT-3'
mRNA	Anti sense primer	5'-CGCCATGATAAAACCCAACTG-3'
	Probe	FAM-CCGAGAGCGGAAGGCAGCCA-TAMRA
rat IL-4	Sense primer	5'-CAGGGTGCTTCGCAAATTTTAC-3'
mRNA	Anti sense primer	5'-CACCGAGAACCCCAGACTTG-3'
	Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA
rat IL-5	Sense primer	5'-CAGTGGTGAAAGAGACCTTGATACAG-3'
mRNA	Anti sense primer	5'-GAAGCCTCATCGTCTCATTGC-3'
	Probe	FAM-TGTCACTCACCGAGCTCTGTTGACG-TAMRA
rat IL-9	Sense primer	5'-GACGACCCATCATCAAAATGC-3'
mRNA	Anti sense primer	5'-CTGTGACATTCCCTCCTGGAA-3'
	Probe	FAM-TTGTGCCTCCCCATCCCATCTGAT- TAMRA

For measuring rat IL-13 mRNA, a primer probe kit from Applied Biosystems (Rn00587615-A1 1113) was used. GAPDH mRNA was measured using Rodent GAPDH Control Reagents (VIC™ Probe) from Applied Biosystems.

ANOVA followed by Dunnet's multiple comparison tests. P values if less than 0.05 were considered significant.

Results and Discussion

As shown in Fig. 2 and the previous reports,[18,28,51] provocation with TDI increased the allergy sensitive gene expressions of H1R and Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 in TDI-sensitized rats. Oral administration of the crude extract (60 mg/rat) from green tea Yabukita for 3 weeks, significantly suppressed the TDI-induced mRNA elevations of IL-4, IL-5, IL-9, and IL-13 (Fig. 2B-E). It also

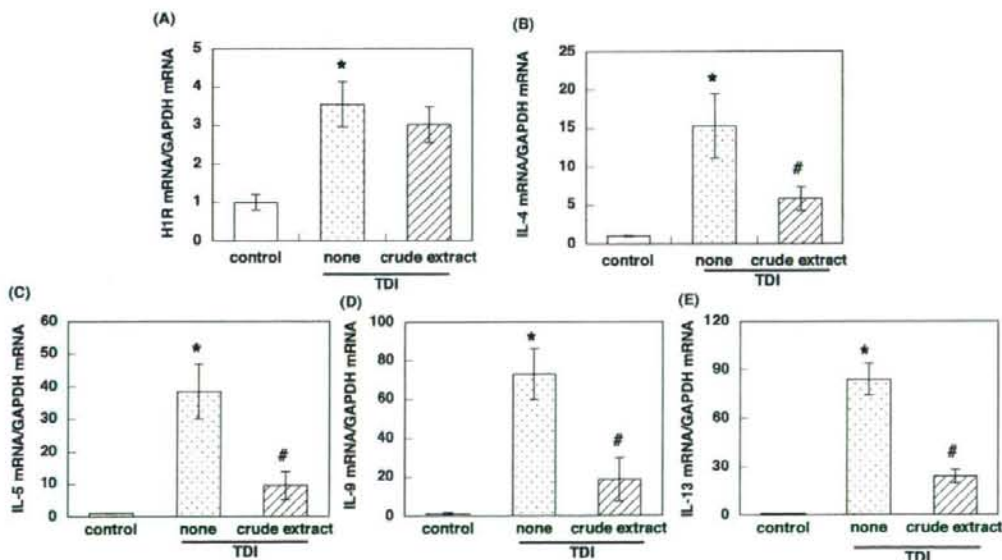

Figure 2. Suppression of gene expressions of H1R and Th2 cytokines by green tea extract in nasal allergy model rats.
Nasal mucosa was collected 4 h after provocation with TDI. The mRNA levels of H1R (A) and Th2 cytokines (B to E) were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. Data are presented as means with standard errors. *$p<0.05$ *vs* control, #$p<0.05$ *vs* TDI (n = 4).

tended to suppress TDI-induced H1R mRNA elevation (Fig. 2A). To identify the active ingredient in the extract from Yabukita, which is the most cultivated variety of green tea and drunk most regularly in Japan, we investigated whether the suppressive effect of the tea extract on the elevations of these gene expressions can be seen *in vitro*. The tea extract showed the maximum inhibitory effect on the stimuli-induced elevations of Th2 cytokine mRNAs after 6 h (data not shown). Therefore, we treated cells with the tea extract 6 h before stimulation. Stimulation of cells through IgE-antigen (Ag) interaction caused elevations of IL-4, IL-9, and IL-13 mRNAs, and treatment with the tea extract 6 h before stimulation decreased these mRNA levels in RBL-2H3 cells in a dose-dependent manner (Fig. 3A-C). PMA-induced H1R mRNA elevation was also dose-dependently suppressed by the addition of the tea extract 6 h before PMA stimulation in HeLa cells (Fig. 3D).

To identify the active constituent, the crude extract from green tea was separated by chromatography using TOYOPEARL HW40EC and DIAION HP20 columns (Table 1) and the fraction with a suppressive effect on the stimuli-induced elevations of Th2 cytokine mRNAs in RBL-2H3 cells was determined. The MIX fraction (100 μg/ml) that reconstitutes all the fractions according to the rate of their recoveries showed a similar inhibitory effect on IgE-stimulated Th2 cytokine gene expressions as the original crude extract in RBL-2H3 cells (Fig. 4). When fraction 1 was omitted from the MIX fraction, no suppressive effect on IgE-stimulated elevations of mRNA of any Th2 cytokines was observed (Fig. 4). Omissions of fraction 3 or 4 containing gallated catechins, caffeine, or polyphenol glycosides from the MIX fraction had no suppressive effect on the IgE/Ag-induced mRNA elevations of these cytokines. These data suggest that the active ingredient was in the fraction 1. As fraction 1 is a mixture of the eluates with 80% ethanol and acetone, we further investigated which fraction contains the active substance. As shown in Fig. 5, the eluate with 80% ethanol suppressed the IgE-stimulated elevations of IL-4, IL-9, and IL-13 mRNAs as much as fraction 1. The eluate with acetone did not inhibit the mRNA elevations of

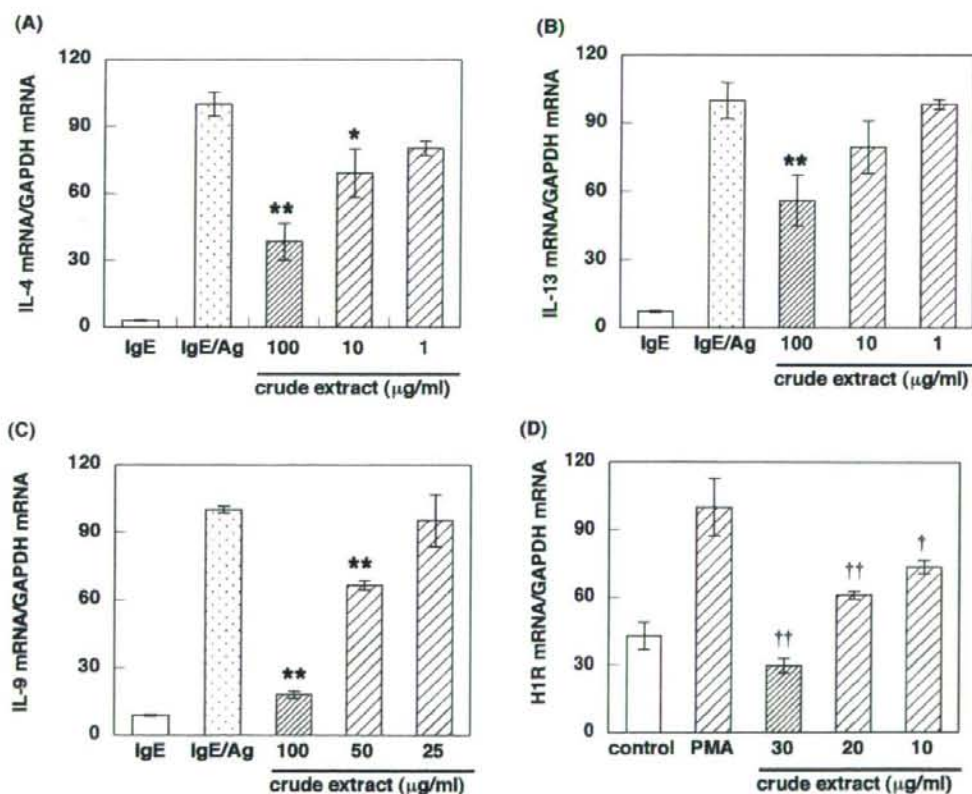

Figure 3. Suppressions of gene expression of Th2 cytokines and H1R by green tea extract in RBL-2H3 and HeLa cells.
For (A) to (C), RBL-2H3 cells were pre-treated with crude tea extract 6 h before monoclonal anti-DNP IgE treatment. After 12 h incubation, cells were stimulated with DNP-albumin for 1h, and total RNA was extracted. For (D), HeLa cells were pre-treated with crude tea extract 24 h before PMA treatment. After 3 h stimulation with PMA, cells were harvested and total RNA was isolated. The mRNA levels of Th2 cytokines (A to C) and H1R (D) were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. Data are presented as means with standard errors. $^*p<0.05$ and $^{**}p<0.01$ *vs* IgE/Ag, $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ *vs* PMA (n = 4).

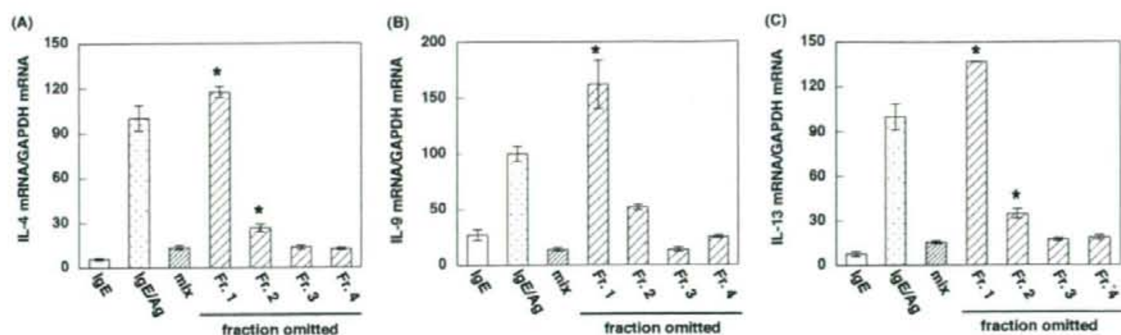

Figure 4. Suppression of gene expression in RBL-2H3 cells of Th2 cytokines by fractions obtained by column chromatography.
The fractions obtained from TOYOPEARL HW40EC column were reconstituted and designated as MIX fraction. RBL-2H3 cells were pre-treated with the fraction that one of the fractions was omitted from the MIX fraction 6 h before monoclonal anti-DNP IgE treatment. After 12 h incubation with monoclonal anti-DNP IgE, cells were stimulated with DNP-albumin for 1 h, and total RNA was extracted. The mRNA levels of IL-4 (A), IL-9 (B), and IL-13(C) were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. Data are presented as means with standard errors. $^*p<0.05$ *vs* MIX (n = 4).

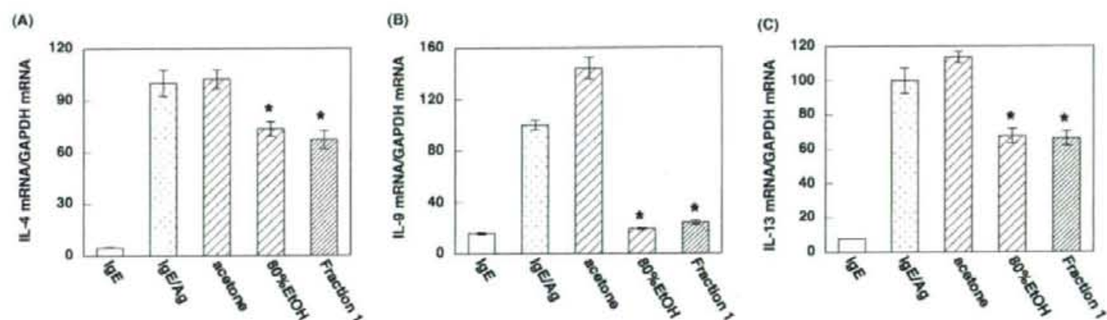

Figure 5. The eluate with 80% ethanol from the TOYOPEARL HW40EC column contains the active compound.
Eluates with 80% ethanol and acetone were reconstituted and designated as fraction 1. RBL-2H3 cells were pre-treated with each fraction 6 h before monoclonal anti-DNP IgE treatment. After 12 h incubation with monoclonal anti-DNP IgE, cells were stimulated with DNP-albumin for 1 h, and total RNA was extracted. The mRNA levels of IL-4 (A), IL-9 (B), and IL-13(C) were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. Data are presented as means with standard errors. $^*p<0.05$ *vs* IgE/Ag (n = 4).

these cytokines (Fig. 5).

As the 80% ethanol eluate from the TOYOPEARL HW40EC column contained gallated catechins and the main constituent was EGCG (Table 1), we investigated the effect of EGCG on the IgE-stimulated mRNA elevations of Th2 cytokines in RBL-2H3 cells. Pure EGCG (20 μg/ml) and the eluate with 80% ethanol containing the same amount of EGCG suppressed IgE-stimulated IL-4 gene expressions to the same extent (Fig. 6A). However, the potencies of the inhibitory effects of pure EGCG and the 80% eluate on elevations of the IL-9 or IL-13 mRNA levels were different (Fig. 6B and C). These data suggest that EGCG is the compound responsible for the suppression of IgE-stimulated IL-4 mRNA elevation, but that another gallated compound like ECG is likely to be involved in the suppressions of IgE-stimulated mRNA elevations of IL-9 and IL-13. The effect of EGCG on histamine- or PMA-induced H1R mRNA elevation was also investigated. We previously reported that stimulation with histamine or PMA caused elevation of H1R mRNA in HeLa cells.[52] Pure EGCG and the eluate with 80% ethanol containing the same amount of EGCG suppressed PMA-induced H1R mRNA elevation to similar degrees (Fig. 6D). As shown in Fig. 7, pure EGCG suppressed IgE-stimulated IL-4 gene expression and histamine- or PMA-induced H1R gene expression dose-dependently.

These data suggest that EGCG may be responsible for the suppression of stimuli-induced IL-4 and H1R mRNA elevations *in vivo*. To confirm this, we re-investigated the suppressive effect of EGCG using TDI-sensitized rats. Pretreatment with EGCG (9 mg/rat) once daily for 3 weeks significantly suppressed the TDI-induced elevations of H1R and IL-4 mRNAs (Fig. 8A and B).

The involvements of nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T-cells (NFAT) signalings in IL-4 gene expression have been reported.[53-55] The binding sites of these transcription factors

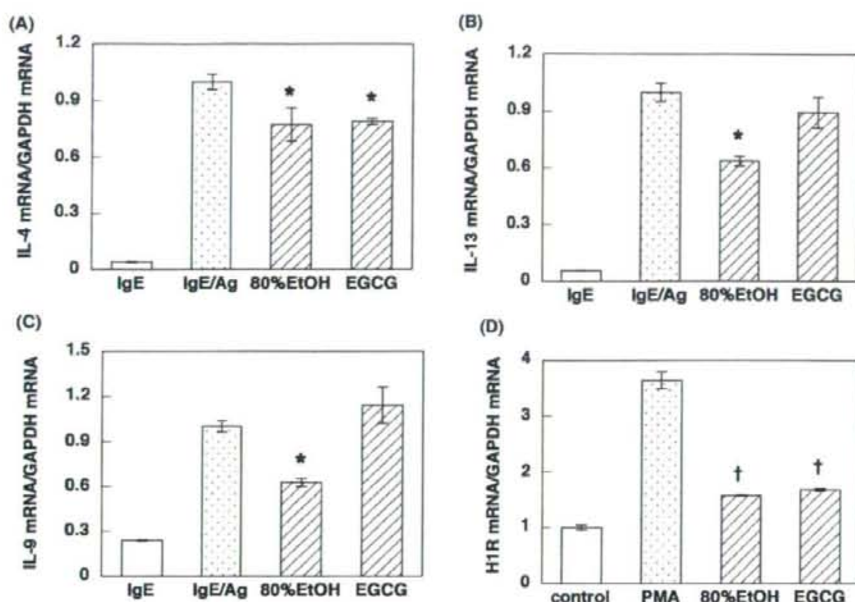

Figure 6. Suppression of gene expressions of IL-4 and H1R by green tea extract in RBL-2H3 and HeLa cells.
For (A) to (C), RBL-2H3 cells were pre-treated with EGCG or the 80% ethanol eluate fraction containing the same amount of EGCG 6 h before monoclonal anti-DNP IgE treatment. After 12 h incubation, cells were stimulated with DNP-albumin for 1 h, and total RNA was extracted. For (D), HeLa cells were pre-treated with EGCG or the 80% ethanol eluate fraction containing the same amount of EGCG 24 h before PMA treatment. After 3 h stimulation with PMA, cells were harvested and total RNA was isolated. The mRNA levels of IL-4 (A), IL-13 (B), IL-9 (C), and H1R (D) were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. Data are presented as means with standard errors. $^{*}p<0.05$ *vs* IgE/Ag, $^{\dagger}p<0.05$ *vs* PMA (n = 4).

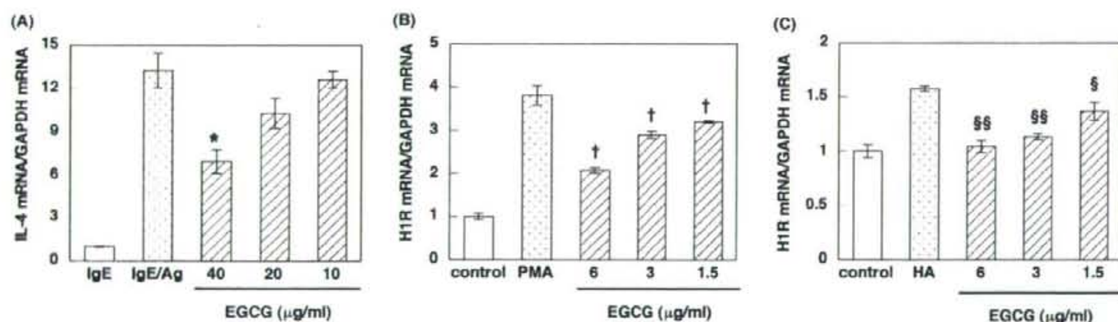

Figure 7. Suppression of gene expressions of IL-4 and H1R by EGCG in RBL-2H3 and HeLa cells.
For (A), RBL-2H3 cells were pre-treated with EGCG at various concentrations 6 h before monoclonal anti-DNP IgE treatment. After 12 h incubation, cells were stimulated with DNP-albumin for 1 h, and total RNA was extracted. For (B) and (C), HeLa cells were pre-treated with EGCG 24 h before PMA (B) or histamine (C) treatment. After 3 h stimulation with PMA or histamine, cells were harvested and total RNA was isolated. The mRNA levels of IL-4 and H1R were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. Data are presented as means with standard errors. $^{*}p<0.05$ *vs* IgE/Ag, $^{\dagger}p<0.05$ *vs* PMA, $^{\S}p<0.05$ and $^{\S\S}p<0.01$ *vs* histamine (n = 4).

are shown in the IL-4 promoter region in humans.[56] On the other hand, EGCG has been reported to inhibit NF-κB activity.[57-59] It is also reported to inhibit antigen- or A23187-induced histamine release by inhibiting tyrosine phosphorylation of signal proteins including $pp125^{FAK}$ in RBL-2H3 cells.[41] Furthermore, EGCG inhibits the activation of AP-1 induced by PMA by inhibiting a c-Jun NH_2-terminal kinase-dependent pathway.[60] These findings suggest that the effect of EGCG on IL-4 gene expression is due to inhibit these signalings. In this study, we did not check the effect of EGCG on IL-5 gene expression in RBL-2H3 cells, although we observed that crude tea extract suppressed TDI-induced

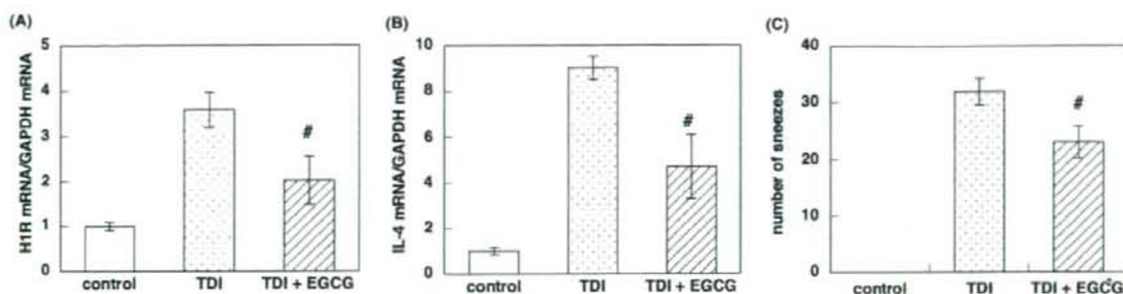

Figure 8. Suppression of gene expressions of H1R (A) and IL-4 (B) and allergic symptom (C) by EGCG in nasal allergy model rats.
Nasal mucosa was collected 4 h after provocation with TDI. The mRNA levels of H1R (A) and IL-4 (B) were determined by real-time RT-PCR. The mRNA level was normalized by GAPDH mRNA. For (C), number of sneezes in 10 min after TDI-provocation was counted. Data are presented as means with standard errors. $^{\#}p<0.05$ *vs.* TDI (n = 3).

up-regulation of the IL-5 gene. As suppression of TDI-induced IL-5 production by EGCG was reported in TDI-sensitized mice,[61] it is likely that the suppressive effect of tea extract on IL-5 gene elevation is due to EGCG. The number of sneezes was also significantly decreased in EGCG-treated rats (Fig. 8C). These data suggest that the active constituent in the extract from green tea is EGCG and that EGCG suppresses TDI-induced up-regulations of H1R and IL-4 genes in the nasal mucosa of TDI-sensitized rats.

Our data are the first to implicate the suppressive effect of EGCG on histamine signaling. In this study, we used HeLa cells to investigate the suppressive effect of tea extract and EGCG because they express endogenous H1R and stimulation of H1R with histamine up-regulates H1R gene expression through protein kinase C dependent signaling pathway.[52] We think this agonist-induced up-regulation of H1R can be a model to explain how provocation of small amount of antigen aggravates allergic symptoms. This agonist-induced up-regulation of the H1R gene consequently increases the number of H1R protein molecules and makes cells more sensitive to histamine stimulation. Accordingly, this "positive feedback circuit" between histamine and H1R exacerbates allergic symptoms. Pretreatment with EGCG could shut down this positive feedback circuit of histamine-induced up-regulation of the H1R gene and reduce the symptoms.

Conclusion

In the present study, we showed that extract from green tea suppressed the TDI-induced up-regulations of the H1R and Th2 cytokines genes in the nasal mucosa of TDI-sensitized rats. We also identified EGCG as the active constituent in tea extract, which suppresses the TDI-induced elevations of H1R and IL-4 mRNAs. Our data suggest that EGCG alleviates nasal symptoms by inhibiting both histamine and IL-4 signaling through the suppressions of TDI-induced H1R and IL-4 gene up-regulations.

Acknowledgements

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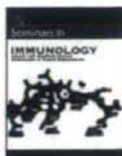

Review

Critical role of the Polycomb and Trithorax complexes in the maintenance of CD4 T cell memory

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ABSTRACT

The maintenance of memory CD4 T cells is crucial for the establishment of immunological memory. The Polycomb (PcG) and Trithorax group (TrxG) genes control key developmental regulators such as the homeobox genes, and these two antagonize each other in the same developmental processes. Recently, PcG gene *Bmi1* has been found to control memory Th1/Th2 cell survival and TrxG gene *MLL* is to control the maintenance of memory Th2 cell function selectively. Therefore, in memory CD4 T cells, PcG and TrxG genes appear to control distinct processes in a distinct manner, which indicates a novel regulatory feature of the PcG/TrxG genes.

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1. Introduction

After antigen recognition by TCR, naïve CD4 T cells undergo clonal expansion and become functionally polarized effector Th cells, such as Th1 and Th2 cells within a few weeks. After antigen clearance, however, most of the effector Th1/Th2 cells are thought to undergo apoptotic cell death during a period known as the contraction phase [1,2]. Some of the effector cells, however, escape cell death, differentiate into memory type Th1/Th2 cells and survive for a long time *in vivo* (memory phase) [3–5]. Various cellular and molecular processes are required for the successful differentiation and maintenance of functional memory type Th1/Th2 cells; cell survival/escape from cell death and proliferation/homeostatic proliferation at both contraction and memory phases, and the maintenance of Th1/Th2 cell function at memory phase [5]. The idea of memory stem cells is also considered, but this has not been experimentally addressed very well.

The Polycomb group (PcG) gene products are reported to localize in the nucleus as heterogeneous multimeric protein complexes and they appear to maintain, through silencing mechanisms, early determined gene expression patterns of key developmental regulators such as the homeobox genes both in invertebrates and vertebrates (reviewed in [6,7]). The PcG gene *Bmi1* has recently been implicated in the maintenance of hematopoietic [8,9], neural [10] and cancer stem cells [11]. The Trithorax group (TrxG) gene products are known to antagonize the effect of PcG gene products in the early developmental processes, and control nuclear regulatory mechanisms that establish the epigenetic transcriptional memory [12].

This review focuses on the recently identified molecular mechanisms by which PcG and TrxG gene products directly control the differentiation and the maintenance of functional memory CD4 T cells. A distinct view of the mode of regulation by PcG/TrxG gene products from that in the early developmental processes has emerged.

2. Establishment of an experimental system for the molecular analysis of memory Th cells

One of the difficulties in studying the molecular events operating in memory Th cells is the limitations in the preparation of substantial numbers of antigen-specific memory Th cells generated *in vivo*. To overcome this issue, an *in vivo* experimental system was established in which antigen-specific memory Th1 and Th2 cells are generated and maintained quite efficiently [13]. In brief, naïve CD4 T cells from DO11.10 OVA-specific TCR transgenic (Tg) mice are stimulated with a specific OVA peptide plus APC for 5 days *in vitro* under either Th1 or Th2 culture conditions, and then transferred intravenously into normal syngeneic BALB/c or BALB/c *nu/nu* recipient mice (Fig. 1A). The transferred DO11.10 Tg T cells can be monitored by the staining with clonotypic KJ1 mAb, which is specific for a donor-derived TCR Tg T cells. A week after cell transfer of the effector Th2 cells into normal BALB/c mice, ~25% of the splenic CD4 T cells are donor derived [13]. The numbers of KJ1$^+$ cells are decreased to approximately 10% at the 2 weeks time point, and this level is maintained at least for 16 weeks. Similar kinetics are

Abbreviations: PcG, Polycomb group; PRC, Polycomb repressive complex; TrxG, Trithorax group; Tg, transgenic.

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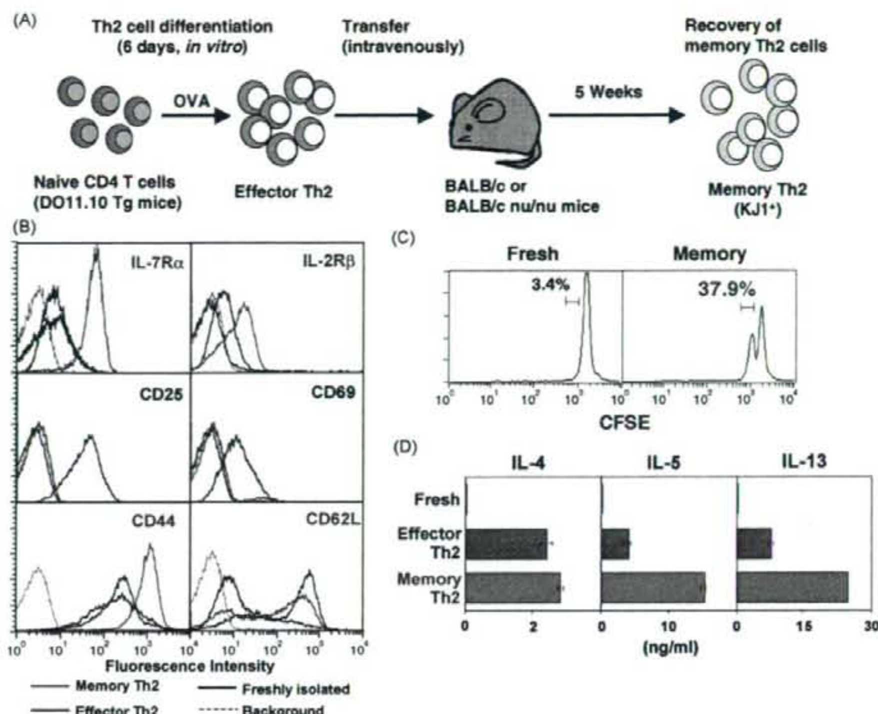

Fig. 1. Generation and phenotypic characterization of OVA-specific memory Th2 cells. (A) The generation of antigen-specific memory Th2 cells by adoptive cell transfer. Splenic CD4 T cells from DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15) plus APC under Th2 conditions for 5 days *in vitro*, and then transferred intravenously into normal syngeneic BALB/c or BALB/c *nu/nu* recipient mice. The donor-derived cells (memory Th2 cells) were identified by the staining with anti-clonotypic mAb for the Tg TCR, JK1. (B) Expression profiles of cell surface maker antigens on *in vivo* generated memory Th2 cells. (C) Memory Th2 cells rapidly proliferated in response to the antigen. The cells were labeled with CFSE and stimulated with OVA peptides plus APC for 16 h. The cell division of CFSE-labeled cells was analyzed by flow cytometry. (D) Th2 cyokine production profiles of the *in vivo* generated memory Th2 cells upon *in vitro* antigenic restimulation.

observed when effector Th2 cells are transferred into BALB/c *nu/nu* recipient mice (unpublished observation).

The expression profiles of the surface makers on the *in vivo* generated memory Th2 cells are shown in Fig. 1B. The expression levels of IL-7 receptor (R)α and IL-2 receptor (R)β chains are higher in memory Th2 cells in comparison to those of freshly isolated KJ1+ CD4 T cells from DO11.10 Tg mice or *in vitro* generated effector Th2 cells. The activation markers, CD69 and CD25 (IL-2Rα chain), are not significantly expressed in either memory or naïve populations, whereas effector Th2 cells expressed these makers substantially. The high-level expression of CD44 is observed in the memory Th2 cells. Two subpopulations with high and low expression of CD62L (phenotypically central and effector memory cells, respectively) are observed in memory Th2 cells.

Memory T cells proliferate rapidly in response to a low concentration of antigens in comparison to naïve T cells [14]. *In vivo* generated memory Th2 cells from the spleen were labeled with CFSE *in vitro*, and stimulated with two different doses of OVA peptides and APC for 16 h. Freshly isolated CD4 T cells from DO11.10 Tg mice do not proliferate (3.4%) during the first 16 h after stimulation, whereas substantial numbers of memory Th2 cells (37.9%) divided once in response to the antigenic peptide (Fig. 1C). Similar results are obtained in Th1 cells (unpublished observation).

Cytokine production profiles are also maintained in the *in vivo* generated memory Th2 cells (Fig. 1D). After restimulation with antigenic peptide *in vitro*, the *in vivo* generated memory Th2 cells produced large amounts of Th2 cytokines (IL-4, IL-5 and IL-13) and the levels are equivalent or higher than those of effector Th2 cells.

Taken as a whole, these results indicate that the *in vivo* generated memory Th2 cells possess the phenotypic and functional properties that are typical for memory cells.

3. Molecular basis for the maintenance of memory CD4 T cells

In contrast to CD8 memory T cells, CD4 memory T cells may not require any specific cytokine signals for their homeostatic maintenance [15,16]. Memory CD4 T cells that lack common γ-chain (γc) could survive normally *in vivo* [17]. However, a role for IL-7 in memory CD4 T cells is demonstrated when the TCR-mediated signals are impaired [18]. Therefore, IL-7 may play a role in the regulation of the generation and survival of CD4 memory T cells [18–20]. The γc cytokines such as IL-7, IL-2, and IL-15 transduce signals through the recruitment of Jak1 and Jak3, which phosphorylate STAT5 [21]. The proto-oncogenes Pim1 and Pim2, which are transcriptional targets of STAT5, are preferentially expressed in the central memory T cell subset [22]. In addition, a higher level of STAT5 phosphorylation is induced by IL-2/IL-7-treatment in central memory T cells in comparison to effector memory T cells. These results suggest that central memory T cells are more sensitive to the γc cytokines to be activated and proliferate.

Recently, the upregulation of several target genes (Bim, Gadd45α, p130 proteins and Fas ligand) of the forkhead-family

transcription factor, FOXO3a was reported in CD4+ effector memory T cells [23]. The activity of FOXO3a is suppressed by Akt- and IKK-mediated Ser/Thr-phosphorylation [24,25]. Stimulation via the TCR/CD28 and cytokines activated Akt through the PI3K-dependent signaling pathway and regulated phosphorylation of FOXO3. TCR/CD28-mediated signals induced phosphorylation on Ser315 of FOXO3a, whereas IL-2/IL-7 stimulation results in the phosphorylation on Thr315 [23]. The preferential activation of these cascades in central memory T cells may therefore support the survival of these cells.

The OX40-dependent signaling either from APC or other activated CD4 T cells are reported to be required for the sufficient generation of memory CD4 T cells, at least under Th2 conditions [26,27]. GATA3 is required for the maintenance of Th2 cytokine production [28–30] and chromatin remodeling of the Th2 cytokine gene loci [28]. Memory Th2 cells maintain the Th2 features, such as selective Th2 cytokine production, a high-level expression of GATA3 mRNA and histone modifications of the Th2 cytokine gene loci in an IL-4-independent manner [13]. However, the molecules that control the maintenance of these Th2 features in memory Th2 cells have only recently been identified.

Table 1
Major components of the Polycomb/Trithorax complexes.

Drosophila protein	Protein domains	Mouse protein homologues
Polycomb group		
PC	Chromodomain	M33 (CBX2), CBX4, 6–8
PH	SAM	PHC1–3
PSC	RING	BMI1, Mel18
SCE	RING	RING1A/B
SCM	SAM, MBT, Zinc-finger	SCMH1, 2
E(Z)	SET	EZH1, 2
ESC/ESCL	WD40	EED
SU(Z)12	Zinc-finger	SUZ12
PHO/PHOL	Zinc-finger	YY1
ASX	PHD	ASXL1, 2
SFMBT	SAM, MBT	L3MBTL2, MBTD1
Trithorax group		
TRX	PHD, SET	MLL1, WBP7
ASH1	PHD, SET, BAH	ASH1
ASH2	PHD, SPRY	ASH2L
BRM	SNF2, Bromodomain	SMARCA4
MOR	SWIRM, SAINT	SMARCC1, 2
OSA	BRIGHT	ARID1B

Ref. [33].

4. PcG and TrxG gene products control cellular memory

In general, the gene expression in eukaryotic cells is maintained by epigenetic changes in the chromatin structure [31]. These changes include covalent posttranslational modification of nucleosomal histones, DNA methylation and remodeling of the nucleosome structure [32]. Recent studies provide substantial evidence indicating that PcG and TrxG proteins are key components for the maintenance of chromatin structures. The members of PcG and TrxG family proteins are summarized in Table 1 [33].

The PcG gene products were first identified in *Drosophila melanogaster* as molecules that are required for maintaining proper expression pattern of *homeotic* (*Hox*) genes [34]. The PcG gene is required for the maintenance of the repressed state of its target genes. PcG silencing involves at least two kinds of multimeric heterogenous protein complex, called Polycomb repressive complex 1 (PRC1) and PRC2 (Fig. 2A). The mammalian PRC1 complex has been isolated and identified from Hela cells [35]. The purified complex contains Ring1A/B, HPC1/2/3, HPH1/2, Bmi1 and Mel18. The PRC1 contains Polycomb (Pc) molecule, and the PRC1 is able to bind specifically to the tri-methylated histone H3-K27 via the chromodomain of the Pc molecules [36]. The key component of the mammalian PRC2 complex is the Enhancer of Zeste homolog (Ezh2) having an intrinsic histone methyltransferase activity for histone

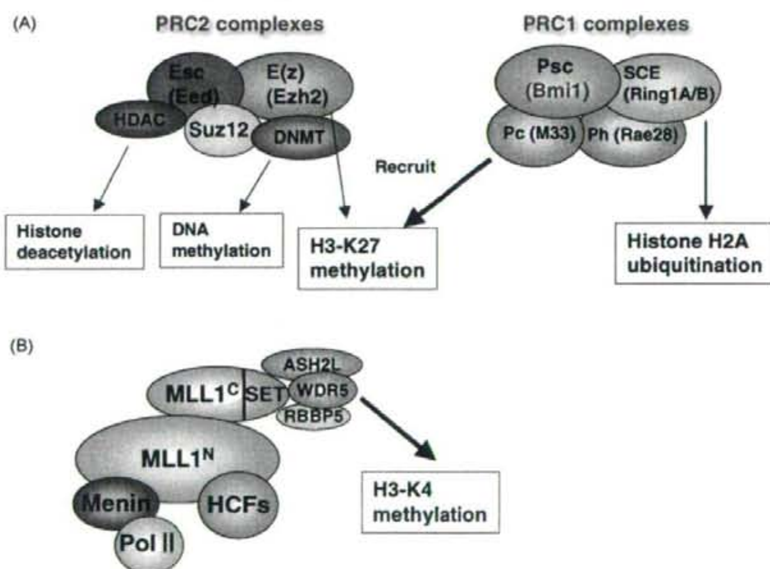

Fig. 2. Schematic representation of PcG and TrxG molecule complexes. (A) PcG proteins form at least two types of multiprotein complexes Polycomb repressive complex 1 (PRC1) and PRC2. E(z) has an intrinsic activity of tri-methylation of histone H3-K27 [51–54]. SCE (Ring 1B) monoubiquitinates histone H2A-K119 [55,56]. The chromodomain of Pc recognizes the tri-methylated histone H3-K27 [36]. PRC2 contains DNMTs (DNA methyl transferases) and HDACs (histone deacetylases). (B) A proposed Trx complex containing MLL1. MLL1 is reported to be associated with ASH2L, RbBP5, WDR5, and Menin [57]. RbBP5 and WDR5 are the WD40 repeat containing protein. The MLL complex has an activity of tri-methylation of histone H3-K4.

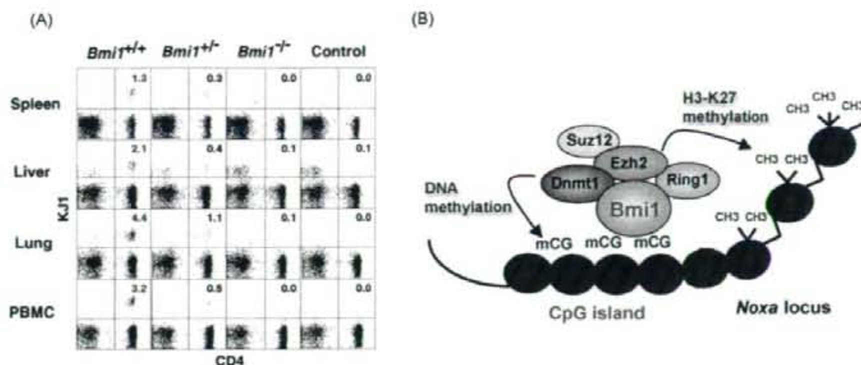

Fig. 3. Bmi1 regulates memory Th2 cell generation via direct binding to the *Noxa* gene. (A) Impaired generation of memory Th2 cells from *Bmi1*$^{-/-}$ effector Th2 cells. *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$ or *Bmi1*$^{-/-}$ effector Th2 cells with DO11.10 Tg background were intravenously transferred into syngeneic BALB/c mice. Five weeks later, the number of donor-derived KJ1^{+} memory Th2 cells was determined by flow cytometry. (B) A proposed Bmi1 containing PcG complex that regulates directly the expression of *Noxa*. Dnmt1, Ring1, Ezh2, and Suz12 are recruited to the CpG island of the *Noxa* gene locus, and the methylation of genomic DNA and histone H3-K27 of this region was detected [47].

H3-K27. Interestingly, DNA methyltransferase 1 (DNMT1) and histone deacetylases (HDACs) are also components of PRC2, and thus PRC2 appears to be able to negatively regulate the expression of target genes. The mammalian PRC2 complex contains at least two other PcG molecules, namely Suz12 and Eed.

The major role of TrxG gene products in the regulation of the *Hox* gene expression is to prevent PcG gene product-mediated silencing [37,38]. The mammalian homolog of *Drosophila trithorax* (*trx*) gene, *Mixed-Lineage Leukemia 1* (*MLL1*) gene is isolated as a common target of chromosomal translocations observed in human acute leukemias [39–43]. MLL1 forms a multi-component complex containing other TrxG protein, ASH2L (Fig. 2B), and has a histone methyltransferase activity specific for histone H3-K4, a modification typically associated with the transcriptionally active regions of chromatin [44,45].

5. Essential role of Bmi1 in the regulation of memory T cell survival

Memory T cells have several features associated with stem cells, and the similarity of the gene expression pattern between memory T/B cells and long-term hematopoietic stem cells is reported [46]. Similar to hematopoietic stem cells, memory T cells appear to possess the ability to proliferate in response to homeostatic signals. The PcG gene *Bmi1* has recently been implicated in the maintenance of hematopoietic [8,9], neural [10] and cancer stem cells [11]. The role of Bmi1 in the generation and maintenance of memory Th1/Th2 cells was investigated using the memory Th cell generation system stated above [47]. A *Bmi1* gene dose-dependent decrease in the numbers of memory Th2 cells is observed in all tissues tested (spleen, liver, lung, and peripheral blood mononuclear cells; PBMC) (Fig. 3A). Memory Th1 cell generation from *Bmi1*$^{-/-}$ effector Th1 cells is also impaired. This is also observed when they were transferred to lymphopenic BALB/c *nu/nu* mice [47]. These results clearly indicate that Bmi1 expression is required for the generation of memory Th1/Th2 cells.

Bmi1 has been reported to control cell proliferation and stem cell self-renewal by repressing the *Ink4a/Arf* locus [48]. This locus codes for two proteins, p16^{ink4a} and p19^{arf} (Ink4a and Arf), through the use of alternative reading frames. Ink4a is a cyclin D-dependent kinase inhibitor that induces cell cycle arrest following Rb activation. Arf induces p53 activation and p53-mediated cell death [49]. Although the increased expression of mRNA is observed in the *Ink4a/Arf* in *Bmi1*$^{-/-}$ memory Th2 cells, the deletion of the *Ink4a* and *Arf* genes failed to restore memory Th2 cell generation in *Bmi1*$^{-/-}$ [47]. Among the pro-apoptotic genes (*Bax*, *Puma*, *Noxa*, *Bim*, *Bad*, *Fas* and *Fas ligand*) that increased in *Bmi1*$^{-/-}$ Th2 cells, the level of *Noxa* mRNA remained high in *Bmi1*$^{-/-}$/*Ink4a*$^{-/-}$/*Arf*$^{-/-}$ Th2 cells. Noxa is a member of BH3-only protein family that initiates programmed cell death in various cells including lymphocytes. Bmi1 binds to the CpG islands at *Noxa* gene and suppresses the expression of *Noxa* through the maintenance of H3-K27 histone methylation. The other PcG family proteins, such as Ring1B and Suz12 also bind to the Noxa gene locus (Fig. 3B). In addition, Bmi1-dependent recruitment of Dnmt1 at the *Noxa* gene locus is observed. Furthermore, Noxa-deletion significantly rescued the effects of Bmi1-deficiency on memory Th2 cell generation. Mel-18, Mph1/Rae28, Ring1B and M33 are also found to be involved in the regulation of memory Th2 cell generation (unpublished observation). Therefore, Bmi1 containing PcG complex regulates memory Th cell survival at least in part through the regulation of *Noxa* gene expression.

6. Maintenance of memory Th2 cell function regulated by TrxG molecule, MLL1

Some of the effector Th cells are maintained as memory Th cells for a long time *in vivo* [4]. In memory Th2 cells, GATA3 is required for the maintenance of Th2 cytokine production [28–30] and chromatin remodeling of the Th2 cytokine gene loci [28]. Memory Th2 cells maintain the Th2 features, such as selective Th2 cytokine production upon recall stimulation, high-level expression of GATA3 mRNA and histone modifications of the Th2 cytokine gene loci in an IL-4-independent manner [13]. To maintain a high-level expression of GATA3 and subsequent Th2 cytokine production, MLL1, a member of TrxG protein, has been found to play a crucial role [50]. MLL is involved in one of the nuclear regulatory mechanism that establishes an epigenetic transcriptional memory system [12]. MLL1 forms a multi-component complex and mediates its epigenetic transcriptional effector functions via the SET domain-dependent histone methyltransferase activity [44,45] (Fig. 2B). MLL1 specifically methylates lysine 4 (K4) present on histone H3, a modification typically associated with the transcriptionally active regions of chromatin. A substantial decrease in the transcription of GATA3 accompanied by decreased methylation levels of H3-K4 at the GATA3 gene locus are detected in MLL1-knockdown Th2 cell lines and in *in vivo* generated memory Th2 cells with an *MLL1* heterozygous background [50]. MLL1 appears to regulate the memory Th2 responses through the control of chromatin remodeling at the

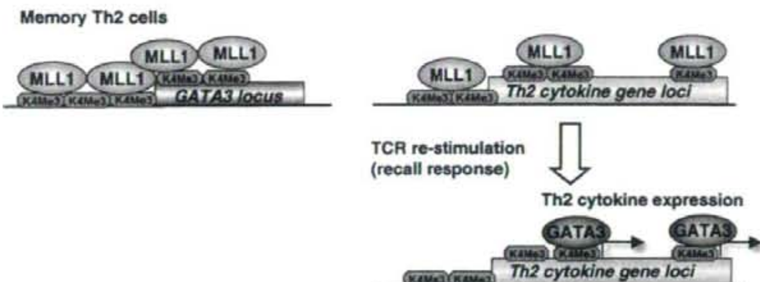

Fig. 4. MLL controls the maintenance of open chromatin of the GATA3 and Th2 cytokine gene loci in memory Th2 cells. In memory Th2 cells, MLL binds to the GATA3 gene locus and Th2 cytokine gene loci to maintain the open chromatin of these loci accompanied with histone H3-K4 methylation. Upon TCR restimulation during recall responses, GATA3 plays an important role in the transcription of Th2 cytokine genes. In memory Th2 cells, GATA3 does not play a major role in the regulation of chromatin status, but still plays an important role as a transcription factor for the Th2 cytokine gene.

GATA3 locus and subsequent GATA3 expression in Th2 cells (Fig. 4). In addition to the GATA3 gene locus, MLL1 is also found to be involved in the maintenance of activated chromatin status at the Th2 cytokine gene loci (Fig. 4). The direct binding of MLL1 at specific regions on the IL-4 and IL-13 gene loci as well as GATA3 gene locus is detected. Th2 cytokine production was severely reduced in *MLL1*-knockdown Th2 cell lines and *MLL1* heterozygous memory Th2 cells accompanied by decreased levels of H3-K9/14 acetylation and H3-K4 methylation. The antigen-induced allergic airway inflammatory responses *in vivo* are also compromised in $MLL1^{+/-}$ memory Th2 mice, thus suggesting a physiological role for MLL1 in the regulation of allergic reactions. Therefore, the maintenance of transcriptional expression of GATA3 in established Th2 cells including memory Th2 cells is epigenetically regulated by TrxG molecule MLL1.

7. Concluding remarks

In the past a few years, new insights into the molecular requirement for the epigenetic regulation of the function of CD4 T cells have emerged. PcG and TrxG gene products counteract each other in the epigenetic function during the development in *Drosophila* and mammals. In contrast, PcG and TrxG gene products have been found to regulate different processes during the generation and the maintenance of memory CD4 T cells. The mode of regulation by PcG and TrxG is also distinct. PcG and TrxG complexes are heterogenous and the components in the complexes appear to be different in different cell types and also in the different developmental stages. Therefore, the analyses of the precise molecular actions of a particular complex formed in a specific process during the initiation and the maintenance of functional memory Th1/Th2 cell would be most important. Moreover, similar to Th1/Th2 cells, further investigation of the regulation by RcG and TrxG molecules in other functionally distinct CD4 T cell subsets, such as Th17, follicular helper T (Tfh), regulatory T (Treg) and Th9 would also be intriguing.

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Human T_h1 differentiation induced by lipoarabinomannan/lipomannan from *Mycobacterium bovis* BCG Tokyo-172

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Abstract

Mycobacterium tuberculosis **(tubercle bacilli) and the related acid-fast bacteria including** ***Mycobacterium bovis*** **Bacille Calmett–Guerin (BCG) have a characteristic cell wall (CW) containing various lipoglycans and glycolipids. Such lipoglycans have been reported to activate type-I inflammatory responses via dendritic cells (DCs) through Toll-like receptor 2. In this study, lipoglycans, lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannoside (PIM), were purified from the CW fractions of** ***M. bovis*** **BCG Tokyo-172, and the effect on the differentiation of human peripheral blood naive CD4 T cells into T_h1 and T_h2 was examined. LAM/LM molecules enhanced T_h1 differentiation under both T_h1 and T_h2 conditions, whereas some other glycolipids and phospholipid enhanced T_h2 differentiation under T_h2 conditions. Other components had little effect under the given conditions. Even in highly purified CD4 T cell cultures, LAM/LM enhanced T_h1 generation only under T_h1 culture conditions. These results indicate that LAM/LM possesses a potent augmenting activity in T_h1 differentiation in human CD4 T cells. LAM/LM appeared to act directly on naive CD4 T cells to enhance T_h1 differentiation under T_h1 culture conditions, while acting indirectly to up-regulate the generation of T_h1 cells via IL-12/DCs under T_h1 and T_h2 conditions. Therefore, these results provide the first evidence indicating that LAM/LM from** ***M. bovis*** **BCG may possess a potent modulating activity in the human system, and thus supporting the strategy for the use of BCG components in the vaccine development for such T_h2 diseases as allergic asthma and rhinitis.**

Introduction

The mycobacterial cell envelope consists of diverse lipophylic components such as mycoloyl glycolipids, lipomannan (LM)/lipoarabinomannans (LAM), lipopeptides and phosphatidylinositol mannosides (PIMs) or cardiolipin as shown schematically in Fig. 1 (1, 2). LAM is a major amphipathic molecule in the cell wall (CW) components of mycobacteria and is regarded as a modulin acting through its diverse immunoregulatory and anti-inflammatory effects, which may support the survival of the mycobacteria within the infected hosts. These effects are mediated by the inhibition of IFN-γ-dependent activation of macrophages (3, 4), inhibition of antigen-induced T cell proliferation (5) and scavenging of oxygen-derived free radicals (6). LAM acts as a virulence factor responsible for macrophage deactivation by mannose receptor down-regulation and also is implicated in phagocytosis of mycobacteria (7). Furthermore, PIMs, that are assumed to be precursors of LM and LAM have recently been proposed to recruit NKT cells, which play a primary role in the granulomatous response in mycobacterial infection (8, 9). The precursor–product relationship of phosphatidylinositol (PI), PIMs, LM and LAM has recently been proposed based on biosynthetic (1, 10) and genetic studies (11, 12), but the details of this pathway remain unclear. On the other hand, however, the structures of LAM from many species of mycobacteria, nocardia and rhodococcus including *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *M. bovis* Bacille Calmett–Guerin (BCG) and *Mycobacterium smegmatis* have been vigorously investigated over the last

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