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CRTH2-dependent, STAT6-independent induction of cedar pollen dermatitis

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Clinical and
Experimental
Allergy

Summary

Background Airborne contact dermatitis to cedar pollen is a recently identified disease that generally affects individuals with cedar pollinosis of the nasal and/or ocular symptoms, as well as some patients with atopic dermatitis.

Objective To elucidate the pathological mechanisms of cedar pollen dermatitis.

Methods We established a mouse model of cedar pollen dermatitis by epicutaneous sensitization with Japanese cedar pollen antigen (Ag).

Results Histologically, there was marked dermal cellular infiltrate, including eosinophils and mast cells, with epidermal thickening. The induction of dermatitis was accompanied by production of cedar pollen-specific IgE. In the lesional skin, IL-13, IL-18, eotaxin/chemokine (C-C motif) ligand (CCL) 11, regulated upon activation, normal T cell expressed and secreted/CCL5, macrophage-derived chemokine/CCL22 and thymus and activation-regulated chemokine/CCL17, but not IL-4 and IFN- γ , were produced. Mast cell-deficient WBB6F1-W/W^v mice failed to develop cedar pollen dermatitis, although regional lymph node cells proliferated in response to *Cryptomeria japonica* (Cry j) 1 and Cry j2 Ags *in vitro*. Surprisingly, the induction of dermatitis was independent of STAT6/IgE. In contrast, mice deficient in CRTH2, a receptor for prostaglandin D2 (PGD2), showed diminished inflammation. Consistent with this, ramatroban, a CRTH2 antagonist, significantly inhibited inflammatory cell infiltration.

Conclusion These data suggest that PGD2-CRTH2 signalling contributes to inflammation in cedar pollen dermatitis, and unlike cedar pollinosis of the nasal mucosa, STAT6 is not a therapeutic target for treatment.

Keywords atopic dermatitis, cedar pollen, CRTH2, Cry j, IL-18, mast cells, prostaglandin D2, STAT6

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Introduction

Airborne Japanese cedar pollen has been increasing annually, although yearly variations are seen, thus leading to a marked increase in the number of patients with pollinosis [1] characterized by seasonal rhinoconjunctivitis due to type I allergy to cedar pollen. Inhalation of cedar pollen leads to sensitization through the mucosal tissues of the upper respiratory tract, followed by the generation of cedar pollen-specific IgE. Subsequent exposure to cedar pollen antigen (Ag) by mucosal tissues results in the development of nasal and/or ocular symptoms via the activation of mast cells through Fc ϵ R1 receptors.

Japanese cedar pollen dermatitis is a recently identified disease characterized by itchy erythema of the skin during

the Japanese cedar pollen season (February–April) [2]. It has been postulated that cedar pollen dermatitis is triggered by contact of cedar pollen Ag with the skin, i.e. airborne contact dermatitis, as skin symptoms characteristically appear on exposed areas such as the face.

Japanese cedar pollen is globe shaped with a short papilla, measuring 30 μ m in diameter, that ruptures in water, thus resulting in the escape of the intragrain plasma to the outside of the pollen particle. Two allergenic substances, *Cryptomeria japonica* (Cry j) 1 and 2, have been extracted [3]. Cedar pollen Ag cannot penetrate the stratum corneum of the epidermis of normal skin because of the high molecular weight of Cry j1 and Cry j2 Ags (approximately 40 000). Thus, it is uncertain whether the skin surface is the initial site of sensitization in cedar pollen dermatitis. On the other

hand, disruption of the skin barrier probably accelerates the epicutaneous challenge step. Indeed, in patients with Japanese cedar pollen dermatitis, patch testing with cedar pollen Ag to mechanically injured skin, such as scratching or tape stripping, induces a positive reaction after 48 h [2, 4]. Moreover, in some patients with atopic dermatitis, which is characterized by impaired skin barrier function, Japanese cedar pollen can preferentially cause seasonal exacerbation of dermatitis in exposed areas [4].

Despite these clinical observations on cedar pollen dermatitis, the pathological mechanisms of the disease have yet to be elucidated. Japanese cedar pollen dermatitis generally affects individuals who have a history of pollinosis, but whether cedar pollen-specific IgE is required for the development of dermatitis is uncertain. There may be a difference in the underlying mechanisms between mucosal cedar pollinosis and cedar pollen dermatitis, as only a minor population of patients with rhinoconjunctivitis to cedar pollen responds to skin patch testing for cedar pollen Ags [2]. In this study, we attempted to establish a mouse model of cedar pollen dermatitis by epicutaneous sensitization and challenge. We also aimed to elucidate the immunological characteristics of cedar pollen dermatitis and to identify effective therapeutic targets.

Materials and methods

Mice

Six- to 8-week-old BALB/c, C57BL/6J, WBB6F1-W/W^v and WBB6F1-SI/SId mice were purchased from Sankyo Labo Service Co. (Tokyo, Japan). CRTH2 (–/–) and STAT6 (–/–) mice were backcrossed with BALB/c mice as described previously [5, 6]. Experiments were performed according to local ethical guidelines.

Induction of cedar pollen dermatitis in mice

Japanese cedar pollen Ag (Cry j1, 76.0 µg/vial + Cry j2, 18.85 µg/vial) was obtained from Torii Pharmaceutical Co. Ltd (Tokyo, Japan). Cedar pollen dermatitis was induced as described previously, with some modifications [7–9]. The dorsal skin was shaved and treated with Epilat[®] (Kanebo Ltd, Tokyo, Japan) to remove hair. After tape stripping with Cello Tape 10 ×, cedar pollen Ag was dissolved in 100 µL phosphate-buffered saline (PBS) and applied to the dorsal skin with adhesive tape for patch testing (Torii Pharmaceutical Co. Ltd). Patches were secured to the skin with Opsite Flexigrid[®] (Smith & Nephew Wound Management KK, Tokyo, Japan) and Benefact[®] (Nippon Sigmax Co. Ltd, Tokyo, Japan). Patches were left on for three 1-week periods with a 2-week interval between applications (Fig. 1a). Blood and skin samples were obtained on day 51.

Histological assessment

Skin samples were fixed in 10% formalin and embedded in paraffin. Serial 5 µm sections were stained with Giemsa

stain or Toluidine blue. At least 10 random fields were examined at × 400 magnification. The number of cells in the dermis was counted and expressed as cell number per millimetre basement membrane.

Measurement of serum immunoglobulin concentrations

Peripheral blood was obtained by cardiac puncture. Serum was collected by centrifugation and stored at –20 °C until use.

Concentrations of IgE and IgG2a were determined by ELISA kits obtained from Yamasa Co. (Chiba, Japan) and Bethyl Laboratories Inc. (Montgomery, TX, USA), respectively. For measurement of Japanese cedar pollen-specific IgE, 96-well flat-bottomed microtitre plates were coated with Japanese cedar pollen Ag in 0.1 M carbonate buffer (pH 9.6) (10 µg/mL, 50 µL/well) at 4 °C overnight. Plates were then washed with PBS containing 0.05% Tween 20 and, after addition of serially diluted serum samples (100 µL/well), were incubated at 4 °C overnight. After washing, plates were reacted with biotin-conjugated anti-mouse IgE (BD Biosciences, San Jose, CA, USA) (2 µg/mL, 100 µL/well) for 1 h at room temperature. These were then reacted with streptavidin-peroxidase (Zymed Laboratories Inc., South San Francisco, CA, USA) (100 µL/well) for 1 h followed by incubation with tetramethylbenzidine for 30 min. The reaction was stopped with 2 M H₂SO₄ and the absorbance was read at 450 nm.

Measurement of cytokines and chemokines in the skin

Excised skin specimens (8 mm in diameter) were cut into small pieces and homogenized with PBS containing 0.1% Tween 20 and centrifuged at 25 000 g for 10 min. Concentrations of cytokines and chemokines in the supernatants were determined by ELISA according to the manufacturer's instructions. ELISA kits for IL-4 and IFN-γ were obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). ELISA kits for IL-5, IL-13, thymus and activation-regulated chemokine (TARC)/chemokine (C-C motif) ligand (CCL) 17, macrophage-derived chemokine (MDC)/CCL22, regulated upon activation, normal T cell expressed and secreted (RANTES)/CCL5, eotaxin/CCL11 and IP-10/CXCL10 were obtained from R&D Systems Inc. (Minneapolis, MN, USA). ELISA kits for IL-18 were purchased from Medical and Biological Lab. Co. Ltd (Nagoya, Japan).

Lymphocyte proliferation assay

Single cell suspensions were prepared from regional lymph nodes (LN) and were cultured with Cry j1 or Cry j2 Ag (Hayashibara Biochemical Laboratories Inc., Okayama, Japan) for 72 h at 37 °C. Proliferation of immune LN cells in response to Cry j Ags was determined by CellTiter96 Aqueous One Solution Cell Proliferation Assay Kit (Promega Co., Madison, WI, USA).

Statistical analyses

Student's *t*-test was used to assess the statistical significance of differences between the mean values.

Results

Epicutaneous sensitization with Japanese cedar pollen induces eczematous reaction in mice

BALB/c mice were epicutaneously sensitized with Japanese cedar pollen Ag on the dorsal skin, as described in 'Materials and methods' (Fig. 1a). Histological examination of the skin prepared on day 51 showed acanthosis and

inflammatory cellular infiltrate in the epidermis and dermis (Fig. 1b). The cellular infiltrate largely consisted of mononuclear cells and numerous eosinophils. These features suggest a subacute eczematous reaction and resemble the histological changes in human cedar pollen dermatitis [2]. In addition, the number of dermal mast cells, including degranulated mast cells, was significantly increased in the dermis (Figs 1b and c).

Peripheral blood was collected in order to determine whether induction of dermatitis was accompanied by elevated levels of IgE. We found that 100 µg of cedar pollen Ag per site most effectively induced total serum IgE and cedar pollen-specific IgE production on day 51 (Fig. 2). On the other hand, total IgG2a levels did not change.

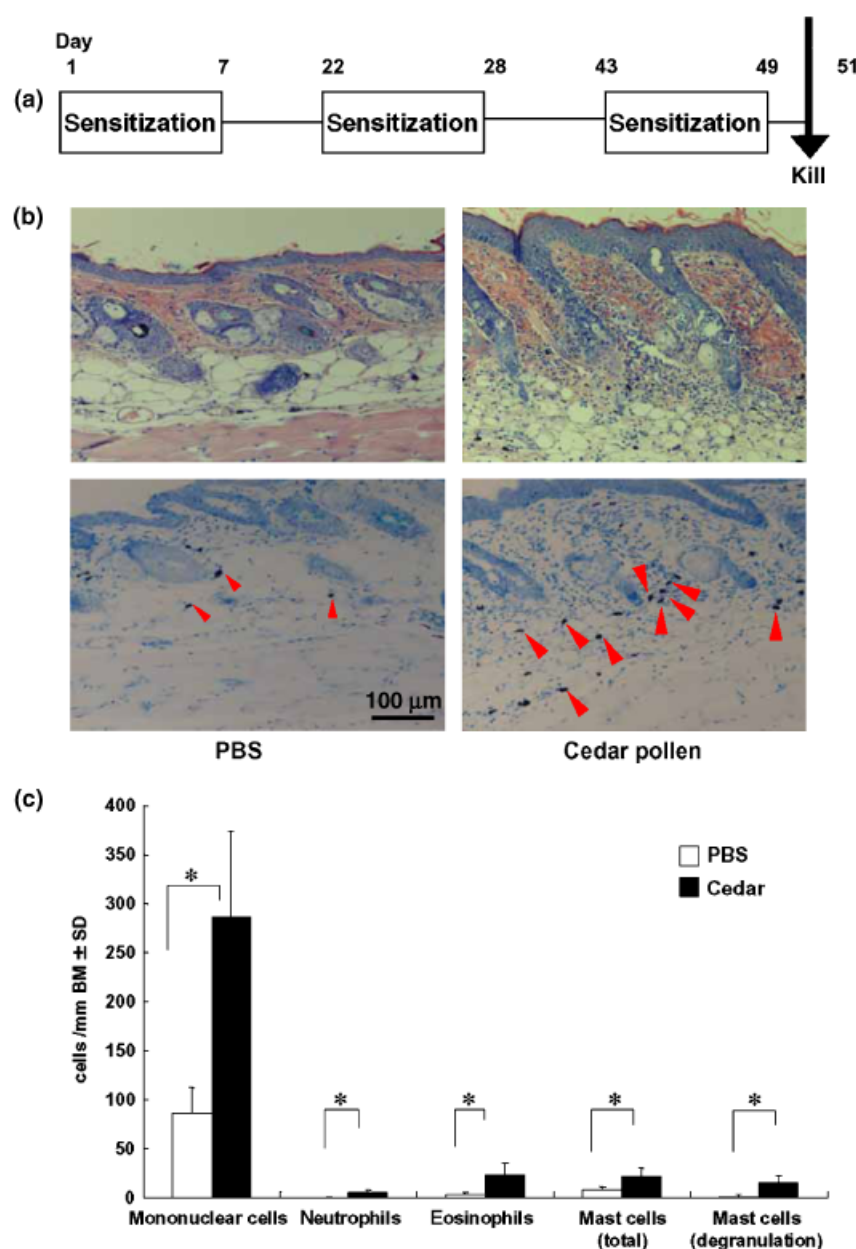


Fig. 1. Epicutaneous exposure to Japanese cedar pollen induces dermatitis. (a) Protocol for induction of dermatitis. (b) Histological features of lesional skin in BALB/c mice. The cellular infiltrate comprising mononuclear cells and eosinophils and epidermal thickening is observed. Occasionally, mononuclear cells infiltrated into the epidermis (upper panel: Giemsa staining). Dermal mast cells also significantly increased (lower panel: Toluidine blue staining). (c) Cell populations in infiltrates from inflammatory skin. **P* < 0.05. SD, standard deviation; PBS, phosphate-buffered saline.

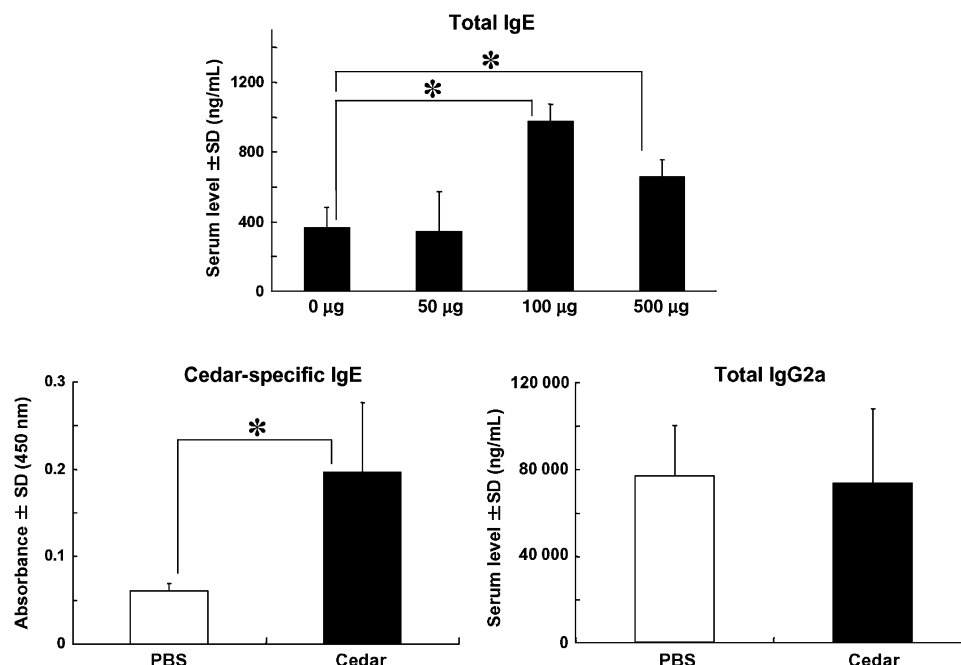


Fig. 2. Serum IgE production in cedar pollen dermatitis. Mice treated with Japanese cedar pollen were killed on day 51. The application of Japanese cedar pollen antigen induced a significant elevation of total serum IgE and cedar pollen-specific IgE. Total IgG2a production was not affected. One group consisted of at least five mice. Representative results of three separate experiments are shown. * $P < 0.05$. SD, standard deviation; PBS, phosphate-buffered saline.

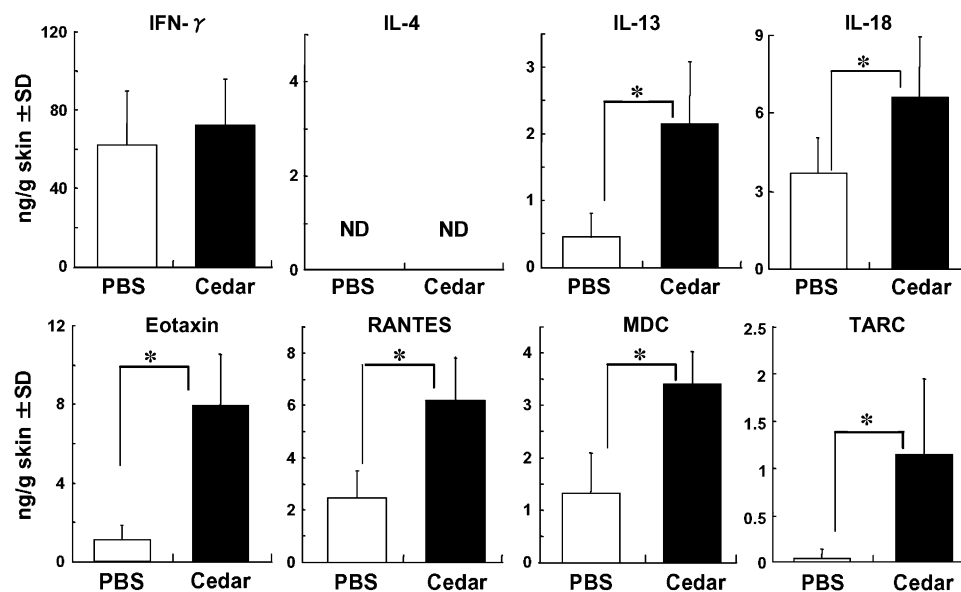


Fig. 3. Levels of cytokines and chemokines in lesional skin. The dorsal skin was removed on day 51 by punching and supernatants were prepared from skin homogenates. Cytokines and chemokines in the supernatants were measured by ELISA. Each group consisted of more than five mice. * $P < 0.05$. SD, standard deviation; PBS, phosphate-buffered saline; RANTES, regulated upon activation, normal T cell expressed and secreted; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine.

Local cytokine and chemokine profiles in the skin

Production of cytokines and chemokines in the lesional skin was assessed by ELISA. The levels of IL-13, IL-18, eotaxin/CCL11, MDC/CCL22, TRAC/CCL17 and RANTES/

CCL5 were elevated after the induction of cedar pollen dermatitis (Fig. 3). On the other hand, IFN- γ and IP-10/CXCL10 levels (data not shown) were not elevated. IL-4 and IL-5 were below detectable levels.

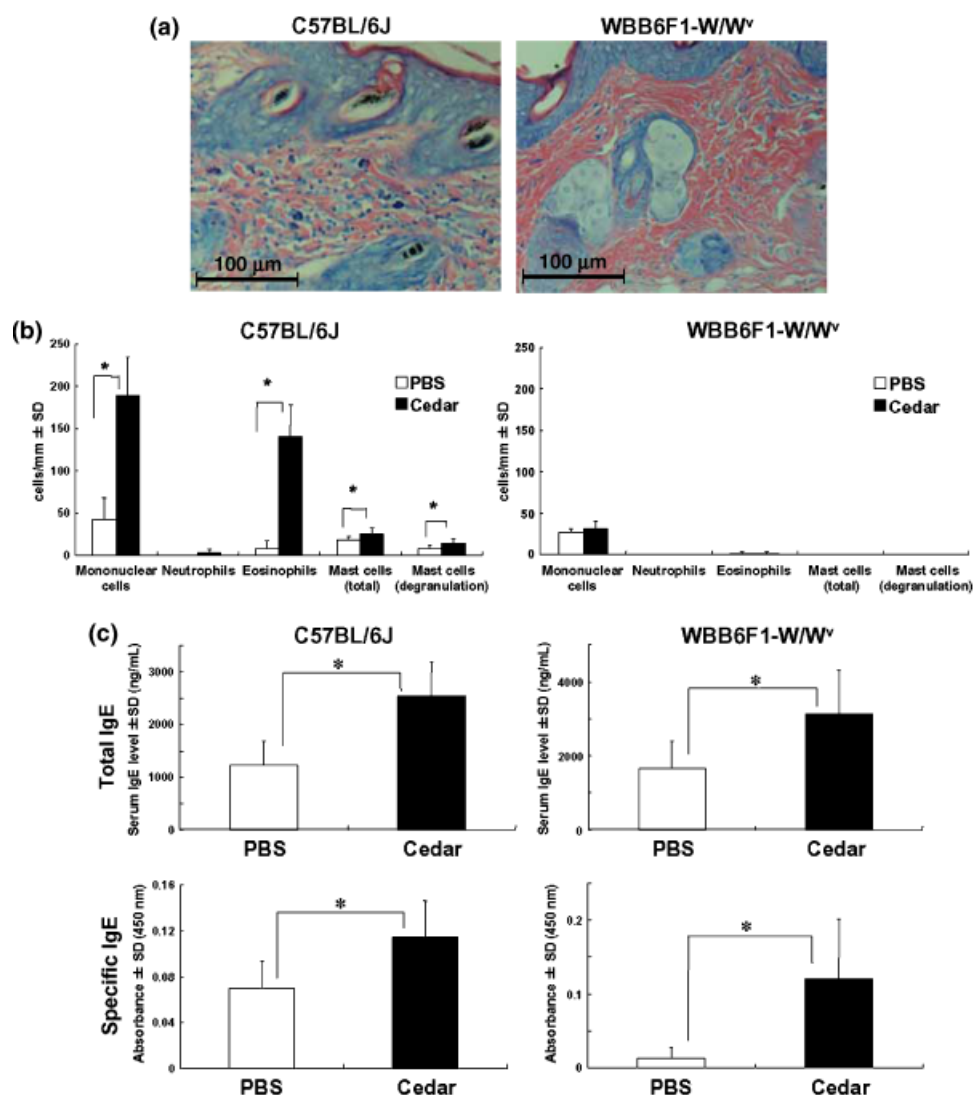


Fig. 4. Cedar pollen dermatitis is dependent on mast cells. (a and b) Mast cell-deficient WBB6F1-W/W^v mice failed to develop skin inflammation, while marked infiltration by mononuclear cells and eosinophils were induced in wild-type C57BL/6 mice. (c) Serum levels of total IgE and cedar pollen-specific IgE were elevated irrespective of mast cells. Each group consisted of at least 5 mice. * $P < 0.05$. SD, standard deviation; PBS, phosphate-buffered saline.

Cedar pollen dermatitis is dependent on cutaneous mast cells

The development of cedar pollen dermatitis was not limited to BALB/c mice, as demonstrated by the marked skin inflammation seen in C57BL/6J mice treated with cedar pollen Ag (Figs 4a and b). This was also accompanied by increased serum levels of total IgE and cedar pollen-specific IgE (Fig. 4c).

We next attempted to induce cedar pollen dermatitis in mast cell-deficient mice. Notably, cellular infiltration was much lower in mast cell-deficient WBB6F1-W/W^v mice than in wild-type C57BL/6J mice (Figs 4a and b), and was almost absent in WBB6F1-Sld/Sld mice (data not shown), despite the fact that elevated total serum IgE and cedar

pollen-specific IgE were observed in the absence of mast cells (Fig. 4c). Thus, mast cells appear to be essential for the induction of Japanese cedar pollen dermatitis.

Antigen-specific lymphocytes are induced by the epicutaneous sensitization of cedar pollen antigen

Abdominal LN cells were incubated with cedar pollen Ags (Cry j1 or Cry j2), and proliferation of LN cells was assessed. LN cells from BALB/c (Fig. 5) and C57BL/6J (data not shown) mice proliferated in response to both Cry j1 and Cry j2 *in vitro*. Similarly, LN cells from mast cell-deficient mice responded to Ag stimulation despite the fact that dermatitis could not be induced in this strain.

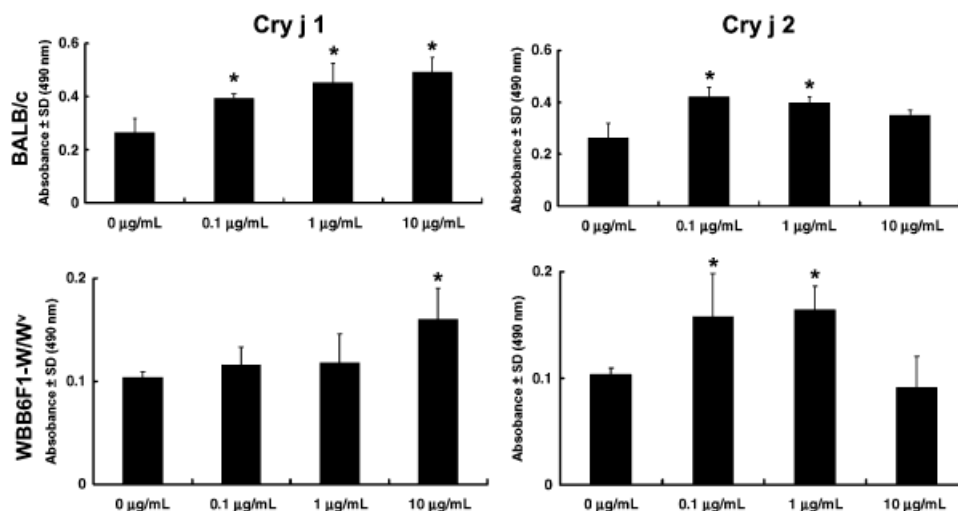


Fig. 5. Proliferation assay of regional lymph node (LN) cells. Mice that showed cedar pollen dermatitis were killed on day 29 and regional LN cells were collected. Cells were then incubated with *Cryptomeria japonica* (Cry j) 1 or Cry j2 at the indicated concentrations for 72 h *in vitro*. LN cells from BALB/c and WBB6F1-W/W^v mice exhibited cell proliferation in response to cedar pollen antigens. * $P < 0.05$. SD, standard deviation.

These data suggest that induction of Ag-specific lymphocytes is not sufficient for the development of cedar pollen dermatitis, further confirming the importance of mast cells.

STAT6 is dispensable for cedar pollen dermatitis

The cytokine profile characterized by increased IL-13, eotaxin/CCL11, MDC/CCL22 and TARC/CCL17, but not IFN- γ and IP-10/CXCL10, suggests that Japanese cedar pollen dermatitis is a T-helper (Th) type 2-dependent inflammation. Therefore, we postulated that a deficiency in STAT6, which is an essential signaling molecule for the Th2-type response [10, 11], would ameliorate dermatitis. However, STAT6 (–/–) mice showed comparable levels of inflammatory cellular infiltrate to wild-type mice (Fig. 6a). Notably, STAT6 (–/–) mice did not produce IgE (Fig. 6b), indicating that IgE is not required for the development of cedar pollen dermatitis.

Chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTH2) plays an important role in cedar pollen dermatitis

CRTH2 is a receptor for prostaglandin D2 (PGD2), which is secreted from activated mast cells [12]. We thus sought to determine the contribution of CRTH2 in cedar pollen dermatitis. As seen in Fig. 7a, CRTH2 (–/–) mice showed decreased inflammatory cell infiltration in the skin. To further confirm the involvement of CRTH2, the therapeutic effects of ramatroban, a CRTH2 antagonist [13], were tested. Ramatroban, a drug originally developed as a thromboxane A2 receptor antagonist, but later found to be a CRTH2 antagonist [13], has been widely used for the

treatment of human allergic rhinitis in Japan and has been demonstrated to be clinically effective [14, 15]. BALB/c mice were administered p.o. with ramatroban after sensitization, i.e. on days 22–28 and on days 43–49. As expected, oral administration of ramatroban significantly inhibited the cellular infiltration induced by cedar pollen (Fig. 7b). Serum levels of IgE were also reduced by administration of ramatroban (667 ± 166.1 vs. 215 ± 133.6 ng/mL, positive control and ramatroban-treated groups, respectively).

Discussion

Japanese cedar pollen dermatitis is a relatively uncommon, but increasingly prevalent, disease that is thought to occur in association with cedar pollinosis. In this study, we established a mouse model of cedar pollen dermatitis. The dermatitis induced by epicutaneous application of cedar pollen Ags was accompanied by the production of cedar pollen-specific IgE. This model may also share some similarities with human atopic dermatitis, in particular, extrinsic atopic dermatitis characterized by elevated serum IgE against aeroallergens, although skin symptoms of atopic dermatitis are not necessarily exacerbated by contact with allergens but by irritative substances due to skin barrier dysfunction. We have not been able to determine whether these mice also exhibited hyperresponsiveness of the upper respiratory tract. However, prior evidence has revealed that nasal or bronchial allergic responses are observed after epicutaneous sensitization of protein Ags, such as *Aspergillus fumigatus* Ag or ovalbumin (OVA), in association with increased production of Ag-specific IgE [9, 16].

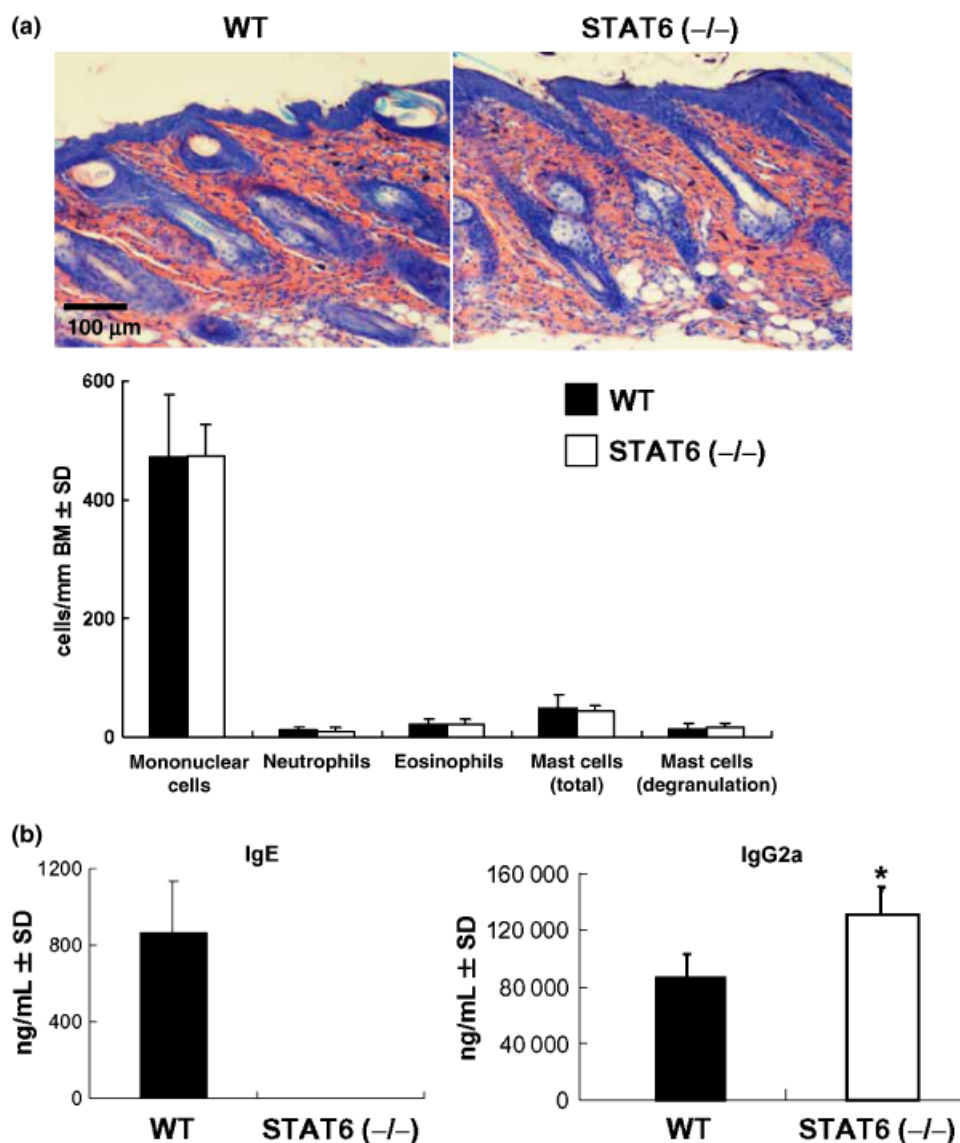


Fig. 6. STAT6 is not involved in cedar pollen dermatitis. (a) Dermal inflammatory infiltrates in STAT6 (-/-) mice were comparable to those in wild-type littermates (Giemsa staining) ($n = 5$). (b) STAT6 (-/-) mice did not produce IgE, while levels of IgG2a were higher in STAT6 (-/-) mice than in wild-type BALB/c mice. * $P < 0.05$. WT, wild-type mice; SD, standard deviation.

Epicutaneous exposure of protein Ags with or without disruption of the stratum corneum generally induces Th2-type cytokine production [7–9]. Consistent with this, we demonstrated local production of IL-13, eotaxin/CCL11 and MDC/CCL22 in lesional skin. IFN- γ production did not change. Surprisingly, deficiency of STAT6 did not influence the development of cedar pollen dermatitis. A previous report also demonstrated that *A. fumigatus* Ag induced skin inflammation independent of STAT6, which is notably essential for nasal allergic responses [16]. This evidence suggests that the nature of immunological reactions differs in skin and nasal mucosa.

PGD2 exerts its biological activities through DP and/or CRTH2 receptors. CRTH2 and DP are members of the G protein-coupled seven-transmembrane receptor family.

CRTH2 is coupled with Gi, whereas Gs is associated with DP [12]. In humans, CRTH2 is expressed in Th2 cells, eosinophils, basophils and monocytes [17]. On treatment with PGD2, these cells exhibit chemotaxis and/or Ca^{2+} mobilization [12, 18]. An increase in the percentage of CRTH2⁺ cells among CD4⁺ cells in blood is observed in patients with atopic dermatitis [19]. In human allergic rhinitis, CRTH2 is apparently involved in the inflammation of mucosal tissues [20]. In addition, in a number of animal models, antagonism of CRTH2 has been demonstrated to exert inhibitory effects on inflammatory reactions [21–24]. Consistent with these observations, an agonist of CRTH2 increases allergic skin inflammation induced by epicutaneous exposure of OVA [25], which is a mouse model of atopic dermatitis. In this study, we

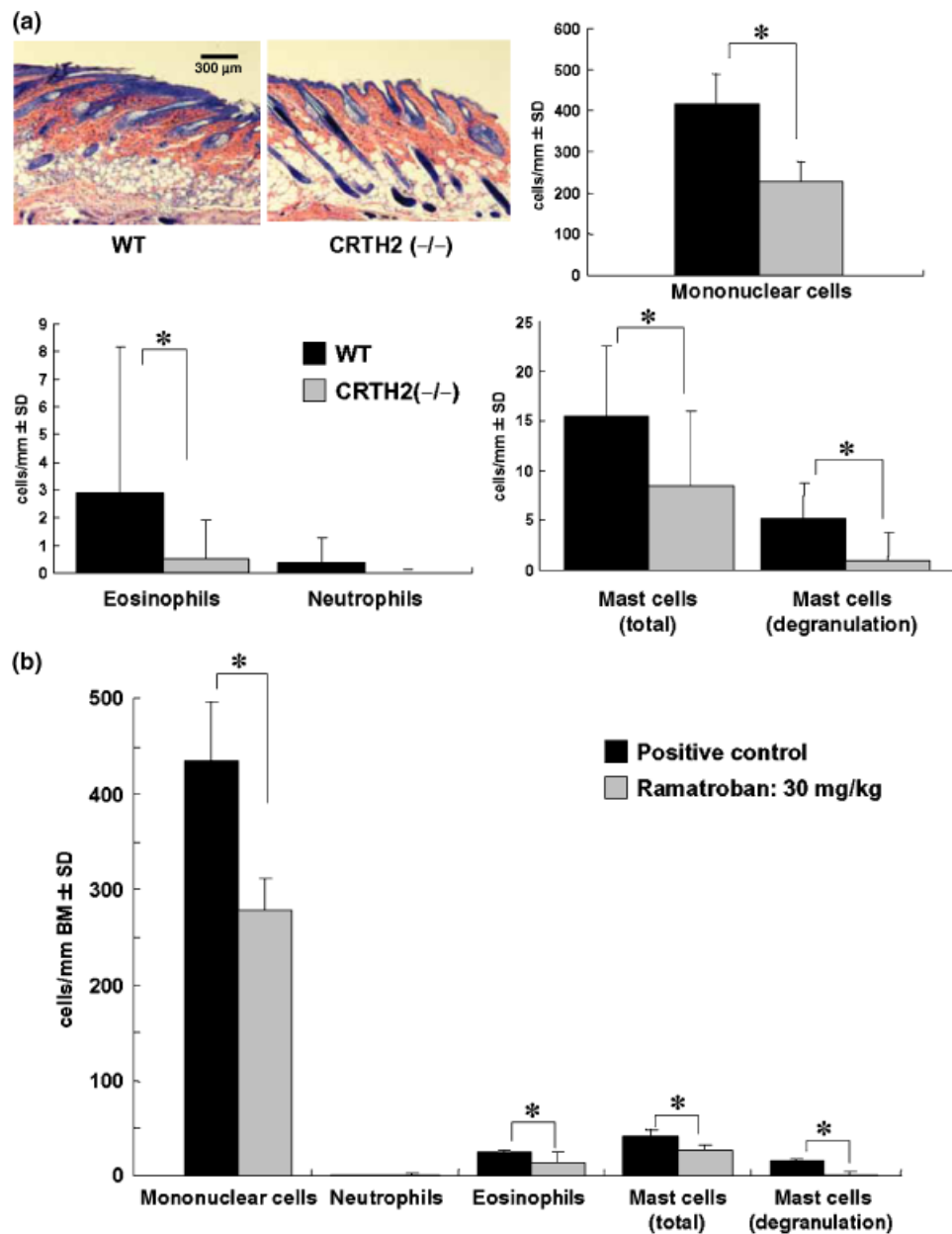


Fig. 7. CRTH2 is essential for cedar pollen dermatitis. (a) In CRTH2 (-/-) mice, cellular infiltration was less than in wild-type (WT) mice ($n = 6$). (b) Ramatroban was orally administered to WT BALB/c mice on days 22–28 and on days 43–49, and mice were killed on day 51. Reductions in cellular infiltrates were observed in mice treated with ramatroban ($n = 6$). * $P < 0.05$. WT, wild-type mice; SD, standard deviation.

confirmed the partial involvement of CRTH2 in cedar pollen dermatitis. CRTH2-deficient mice showed impaired skin inflammation (Fig. 7a) and ramatroban, a CRTH2 antagonist, clearly inhibited skin responses to cedar pollen (Fig. 7b). Considering that CRTH2 was originally isolated as a surface molecule preferentially expressed on human Th2 cells [17] and that STAT6 mediates Th2-type inflammation, it appears to be somewhat inconsistent that cedar pollen dermatitis is dependent on CRTH2 but not on STAT6. This may be because, unlike in humans, CRTH2 appears to be expressed not only on Th2 cells but also on

Th1 cells in mice. In a model of IgE-mediated chronic skin inflammation, defects in cytokine/chemokine production in CRTH2 (-/-) mice were not limited to Th2-type reactions [5].

Cedar pollen dermatitis appears to be almost entirely dependent on mast cells. It is possible that mast cells contributed to inflammation by PGD2 secretion via IgE-Ag interaction, leading to the infiltration of effector cells such as CRTH2⁺ cells. However, the involvement of IgE-mediated mast cell activation is unlikely, as STAT6-deficient mice showed comparable skin inflammation to

wild-type mice in the absence of IgE (Figs 6a and b). Cedar pollen Ag may be capable of directly activating mast cells, as observed in the mast cell activation by *Dermatophagoides farinae* [26]. Alternatively, mast cell activation might be induced by the products of complements or by CD200R3, a CD200R-like protein, which has been recently demonstrated to activate mast cells independent of IgE [27]. Further study is needed to clarify the mechanisms of IgE-independent PGD2 release from mast cells in cedar pollen dermatitis.

It is notable that IL-18 production is elevated during skin inflammation. IL-18 is a pro-inflammatory cytokine that has been shown to induce atopic dermatitis-like skin responses accompanied by Th2-type cytokine production, such as IL-13 and dermal mastocytosis, independently of STAT6 [28, 29]. This cytokine is generated and stored in keratinocytes in the form of pro-IL-18. For cleavage and activation of pro-IL-18, specific enzymes such as caspase-1 are required. However, keratinocytes have been shown to lack the enzymes to convert pro-IL-18 to its active form. Thus, it was intriguing to note that mast cell chymase is capable of cleaving pro-IL-18 [30]. In addition to the production of PGD2, mast cells may contribute to cedar pollen dermatitis via the activation of IL-18, which then mediates IL-3-induced dermal mastocytosis and Th2 cytokine production [28].

Considering that CRTH2 appears to contribute to allergic rhinitis [20], the present findings suggest that CRTH2 is a potential therapeutic target for both nasal cedar pollinosis and cedar pollen dermatitis in humans. Conversely, therapies targeted against STAT6 may be ineffective, although nasal allergies are dependent on STAT6 and IgE [16, 31].

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