期間中のアレルゲン投与に際する副作用予防目的での前処置として、毎回アレルゲン注射日の朝に第2世代ヒスタミンH1受容体拮抗薬（ロラタジンなど）を内服させた。受診時にはこの内服が確実に行われたか否かを毎回問診にて確認することとした。注射の実際は、スギ標準化アレルゲンの0.2ＪＡU/mlの0.1 mlを開始量とした。各受診日において、これを増量しながら1時間ごとに1日3回皮下注射をアレルゲンを迅速に吸収させる目的で揉み込みながら反復し、週1回から2回施行することとし、5週で2000JAU/mlの0.1mlにまで到達させることを目標として行った。副反応の発現に備え、各アレルゲン注射後の1時間は施行医師の近くに患者を座位安静状態にて待機させることとした。注射後の皮膚反応が著明となるか、または全身的副反応が生じた場合にはただちに処置するとともに、次回注射はその半分程度にもどして増量ペースを再設定することとした。設定維持量に到達しない場合でも、5週目の最終注射量を維持量として、それ以降は維持療法を2週に1回のペースで行い、その際には注射部位を揉みこまない通常の施行法にておこなった。

C. 研究結果

成人スギ花粉症患者11例中10例で、5週以内で目標維持量である2000JAU/mlの0.1mlに到達でき、円滑に維持療法に移行することができた。

予期される副作用として、アレルゲン注射部位局所の腫脹は全例でみられたが、局所への副腎皮質ステロイド軟膏の塗布等で制御可能であった。また3例でアナフィラキシーを生じたがアドレナリンの皮下注射にて速やかに改善した。アナフィラキシーを生じた症例には各々に要因として、1．喘息合併例であった、2．患者の受診日程違反で受診間隔があきすぎていた、3．注射後の安静が守られておらず激しく体動していた、などが存在した。逆にスギ花粉症のみの症例で、週1回以上の受診スケジュールが守られ、注射後の座位での安静状態が守られていた通常の施行症例においてはアナフィラキシーをふくめ、全身的な副作用はみとめられなかった。

D. 考察

成人スギ花粉症患者11例中10例で、5週以内で目標維持量に到達でき、速やかに維持療法に移行できた。 局所の腫脹は全例でみられた。プロトコール違反として喘息合併、患者の受診日程違反、注射後の体動のあったアナフィラキシーなどの全身的副作用を生じたがアドレナリンの皮下注射にて速やかに改善した。以上の研究結果は、現在使用可能なスギ標準化アレルゲンを用いて、クラスター方式免疫療法は導入治療期間の短縮と治療の完遂性の視点から選択可能なオプションであって、向後の舌下あるいはペプチド免疫療法を含む免疫療法の新規アプローチの臨床応用の際にも考慮してよい施行方法であるものと考えられた。また現状の日常臨床においても、治療期間の短縮を要望するケースなどにおいては、喘息症例を除外し、週1回以上の導入通院を遵守させ、かつ注射後の待機安静維持を確保したうえであれば、充分に選択可能な方法であるものと考えられた。

E. 結論

スギ花粉症において、スギ標準化アレルゲンを用いたクラスター方式免疫療法は、プロトコールを正確に遵守しさえするならば、短期間で高濃度の維持量に達せられる有益性のある治療手段である。安全性の点で現在の治療用アレルゲンでは厳格なプロトコール遵守の管理が必要であるという課題を残したが、今後、舌下あるいはペプチド免疫療法における治療スケジュールの検討上、ひとつのオプションとして応用可能であると考える。

F. 健康危険情報

該当事項なし

G. 研究発表

1. 論文発表

該当事項なし

2. 学会発表

該当事項なし

H. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

該当事項なし

2. 実用新案登録

該当事項なし

3. その他

該当事項なし

リアルタイムモニター飛散数の情報のあり方の研究と舌下ペプチド・アジュバント療法の臨床研究
スギ特異的免疫療法の スギおよびヒノキ 特異的 IL-5 産生への効果とヒノキ感作の意義

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研究要旨
舌下免疫療法を施行する上で、投与抗原を吟味することは必須である。これまでの QOL 研究で、標準化スギ花粉エキスを用いた特異的免疫療法はスギ花粉飛散期の症状および QOL を有意に改善する一方、ヒノキ花粉飛散期にはその効果が減弱することが明らかになり、ヒノキ抗原の特異性が示唆されている。今回は、免疫療法の有無によるスギおよびヒノキ粗抗原に対する IL-5 産生量を比較し、さらにヒノキ感作のヒノキ特異的 IL-5 産生とヒノキ花粉飛散期における鼻眼症状に与える影響について検討した。免疫療法群の Cry j 1-および Cha o 1-誘導 IL-5 産生量は非免疫療法群に比較して有意に低かった。一方、スギ粗抗原特異的 IL-5 産生量に関しては、いずれの濃度においても免疫療法群は非免疫療法群と比較して有意な低値を示した。一方ヒノキ粗抗原特異的 IL-5 産生量に関しては、10 μg/ml では免疫療法群で有意な IL-5 産生抑制を示したが、50 μg/ml および 100 μg/ml では二群間で有意な差を認めなかった。ヒノキに対する感作陰性例は、感作陽性例と同等の Cha o 1 およびヒノキ粗抗原に特異的な IL-5 産生を示した。ヒノキ感作陰性例はヒノキ感作陽性例と比較してヒノキ飛散期の眼症状が有意に軽減していたが、鼻症状に関しては有意な差を認めなかった。スギ特異的免疫療法のヒノキ花粉飛散期における作用の減弱化の因子のひとつとして、ヒノキ花粉にのみ存在する抗原に対する免疫寛容の不誘導が示唆された。さらにヒノキ花粉飛散期にはヒノキ IgE 非依存的なアレルギー性炎症が誘導されている可能性が示唆された。

A. 研究目的

これまでの QOL 研究で、標準化スギ花粉エキスを用いた特異的免疫療法はスギ花粉飛散期の症状および QOL を有意に改善する一方、ヒノキ花粉飛散期にはその効果が減弱することを明らかにした。また昨年度の研究で、標準化スギ花粉エキスを用いた特異的免疫療法を施行した患者由来の末梢血単核細胞（PBMC）はスギ主要アレルゲン Cry j 1 特異的 IL-5 産生のみならずヒノキ主要アレルゲン Cha o 1 特異的 IL-5 産生に関しても有意な抑制を示し、さらに非免疫療法群のみならず免疫療法群においても Cry j 1 特異的 IL-5 量と Cha o 1 特異的 IL-5 産生量は有意な正の相関を示した。これらの結果は、免疫療法群でのヒノキ飛散期における症状および QOL の増悪のファクターとしては、累積飛散によるアレルギー性炎症の惹起や、Cha o 1 以外のヒノキアレルゲンによる影響などが考えられた。そこで今回は、免疫療法の有無によるスギおよびヒノキ粗抗原に対する IL-5 産生量を比較し、さらにヒノキ感作のヒノキ特異的 IL-5 産生とヒノキ花粉飛散期における鼻眼症状に与える影響について検討した。

B. 研究方法

2008 年 5 月前後に皮下注射法による免疫療法施行（全例 2000 JAU/ml で維持: SIT 群, n=19）および非施行（非 SIT 群: n=46）のスギ花粉症患者より採血を行い、PBMC を採取した。10 μg/ml の Cry j 1 あるいは Cha o 1 、あるいは 10 μg/ml、50 μg/ml、100 μg/ml のスギおよびヒノキ粗抗原にて刺激し、培養 72 時間後に上清を回収し、IL-5 を測定した。スギ花粉症患者のヒノキ特異的 IgE 抗体価を CAP にて測定し、クラス 2 以上を陽性とした。スギ花粉症患者の鼻眼症状を鼻アレルギー日記に記載し、スギおよびヒノキ飛散期における症状スコアの推移を検討した。

（倫理面への配慮）ボランティアからの検体（末梢血）採取に関しては、学術的な意義について十分な説明を行い、同意・協力が得られた上で採取保存する。

C. 研究結果

昨年度の結果と同様に、SIT 群の Cry j 1-および Cha o 1-誘導 IL-5 産生量は非 SIT 群に比較して有意に低かった。スギ粗抗原特異的 IL-5 産生量に関しては、いずれの濃度においても

SIT 群は非 SIT 群と比較して有意な低値を示した。一方ヒノキ粗抗原特異的 IL-5 産生量に関しては、10 μg/ml では SIT 群で有意な IL-5 産生抑制を示したが、50 μg/ml および 100 μg/ml では二群間で有意な差を認めなかった。ヒノキに対する感作陰性例は、感作陽性例と同等の Cha o 1 およびヒノキ粗抗原に特異的な IL-5 産生を示した。ヒノキ感作陰性例はヒノキ感作陽性例と比較してヒノキ飛散期の眼症状が有意に軽減していたが、鼻症状に関しては有意な差を認めなかった。

D. 考察

免疫療法群と非免疫療法群との間で高濃度のヒノキ粗抗原に対する IL-5 産生に有意な差を認めなかったことは、ヒノキ花粉の中にはスギ花粉と交差反応性のない抗原が存在する可能性が考えられる。またヒノキ飛散期の鼻症状においてヒノキ感作の有無で有意な差を生じなかった要因のひとつとして、ヒノキ感作陰性例においてもヒノキ抗原に対する有意な IL-5 産生がみられたことが挙げられる。ヒノキ感作陰性例であってもヒノキ花粉飛散期には IL-5 などによるアレルギー性炎症が惹起され発症することが考えられ、十分なケアを要すると思われた。

E. 結論

スギ特異的免疫療法のヒノキ花粉飛散期における作用の減弱化の因子のひとつとして、ヒノキ花粉にのみ存在する抗原に対する免疫寛容の不誘導が示唆された。さらにヒノキ花粉飛散期にはヒノキ IgE 非依存的なアレルギー性炎症が誘導されている可能性が示唆された。

G. 研究発表

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H 知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

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ORIGINAL PAPER Clinical Mechanisms in Allergic Disease

Allergen-specific immunotherapy alters the expression of B and T lymphocyte attenuator, a co-inhibitory molecule, in allergic rhinitis

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Clinical and Experimental Allergy

Summary

Background B7/CD28 family co-signalling molecules play a key role in regulating T cell activation and tolerance. Allergen-specific immunotherapy (SIT) alters allergen-specific T cell responses. However, the effect of SIT on the expression of various co-signalling molecules has not been clarified.

Objective We sought to determine whether SIT might affect the expression of three co-inhibitory molecules, programmed death (PD)-1, B7-H1 and B and T lymphocyte attenuator (BTLA), in Japanese cedar pollinosis (JCP).

Methods Peripheral blood mononuclear cells (PBMCs) were isolated from JCP patients who had or had not received SIT. PBMC were cultured in the presence or absence of Cry j 1, after which the cell surface expression of PD-1, B7-H1 and BTLA, as well as IL-5 production, were determined. In addition, the effect of BTLA cross-linking on IL-5 production was examined.

Results After Cry j 1 stimulation, no significant differences in PD-1 and B7-H1 expression were observed between SIT-treated and SIT-untreated patients. BTLA expression was down-regulated in untreated patients after Cry j 1 stimulation and up-regulated in SIT-treated patients. Up-regulation of BTLA in SIT-treated patients was particularly apparent in a $CD4^+$ T cell subset. IL-5 production was clearly reduced among SIT-treated patients, and the observed changes in BTLA expression correlated negatively with IL-5 production. Moreover, immobilization of BTLA suppressed IL-5 production in JCP patients.

Conclusion These results suggest that both IL-5 production and down-regulation of BTLA in response to allergen are inhibited in SIT-treated patients with JCP. BTLA-mediated co-inhibition of IL-5 production may contribute to the regulation of allergen-specific T cell responses in patients receiving immunotherapy.

Keywords allergen immunotherapy, allergic rhinitis, BTLA, Cry j 1, IL-5
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Introduction

Allergen-specific immunotherapy (SIT) is an effective treatment for IgE-mediated, type 2 T helper (Th2)-biased allergic diseases, particularly allergic rhinitis [1]. Unlike pharmacotherapy, SIT is unique in that it can alter the natural course of allergic disease by preventing new sensitization/onset and providing long-term remission after discontinuation of treatment [2–4].

A number of studies have shown that SIT alters allergen-specific T cell responses resulting in immune tolerance [1, 5]. For example, SIT suppresses allergen-specific Th2 immunity, including IL-4 and IL-5 production,

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systemically and at local sites [6–8]. SIT alters the immune response to favour Th1 immunity, such as IFN-γ production [9]. In addition, SIT induces immune suppression by activating regulatory T cells and cytokines, such as IL-10 and TGF-β [5, 10].

The activation, proliferation and cytokine production of antigen-specific T cells are regulated by two distinct signals from antigen-presenting cells (APC) [11, 12]. The first signal is provided by interaction of the antigen/major histocompatibility complex with a T cell receptor (TCR). The second signal is delivered by co-signalling molecules. Among these, molecules from the B7/CD28 family play a key role in the regulation of T cell activation and tolerance [12]. B7-1 (CD80) and B7-2 (CD86), as well as their ligands (CD28 and CTLA-4) were the first-discovered B7/CD28 family molecules and therefore have been the most extensively characterized in allergic rhinitis [13–15]. For example, we have previously shown that the expression of both CD80 and CD86 is increased within the nasal mucosa of patients with perennial allergic rhinitis, compared with control subjects following nasal provocation with house dust [14].

In addition to the original B7/CD28 family molecules, new B7 family members, such as inducible co-stimulator (ICOS) L, programmed death (PD)-L1, PD-L2, B7-H3 and B7-H4, have been identified [12]. New CD28 family members, including ICOS, PD-1 and B and T lymphocyte attenuator (BTLA), have also been identified [12]. Among the new CD28 family members, ICOS delivers a range of co-stimulatory signals that augment T cell differentiation and cytokine production and provide critical signals for Ig production [12, 16]. Conversely, both PD-1 and BTLA possess an immunoreceptor tyrosine-based inhibition motif (ITIM) within their cytoplasmic domain, and display co-inhibitory signals that suppress T cell activation [17, 18]. One report has demonstrated enhanced CD86 expression in $CD14^+$ cells after recall stimulation with PLA_2 in patients exposed to bee-venom SIT, as well as suppressed IL-10 production by peripheral blood mononuclear cell (PMBC) following blockade with CD86 in patients exposed to SIT [19]. However, little is known regarding the potential role of co-inhibitory molecules in these responses to SIT.

In the present study, we investigated the expression and characteristics of the co-inhibitory molecules, PD-1 and BTLA, along with B7-H1 (a ligand of PD-1), in allergen-stimulated PBMC from patients with Japanese cedar pollinosis (JCP). We believe that the findings presented here are the first to demonstrate altered BLTA expression in response to Cry j 1, the major allergen of *Cryptomeria japonica* pollen, potentially explaining the beneficial effect of SIT in JCP and providing a basis for future therapeutic approaches aimed at the regulation of BTLA expression to limit allergic diseases.

Materials and methods

Antigens and reagents

Cry j 1 was purified and concentrated from the crude extracts of *C. japonica* pollen, as previously described [20]. The flourescein isothiocyanate (FITC)-labelled anti-human PD-1 (clone MIH4, mouse IgG1), B7-H1 (clone MIH1, mouse IgG1) and BTLA (clone MIH27, mouse IgG2b) were generated as previously described [21, 22]. The purification of anti-human BTLA (clone MIH26, mouse IgG2b) is also described elsewhere [22]. Cy5-labelled anti-human CD4, PE-labelled anti-human CD8 and CD19, as well as their respective control mouse IgG isotypes, were purchased from BD Biosciences (San Jose, CA, USA). Biotinylated anti-human CD203c mAb (FR3-16A11), anti-biotin microbeads and MS columns were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Patients

Twenty-one patients with JCP (three males and 18 females: aged 21–57, mean age 42.9 years) were enrolled in the study. Written informed consent was obtained from each subject. Sensitization to Japanese cedar pollen was confirmed by the presence of specific-IgE antibodies, as determined by CAP (Pharmacia, Uppsala, Sweden), ranging in concentration from 1.8 to > 100 UA/mL (mean 21.3±27.2). Eleven patients received SIT using a standardized extract of *C. japonica* pollen (Torii Co., Tokyo, Japan) over a period of at least 2 years. A maintenance concentration of 2000 JAU/mL was archived in all the patients treated with SIT. The mean maintenance dose of the extract was 509.0 JAU. None of the patients had used immunosuppressive drugs, including oral steroids, during the pollen season. No significant differences in age or sex existed among the SIT-treated and untreated patients. Clinical characteristics of groups of patients are shown in Table 1. Comparison of naso-ocular symptoms and rhinitis-related quality of life (QOL) during the pollen-dispersed season between SIT-treated patients and SIT-untreated patients using JRQLQ No.1, the Japanese QOL questionnaire for allergic rhinitis [23]. The study was approved by the Human Research Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Detection of PD-1, B7-H1 and B and T lymphocyte attenuator expression

Heparinized blood was sampled during the season when pollen is dispersed. The PBMCs were then isolated and cultured as previously described [20]. In brief, PBMCs (2×10^6/mL) were incubated in the presence or absence of 10 μg/mL of Cry j 1 at 37 °C in a 5% CO_2/air mixture for

Table 1. Subject characterization

	SIT patients		Non-SIT patients	
No. of patients	11		10	
Sex (male/female)	0/11		3/7	
Age (years)	47 (36–57)		38 (21–53)	
	At enrollment	2008	At enrollment	2008
CAP titre to JCP (UA/mL)	19.6 (1.8–81.1)	16.6 (0–61.3)	23.1 (1.8–100)	30.5 (6.5–100)
Total IgE (IU/mL)	105 (4–232)	161 (0–458)	145 (27–303)	135 (44–304)
Blood eosinophil (μL^{-1})	306 (43–655)	298 (79–532)	205 (48–725)	218 (69–592)

SIT, specific immunotherapy-treated patients; non-SIT, SIT-untreated patients; JCP, Japanese cedar pollinosis.

72 h. Less than 5% of cultured cells had died as judged by trypan blue exclusion test, indicating high cell viability of cultured cells. After incubation, Cry j 1-stimulated and Cry j 1-unstimulated PBMCs were harvested, blocked and stained with FITC-labelled anti-PD-1, B7-H1 or BTLA, as well as PE-labelled anti-CD8, Cy5-labelled anti-CD4, and CD19, in addition to isotype-matched control Abs [13]. The cells were washed and analysed with FACScan equipment using CellQuest software (BD Biosciences). Lymphocytes were gated according to forward scatter and side scatter and at least 10 000 events were acquired and analysed. Cry j 1-induced expression was determined and the percentage of positive cells in cultured PBMC following Cry j 1 stimulation was subtracted from the percentage observed in unstimulated PBMC. To avoid experimental bias, the laboratory investigators were blinded to the sample origin.

Measurement of cytokines

Supernatant was collected after 12 and 72 h of culture and stored at −80 °C until it was used for assay. Levels of IL-5, IL-10 and TGF-β were measured within each sample of culture supernatant by means of Opt EIA sets (BD Biosciences), in accordance with the manufacturer's instructions. Levels of IL-10 were further measured in supernatant after 12 h of culture. The detection limits of these assays were 5 pg/mL for IL-5, 5 pg/mL for IL-10 and 20 pg/mL for TGF-β. Cry j 1-induced production was determined by subtracting the cytokine levels measured following Cry j 1 stimulation from those measured in the absence of stimulation. In order to determine whether the productions were due to basophil responsiveness, containing basophils were removed from PBMC by immunomagnetic negative selection using biotinylated anti-human CD203c mAb (FR3-16A11), anti-biotin microbeads and MS columns. Complete depletion of basophils was confirmed by Kimura's staining [24].

Proliferative responses

After 72 h of incubation with or without Cry j 1, proliferative responses were measured by means of BrdU incorporation (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. Proliferation was estimated by the stimulation index calculated as follows: the ratio between mean OD at 450 nm obtained in the culture with Cry j 1 and that obtained in the antigen-free culture.

Cross-linking of B and T lymphocyte attenuator

PBMCs (2×10^6/mL) from JCP patients without SIT were stimulated with 10 μg/mL of Cry j 1 in the presence of immobilized anti-BTLA mAb (MIH26) or control mouse IgG2b. Immobilization was performed by incubation of 20 μg/mL of each Ab diluted in PBS, followed by washing using complete culture medium as previously described [22]. After 72 h of incubation, IL-5 production and proliferation were determined as described above.

Statistical analysis

The non-parametric Wilcoxon's signed-rank test and Mann–Whitney's *U*-test were used. Correlation analysis was performed using Spearman's correlation coefficient by rank. A level of $P<0.05$ was considered statistically significant. Values were given as means±standard deviation. Statistical analysis was performed using StatView™ software (version 4.5; Abacus Inc., Berkeley, CA, USA).

Results

Clinical efficacy of specific immunotherapy in Japanese cedar pollinosis

Analysis using JRQLQ revealed that cedar immunotherapy was clinically effective against not only naso-ocular symptoms but also rhinitis-related QOL in SIT-treated patients in two consecutive seasons (Fig. 1). Levels of serum total IgE ($P=0.360$), JCP-specific IgE titre ($P=0.725$) and blood eosinophil counts ($P=0.205$) were similar between SIT-treated and SIT-untreated patients at the enrollment of the study. And these levels were still

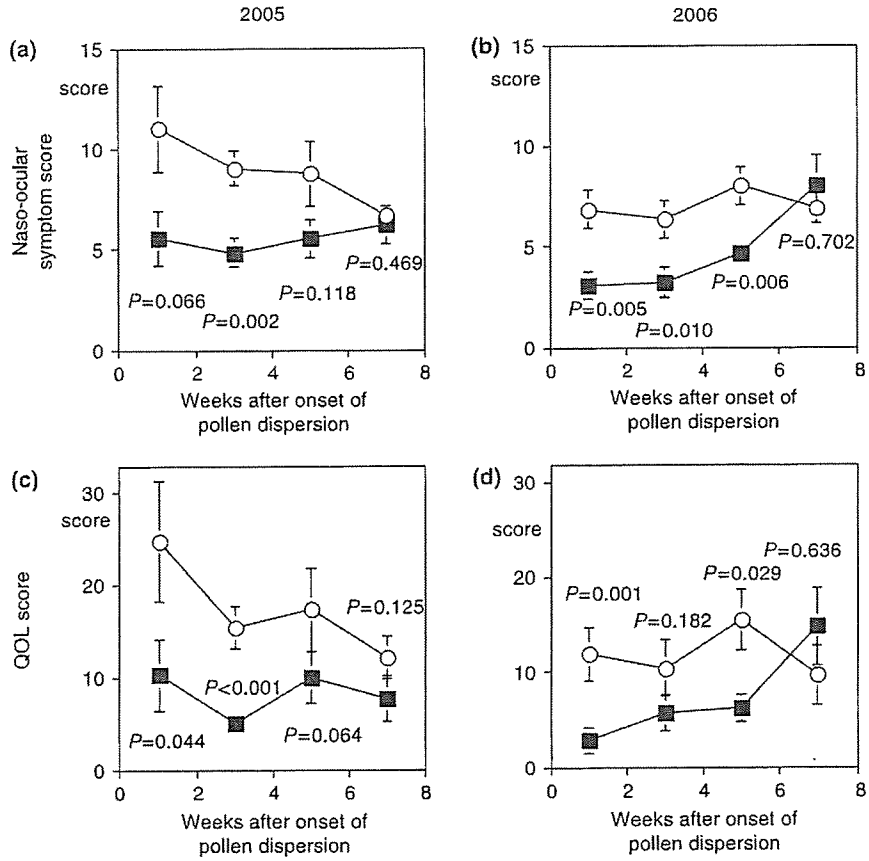

Fig. 1. Effect of cedar immunotherapy on symptom and quality of life (QOL) in Japanese cedar pollinosis (JCP). Naso-ocular symptoms (a, b) and rhinitis-related QOL (c, d) were compared between specific immunotherapy (SIT)-treated patients (closed square) and SIT-untreated patients (open circle) during the pollen-dispersed season in 2005 (a, c) and 2006 (b, d). The *x*-axis denotes weeks after the onset of cedar and cypress pollen dispersion. The *y*-axis denotes scores. The *P*-values were determined using the Mann–Whitney's *U*-test.

similar between the groups in 2008 (serum total IgE: $P = 0.833$, JCP-specific IgE titre: $P = 0.205$, blood eosinophil counts: $P = 0.181$). However, the levels of JCP-specific IgE titre in 2008 were significantly elevated as compared with those at the enrollment in SIT-untreated group ($P = 0.011$) whereas the levels between before- and after-treatment were not significantly different in SIT-treated group ($P = 0.790$) (Table 1).

Changes in co-inhibitory molecule expression in response to Cry j 1 in peripheral blood mononuclear cells from patients with Japanese cedar pollinosis

The percentage of cells expressing PD-1, B7-H1 and BTLA in PBMC after exposure or no exposure to *in vitro* Cry j 1 stimulation is summarized in Table 2. The baseline expression of these molecules in the absence of Cry j 1 stimulation was similar among SIT-treated and SIT-untreated patients. In addition, the percentage of positive cells observed after stimulation with Cry j 1 did not differ significantly in each molecule. However, when we focused on changes in expression in response to Cry j 1 in each patient, BTLA expression was reduced in SIT-untreated patients, but not in SIT-treated patients (Fig. 2).

Table 2. Percentage of positive cells expressing co-inhibitory molecules with or without Ag stimulation

Molecule	Stimulation	SIT ($n = 11$)	Non-SIT ($n = 10$)	*P*-value
PD-1	Ag (−)	1.06±0.86	0.86±0.72	0.860
	Ag (+)	1.66±1.47	1.51±1.49	0.888
	Change	0.60±0.74	0.65±1.03	0.805
B7-H1	Ag (−)	1.08±1.07	0.97±0.95	0.778
	Ag (+)	1.55±1.45	1.06±1.04	0.481
	Change	0.47±0.48	0.09±0.90	0.067
BTLA	Ag (−)	2.73±2.50	4.91±4.22	0.460
	Ag (+)	3.70±2.87	2.67±2.33	0.503
	Change	0.97±1.17	−2.24±2.21	<0.001

SIT, specific immunotherapy-treated patients; non-SIT, SIT-untreated patients; PD, programmed death; BTLA, B and T lymphocyte attenuator.

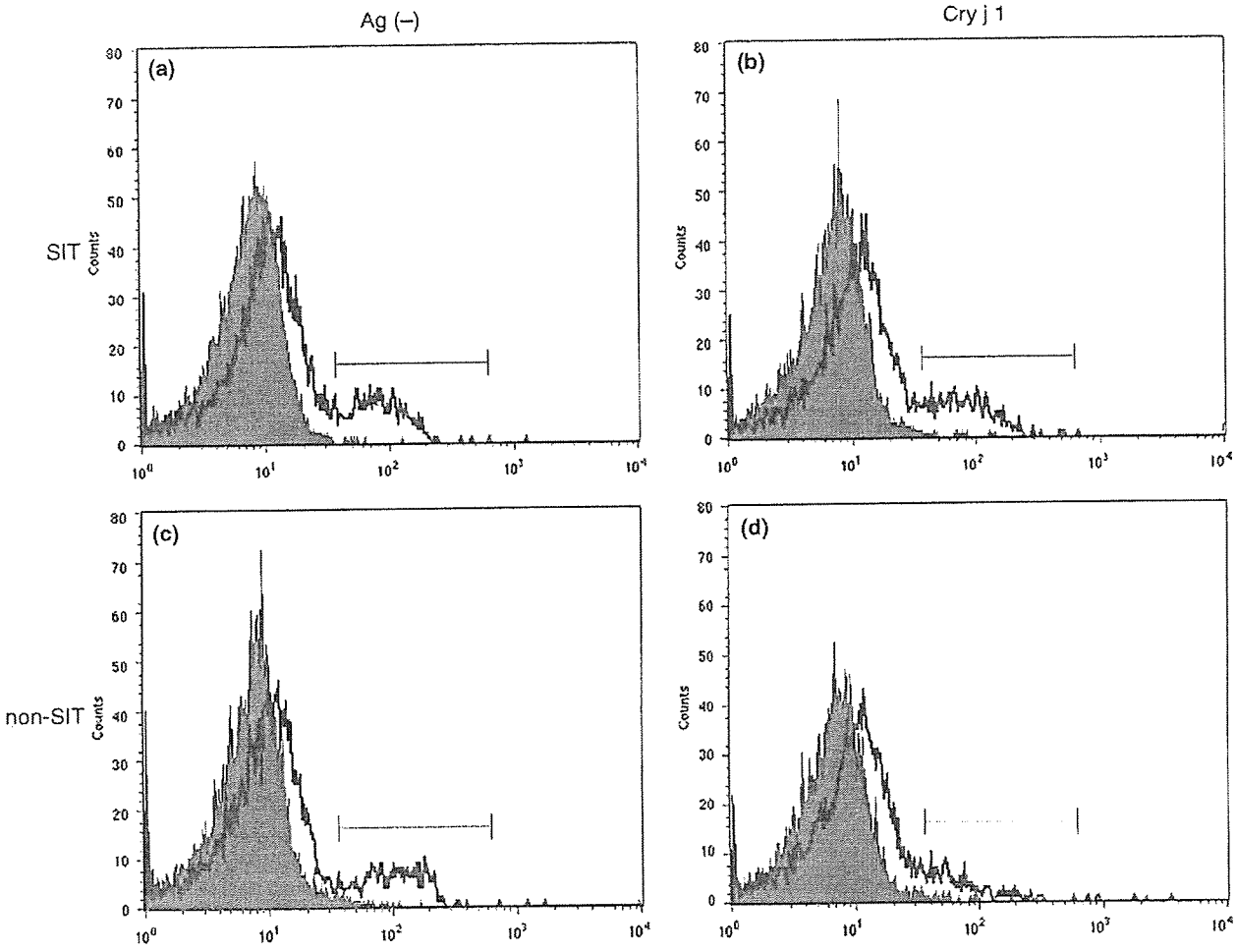

Fig. 2. Typical histogram of B and T lymphocyte attenuator (BTLA) expression. Peripheral blood mononuclear cells (PBMCs) from SIT-treated (a, b) and SIT-untreated (c, d) patients were incubated in the presence or absence of Cry j 1 for 72 h. After incubation, Cry j 1-stimulated (b, d) and unstimulated (a, c) PBMC were harvested, blocked and stained with FITC-labelled anti-BTLA mAb (MIH27, open histogram) or control mouse IgG2b (shaded histogram). Lymphocytes were gated according to forward scatter and side scatter and at least 10 000 events were acquired and analysed. The *x*-axis (log scale) shows fluorescence intensity and *y*-axis shows cell counts. SIT, specific immuno therapy-treated patients; non-SIT, SIT-untreated patients, FITC, fluorescein isothiocyanate.

Significantly different responses in terms of BTLA expression following Cry j 1 stimulation were observed among STI-treated and untreated patients ($P<0.001$). A tendency toward enhanced B7-H1 expression was seen in SIT-treated patients, compared with untreated patients ($P=0.067$). On the other hand, changes in PD-1 expression did not differ among patients from both groups ($P=0.805$) (Table 2).

Phenotype analysis of cells expressing co-inhibitory molecules

BTLA is known to be expressed on both B and T cells. In SIT-treated patients, the percentage of $CD4^+$ cells expressing BTLA was significantly increased after recall stimulation with Cry j 1 ($P=0.003$, Fig. 3a). Conversely, the percentage was significantly reduced in SIT-untreated patients ($P=0.017$, Fig. 3d). On the other hand, a significant change in BTLA expression on $CD8^+$ cells was not observed in either SIT-treated ($P=0.154$) or SIT-untreated ($P>0.999$) patients (Figs 3b and e). A reduced expression on $CD19^+$ cells was observed in both SIT-treated ($P=0.091$) and SIT-untreated ($P=0.037$) patients following stimulation (Figs 3c and f).

PD-1 and B7-H1 are also known to be expressed on both B and T cells. The percentage of $CD4^+$ ($P=0.037$) but not $CD8^+$ ($P=0.161$) or $CD19^+$ ($P=0.110$) cells expressing PD-1 was significantly increased in SIT-treated patients after recall stimulation with Cry j 1 (Figs 4a–c). On the other hand, a tendency toward enhanced PD-1 expression on $CD4^+$ ($P=0.086$) and $CD19^+$ ($P=0.093$) but not $CD8^+$ ($P>0.999$) cells were seen in SIT-untreated

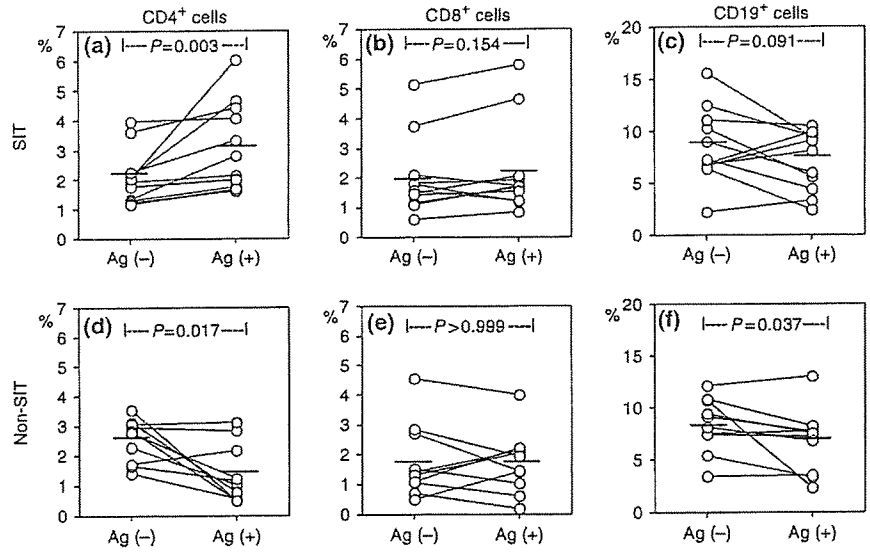

Fig. 3. Phenotype analysis of cells expressing B and T lymphocyte attenuator (BTLA). The peripheral blood mononuclear cells (PBMCs) from patients with (a–c) or without (d–f) specific immunotherapy (SIT) were incubated in the presence or absence of Cry j 1 for 72 h, after which the percentage of CD4+ (a, d), CD8+ (b, e) and CD19+ (c, f) cells expressing BTLA in cultured PBMC was determined by flow cytometry. The *P*-values were determined using the Wilcoxon's signed-rank test.

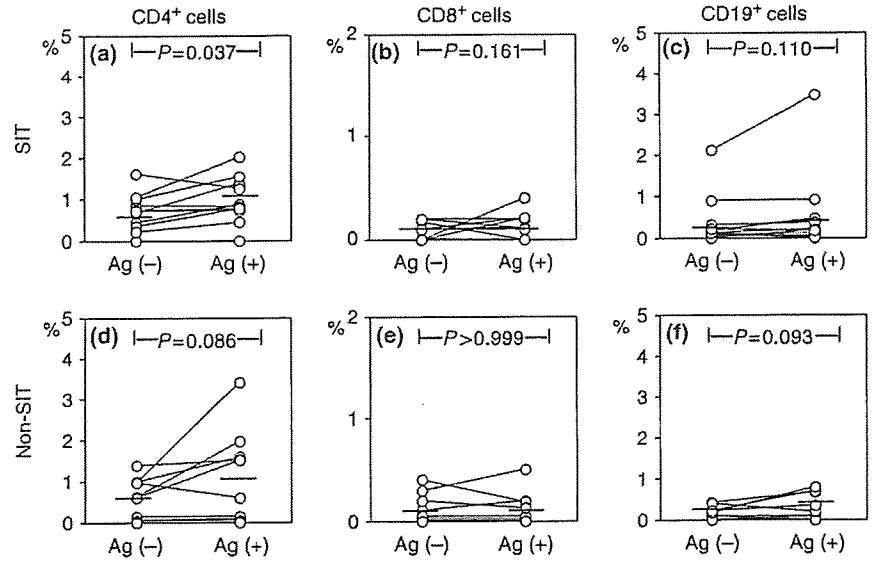

Fig. 4. Phenotype analysis of cells expressing programmed death (PD)-1. The peripheral blood mononuclear cells (PBMCs) from patients with (a–c) or without (d–f) specific immunotherapy (SIT) were incubated in the presence or absence of Cry j 1 for 72 h, after which the percentage of CD4+ (a, d), CD8+ (b, e) and CD19+ (c, f) cells expressing PD-1 in cultured PBMC was determined by flow cytometry. The *P*-values were determined using the Wilcoxon's signed-rank test.

patients (Figs 4d–f). Although a tendency toward overall enhanced B7-H1 expression after recall stimulation with Cry j 1 was seen in SIT-treated patients as compared with untreated patients (Table 2), Cry j 1-induced changes of B7-H1 expression on $CD4^+$, $CD8^+$ or $CD19^+$ cells were not significant in either SIT-treated or SIT-untreated patients (data not shown).

Correlation between Cry j 1-induced interleukin-5 production and changes in B and T lymphocyte attenuator expression

After 72 h of incubation, levels of Cry j 1-induced IL-5 production were significantly reduced in PBMC from SIT-treated patients, compared with untreated patients

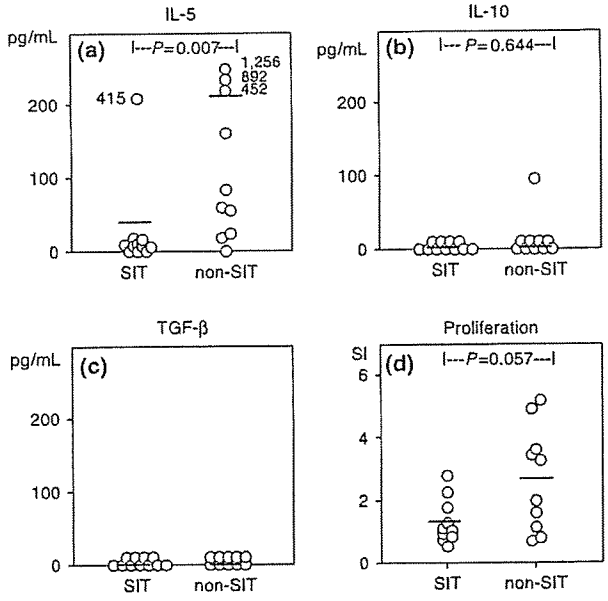

Fig. 5. Comparison of Cry j 1-induced cytokine production and proliferation among specific immunotherapy (SIT)-treated and SIT-untreated Japanese cedar pollinosis (JCP) patients. Peripheral blood mononuclear cells (PBMCs) were incubated in the presence or absence of Cry j 1 for 72 h, after which the levels of IL-5 (a), IL-10 (b) and TGF-β (c) were determined within the supernatant by ELISA. Changes in production were determined by measuring the differences between the levels observed following Cry j 1 stimulation and those observed in the absence of stimulation. In addition, Cry j 1-induced PBMC proliferation was measured by BrdU incorporation (d). *P*-values were determined using the Mann–Whitney's *U*-test. SIT represents the patients treated with SIT. Non-SIT represents SIT-untreated group of patients.

($P = 0.007$, Fig. 5a). On the other hand, IL-10 or TGF-β production in response to Cry j 1 was not detected in either group (Figs 5b and c). IL-10 production was not detected even after 12 h incubation with Cry j 1. Cry j 1-induced IL-5 production was similar between basophil-depleted PBMC (121.9±77.9 pg/mL) and control PBMC (128.9± 59.6 pg/mL, $P = 0.624$), suggesting that basophil responsiveness in IL-5 production is negligible in our culture system. A trend of suppression in Cry j 1-induced proliferation was seen in PBMC from SIT-treated patients as compared with those from SIT-untreated patients ($P = 0.057$; Fig. 5d).

Interestingly, the observed Cry j 1-induced changes in BTLA expression were significantly and negatively correlated with IL-5 production ($\rho = -0.747$, $P < 0.001$; Fig. 6). However, when we analysed the correlation separately, the correlation was not seen in either SIT-treated ($\rho = -0.243$, $P = 0.416$) or SIT-untreated ($\rho = -0.491$, $P = 0.141$) group because Cry j 1-induced IL-5 production was lost in most of SIT-treated patients. The changes in Cry j 1-induced IL-5 production were significantly and positively correlated with both naso-ocular symptom scores ($\rho = 0.616$, $P = 0.024$) and QOL scores ($\rho = 0.719$, $P = 0.008$). The

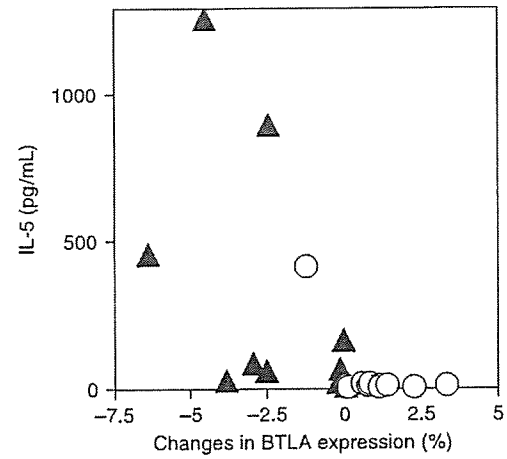

Fig. 6. Relationship between Cry j 1-induced B and T lymphocyte attenuator (BTLA) expression and IL-5 production. Peripheral blood mononuclear cells (PBMCs) were incubated in the presence or absence of Cry j 1 for 72 h, after which the expression of BTLA and IL-5 levels were determined by flow cytometry and ELISA, respectively. Specific immunotherapy (SIT)-treated and SIT-untreated Japanese cedar pollinosis (JCP) patients are represented by the open circles and closed triangles, respectively.

changes in BTLA expression did not correlate with naso-ocular symptom scores ($\rho = -0.063$, $P = 0.760$); however, the changes showed a tendency to inversely correlate with QOL scores ($\rho = -0.450$, $P = 0.067$).

Effect of B and T lymphocyte attenuator cross-linking on Cry j 1-induced interleukin-5 production

Finally, we sought to determine the *in vitro* role of BTLA in Cry j 1-specific PBMC responses. Immobilized anti-BTLA mAb significantly suppressed Cry j 1-induced IL-5 production by PBMC, compared with control mouse IgG2b ($P = 0.016$; Fig. 7a). On the other hand, Cry j 1-induced proliferative responses were not different between immobilized anti-BTLA mAb and the control treatment ($P = 0.879$; Fig. 7b).

Discussion

The key finding of the present study was that BTLA expression is down-regulated after Cry j 1 stimulation in patients not treated with SIT, while it is up-regulated in SIT-treated patients. The up-regulation of BTLA in SIT-treated patients was particularly apparent in a $CD4^+$ T cell subset. Cry j 1-induced changes in BTLA expression were significantly and inversely correlated with IL-5 production by PBMC. Furthermore, cross-linking of BLTA resulted in inhibition of Cry j 1-induced IL-5 production. These results suggest that both IL-5 production and down-regulation of BTLA in response to allergen are inhibited in SIT-treated patients with JCP.

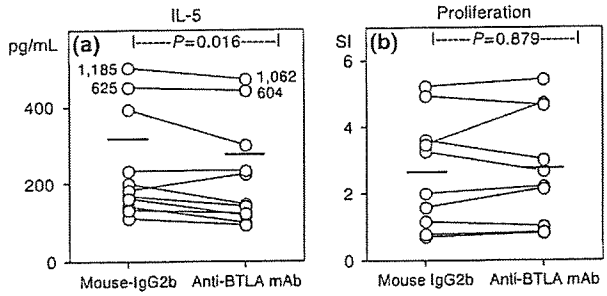

Fig. 7. Effect of cross-linking of B and T lymphocyte attenuator (BTLA) on Cry j 1-induced responses in peripheral blood mononuclear cells (PBMCs). PBMC from patients with Japanese cedar pollinosis (JCP) without immunotherapy were incubated in the presence or absence of Cry j 1, along with either MIH26 or control mouse IgG2b, for 72 h, after which levels of Cry j 1-induced IL-5 (a) and proliferative responses (b) were determined. *P*-values were determined using the Wilcoxon's signed-rank test.

BTLA is a recently identified member of the CD28 family of receptors. Similar to PD-1 and CTLA-4, BTLA contains ITIMs, suggesting that BTLA functions as an inhibitory receptor [17]. In fact, reduced BTLA expression leads to enhanced T cell and B cell responses in mice [17, 25]. However, little is known regarding the potential role of BTLA in human cellular responses. We have previously demonstrated that BTLA ligation inhibits anti-CD3-stimulated proliferation as well as the production of IFN-γ and IL-10 in human $CD4^+$ T cells [22]. Another group has reported suppression of anti-CD3-induced proliferation in response to cross-linking of BTLA, and reduced expression of CD25 and production of IL-2 and IL-4 in addition to IFN-γ and IL-10 in human T cells [26]. In the present study, immobilized anti-BTLA mAb that can cross-link BTLA significantly suppressed Cry j 1-induced IL-5 production by PBMC. This result is consistent with previous reports, and demonstrates for the first time that cross-linking of BTLA also inhibits antigen-specific human PBMC responses. On the other hand, BTLA cross-linking did not alter Cry j 1-induced proliferative responses. We have previously shown that BTLA-induced inhibitory signals depend on the strength of TCR signals [22]. These results suggest that BTLA cross-linking selectively affects Cry j 1-induced IL-5 production in our system. Although, to date, no Ab to block human BTLA is available, the definitive experiment would be to block BTLA (with non-cross-linking Abs or Fab fragments) to see if IL-5 production is restored in SIT patients.

Antigen concentration is one of the key factors regulating *in vitro* cellular responses. We previously reported that PBMC showed proliferative responses to Cry j 1 in a dose-dependent manner, and the samples showed positive responses at 10 μg/mL [20]. Another investigation used Cry j 1 at the concentration of 25–50 μg/mL with substantial results [27]. Thus, we think that the concentration of 10 μg/mL of Cry j 1 is appropriate concentration used in the present study.

Similar to other co-stimulatory molecules, the expression of BTLA is known to change upon activation [22, 25, 26, 28]. The expression of BTLA is up-regulated on T cells and down-regulated on B cells upon activation in mice [25, 28]. In humans, we have demonstrated constitutive BTLA expression on the surface of both CD4 and CD8T cells at high levels, which gradually declines after stimulation with anti-CD3 and anti-CD28 mAb [22]. The relationship between BTLA expression and pathogenesis has been investigated in several human diseases [26, 29, 30]. For example, increased BTLA expression has been demonstrated on $CD4^+$ and $CD8^+$ T cells within pleural fluid in lung cancer patients [26]. An association between a BTLA gene polymorphism and risk of rheumatoid arthritis has also been reported [29]. The baseline expression of BTLA did not differ significantly among SIT-treated and SIT-untreated patients. However, down-regulation of BTLA was only observed in untreated patients in the present study. Although the precise mechanism by which the expression of BTLA is down-regulated remains unknown, signals through the TCR, as well as cytokines produced by Cry j 1-specific T cells and/or pro-inflammatory cytokines/chemokines secreted by monocytes or B cells may all function to regulate BTLA expression. Identification of mechanism regulating BTLA expression must be made in future investigation.

BTLA was originally cloned from murine Th1 cells, and is predominantly expressed by B cells, followed by T cells and APC in mice [17, 25]. In humans, we have recently reported BTLA expression on $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells in freshly isolated human PBMC. Unlike in mice, polarized human Th1 and Th2 cells consist of both BTLA-positive and BTLA-negative populations; however, BTLA expression diminishes with extended length of culture [22]. The present results are consistent with those of previous reports and suggest that BTLA expression persists on human $CD4^+$, $CD8^+$ and $CD19^+$ cells during short-term culture. Furthermore, only $CD4^+$ cells bearing BTLA were significantly increased after recall stimulation with Cry j 1, suggesting that SIT selectively enhances BTLA expression on $CD4^+$ T cells.

A significant difference in Cry j 1-induced IL-5 production by PBMC was seen among SIT-treated and untreated patients. Inhibition of both local and systemic IL-5 production is known to correlate with clinical efficacy [6, 8]. The present result is consistent with a report by Kakinoki et al. [6] demonstrating that the production of IL-5 following Cry j 1-stimulation by PBMC is significantly reduced in good responders, compared with poor responders to SIT in patients with JCP. On the other hand, Cry j 1-induced IL-10 and TGF-β production was not detected in PBMC from SIT-treated patients. One of the reasons why IL-10 production was not induced in our immunotherapy

is that, although our immunotherapy was clinically effective (Fig. 1) and 2000 JAU/mL of JPC extract is maximal concentration commercially available in Japan, the maintenance dose is relatively low (the mean maintenance dose of the extract was 509.0 JAU which is equivalent to 0.37–1.07 μg of Cry j 1) as compared with other reports [1–5]. Moreover, the observed changes in BTLA expression were significantly and negatively correlated with IL-5 production after Cry j 1-stimulation in the present study. This result confirms the result of our cross-linking study and suggests that BTLA has an inhibitory role with regard to Cry j 1-specific IL-5 production in JCP.

In the present study, the expression of co-inhibitory molecules and cytokine production was determined during the pollen season after at least 2 years of SIT treatment. It is interesting to determine the effect of SIT during the first pollen season after starting this therapy. Our preliminary results showed that, as compared with SIT-untreated patients ($n = 10$), PBMC from the newly SIT-treated patients during the first pollen season after starting cedar immunotherapy ($n = 5$) produced significantly less amount of IL-5 (36.7±49.4 vs. 433.6±581.2 pg/mL, $P = 0.020$). On the other hand, Cry j 1-induced changes in BTLA (−1.42±0.80% vs. −3.42±0.97%, $P = 0.178$), PD-1 (0.27±0.38% vs. 0.73±0.33%, $P = 0.390$) and B7-H1 (0.10±0.24% vs. 0.01±0.34%, $P = 0.125$) expression as well as Cry j 1-induced IL-10 (2.4±1.5 vs. 0.2±0.2 pg/mL, $P = 0.137$) and TGF-β (0±0 vs. 0±0 pg/mL) production was not statistically different between the groups. These results suggest that period after reaching of maintenance dose is one of the factors regulating BTLA expression. In addition, the direct effect of SIT on expression of co-inhibitory molecules after reaching the maintenance dose, before the pollen season should be determined.

In conclusion, the present study shows an increase in BTLA-bearing $CD4^+$ T cells in patients treated with SIT. Furthermore, the alterations in BTLA expression were associated with allergen-specific IL-5 production. These results suggest that protection of BTLA down-regulation is a key mechanism of SIT. T cell co-signalling molecules are potential targets in the treatment of allergic airway disease [31]. The present study adds support to this and further suggests that regulation of BTLA expression may be of therapeutic benefit in the treatment of allergic airway disease.

Acknowledgements

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CRTH2 Plays an Essential Role in the Pathophysiology of Cry j 1-Induced Pollinosis in Mice[1]

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PGD_2 is the major prostanoid produced during the acute phase of allergic reactions. Two PGD_2 receptors have been isolated, DP and CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), but whether they participate in the pathophysiology of allergic diseases remains unclear. We investigated the role of CRTH2 in the initiation of allergic rhinitis in mice. First, we developed a novel murine model of pollinosis, a type of seasonal allergic rhinitis. Additionally, pathophysiological differences in the pollinosis were compared between wild-type and *CRTH2* gene-deficient mice. An effect of treatment with ramatroban, a CRTH2/T-prostanoid receptor dual antagonist, was also determined. Repeated intranasal sensitization with Cry j 1, the major allergen of *Cryptomeria japonica* pollen, in the absence of adjuvants significantly exacerbated nasal hyperresponsive symptoms, Cry j 1-specific IgE and IgG1 production, nasal eosinophilia, and Cry j 1-induced in vitro production of IL-4 and IL-5 by submandibular lymph node cells. Additionally, CRTH2 mRNA in nasal mucosa was significantly elevated in Cry j 1-sensitized mice. Following repeated intranasal sensitization with Cry j 1, *CRTH2* gene-deficient mice had significantly weaker Cry j 1-specific IgE/IgG1 production, nasal eosinophilia, and IL-4 production by submandibular lymph node cells than did wild-type mice. Similar results were found in mice treated with ramatroban. These results suggest that the PGD_2-CRTH2 interaction is elevated following sensitization and plays a proinflammatory role in the pathophysiology of allergic rhinitis, especially pollinosis in mice. *The Journal of Immunology,* 2008, 180: 5680–5688.

Pollinosis, a type of seasonal allergic rhinitis, is the most common allergic respiratory disease and is a global health problem that is increasing in prevalence (1–3). For example, as much as 10–20% of the Japanese population suffers from Japanese cedar pollinosis (JCP).[3]

Intensive and extensive studies on pollinosis have greatly improved the understanding of its etiology and pathology (4). Mouse models of allergic rhinitis have contributed to these advances. However, these mouse models usually use adjuvants and/or strong Ags to efficiently sensitize animals (5–9). To further examine the pathophysiological mechanism underlying pollinosis, a murine model that naturally mimics human pollinosis by intranasal administration of pollen extracts in the absence of adjuvants is needed.

Prostanoids are thought to participate in allergic inflammation (10). PGD_2 is one of the most important of these and it plays roles in allergic respiratory diseases including allergic rhinitis (10–15). For example, nebulized PGD_2 enhances Th2-type inflammatory responses and eosinophilia, leading to the development of airway hyperresponsiveness (14). PGD_2 acts via the D-prostanoid receptor (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (16). The expression patterns and signaling pathways utilized by DP and CRTH2 are different, suggesting that they have distinct roles in allergic responses (16, 17). It appears that signals via DP promote eosinophil survival, whereas signals via CRTH2 mediate shape changes, chemotaxis, and degranulation by eosinophils (16, 18, 19).

The role of CRTH2 in allergic airway inflammation in vivo remains controversial (17). CRTH2 has been found to participate in the recruitment of eosinophils from the bone marrow into the bloodstream (19, 20), in eosinophilic airway inflammation (11), and in airway eosinophilia and hyperresponsiveness (21), suggesting that it plays a proinflammatory role in vivo. On the other hand, mice deficient for CRTH2 ($CRTH2^{-/-}$) show eosinophil recruitment and IL-5 production by splenocytes in an asthma model, suggesting that CRTH2 mediates antiinflammatory signals (22). In human nasal mucosa, CRTH2 is expressed in eosinophils and a subset of T cells (23). We have recently reported that there is a close correlation between the number of eosinophils infiltrating into nasal mucosa and the amount of CRTH2, but not DP, in nasal mucosa (12). Also, $CRTH2^{-/-}$ mice have been found to show reduced eosinophil infiltration into skin in a model of chronic allergic skin inflammation (24).

In this study, we established a novel murine model of pollinosis and used it to determine the pathophysiological role of CRTH2 in

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[3] Abbreviations used in this paper: JCP, Japanese cedar pollinosis; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DP, D-protanoid receptor; TP, T-prostanoid receptor; WT, wild type.

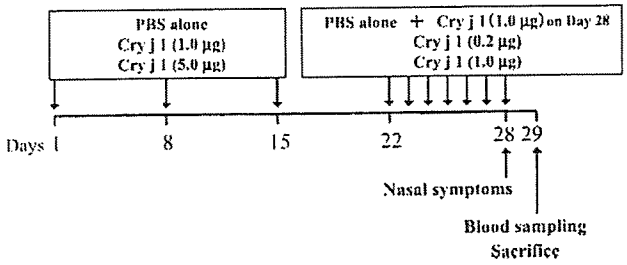

FIGURE 1. Experimental design used to investigate the effect of nasal exposure to Cry j 1 in mice. BALB/c mice (6–9 per group) were sensitized by intranasal administration of either 1.0 or 5.0 μg of Cry j 1 in 10 μl PBS in the absence of adjuvants once a week for 3 wk (on days 1, 8, and 15). One week after the third sensitization, mice were challenged by intranasal administration of one fifth Cry j 1, respectively every day for 7 consecutive days (on days 22 to 27). As a control, mice were treated with PBS, except for the final challenge, where mice were treated with 1.0 μg of Cry j 1. Immediately after the final nasal challenge, nasal symptoms were observed for 10 min, and 16 h after the final nasal challenge, peripheral blood was collected, and the specific Ab content in the serum was measured. After the blood sampling, mice were sacrificed, and the nose and submandibular lymph nodes were obtained for further analysis.

the disease. In this model, intranasal sensitization with Cry j 1, the major allergen of *Cryptomeria japonica* pollen, in the absence of adjuvant induced allergic rhinitis closely resembling human pollinosis. We found that a lack of CRTH2 in the mutant mice greatly reduces allergic pathophysiologies in this model.

Materials and Methods

Animals and Ags

BALB/c mice were purchased from Charles River Laboratories Japan and CLEA Japan. Homozygous CRTH2-deficient and wild-type BALB/c mice were obtained as described previously (24). Female mice (7–11 wk old) were used in all the experiments. The mice were maintained in specific pathogen-free conditions at Okayama University and Tokyo Medical and Dental University in accordance with the guidelines set forth by the university committees. All experimental protocols and procedures in the present study were approved by the University Animal Care and Use Committees. Cry j 1 was purified from crude extracts of *C. japonica* pollen as described previously (25). Endotoxin contamination was considered to be negligible due to a negative result in the Endospec ES test (Seikagaku). Ramatroban was obtained from Bayer Yakuhin. Protein concentrations were determined using a bicinchoninic acid assay (Pierce) according to the manufacturer's instructions.

Sensitization of mice

Mice (6–9 animals per group) were sensitized by intranasal application of serial doses of Cry j 1 in 10 μl PBS in the absence of adjuvants using a microsyringe (Hamilton Medical). The low-dose sensitization consisted of a series of administrations of 1.0 μg of Ag once a week for 3 wk (on days 1, 8, and 15), followed by administration of 0.2 μg Ag every day for 7 consecutive days (on days 22 to 28). For high-dose sensitization, 5.0 and 1.0 μg of Cry j 1 were administered once a week for 3 wk (on days 1, 8, and 15) and every day for 7 consecutive days (on days 22 to 28), respectively. As a control, mice were treated with PBS instead of the Ag at all points except for the final challenge, where the mice were administered 1.0 μg of Cry j 1 (Fig. 1). Immediately after the final nasal challenge, the frequencies of sneezing and nasal rubbing were counted in a blinded manner for 10 min. Peripheral blood was collected from the tail vein 16 h after the final nasal challenge, and then sera were prepared by centrifugation at 200 × *g*, and the levels of Cry j 1-specific Ab in the serum were determined by ELISA. The mice were then sacrificed, and the nose and submandibular lymph nodes were isolated for further immunological and histological analyses.

To determine whether the effect of CRTH2 deficiency is at the level of sensitization or amplification of allergic cascade, outcomes of pollinosis were compared with CRTH2$^{-/-}$ mice sensitized and subsequently challenged with Cry j 1 and nonsensitized CRTH2$^{-/-}$ mice with a single challenge with Cry j 1.

Ramatroban treatment

Ramatroban was suspended in 5% methyl cellulose and administered orally at a dose of 30 mg/kg body weight once a day from 1 day before the first sensitization to the final challenge (day 0 to day 28). Control mice were given 5% methyl cellulose alone.

Ab determination

The levels of Cry j 1-specific IgE, IgG1, and IgG2a were determined by ELISA as previously described (26). The levels of Cry j 1-specific IgE were measured using biotinylated Cry j 1 (Hayashibara Biochemical Laboratories) as a detecting reagent. The titers of Ag-specific Abs were estimated according to the mean OD at 450 nm of serum dilutions of 1/20 for IgE and 1/100 for IgG1 and IgG2a.

In vitro culture of submandibular lymph node cells and measurement of cytokine production

Submandibular lymph nodules from mice were dispersed and filtered through a 70-μm cell strainer (BD Biosciences) to yield a single-cell suspension. Lymph node cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS (Invitrogen), 100 μg/ml streptomycin, 100 U/ml penicillin, and 20 mM L-glutamine (Sigma-Aldrich). Cells (4×10^5 cells/200 μl) were cultured in the presence or absence of 10 μg/ml Cry j 1 in 96-well flat-bottom plates (BD Biosciences) at 37°C in humidified atmosphere of 5% CO_2 and 95% air. After 72 h of culture, supernatants were harvested. The levels of IL-4, IL-5, and IFN-γ in the culture supernatant were measured using OptEIA sets (BD Biosciences). The levels of IL-13 were measured using DuoSet ELISA development kit (R&D Systems). The detection limits for IL-4, IL-5, IL-13, and IFN-γ in this system were 10, 30, 40, and 60 pg/ml, respectively.

Histological examination

Histological examination was performed as previously described (26). Coronal nasal sections were stained with H&E and Luna solution to detect mononuclear cells and eosinophils, respectively. A blind test was conducted to determine the numbers of infiltrating cells in the posterior part of nasal septum using a high-power (10 × 40) microscopic field.

To determine the infiltration of T cells into nasal mucosa, immunohistochemistry for CD3 was examined. Paraffin-embedded nasal tissues were sectioned into 5-μm slices, deparaffinized, rehydrated and retrieved with microwave. Endogenous peroxidase activity was quenched with 3% H_2O_2, and nonspecific protein binding was blocked with normal rabbit serum (DAKO Japan) for 60 min. After this, the tissue sections were incubated with goat anti-mouse CD3-ε polyclonal Ab (sc-1127: Santa Cruz Biotechnology) or control goat IgG Ab (M-20: Santa Cruz Biotechnology) overnight at 4°C. To detect the reaction, N-Histofine Simple Stain MAX PO (G) (Nichirei Biosciences) and diaminobenzidine substrate (DAKO Japan) was used according to the manufacturers' instructions.

Real-time quantitative PCR in nasal mucosa

Mucosal tissues were removed from nasal septum 16 h following the final nasal challenge, immediately soaked in buffer containing guanidine isothiocyanate from the RNeasy Mini Kit (Qiagen), and stored at −80°C until use. Extraction of total cellular RNA, reverse transcription to generate cDNA, and real-time quantitative PCR were performed using a Chromo4 Real-Time PCR detector (Bio-Rad Laboratories, Hercules) and QuantiTect SYBR Green PCR reagents (Qiagen) as described previously (12). The primer sequences for CRTH2 and GAPDH are shown in Table I. Standard curves for both CRTH2 and GAPDH were generated using a PCR fragment of CRTH2 and plasmid DNA of GAPDH as a standard, respectively. Then absolute copy number of CRTH2 and GAPDH for each sample was calculated, and samples were reported with a CRTH2 copy number relative to GAPDH.

Relative amounts of IL-4, IL-5, IL-13, IFN-γ, IL-1β, IL-6, TNF-α, RANTES, and eotaxin mRNA in nasal mucosa were also measured. The primers used are listed in Table I.

Statistical analysis

Statistical significance was determined by nonparametrical Mann-Whitney *U* tests. p values of <0.05 were considered to indicate statistical significance. Values are shown as means ± SEM.

Table I. *Primary sequences used for real-time PCR amplifications*

	Forward Primer	Reverse Primer	Amplification Size (bp)	Genbank Accession No.
IL-4	CCTCACAGCAACGAAGAACA	CTGCAGCTCCATGAGAACAC	133	NM_021283
IL-5	TCAGCTGTGTCTGGGCCACT	TTATGAGTAGGGACAGGAAGCCTCA	133	NM_010558
IL-13	TGCTTGCCTTGGTGGTCTC	CAGGTCCACACTCCATACC	151	NM_008355
IFN-γ	GCGTCATTGAATCACACCTG	ACCTGTGGGTTGTTGACCTC	103	NM_008337
IL-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACCAGCAGGTTA	105	NM_008361
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC	112	NM_031168
TNF-α	ATGAGCACAGAAAGCATGATC	TCCACTTGGTGGTTTGCTACG	305	NM_013693
RANTES	AGATCTCTGCAGCTGCCCTCA	GGAGCACTTGCTGCTGGTGTAG	170	NM_013653
Eotaxin	CAGATGCACCCTGAAAGCCATA	TGCTTTGTGGCATCCTGGAC	96	NM_011330
CRTH2	TCTCAACCAATCAGCACACC	CCTCCAAGAGTGGACAGAGC	173	NM_009962
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	NM_008084

Results

Induction of nasal symptoms in Cry j 1-sensitized mice

We first attempted to generate a mouse model mimicking human allergic rhinitis, especially pollinosis, which causes symptoms of nasal symptoms, including sneezing and nasal rubbing, by intranasal administration of Cry j 1. We found a significant and dose-dependent increase in the frequency of sneezing in BALB/c mice sensitized with Cry j 1. Mice that were treated with PBS alone sneezed 1.8 ± 0.3 (mean ± SEM) times in the 10 min following the final Ag administration, whereas they sneezed 5.8 ± 1.2 times and 15.7 ± 2.7 times when treated with low and high doses of Ag, respectively (Fig. 2*A*). Similarly, immediately after the final Ag challenge, nasal rubbing was observed more frequently in mice sensitized with a high dose of Cry j 1 than in control mice (37.3 ± 5.8 vs 11.2 ± 2.7 times in 10 min). At a low dose of Cry j 1, there was no significant increase in the frequency of nasal rubbing (Fig. 2*B*).

Development of Th2-type immune responses in Cry j 1-sensitized mice

To further characterize the pathogenesis of immune responses caused by Cry j 1, we monitored several parameters associated with pollinosis. Nasal challenge with a low or high dose of Cry j 1 caused a considerable increase in the concentration of Cry j 1-specific IgE in sera when measured 1 day after the final challenge (Fig. 2*C*). There was also a significant elevation in the concentration of Cry j 1-specific IgG1 (Fig. 2*D*). The concentration of Cry j 1-specific IgE and IgG1 was not appreciably different at the low and high doses of Cry j 1. Cry j 1, however, had little effect on the level of Cry j 1-specific IgG2a (Fig. 2*E*).

Eosinophil infiltration into nasal mucosa, another characteristic of pollinosis, is rarely seen in the nasal mucosa in control mice (Fig. 3*A*). On the contrary, there was a marked accumulation of eosinophils not only in the lamina propria but also in the epithelial layer in mice 1 day after the final challenge (Fig. 3, *B* and *C*). Eosinophil numbers per field following intranasal Cry j 1 sensitization/challenge at both low and high doses were significantly higher than in control mice (Fig. 3*D*). The nasal mucosa of Cry j 1-sensitized mice also showed severe infiltration by mononuclear cells. The nasal septum of mice treated with low and high doses of Cry j 1 contained more mononuclear cells per field (59.8 ± 9.0 ($p = 0.055$) and 80.2 ± 9.1 ($p = 0.016$), respectively) than did control mice (39.8 ± 4.7).

We next examined the in vitro production of cytokines in culture by cells isolated from submandibular lymph nodes from mice treated in vivo with or without Cry j 1. The amounts of IL-4 and

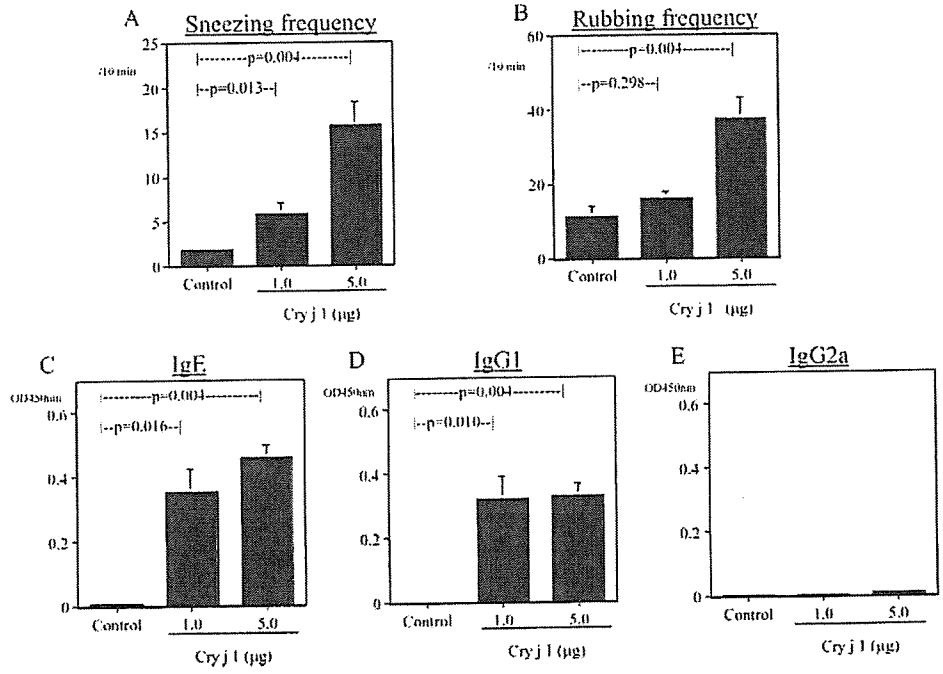

FIGURE 2. Nasal hyperresponsive symptoms and Ab production in mice following intranasal sensitization and challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of Cry j 1. Nasal allergic symptoms, including the frequency of sneezing (*A*) and rubbing (*B*), were determined for the 10 min immediately following the final nasal challenge (day 28). Mean frequencies ± SEM are shown. Serum samples were obtained 16 h after the final intranasal challenge. Cry j 1-specific IgE (*C*), IgG1 (*D*), and IgG2a (*E*) levels were determined by ELISA. Mean OD values ± SEM are shown. Results are representative of two independent experiments.

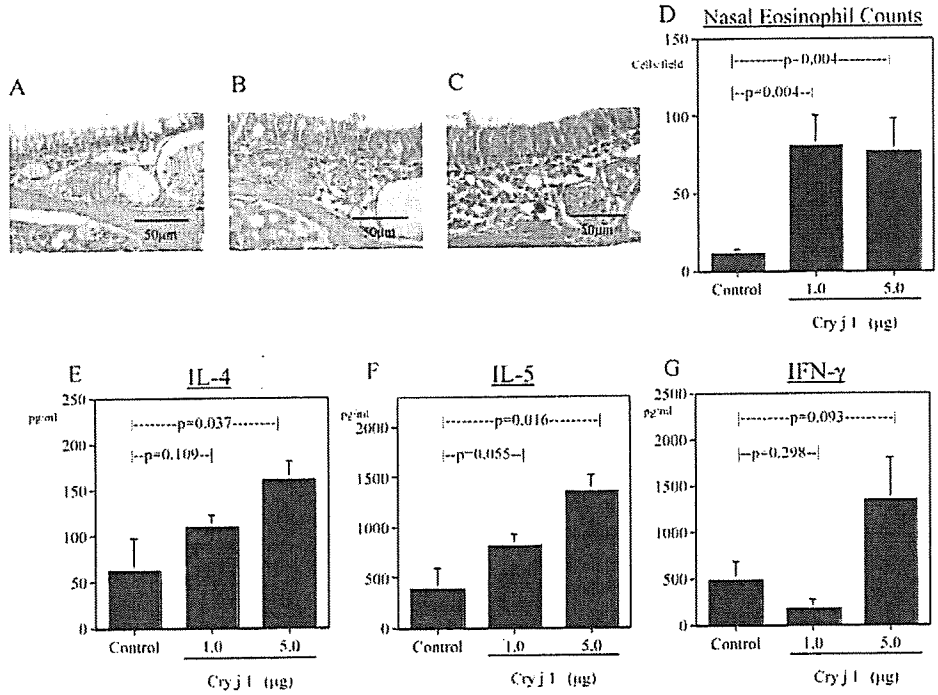

FIGURE 3. Nasal eosinophilia and cytokine production by submandibular lymph node cells following intranasal sensitization and challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of PBS (*A*), low-dose Cry j 1 (*B*), or high-dose Cry j 1 (*C*) according to the schedule shown in Fig. 1. Sixteen hours after the final challenge, nasal sections were collected, fixed, and decalcified, and eosinophils in the nasal mucosa were detected by Luna stain. *D*, The numbers of eosinophils in the posterior portion of the nasal septum were determined per high-power (10 × 40) microscopic field. Mean numbers of infiltrating cells per field ± SEM are shown. Sixteen hours after the final challenge, submandibular lymph node cells were isolated and cultured in the absence or presence of Cry j 1 for 72 h. IL-4 (*E*), IL-5 (*F*), and IFN-γ (*G*) were measured by ELISA. Mean concentrations ± SEM are shown. Results are representative of two independent experiments.

IL-5 produced by the cells were in proportion to the doses used for in vivo sensitization (Fig. 3, *E* and *F*). IFN-γ production was slightly enhanced in lymph node cells from mice treated with a high dose of Cry j 1 compared with control mice, but the increase was not statistically significant (Fig. 3*G*).

CRTH2 mRNA expression in nasal mucosa of Cry j 1-sensitized mice

We next measured the expression of CRTH2 at sites of nasal inflammation. Control mice treated with PBS expressed a low level of CRTH2 mRNA in the mucosal tissue of the nasal septum. In mice treated with Cry j 1, the level of CRTH2 mRNA was significantly increased (Fig. 4). Thus, we further investigated whether CRTH2 is positively or negatively involved in the pathophysiology of pollinosis using CRTH2$^{-/-}$ mice.

Impaired pathophysiology of pollinosis in Cry j 1-sensitized CRTH2$^{-/-}$ mice

A high dose of Cry j 1 was administered to both wild-type (WT) and CRTH2$^{-/-}$ mice, and the nasal hyperresponsive symptoms were examined immediately after the final nasal challenge. Notably, the number of sneezes in 10 min by the Cry j 1-sensitized mutant mice was significantly lower than by the WT mice (Fig. 5*A*). Nasal rubbing was also significantly lower in the CRTH2$^{-/-}$ mice than in the WT mice (Fig. 5*B*).

The level of Cry j 1-specific IgE in serum samples collected on the day following the final Ag challenge was significantly lower for mutant mice than for WT mice (Fig. 5*C*). Production of Cry j 1-specific IgG1 was similarly reduced in CRTH2$^{-/-}$ mice compared with WT mice (Fig. 5*D*). In contrast, serum levels of Cry j 1-specific IgG2a were the same in the two mouse strains (Fig. 5*E*).

The number of eosinophils infiltrating into the nasal septum following administration of Cry j 1 was also significantly lower in the CRTH2$^{-/-}$ mice than in the WT mice (Fig. 6*A–C*). Although the number of mononuclear cells infiltrating the nasal septum was not significantly different in the mutant and WT mice (Fig. 6*D*), the number of infiltrating CD3$^+$ cells was significantly reduced in CRTH2$^{-/-}$ mice as compared with WT mice (Fig. 6*E*). These

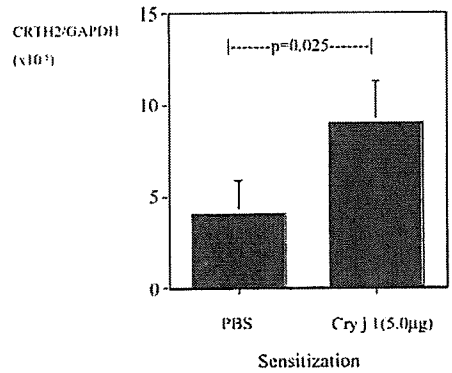

FIGURE 4. Relative amounts of CRTH2 mRNA in nasal mucosa. Mice (6 per group) were sensitized by intranasal administration of 5.0 μg of Cry j 1 once a week for 3 wk. One week after the third sensitization, mice were challenged by intranasal administration of 1.0 μg of Cry j 1 each day for 7 consecutive days. Control animals were treated with PBS at all steps except for the final challenge, where they were treated with 1.0 μg of Cry j 1. Sixteen hours after the final challenge with Cry j 1, mucosal tissues were removed from the nasal septum. The CRTH2 mRNA levels were estimated using real-time quantitative PCR. Results are the mean amounts of mRNA ± SEM.

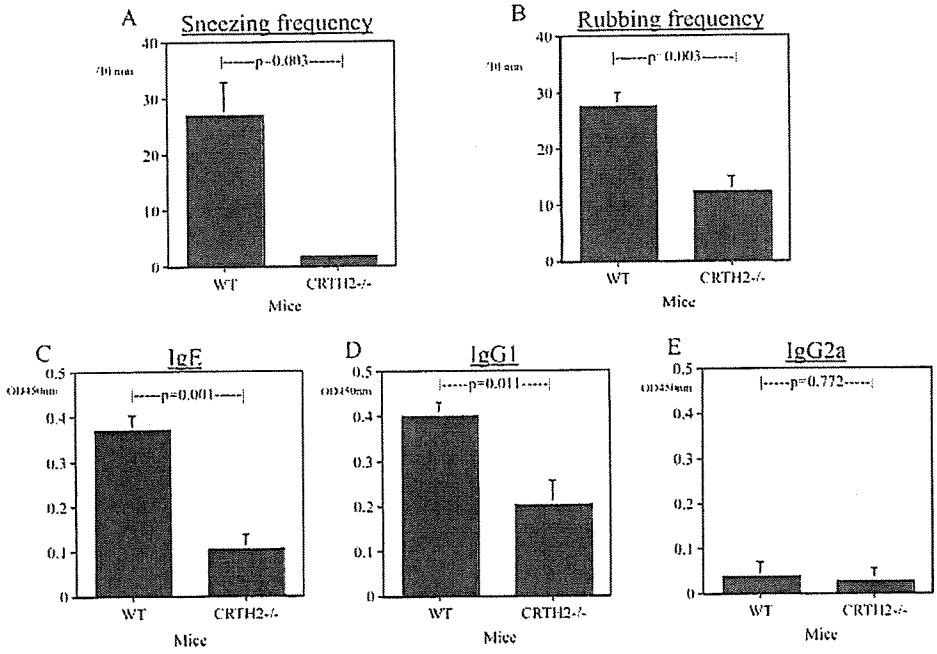

FIGURE 5. Nasal symptoms and Ab production in WT and CRTH2$^{-/-}$ mice following the final nasal challenge with Cry j 1. Mice were sensitized and challenged by intranasal administration of Cry j 1. Sneezing (*A*) and rubbing (*B*) frequency were measured for 10 min following the final nasal challenge (day 28). Mean frequencies ± SEM are shown. Sixteen hours after the final nasal challenge, blood was sampled from mice, and levels of serum Cry j 1-specific IgE (*C*), IgG1 (*D*), and IgG2a (*E*) were determined by ELISA. Mean ODs ± SEM are shown. Results are representative of two independent experiments.

results suggest that CRTH2 deficiency affects infiltration of not only eosinophils but also T cells.

To clarify the link between CRTH2 deficiency and the relief of allergic symptoms, we further investigated cytokine production in vitro by cells from submandibular lymph nodes obtained the day after the final Ag challenge. The amount of IL-4 was 5-fold lower in CRTH2$^{-/-}$ mice than in WT mice (Fig. 6*F*). Additionally, there was a slight reduction in the amount of IL-5 (Fig. 6*G*) and a slight increase in the amount of INF-γ (Fig. 6*I*) in the cells from CRTH2$^{-/-}$ mice, but the differences were not significant. On the contrary, the levels of IL-13 were significantly higher in CRTH2$^{-/-}$ mice as compared with WT mice (Fig. 6*H*).

Additionally, mRNA levels of Th2 cytokines (IL-4, IL-5, and IL-13), Th1 cytokine (IFN-γ), proinflammatory cytokines (IL-1β, IL-6 and TNF-α), and eosinophil-chemotactic chemokines (RANTES and eotaxin) in nasal mucosa were determined. The

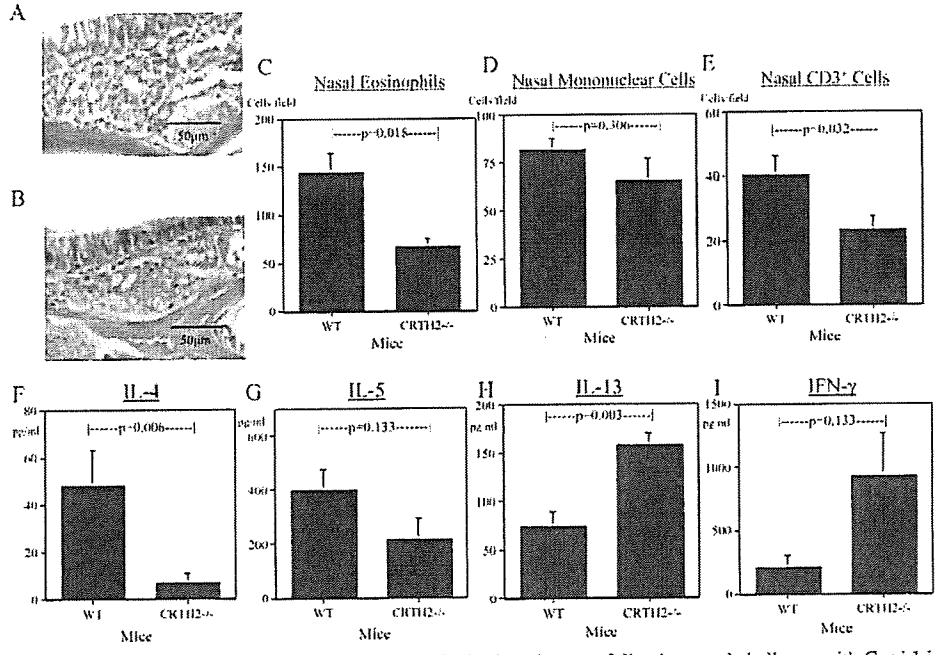

FIGURE 6. Histological changes and cytokine production by submandibular lymphocytes following nasal challenge with Cry j 1 in WT and CRTH2$^{-/-}$ mice. WT (*A*) and CRTH2$^{-/-}$ (*B*) mice were sensitized and challenged by intranasal administration of Cry j 1. Sixteen hours following the final nasal challenge with Cry j 1, nasal sections were collected, fixed, and decalcified, and eosinophils in nasal mucosa were detected by Luna stain. *C*, The number of eosinophils in the posterior portion of the nasal septum was determined per high-power (10 × 40) microscopic field. Mean numbers of infiltrating eosinophils per field ± SEM are shown. Numbers of mononuclear cells (*D*) and CD3$^+$ cell (*E*) in the nasal septum were also determined. Sixteen hours after the final challenge with Cry j 1, submandibular lymph node cells were isolated and cultured with Cry j 1 for 72 h. IL-4 (*F*), IL-5 (*G*), IL-13 (*H*), and IFN-γ (*I*) were measured by ELISA. Mean concentrations ± SEM are shown. Results are representative of two independent experiments.

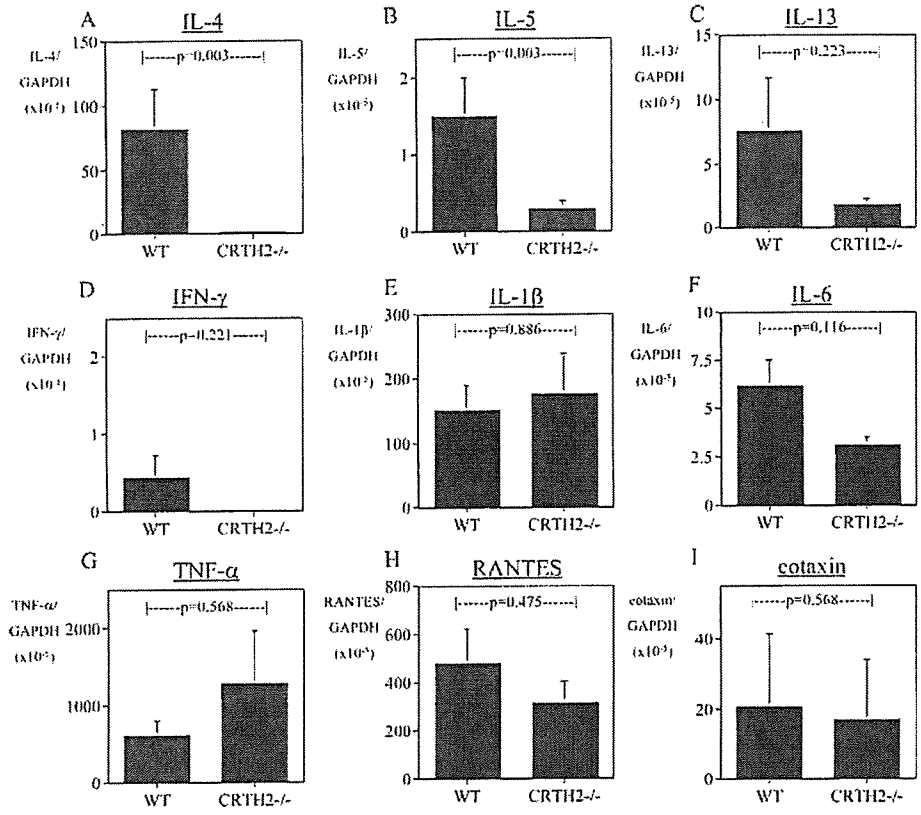

FIGURE 7. Relative amounts of cytokines/chemokines mRNA in nasal mucosa following nasal challenge with Cry j 1 in WT and CRTH2$^{-/-}$ mice. Sixteen hours after the final nasal challenge with Cry j 1, mucosal tissues were removed from nasal septum. Relative amounts of IL-4 (*A*), IL-5 (*B*), IL-13 (*C*), IFN-γ (*D*), IL-1β (*E*), IL-6 (*F*), TNF-α (*G*), RANTES (*H*), and eotaxin (*I*) mRNA were compared between WT and CRTH2$^{-/-}$ mice. Results are the mean amounts of mRNA ± SEM.

levels of IL-4 and IL-5 mRNA were significantly lower in CRTH2$^{-/-}$ mice as compared with WT mice, whereas the levels of other cytokines/chemokines were similar between CRTH$^{-/-}$ and WT mice (Fig. 7). These results suggest that reduced nasal eosinophilia in CRTH2 deficiency is associated with reduced levels of IL-5 but not RANTES or eotaxin in this model. Additionally,

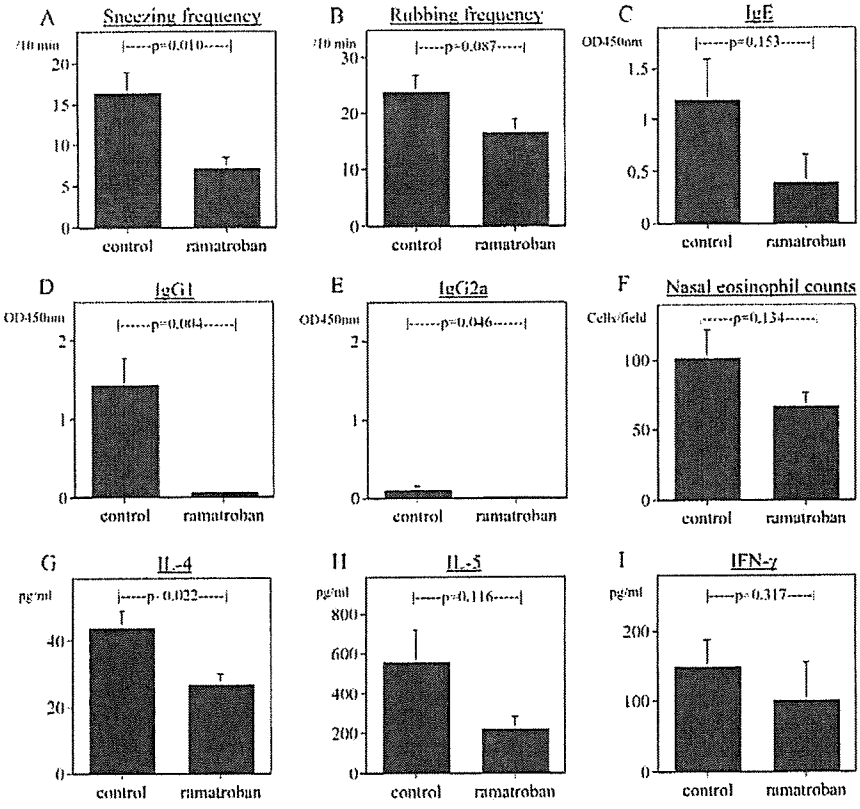

FIGURE 8. Effects of ramatroban on murine JCP. Ramatroban (30 mg/kg body weight), suspended in 5% methyl cellulose, was given orally once a day from 1 day before the first sensitization to the final challenge (day 0 to day 28). Control mice were given 5% methyl cellulose alone. After the final intranasal challenge, the frequencies of sneezing (*A*) and rubbing (*B*) were counted, and serum levels of Cry j 1-specific IgE (*C*), IgG1 (*D*), IgG2a (*E*), and nasal eosinophil count (*F*), as well as Cry j 1-induced IL-4 (*G*), IL-5 (*H*) and IFN-γ (*I*) were determined as described in *Materials and Methods*. Results are expressed as means ± SEM.