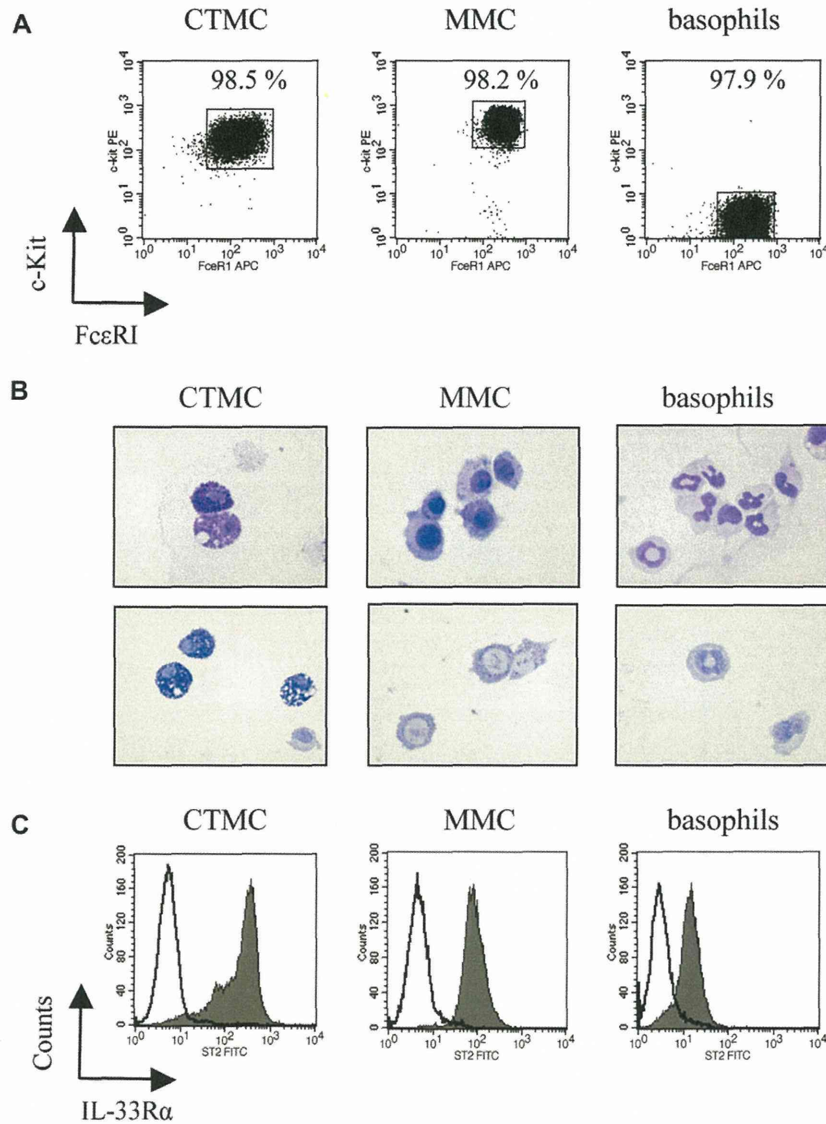

FIG E5. Preparation and characterization of bone marrow-derived CTMCs, MMCs, and basophils. **A** and **B,** Flow cytometric analysis of the expression of FcεRI and c-Kit by bone marrow–derived and sorted CTMCs, MMCs, and basophils. Fig E5, *A, Numbers above the outlined areas* indicate percentages of cells. Fig E5, *B,* Diff-Quik *(upper)* and toluidine blue *(lower)* staining of sorted CTMCs, MMCs, and basophils (original magnification ×400). **C,** Flow cytometric analysis of the expression of the IL-33Rα chain by sorted CTMCs, MMCs, and basophils. *Filled histograms,* IL-33Rα chain; *lines,* unstained cells.

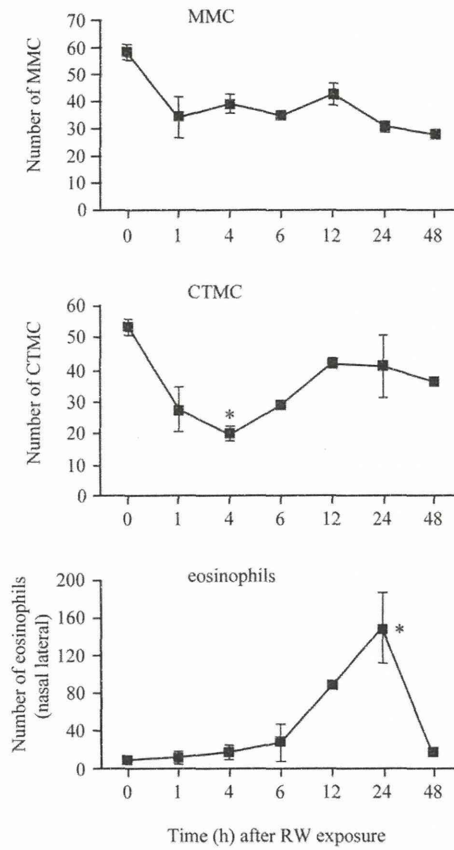

FIG E6. Kinetics of the number of MMCs, CTMCs, and eosinophils in the nose. Ragweed *(RW)*-immunized WT mice were nasally administered with single ragweed. Kinetics of the numbers of MMCs, CTMCs, and eosinophils in the nose (3 mice per time point) are shown. Data are representative of 2 independent experiments. Means and SEMs of 3 mice are shown. *$P < .05$ compared with before ragweed exposure (0 hour).

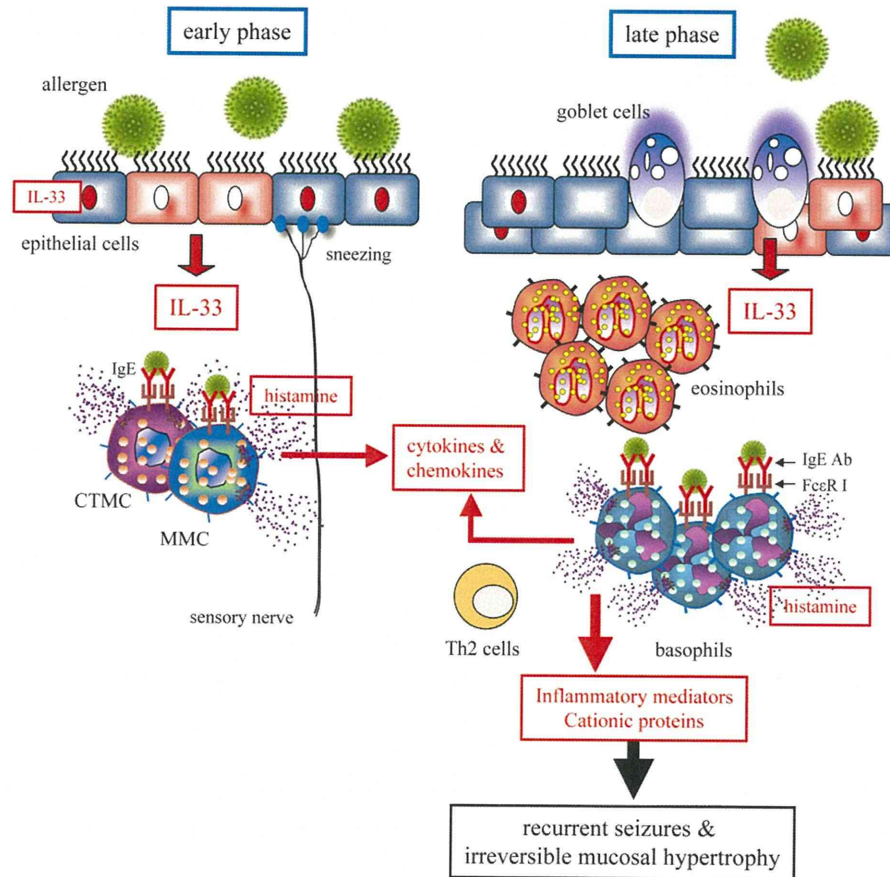

FIG E7. Schematic representation of contribution of IL-33 to allergic responses in AR. Ragweed pollen–driven endogenous IL-33 from nasal epithelial cells contributes to both early-phase (sneezing) and late-phase (nasal accumulation of eosinophils and basophils) responses in AR by increasing histamine release and inducing production of chemoattractants from mast cells and basophils, respectively. This process, together with the contribution of IL-33 to stimulate T_H2 cells, eosinophils, basophils, and mast cells to produce allergic inflammatory mediators, might lead to the recurrent seizures and irreversible mucosal hypertrophy seen in patients with AR.

TABLE E1. Characteristics of the patients with AR and control subjects

Characteristics	Cases	Control subjects
Age (y), mean (range)	39.9 (10-62)	44.5 (24-65)
Total subjects and sex (male/female)	10 (9/1)	5 (3/2)
Serum total IgE (IU/mL), mean ± SEM	478.8 ± 166.9	32.5 ± 7.8
Atopic sensitization (RAST), no. (%)		
Dermatophagoides pteronyssinus positive	10 (100)	0 (0)
JC pollen positive	5 (50)	0 (0)
Japanese cypress pollen positive	5 (50)	0 (0)
Cocksfoot pollen positive	3 (33.3)	0 (0)
Grey alder pollen positive	2 (20)	0 (0)
Common ragweed pollen positive	1 (10)	0 (0)
Mugwort positive	1 (10)	0 (0)

TABLE E2. Characteristics of the patients with JC pollinosis and control subjects

Characteristics	Cases	Control subjects
Age (y), mean (range)	27.8 (20-32)	23.8 (19-34)
Total subjects and sex (male/female)	13 (8/5)	11 (5/6)
Serum total IgE (IU/mL), mean ± SEM	93.8 ± 17.0	40.0 ± 10.5
Atopic sensitization (RAST), no. (%)		
JC pollen positive	13 (100)	0 (0)
Dermatophagoides pteronyssinus positive	0 (0)	0 (0)
Dermatophagoides farinae positive	0 (0)	0 (0)
Cocksfoot pollen positive	0 (0)	0 (0)
Candida albicans positive	0 (0)	0 (0)
Aspergillus fumigatus positive	0 (0)	0 (0)
Common ragweed pollen positive	0 (0)	0 (0)

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ORIGINAL ARTICLE Basic Mechanisms in Allergic Disease

COX/PGE_2 axis critically regulates effects of LPS on eosinophilia-associated cytokine production in nasal polyps

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Clinical & Experimental Allergy

Abstract
Background Lipopolysaccharide (LPS) has shown heterogeneous effects on eosinophilic inflammation in airways. However, little is known about how LPS regulates pathogenesis of chronic rhinosinusitis with nasal polyps, a major form of eosinophilic inflammation in the upper airway.
Objective We sought to investigate the effect of LPS on cytokine production by dispersed nasal polyp cells (DNPCs).
Methods Either diclofenac-treated or untreated DNPCs were cultured with or without staphylococcal enterotoxin B (SEB) in the presence or absence of LPS, after which the levels of IL-5, IL-13, IL-17A and IFN-γ within the supernatant were measured. The effects of PGE_2 on LPS-induced responses by diclofenac-treated DNPCs were also examined. LPS-induced PGE_2 production and mRNA expression of COX-1, COX-2 and microsomal PGE_2 synthase-1 (m-PGES-1) were measured.
Results Staphylococcal enterotoxin B induced IL-5, IL-13, IL-17A and IFN-γ production by DNPCs. Pre-treatment with LPS prior to SEB stimulation inhibited production of these cytokines. After stimulation with LPS, PGE_2 production and expression of COX-2 and m-PGES-1 mRNA by DNPCs increased significantly. In the presence of diclofenac, the suppressive effects of LPS were eliminated. LPS pre-treatment enhanced SEB-induced IL-5, IL-13 and IL-17A production in diclofenac-treated DNPCs, while addition of PGE_2 inhibited IL-5, IL-13 and IFN-γ production. LPS alone induced IL-5, IL-13 and IFN- γ production by diclofenac-treated DNPCs, while the addition of EP2 and EP4 receptor-selective agonists, as well as PGE_2 itself, inhibited IL-5 and IL-13 production.
Conclusions and Clinical Relevance These results suggest that the regulatory effects of LPS on eosinophilic airway inflammation are controlled via the COX-2/PGE_2 axis. For clinical implications, indiscreet use of non-steroidal anti-inflammatory drugs should be avoided in patients with chronic rhinosinusitis with nasal polyps.

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Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNPs) is characterized by eosinophilic inflammation, and is often associated with asthma and aspirin sensitivity [1]. Although the precise aetiology and pathophysiology underlying this disease remains poorly understood, imbalances in local Th1, Th2, Th17 and Treg responses appear to be involved [2, 3].

Components and products derived from microbes including viruses, fungi and bacteria can exert cellular responses in CRSwNP [4–7]. For example, we demonstrated that *Staphylococcus aureus* enterotoxin B (SEB) and crude extracts of fungi including *Aspergillus*, *Alternaria* and *Candida* induced IL-5 and IL-13 production by dispersed nasal polyp cells (DNPCs) [5, 7]. In addition, regulatory role of COX pathway in these responses has been investigated [3, 5, 7].

Lipopolysaccharide (LPS), a ubiquitous cell wall component of gram-negative bacteria, is known to participate in the pathogenesis of CRSwNPs, particularly mucin production [8]. Exposure to LPS showed heterogeneous

effects on eosinophilic inflammation in the airway [9–12]. Experimental studies have demonstrated that exposure to LPS suppresses eosinophilic inflammation by immune deviation towards Th1 responses or triggering nitric oxide synthase 2 activity [9, 10]. In contrast, a significant increase in eosinophil count was seen in nasal/bronchial lavage fluid following exposure to allergen and LPS in patients with allergic asthma [11, 12]. However, little is known about whether or not LPS affects eosinophilia-associated cytokine production reflecting Th responses in CRSwNP.

To determine whether or not the exposure to LPS affects the pathogenesis of CRSwNP, we investigated the effects of LPS on SEB-induced Th1, Th2 and Th17-associated cytokine production using a recently developed *ex vivo* model [3, 5]. In addition, we investigated the role of COX metabolism, particularly PGE_2, in the regulatory effects of LPS on SEB-induced cytokine production by DNPCs, as LPS is known to induce COX expression and PGE_2 production in various cells and organs including nasal epithelial cells from patients with CRS [13–15]. We believe that the present findings provide new insight into the role of LPS in the pathogenesis of eosinophilic airway inflammation, and a basis for the critical role of PGE_2 on the action of LPS.

Materials and methods

Patients

Fourteen Japanese patients (age range, 15–72 years; median age, 41.5 years; 11 men and 3 women) with CRSwNP were studied. CRSwNP was defined using the diagnostic criteria of Benninger et al. [16]. Four patients were asthmatic, and none were thought to exhibit aspirin intolerance based on their history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs. All patients were resistant to medical treatment, including macrolide therapy, and thus had endonasal sinus surgery. None of the participants received systemic glucocorticoids for a period of at least 8 weeks prior to surgery, and none received pharmacotherapy for sinusitis, such as macrolide antibiotics or intranasal glucocorticoids, for a period of at least 3 weeks prior to surgery. Informed consent for participation in the study was obtained from each patient, and the study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Antigen and reagents

We purchased the following study materials: SEB (Toxin Technology, Sarasota, FL, USA); LPS, RPMI-1640, L-glutamine-penicillin-streptomycin solution, protease, collagenase (Type I), hyaluronidase, DNase I, and SQ22536 (Sigma, St. Louis, MO, USA); diclofenac sodium (Wako Pure Chemicals, Osaka, Japan); FCS (Invitrogen, Carlsbad, CA, USA); red blood cell lysis buffer (Roche, Indianapolis, IN, USA); and PGE_2 (Cayman, Ann Arbor, MI, USA). The receptor-selective agonists for EP1, (ONO-DI-004), EP2 (ONO-AE1-259-01), EP3 (ONO-AE-248) and EP4 (ONO-AE1-329) were provided by Ono Pharmaceuticals (Osaka, Japan). PGE_2 and EP receptor-selective agonists were dissolved to a stock concentration of 10^{-2} M in DMSO (Sigma) and stored at −80°C until use.

Preparation of DNPCs

Dispersed nasal polyp cells were prepared from nasal polyps by enzymatic digestion, as described previously [5]. Briefly, minced nasal polyps were incubated for 2 h at 37°C in RPMI 1640 (1 g tissue per 4 ml) containing 2.0 mg/ml protease, 1.5 mg/ml collagenase, 0.75 mg/ml hyaluronidase and 0.05 mg/ml DNase. The cell suspension was then filtered through a 70-μm cell strainer (BD Falcon, Bedford, MA, USA) to remove any undigested tissue, and was washed twice with washing medium (RPMI 1640 supplemented with 2% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). The cell pellet was resuspended in erythrocyte lysis buffer and washed with washing medium. After washing, DNPCs were suspended in culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). 8.5 ± 5.3%, 11.7 ± 8.9%, 8.9 ± 8.2%, 8.5 ± 6.8%, 7.8 ± 11.1%, 10.9 ± 10.5%, 15.5 ± 6.7% and 21.6 ± 7.7% cells in DNPC express c-kit, ECP/EPX, CD79α, CD68, CD4, CD8, cytokeratin and vimentin respectively [5]. DNPCs were immediately used for the following experiments after the preparation.

Cell cultures and cytokine determination

In flat-bottomed 48-well culture plates (Asahi Techno Glass, Tokyo, Japan), 500 μl of 1 × 10^6/ml DNPCs were stimulated with 1 ng/ml SEB at 37°C in a 5% CO_2/air mixture because our preliminary study indicated that 72 h incubation is the optimal period to induce a substantial production of IL-5 by DNPCs without microbial contamination. The culture supernatant was collected after 72 h and stored at −80°C, after which levels of IL-5, IL-13, IL-17A and IFN-γ were determined by ELISA [3, 5]. Levels of IL-5, IL-13 and IFN-γ were measured using Opt EIA sets (BD Biosciences), according to the manufacturer's instructions. Levels of IL-17A were measured using a DuoSet ELISA development kit (R&D Systems, Minneapolis, MN, USA). The detection limit of these assays was 4 pg/ml for IL-5, 2 pg/ml for IL-13, 8 pg/ml for IL-17A and 4 pg/ml for IFN-γ.

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Effects of LPS on SEB-induced cytokine production by DNPCs

Dispersed nasal polyp cells were pre-treated with LPS at 0.2 or 2.0 μg/ml 2 h prior to SEB stimulation to explore the effects of LPS on SEB-induced cytokine production by DNPCs. Alternatively, 0.2 μg/ml LPS was added to the culture at 1, 12 or 24 h after SEB stimulation. To determine the role of COX and PGE_2 in the effect of LPS on SEB-induced cytokine production, DNPCs were pre-treated with 10^{-5} M diclofenac in the presence or absence of 10^{-6} M PGE_2 or control buffer (0.05% DMSO) prior to LPS treatment.

Effects of COX and PGE_2 on LPS-induced cytokine production by DNPCs

Dispersed nasal polyp cells were solely cultured with and without 0.2 μg/ml LPS for 72 h, after which levels of cytokines were determined. To determine whether or not COX and PGE_2 are involved in LPS-induced cytokine production, DNPCs were pre-treated with 10^{-5} M diclofenac or SC-791 (Merk KGaA, Darmstadt, Germany), a selective COX-2 inhibitor, in the presence or absence of 10^{-6} M PGE_2, four EP receptor-selective agonists or control buffer (0.05% DMSO). Our preliminary study showed that DMSO concentrated from 0.001% to 0.1% had no significant effect on the viability of DNPCs for either 24 h or 72 h incubation as determined by trypan blue dye exclusion test. To determine adenylate cyclase activity, DNPCs were incubated with SQ22536, an inhibitor of adenylate cyclase, at 37°C for 1 h. Following incubation, the cells were washed with culture medium twice, after which they were treated with diclofenac in the presence of either PGE_2 or control buffer, then the cells were stimulated with LPS.

Effects of LPS on COX-mediated PGE_2 metabolism in DNPCs

To determine whether or not LPS affects COX-mediated PGE_2 metabolism, 1×10^6/ml DNPCs ($n = 8$) were cultured in the presence or absence of 0.2 μg/ml LPS for 2 and 24 h. Extraction of total cellular RNA, reverse transcription to generate cDNA, and real-time quantitative PCR for COX-1, COX-2 and microsomal PGE_2 synthase-1 (m-PGES-1) was then performed, as described previously [17]. The amounts of GAPDH, for which primers were purchased from Toyobo (Osaka, Japan), were used as an internal control. The absolute copy number for each sample was calculated, and samples were reported as copy numbers relative to GAPDH. The concentration of PGE_2 in the supernatants after 72 h culture with 0.2 μg/ml LPS in DNPCs ($n = 9$) was also determined using a PGE_2 EIA kit (Cayman). The detection limit was 7.8 pg/ml.

Statistical analysis

The data were looked as ratio to baseline, and values are given as medians. Nonparametric Mann–Whitney *U*-test was used to compare data between groups, and Wilcoxon signed-rank test was used for analysis within groups. One-way repeated-measures ANOVA and multiple comparisons with Bonferroni method was used to examine the data among three or more groups. *P* values of less than 0.05 were considered to be statistically significant. Statistical analyses were performed with SPSS software (version 11.0 SPSS, Chicago, IL, USA).

Results

Pre-treatment with LPS inhibits SEB-induced Th1/Th2/Th17 cytokine production by DNPCs

We have previously shown that SEB induced not only Th2-associated IL-5 and IL-13 production, but also Th17-assciated IL-17A production by DNPCs [3, 5]. In the present study, we confirmed that DNPCs produced a substantial amount of IFN-γ in response to SEB ($P < 0.001$, Fig. 1).

Pre-treatment with 0.2 μg/ml LPS 2 h prior to SEB stimulation significantly inhibited SEB-induced IL-5 (25.1% inhibition, $P < 0.001$), IL-13 (30.6% inhibition, $P < 0.001$), IL-17A (13.6% inhibition, $P = 0.022$) and IFN-γ (28.0% inhibition, $P = 0.002$) production by DNPCs (Fig. 1). The presence of asthma did not affect the inhibitory role of LPS on SEB-induced cytokine production (data not shown). On the other hand, addition of 0.2 μg/ml LPS after SEB stimulation had no inhibitory effect on cytokine production, except for IL-5 production with LPS treatment at 1 h after SEB stimulation ($P = 0.012$) (Fig. 2). Rather, addition of LPS at 24 h after SEB stimulation significantly enhanced SEB-induced IFN- γ production ($P = 0.036$, Fig. 2D). Thus, we used LPS at a concentration of 0.2 μg/ml for additional analysis.

LPS induces COX and PGE_2 expression in DNPCs

To determine how pre-treatment with LPS exerts its inhibitory effects on SEB-induced cytokine production, we focused on the COX-mediated PGE_2 pathway. The amount of COX-1 mRNA was not significantly altered after stimulation with LPS (Fig. 3A). However, the amount of COX-2 mRNA in DNPCs increased significantly at 2 h after stimulation with LPS ($P = 0.012$, Fig. 3B), and this increase was sustained at 24 h ($P = 0.012$, Fig. 3B). The amount of m-PGES-1 mRNA was increased at 24 h but not at 2 h post stimulation with LPS ($P = 0.012$, Fig. 3C). At the functional level,

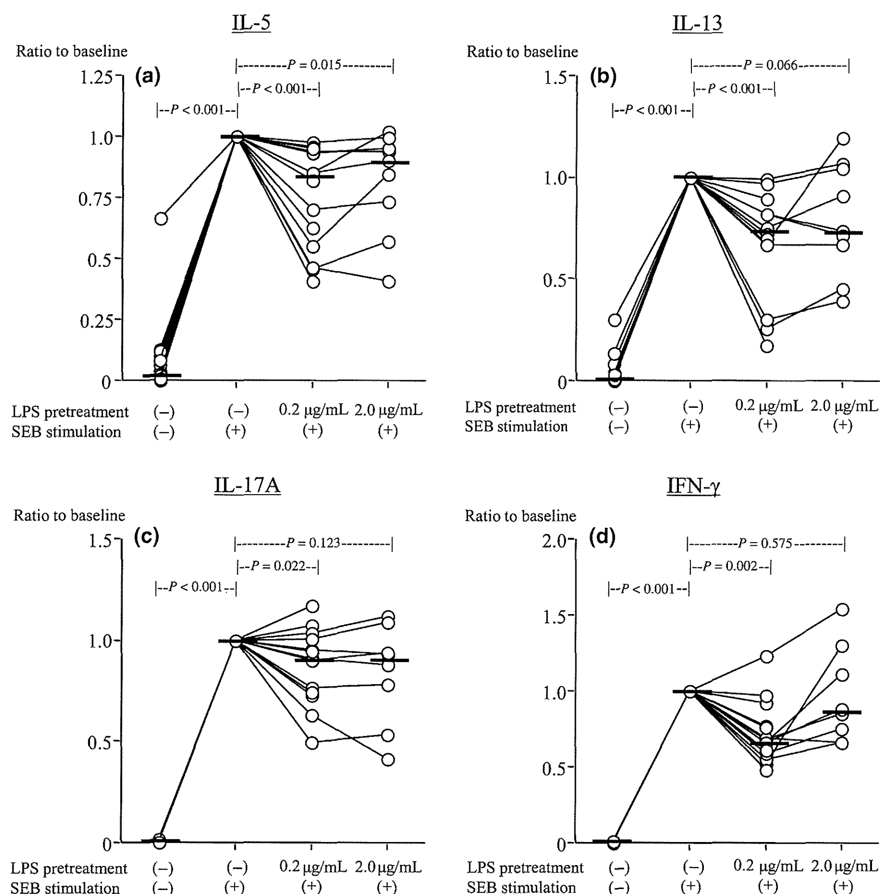

Fig. 1. Effects of pre-treatment with LPS on SEB-induced cytokine production by DNPCs. DNPCs were treated with 0, 0.2 or 2.0 μg/ml LPS 2 h prior to SEB stimulation. After 72 h of incubation with SEB, levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) within the supernatant were determined. The data were looked as differences from baseline. Bars represent median values. P values were determined using Wilcoxon signed-rank test. LPS, Lipopolysaccharide; DNPC, dispersed nasal polyp cells; SEB, staphylococcal enterotoxin B.

DNPCs displayed a significant increase in PGE_2 production in response to LPS for 72 h ($P = 0.021$, Fig. 3D).

COX-derived PGE_2 displays a crucial role in the inhibitory effects of LPS on SEB-induced cytokine production

Based on the above information, we examined whether or not COX blockade affects the inhibitory effects of LPS on SEB-induced cytokine production. Treatment with diclofenac significantly enhanced SEB-induced IL-5 ($P < 0.001$) and IL-13 ($P = 0.012$) production and conversely suppressed SEB-induced IL-17A production ($P < 0.001$), validating our previous studies [3, 5]. There was a trend for enhanced SEB-induced IFN-γ production by diclofenac treatment ($P = 0.075$).

In the presence of diclofenac, the suppressive effects of pre-treatment with LPS on SEB-induced cytokine production were blocked. Rather, LPS pre-treatment significantly enhanced SEB-induced IL-5 ($P = 0.019$, Fig. 4A), IL-13 ($P = 0.006$, Fig. 4B) and IL-17A ($P = 0.026$, Fig. 4C) production. There was a trend for enhanced SEB-induced IFN-γ production ($P = 0.064$, Fig. 4D).

As compared with control buffer, addition of 10^{-6} M PGE_2 significantly reversed the enhancement by diclofenac on SEB-induced IL-5 ($P = 0.008$, Fig. 4A), IL-13 ($P = 0.008$, Fig. 4B) and IFN-γ ($P = 0.008$, Fig. 4D) production by LPS-pre-treated DNPCs. These results were similar to the effect when we pre-treated cells with LPS in Fig. 1. On the other hand, IL-17A production was further increased in the presence of PGE_2 ($P = 0.011$, Fig. 4C).

COX-derived PGE_2 controls LPS-induced cytokine production by DNPCs via EP2/EP4-mediated pathway

Finally, we investigated whether or not LPS alone induced cytokine production in DNPCs. Stimulation with 0.2 μg/ml LPS did not induce significant production of IL-5 ($P = 0.117$, Fig. 5A), IL-13 ($P = 0.209$, Fig. 5B), IL-17A ($P = 0.655$, Fig. 5C) or IFN-γ ($P = 0.062$, Fig. 5D) by DNPCs. However, in diclofenac-treated DNPCs, LPS

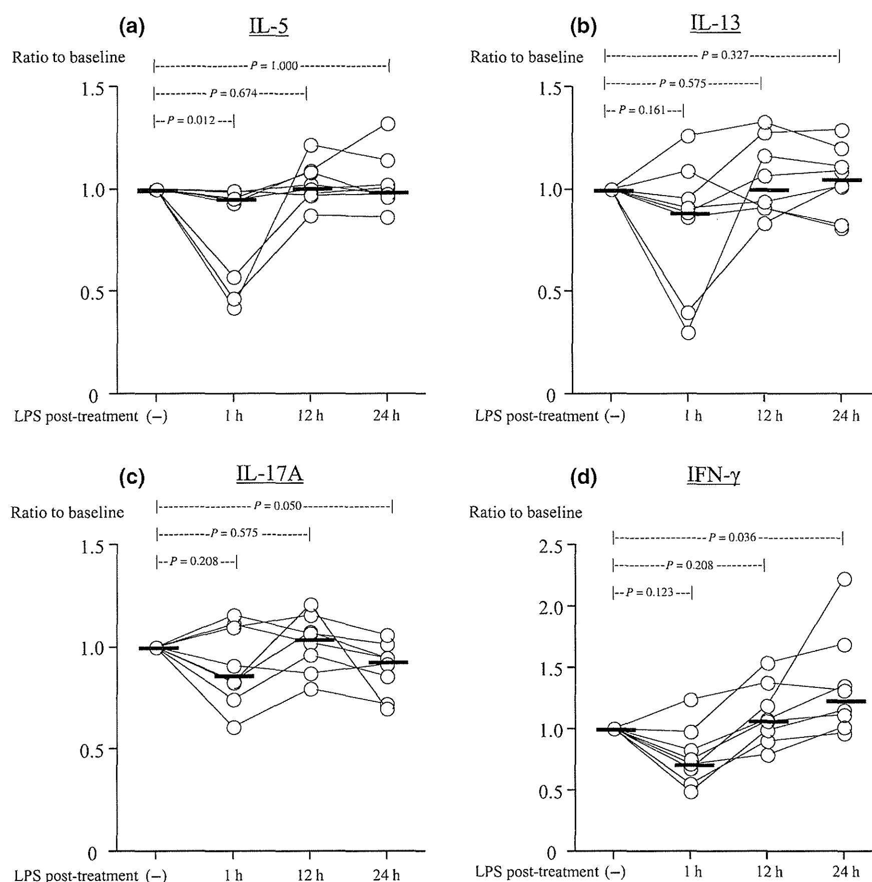

Fig. 2. Effects of post-treatment with LPS on SEB-induced cytokine production by DNPCs. At 1, 12 or 24 h after SEB stimulation, DNPCs were exposed to 0.2 μg/ml LPS. After 72 h of incubation with SEB, levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) were determined. Bars represent median values. The data were looked as differences from baseline. *P* values were determined using Wilcoxon signed-rank test. LPS, Lipopolysaccharide; DNPC, dispersed nasal polyp cells; SEB, staphylococcal enterotoxin B.

significantly induced IL-5 ($P = 0.001$, Fig. 5A), IL-13 ($P = 0.002$, Fig. 5B) and IFN- γ ($P = 0.003$, Fig. 5D) production. LPS stimulation did not affect IL-17A production by DNPCs, even in the presence of diclofenac ($P = 0.593$, Fig. 5C). Treatment with SC-791, a selective COX-2 inhibitor, also significantly induced IL-5 ($P = 0.007$), IL-13 ($P = 0.005$) and IFN- γ ($P = 0.013$) but not IL-17A ($P = 0.944$) production by LPS-stimulated DNPCs, suggesting that LPS-induced COX-2 is involved in the pathogenesis of CRSwNP.

Addition of PGE_2 significantly inhibited LPS-induced IL-5 ($P = 0.002$, Fig. 6A) and IL-13 ($P = 0.022$, Fig. 6B) production, but not IL-17A ($P = 0.109$, Fig. 6C) or IFN- γ ($P = 0.317$, Fig. 6D) production by diclofenac-treated DNPCs. When we used four EP receptor-selective agonists, one-way repeated-measures ANOVA showed that treatment with these agonists significantly altered LPS-induced IL-5 ($P = 0.007$) and IL-13 ($P < 0.001$) production by diclofenac-treated DNPCs. Treatment with EP2 ($P < 0.001$) and EP4 ($P < 0.001$) receptor-selective agonist significantly suppressed the IL-5 production. Treatment with EP2 and the EP4 receptor selective agonist also inhibited the IL-13 production ($P < 0.001$, Fig. 6F). Pre-treatment with SQ22536 significantly reversed the inhibitory effect of PGE_2 on LPS-induced IL-5 ($P = 0.005$) and IL-13 ($P = 0.005$) production (Fig. 7).

Discussion

In the present study, we investigated the regulatory effects of LPS on SEB-induced Th1-, Th2- and Th17-associated cytokine productions in ex vivo model of CRSwNP. Our results demonstrated that exposure to LPS induced a substantial suppression in SEB-induced IL-5, IL-13, IFN-γ and IL-17A production in a dose and phase-dependent fashion, whereas this exposure inversely increased these productions when the COX pathway was blocked. Moreover, LPS itself induced IL-5, IL-13 and IFN-γ production, but not IL-17A production, by DNPCs when the COX pathway was blocked, and addition of PGE_2 blocked LPS-induced IL-5 and IL-13

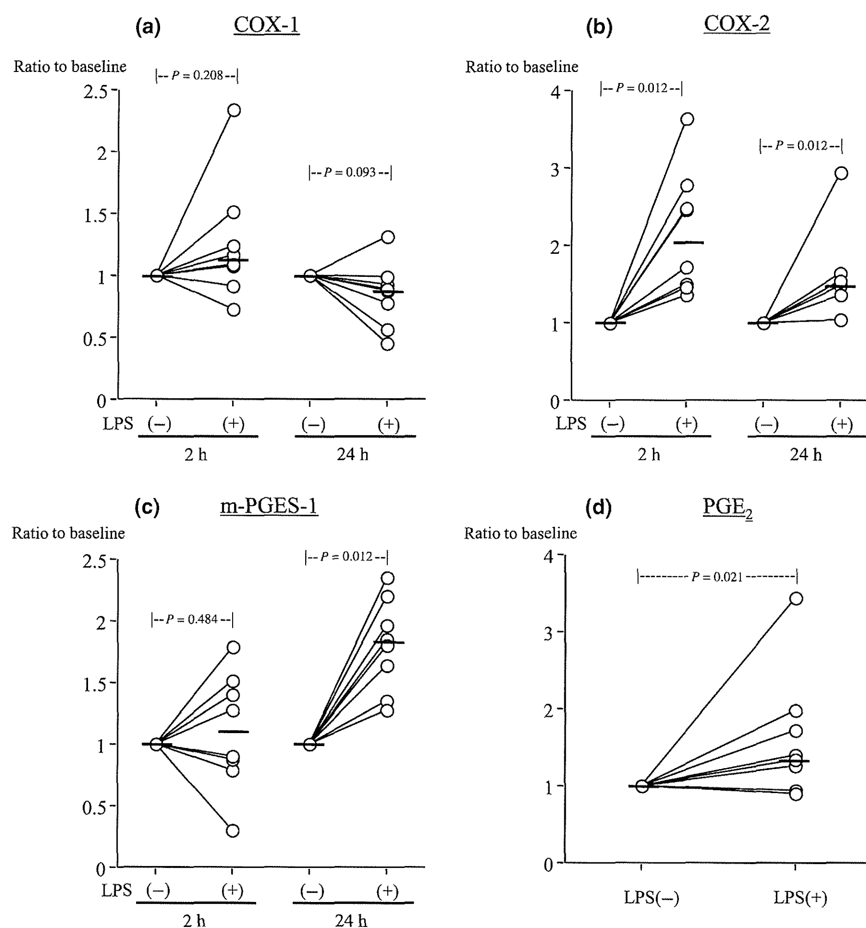

Fig. 3. Effects of LPS on expression of COX-related molecules in DNPCs. DNPCs were cultured with or without 0.2 μg/ml LPS for 2 or 24 h, and relative amounts of COX-1 (A), COX-2 (B) and m-PGES-1 (C) mRNA were determined. Alternatively, levels of PGE_2 after 72 h of incubation with LPS were measured (D). The data were looked as differences from baseline. Bars represent median values. *P* values were determined using Wilcoxon signed-rank test. LPS, Lipopolysaccharide; DNPC, dispersed nasal polyp cells.

production. Taken together with the finding that LPS enhanced expression of COX-2 and m-PGES-1 mRNA, as well as production of PGE_2 in DNPCs, the main advancement in knowledge offered by this study is that the regulatory effect of LPS on the pathogenesis of CRSwNP is critically regulated by COX/PGE_2 axis.

The major Th2 cytokines IL-5 and IL-13 are associated with eosinophilic inflammation [18]. High levels of IL-5 in nasal secretions are a specific biomarker for CRSwNP [19]. It has been reported that the exposure to LPS can either protect or exacerbate eosinophilic inflammation [9–12]. The present results support both findings, and suggest that the detrimental or alleviative effects of LPS on eosinophilic inflammation in CRSwNP are dependent on the activation of the COX pathway. In addition, EP2 and EP4 receptor-selective agonists, as well as PGE_2 itself, cancelled LPS-induced IL-5 and IL-13 production by diclofenac-treated DNPCs. It is known that PGE_2 is able to inhibit eosinophilic inflammation and Th2 cytokine production [8, 20]. Together with the finding that the pre-treatment with SQ22536 significantly cancelled the effect of PGE_2, the present results suggest that LPS-induced PGE_2 through COX-2 activation displays a critical role in controlling eosinophilic inflammation via cAMP-dependent EP2- and EP4-mediated pathways in CRSwNP. In addition, we have previously reported that m-PGES-1 was selectively expressed on CD68+ cells in nasal polyps, suggesting that nasal polyp macrophages are involved in the regulatory effects of LPS on these cytokine productions.

The characterization and role of IFN-γ, the major Th1 cytokine, in the pathogenesis of CRSwNP has been recently clarified [21, 22]. Our results suggest that exposure to LPS inhibits not only Th2 responses but also Th1 responses induced by SEB in DNPCs. The inhibition of SEB-induced IFN-γ production by LPS may be mediated by the induction of PGE_2 by LPS, as PGE_2 is known to suppress IFN-γ production under various conditions [23, 24]. The present findings

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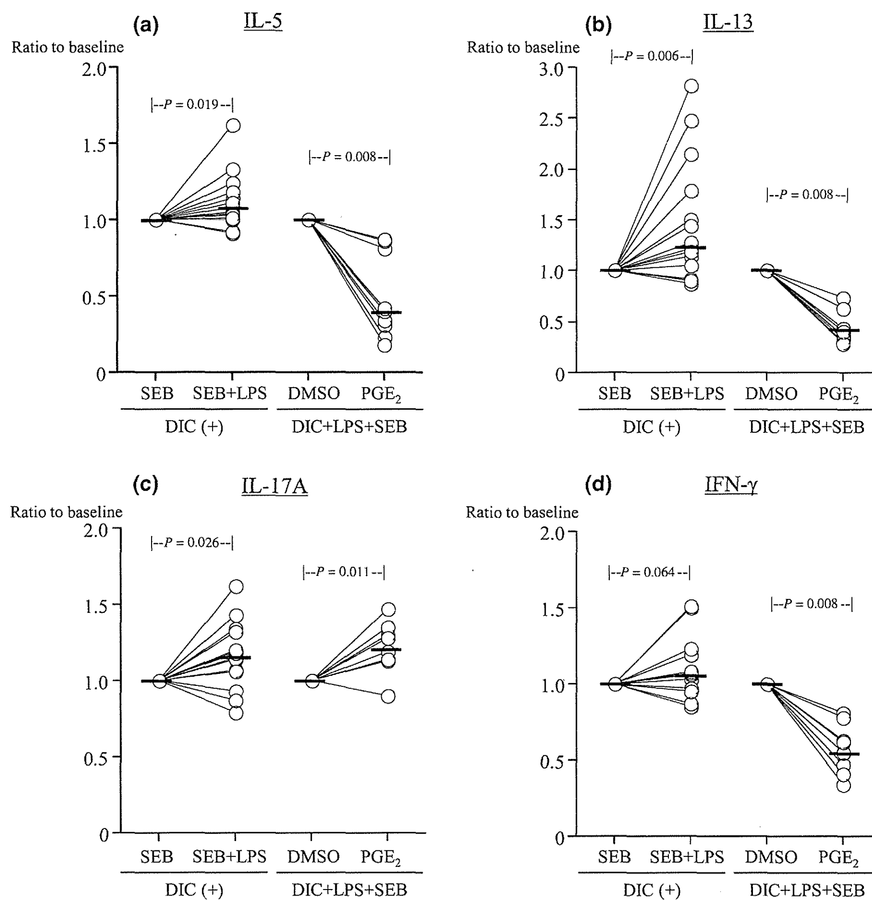

Fig. 4. Effects of LPS and PGE_2 on SEB-induced cytokine production in the presence of diclofenac. Diclofenac-treated DNPCs were exposed or unexposed to LPS prior to SEB stimulation (left side of each graph). Under these conditions, either 10^{-6} M PGE_2 or control buffer was added to the culture (right side of each graph). Levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) were determined and changes from baseline were expressed. The data were looked as differences from baseline. Bars represent median values. *P* values were determined using Wilcoxon signed-rank test. LPS, Lipopolysaccharide; DNPC, dispersed nasal polyp cells; SEB, staphylococcal enterotoxin B.

further suggest that the inhibitory effect of LPS on SEB-induced eosinophilia-associated Th2 cytokine productions is not attributable to Th1/Th2 cross-regulation. In Fig. 1D, one patient showed an outlier response of increased IFN-γ production in response to pre-treatment with 0.2 μg/ml LPS. Since the patient did not exhibit infection on board or increased neutrophilia in nasal polyps, the reason for this outlier response was not clear.

Similar to IL-5 and IL-13, LPS alone did not affect IFN-γ production by DNPCs, but induced IFN-γ production when COX was blocked. On the other hand, unlike IL-5 and IL-13, addition of PGE_2 did not block LPS-induced IFN-γ production in diclofenac-treated DNPCs. Although the production of IFN-γ in response to LPS was modest, these results suggest that prostanoids other than PGE_2 have a potent inhibitory effect on LPS-induced IFN-γ production.

A major Th17 cytokine, IL-17A is expressed by macrophages, CD4+ T cells and eosinophils in NPs, and its expression is correlated with the degree of eosinophilia in sinonasal tissues [3, 25]. Exposure to LPS also inhibits IL-17A production induced by SEB in DNPCs. However, this inhibition is marginal as compared with IL-5, IL-13 and IFN-γ. In addition, LPS did not induce IL-17A production by DNPCs either with or without COX blockade. Although IL-17A can be produced by exposure to LPS both *in vivo* and *in vitro*, our results suggest that the inhibitory effect of LPS on SEB-induced Th17 response was weak as compared with Th1 and Th2 responses [26].

Unlike IL-5, IL-13 and IFN-γ, addition of PGE_2 enhanced the effects of LPS on SEB-induced IL-17A production by diclofenac-treated DNPCs. This is consistent with recent reports showing that PGE_2 promotes IL-17A production and Th17 differentiation [3, 27]. For example, we recently showed that PGE_2 enhanced SEB-induced IL-17A production by diclofenac-treated DNPCs [3]. One of the reasons why pre-treatment with LPS inhibits SEB-induced IL-17A production despite PGE_2

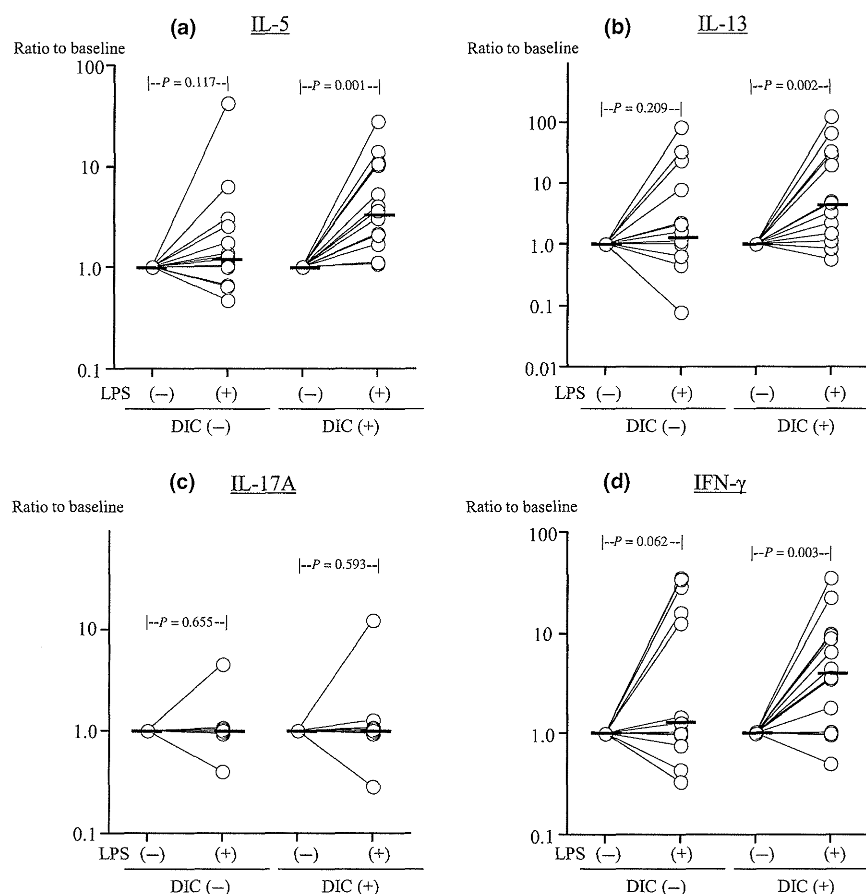

Fig. 5. Effects of diclofenac on LPS-induced cytokine production by DNPCs. Diclofenac-treated or untreated DNPCs were cultured with or without 0.2 μg/ml LPS for 72 h. Levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) were determined. The data were looked as differences from baseline. Bars represent median values. *P* values were determined using Wilcoxon signed-rank test. LPS, Lipopolysaccharide; DNPC, dispersed nasal polyp cells.

further increase IL-17A production from LPS- and SEB-stimulated cells in the presence of diclofenac, may be that LPS-induced prostanoids contrary to PGE_2 may have an inhibitory effect on SEB-induced IL-17A production.

The present study demonstrated that the inhibitory effects of LPS on SEB-induced cytokine productions were dose- and phase-dependent. Exposure to LPS after SEB stimulation had little effects on SEB-induced cytokine production. This is consistent with a recent report demonstrating that exposure to LPS after the first immunization with allergen has little effect on allergen tolerance, thus suggesting that pre-exposure to LPS is critical for the inhibitory effects on SEB-induced cytokine productions [28].

Various concentrations of LPS were used for human *in vitro* and/or *ex vivo* studies. Nanogram quantities of LPS are normally used in subcultured cell lines including human nasal fibroblasts [29]. However, microgram quantities of LPS are often used in freshly isolated bulk cell lines including peripheral blood mononuclear cells and cord blood mononuclear cells [30, 31]. Since DNPCs were also freshly isolated bulk cell lines, we think that 0.2 μg/ml is an acceptable concentration to analyse the effect of LPS in the present study.

In conclusion, LPS can play both a beneficial and a harmful role in the pathology of CRSwNP, and LPS-derived COX-2/PGE_2 axis is critically involved. Several reports demonstrate that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) increases the risk of adult-onset asthma [32, 33]. For clinical implications, although there is no any clinical evidence that NSAIDs treatment can increase symptoms (rhinorrhea, nasal obstruction, anosmia), polyp size or number of polypectomies, the present ex vivo study may suggest that avoidance of indiscreet regular use of NSAIDs is preferable for patients with CRSwNP. In addition, these observations may provide a basis for novel therapeutic approaches targeting LPS and other components of microbes the management of eosinophilic airway diseases such as CRS-NP, allergic rhinitis and bronchial asthma.

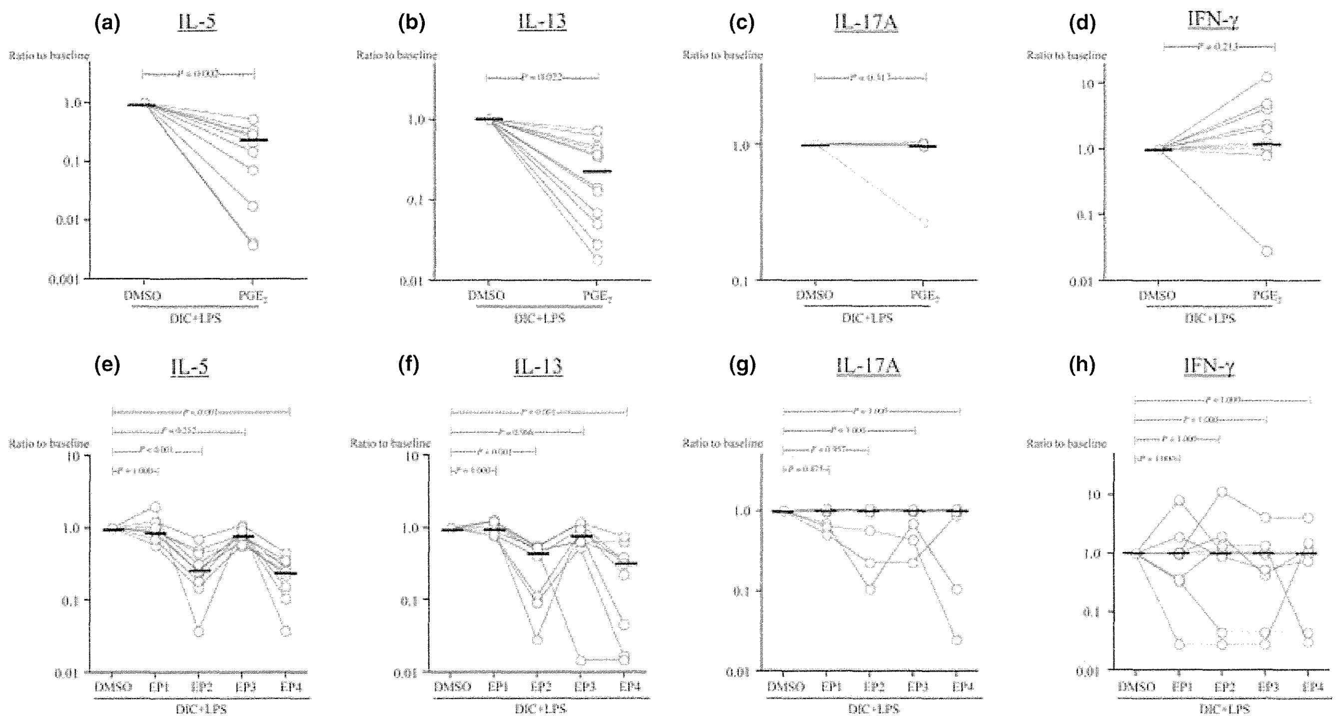

Fig. 6. Effects of PGE_2 and EP-selective agonists on LPS-induced cytokine production by diclofenac-treated DNPCs. Diclofenac-treated DNPCs were cultured with LPS in the presence of either 10^{-6} M PGE_2 (A–D), EP-selective agonists (E–H) or control buffer for 72 h. Levels of IL-5 (A, E), IL-13 (B, F), IL-17A (C, G) and IFN-γ (D, H) within the supernatant were determined. The data were looked as differences from baseline. Bars represent median values. *P* values were determined using Wilcoxon signed-rank test (A–D) and One-way repeated-measures ANOVA and multiple comparisons with Bonferroni method (E–H). LPS, Lipopolysaccharide; DNPC, dispersed nasal polyp cells.

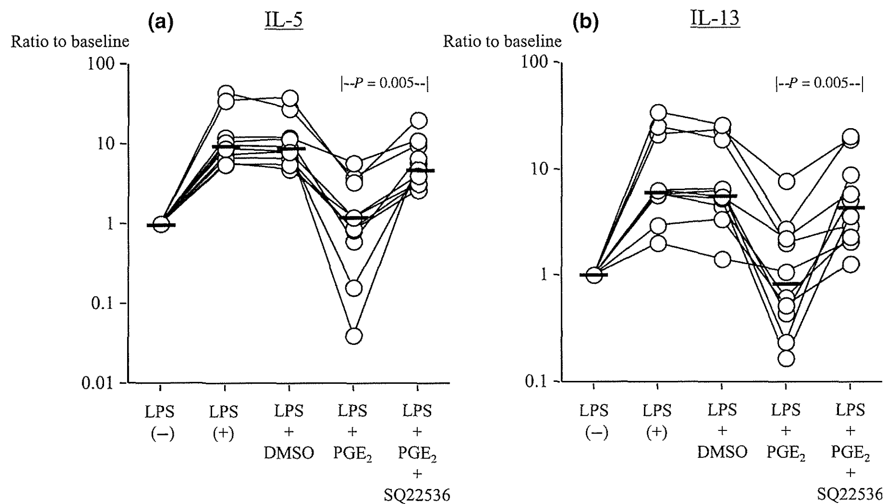

Fig. 7. Reversal of inhibitory effect of PGE_2 on LPS-induced IL-5 and IL-13 production by diclofenac-treated DNPCs with adenylate cyclase inhibitor. DNPCs were pre-treated with SQ22536 at 37°C for 1 h. Following incubation, the cells were washed with culture medium twice, after which they were treated with diclofenac in the presence of either PGE_2 or control buffer, then the cells were stimulated with LPS for 72 h. Levels of IL-5 (A) and IL-13 (B) within the supernatant were determined. Bars represent median values. *P* values were determined using Wilcoxon signed-rank test. LPS, Lipopolysaccharide; DNPC, dispersed nasal polyp cells.

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REVIEW ARTICLE

Sublingual immunotherapy for Japanese cedar pollinosis: current status in Japan

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Clinical & Experimental Allergy Reviews

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Summary

Immunotherapy is not generally used in Japan and very few medical institutions perform it. For radical treatment, immunotherapy should be selected. However, subcutaneous immunotherapy has not yet gained acceptance. Japanese cedar pollinosis is a seasonal allergic rhinitis that is indigenous to Japan and occurs with a nationwide mean prevalence of 25%. Clinical research on sublingual immunotherapy for Japanese cedar pollinosis has made progress and it should come into practical use within 2–3 years. If sublingual immunotherapy becomes practical, it should provide a new treatment option for Japanese cedar pollinosis.

Keywords cytokines, immunotherapy, Japanese cedar pollinosis, subcutaneous immunotherapy, sublingual immunotherapy

Introduction

Japanese cedar pollinosis is a seasonal allergic rhinitis that only occurs in Japan and affects the daily activities of Japanese people for about 3 months from February to early May. The number of patients has increased recently, and rose by 10% over 10 years according to the *Practical guideline for the management of allergic rhinitis in Japan* 2009 (6th Revised Edition) [1, 2]. Unlike grass pollens and ragweed pollens, Japanese cedar pollens can be dispersed for long distances of several tens of kilometres, and there are many patients in urban areas as well as in rural areas. The pollens are dispersed over a wide area from Honshu to Chugoku, Shikoku and Kyushu. The prevalence of Japanese cedar pollinosis is especially high in areas from the Kanto region to the Pacific Ocean side of the Tokai region [3]. The quality of life (QOL) deterioration [4] and decreased social productivity [5] caused by Japanese cedar pollinosis are high. It is hoped that an effective method of treatment will be developed soon.

The principles of treatment of allergic rhinitis are (i) antigen elimination and avoidance, (ii) drug therapy, (iii) surgical therapy and (iv) immunotherapy. As a defence against pollens in Japan, patients wear eyeglasses and facemasks when going outdoors [6]. For drug therapy, prophylactic treatments have been widely used for about 20 years. From about 2 weeks before the predicted date of the first pollen dispersion in the middle of February, i.e. from a time when no symptoms have appeared, administration of drugs such as second-generation antihistamines is started. It has been reported that such prophylactic treatments inhibit worsening of the disease severity [7–9]. In principle, surgical therapy is conducted for patients in whom conventional therapy is ineffective. Furthermore, with the advancement of laser devices, patients no longer need to be hospitalized for these surgical procedures. In Japan, immunotherapy as a radical therapy is only performed in some university hospitals or clinics specializing in allergies. In comparison with Western countries, the use of such treatments is very limited. The reasons for this include problems related to the Japanese healthcare system and the incidences of adverse drug reactions. Standardized treatment extracts for subcutaneous injection to treat Japanese cedar pollinosis first went on the market in 2000, and have therefore only been used for about 10 years.

Under such conditions, attention has been focused on sublingual immunotherapy. If sublingual immunotherapy comes into wide use, it is expected to be a safe and simple treatment with few adverse drug reactions that can be performed at home. If treatment extracts for sublingual immunotherapy are marketed, it is possible that immunotherapy will soon become widespread.

Table 1. Administration schedule for sublingual immunotherapy. Reprinted with permission from Ref. [13].

	Week 1 2 JAU/ ml	Week 2 20 JAU/ ml	Week 3 200 JAU/ ml	Week 4 2000 JAU/ ml	Week 5 2000 JAU/ ml
Day 1	1 drop	1 drop	1 drop	1 drop	
Day 2	2 drops	2 drops	2 drops	2 drops	
Day 3	3 drops	3 drops	3 drops	4 drops	20 drops
Day 4	4 drops	4 drops	4 drops	8 drops	
Day 5	6 drops	6 drops	6 drops	12 drops	
Day 6	8 drops	8 drops	8 drops	18 drops	
Day 7	10 drops	10 drops	10 drops	20 drops	20 drops

The number of drops refers to the amount of allergen solution applied sublingually.

Subcutaneous immunotherapy for Japanese cedar pollinosis

Since its first report by Noon in 1911 [10], immunotherapy by subcutaneous injection has had a history of 100 years. A treatment extract for house dust mites, which was the first immunotherapy in Japan, went on sale in 1963. Progress was not made in the development of treatment extracts thereafter, and the first standardized treatment extract for Japanese cedar pollinosis in Japan only went on the market in 2000. It means that few advances had been made in nearly 40 years since the development of the treatment extract for house dust mites. When patients with Japanese cedar pollinosis are given subcutaneous immunotherapy, improvements in their clinical symptoms and even long-term remission have been reported [11].

Sublingual immunotherapy for Japanese cedar pollinosis

Clinical research on sublingual immunotherapy for Japanese cedar pollinosis started in Nippon Medical School in 2003, following approval from the Ethics Committee of Nippon Medical School. The treatment schedule is shown in Table 1. Sublingual spit, in which the

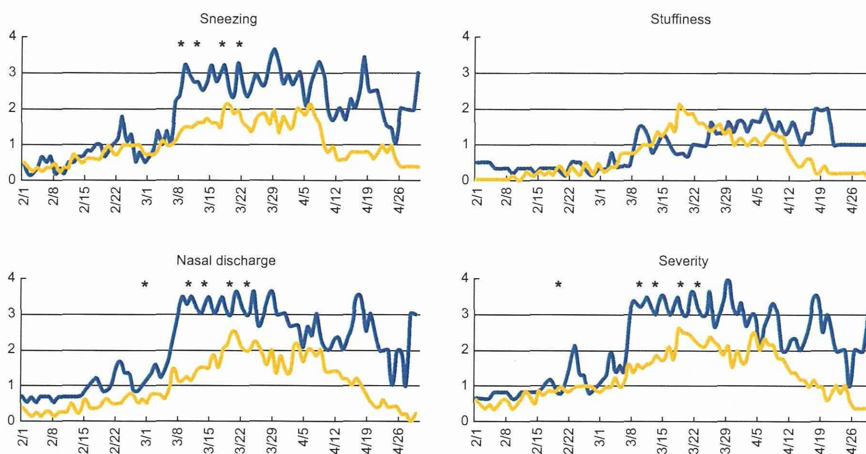

Fig. 1. Changes in the nasal symptom scores in the Nippon Medical School Study. Yellow line, sublingual immunotherapy; blue line, placebo. *$P < 0.05$, significant difference between the two groups.

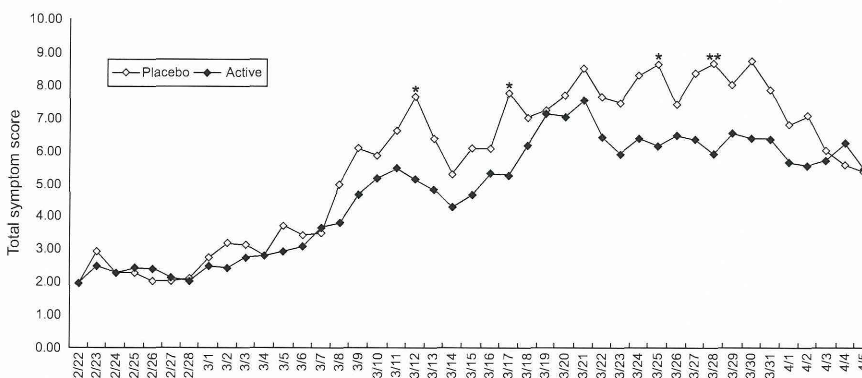

Fig. 2. Mean changes in the total symptom scores in the Ministry of Health, Labour and Welfare Study. *$P < 0.05$, significant difference between the two groups. Reprinted with permission from Ref. [13].

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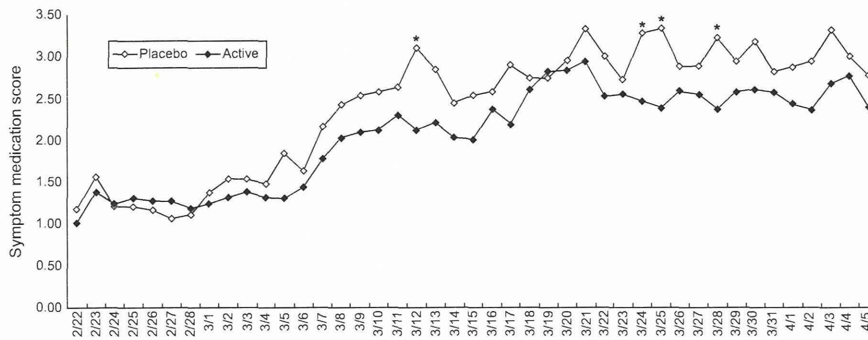

Fig. 3. Mean changes in the symptom medication scores in the Ministry of Health, Labour and Welfare Study. *$P < 0.05$, significant difference between the two groups. Reprinted with permission from Ref. [13].

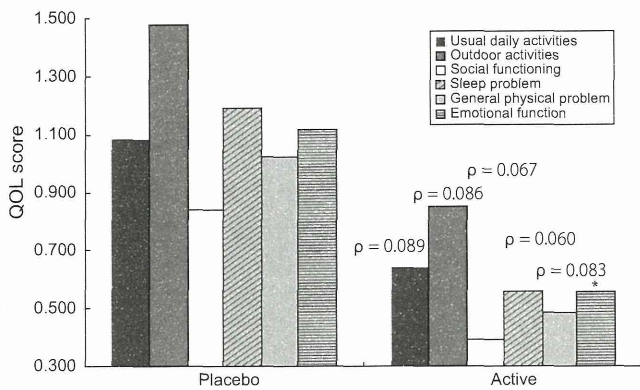

Fig. 4. Mean changes in the deterioration of the quality of life (QOL) domains of the Japanese Rhinitis QOL Standard Questionnaire No. 1 in the Ministry of Health, Labour and Welfare Study. *$P < 0.05$, significant difference between the two groups. Reprinted with permission from Ref. [13].

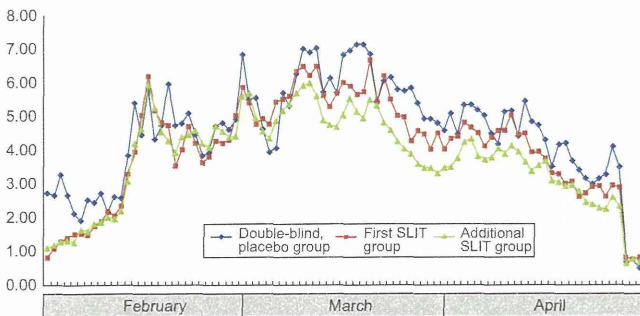

Fig. 5. Mean changes in the total symptom scores in the Nippon Medical School Study when continued for a second year.

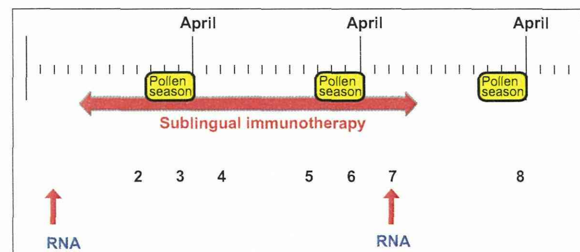

Fig. 6. Collaborative research by the Tokyo Metropolitan Institute of Medical Science, Nippon Medical School and Tokyo Metropolitan Hospitals from 2006 to 2008. The schedule for the sublingual immunotherapy is shown.

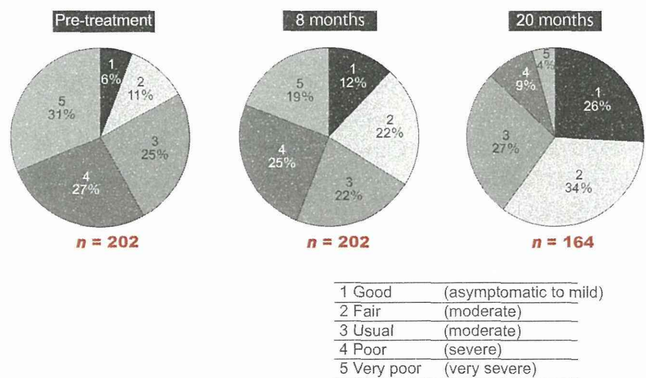

Fig. 7. Changes in the severity of symptoms according to the duration of sublingual immunotherapy in the collaborative research by the Tokyo Metropolitan Institute of Medical Science, Nippon Medical School and Tokyo Metropolitan Hospitals from 2006 to 2008.

prepared extract is dropped onto bits of bread, held sublingually for a few minutes and then expectorated, was adopted in this research. In the initial phase of the research, comparisons were made with a drug treatment group in an open-label study. The results showed that the symptoms were alleviated in the sublingual immunotherapy group during the period when large amounts of Japanese cedar pollens were dispersed and the number of serious cases was increasing [12].

In 2005, we, with the cooperation of a study group of the Japanese Ministry of Health, Labour and Welfare, conducted a multicentre, double-blind, placebo-controlled study for the first time in Japan. In this study, standardized Japanese cedar extract or a placebo was administered sublingually from 22 February

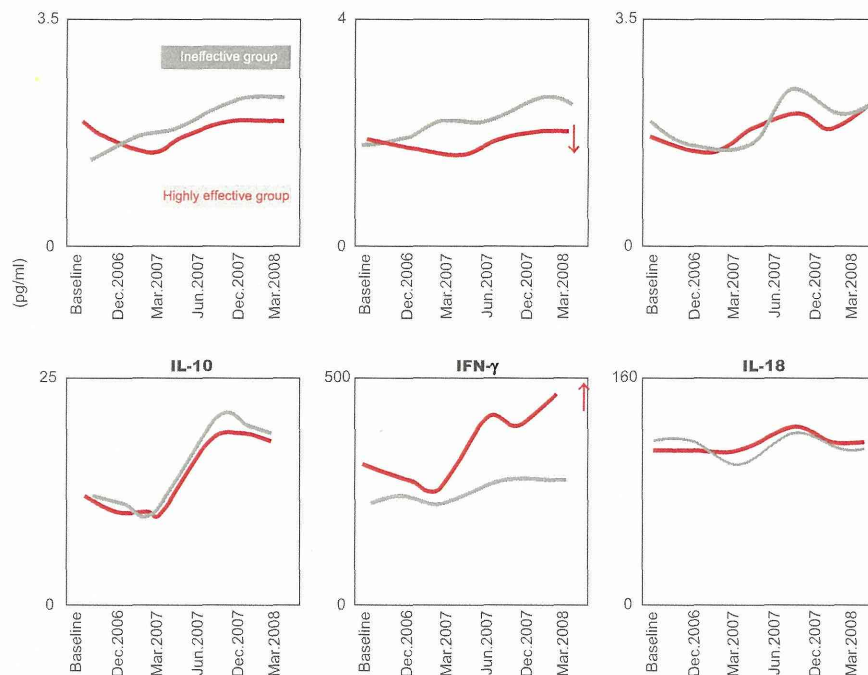

Fig. 8. Mean changes in cytokine levels in two groups showing high efficacy (n = 38) and low efficacy (n = 37) with the duration of sublingual immunotherapy in the collaborative research by the Tokyo Metropolitan Institute of Medical Science, Nippon Medical School and Tokyo Metropolitan Hospitals from 2006 to 2008. The levels of IL-4, IL-5, IL-10, IL-13, IL-18 and IFN-γ were measured.

to 5 April 2005. During the 2005 pollen season, the levels of airborne pollens were very high and it was clear that the symptoms were inhibited in the active treatment group compared with the placebo group in an overall assessment of the results from each region. Inhibitory effects were obtained not only on the symptoms but also on the QOL deterioration [13] (Figs 1–4). Sublingual immunotherapy showed increased efficacy when it was continued from the initial year (Fig. 5).

From 2006 to 2008, collaborative research by the Tokyo Metropolitan Institute of Medical Science, Nippon Medical School and Tokyo Metropolitan Hospitals was started (Fig. 6). As the research was mainly performed in government institutions, only active treatment without a placebo was evaluated. The study participants were 202 patients. When the treatment was continued, it was evident that the efficacy increased from the second year (Fig. 7). Among the patients participating in the study, a group of 38 patients showing especially high efficacy and a group of 37 patients showing especially poor efficacy were selected, and the changes in their serum cytokines were examined. The results showed tendencies towards a decrease in IL-5 and an increase in IFN-γ in the high efficacy group. Unexpectedly, however, IL-10 showed the same changes in both groups (Fig. 8). Further research is necessary on the changes and functions of cytokines after sublingual immunotherapy.

Current status and prospects for sublingual immunotherapy

As sublingual immunotherapy is very safe, it is possible to perform the therapy in facilities that have not prescribed immunotherapy to date. The widespread use of sublingual immunotherapy would be a turning point for allergy treatment in Japan because it is a highly promising method. A phase 3 clinical trial on Japanese cedar extract for sublingual immunotherapy is being conducted by a pharmaceutical company and the results are eagerly awaited.

Conclusions

On an international level, immunotherapy is now considered to be a method of treatment that can achieve cures and long-term remission. However, current immunotherapy by subcutaneous injection in Japan is not widely used because of the risk of adverse drug reactions, such as anaphylaxis, and the necessity of long-term outpatient treatment. In the near future, if sublingual immunotherapy becomes practical, it should become a safer and simpler method for treatment of Japanese cedar pollinosis because few adverse drug reactions will occur and it can be performed at home.

Conflicts of interest

The author has no conflicts of interest to declare.

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