Original article

Regulatory effect of TLR3 signaling on staphylococcal enterotoxin-induced IL-5, IL-13, IL-17A and IFN-γ production in chronic rhinosinusitis with nasal polyps

Mitsuhiro Okano [a, *], Tazuko Fujiwara [a], Shin Kariya [a], Takaya Higaki [a],
Sei-ichiro Makihara [b], Takenori Haruna [a], Yasuyuki Noyama [a], Takahisa Koyama [a],
Ryotaro Omichi [a], Yorihisa Orita [a], Kentaro Miki [a], Kengo Kanai [c], Kazunori Nishizaki [a]

[a] Department of Otolaryngology-Head & Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
[b] Department of Otorhinolaryngology, Kagawa Rosai Hospital, Kagawa, Japan
[c] Department of Otorhinolaryngology, Kagawa Prefectural Central Hospital, Kagawa, Japan

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ABSTRACT

Background: Toll-like receptor 3 (TLR3) is expressed in upper airways, however, little is known regarding whether Toll-like receptor 3 (TLR3) signals exert a regulatory effect on the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP), especially on eosinophilic inflammation. We sought to investigate the effect of Poly(IC), the ligand for TLR3, on cytokine production by dispersed nasal polyp cells (DNPCs).
Methods: DNPCs were pretreated with or without Poly(IC), and were then cultured in the presence or absence of staphylococcal enterotoxin B (SEB), following which the levels of IL-5, IL-10, IL-13, IL-17A and interferon (IFN)-γ in the supernatant were measured. To determine the involvement of IL-10 and cyclooxygenase in Poly(IC)-mediated signaling, DNPCs were treated with anti-IL-10 monoclonal antibody and diclofenac, the cyclooxygenase inhibitor, respectively. Poly(IC)-induced prostaglandin E2 (PGE2) production was also determined.
Results: Exposure to Poly(IC) induced a significant production of IL-10, but not of IL-5, IL-13, IL-17A or IFN-γ by DNPCs. Pretreatment with Poly(IC) dose-dependently inhibited SEB-induced IL-5, IL-13 and IL-17A, but not IFN-γ production. Neutralization of IL-10 significantly abrogated the inhibitory effect of Poly(IC). Treatment with diclofenac also abrogated the inhibitory effect of Poly(IC) on SEB-induced IL-5 and IL-13 production. However, unlike exposure of diclofenac-treated DNPCs to lipopolysaccharide, the ligand for TLR4, exposure of these cells to Poly(IC) did not enhance IL-5 or IL-13 production. Poly(IC) did not significantly increase PGE2 production by DNPCs.
Conclusions: These results suggest that TLR3 signaling regulates eosinophilia-associated cytokine production in CRSwNP, at least in part, via IL-10 production.

Introduction

Toll-like receptors (TLRs) are the major elements of innate immunity, and TLR-mediated signals are known to be associated with the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP).[1–3] For example, nasal polyp fibroblasts produce macrophage inflammatory protein-3alpha (MIP-3alpha) in response to TLR ligands.[4] On the other hand, signals mediated through TLRs have been shown to regulate airway inflammation in a manner that may reflect the concept of the "hygiene (in other words microbial) hypothesis" in which early exposure to microbial stimuli prevents later chronic inflammatory conditions.[5–8] For example, exposure to CpG oligodeoxynucleotide, the ligand for TLR9, reduces the production of IL-6, G-CSF and MIP-1β by turbinate tissue from patients with CRSwNP.[6] We have recently demonstrated that pre-exposure of dispersed nasal polyp cells

* Corresponding author. Department of Otolaryngology-Head & Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikatacho, Okayama 700-8558, Japan.
E-mail address: mokano@cc.okayama-u.ac.jp (M. Okano).
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(DNPCs) to lipopolysaccharide (LPS), the ligand for TLR4, suppresses staphylococcal enterotoxin B (SEB)-induced IL-5, IL-13, IFN-γ and IL-17A production via cyclooxygenase (COX) and prostaglandin E2 (PGE2) pathway.[7]

TLR3 is expressed in intracellular vesicles such as the endosome, and recognizes viral components including dsRNA. Unlike other TLRs, TLR3 initiates a TRIF-dependent, but MyD88-independent, pathway for its actions.[9] In upper airways, TLR3 is found on constitutive cells including epithelial cells and fibroblasts.[10,11] Signaling through TLR3 induces the production of pro-inflammatory cytokines and chemokines including IL-6, IL-8, GM-CSF, TARC, RANTES, TSLP and osteopontin.[10–15] However, little is known regarding whether TLR signals exert a regulatory effect on upper airway inflammation, especially on eosinophilic inflammation.

In the present study, we investigated whether exposure to polyinosinic: polycytidylic acid (Poly(IC)), the ligand for TLR3, affects IL-5, IL-13, IFN-γ or IL-17A production by DNPCs in response to SEB, which is a candidate agent for facilitation of the pathogenesis of CRSwNP.[16] We found that the regulatory effect of TLR3 signaling on SEB-induced cytokine production was mediated by a different pathway than the pathway that mediates the regulatory effect of TLR4 signaling.

Methods

Patients

The study utilized nasal polyps that were surgically excised from 36 Japanese CRSwNPs patients (age range, 20–78 years; mean age, 53.5 years). The presence of CRSwNPs was determined based upon diagnostic criteria reported in a European position paper on rhinosinusitis and nasal polyps.[17] According to the JESREC (Japanese epidemiological survey of refractory eosinophilic chronic rhinosinusitis) criterion, 23 NPs were eosinophilic (over 70 eosinophils per ×400 field).[18] Eleven patients were asthmatic, and four patients were thought to have aspirin exacerbated respiratory disease (AERD) based on a history of asthma attacks precipitated by non-steroidal anti-inflammatory drugs. Exclusion criteria were as described previously.[7] Informed consent for participation in the study was obtained from each patient. The study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry.

Antigen and reagents

The following materials were purchased for the study: Poly(IC), RPMI-1640, L-glutamine-penicillin-streptomycin solution, protease, collagenase, hyaluronidase, DNase I, fetal calf serum (FCS) (Sigma, St. Louis, MO, USA); red blood cell lysis buffer (Roche, Indianapolis, IN, USA); SEB (Toxin Technology, Sarasota, FL, USA); diclofenac sodium (Wako Pure Chemicals, Osaka, Japan); rat anti-human IL-10 mAb (LifeSpan BioSciences, Inc., Seattle, WA, USA), and rat IgG1 (R&D Systems, Minneapolis, MN, USA).

Culture of dispersed nasal polyp cells with Poly(IC)

Dispersed nasal polyp cells were prepared from nasal polyps by enzymatic digestion, as described previously, and suspended in culture medium containing RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.[7] $8.5 \pm 5.3\%$, $11.7 \pm 8.9\%$, $8.9 \pm 8.2\%$, $8.5 \pm 6.8\%$, $7.8 \pm 11.1\%$, $10.9 \pm 10.5\%$, $15.5 \pm 6.7\%$, and $21.6 \pm 7.7\%$ cells in DNPCs express c-kit, ECP/EPX, CD79α, CD68, CD4, CD8, cytokeratin, and vimentin, respectively, suggesting that DNPCs consist of both constitutive cells and inflammatory cells including mast cells, eosinophils, B cells, macrophages, CD4$^+$ T cells, CD8$^+$ T cells, epithelial cells, and fibroblasts/vascular endothelial cells.[7] These DNPCs, in a volume of 500 μl/well (1×10^6 DNPCs/ml) in flat-bottomed 48-well culture plates (Asahi Techno Glass, Tokyo, Japan), were stimulated with 1, 10, or 100 μg/ml of Poly(IC) and were incubated at 37 °C in a 5% CO$_2$ atmosphere. An aliquot of the culture supernatant was collected after 12 and 72 h and was stored at −80 °C for subsequent analysis of the cytokines IL-5, IL-10, IL-13, IL-17A and IFN-γ.

Effects of Poly(IC) on SEB-induced cytokine production by DNPCs

DNPCs were cultured with or without Poly(IC) at 1, 10 or 100 μg/ml for 2 h prior to SEB stimulation (1 ng/ml). The culture supernatant was collected after SEB stimulation for 72 h, after which the levels of IL-5, IL-13, IL-17A and IFN-γ were determined.

Role of IL-10 and COX in the regulatory effect of Poly(IC) on SEB-induced cytokine production by DNPCs

To determine the role of IL-10 in the effect of Poly(IC) on SEB-induced cytokine production, DNPCs were pretreated with or without 100 μg/ml Poly(IC), and were then stimulated with SEB in the presence of either anti-human IL-10 mAb or control rat IgG1 (20 μg/ml) for 72 h. To determine the role of cyclooxygenase (COX), DNPCs were pretreated with 10^{-5} M diclofenac 2 h prior to Poly(IC) treatment.

Effect of Poly(IC) on PGE2 production by DNPCs

DNPCs (1×10^6/ml) were cultured in the presence or absence of 100 μg/ml Poly(IC) for 12 or 72 h. The concentration of PGE2 in the supernatant was determined using a PGE2 EIA kit (Cayman, Ann Arbor, MI, USA). The detection limit was 7.8 pg/ml.

Cytokine determination

Levels of IL-5, IL-10, IL-13, IL-17A and IFN-γ in the culture supernatants were determined using ELISA.[7] Levels of IL-5, IFN-γ, and IL-10 were measured using Opt™ EIA sets (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Levels of IL-17A were measured using a DuoSet™ ELISA development kit (R&D Systems). Levels of IL-13 were measured using paired capture and detection antibodies (BD Biosciences) and recombinant standards (R&D Systems). 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, 4 pg/ml for IFN-γ, and 8 pg/ml for IL-10.

Statistical analysis

Values are given as medians. A nonparametric Mann–Whitney U test was used to compare data between groups, and Wilcoxon signed-rank test was used to analyze data within each group. *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

Poly(IC)-induced cytokine production by DNPCs

DNPCs were stimulated with or without various concentrations of Poly(IC) for 12 or 72 h. Either 12-h or 72-h stimulation of DNPCs with Poly(IC) did not induce the production of IL-5 ($p = 0.462$ and $p = 0.755$, respectively, at 100 μg/ml), IL-13 ($p = 0.236$ and

$p = 0.116$), IFN-γ ($p > 0.999$ and $p = 0.066$) or IL-17A ($p = 0.345$ and $p = 0.414$). However, exposure to Poly (IC) for 12 h did significantly induce a dose-dependent increase in the production of IL-10 compared to unstimulated control cells. The maximum production of IL-10 was detected at 100 µg/ml Poly(IC). Dose-dependent production of IL-10 by Poly(IC) stimulation was also observed following Poly(IC) stimulation for 72 h (Fig. 1). We therefore further investigated the regulatory effect of Poly(IC) on DPNCs.

Effect of exposure of DPNCs to Poly(IC) on SEB-induced Th1/Th2/Th17-related cytokine production by DNPCs

Since SEB is known to be involved in the pathogenesis of CRSwNP, we sought to determine whether exposure of DNPCs to Poly(IC) regulates SEB-induced cytokine production. Consistent with our previous report, the DNPCs produced a substantial amount of IL-5, IL-13, IFN-γ and IL-17A in response to SEB.[7] Pre-exposure to Poly(IC) dose-dependently inhibited SEB-induced IL-5, IL-13 and IL-17A production. SEB-induced IL-5, IL-13 and IL-17A production was inhibited by 50.09% ($p = 0.002$, Fig. 2A), 47.41% ($p = 0.002$, Fig. 2B) and 65.21% ($p = 0.002$, Fig. 2D), respectively, by pretreatment with 100 µg/ml Poly(IC) for 2 h prior to SEB stimulation. Although exposure of DNPCs to Poly (IC) at a concentration of 1 µg/ml resulted in a weak inhibition (22.98% inhibition, $p = 0.041$) of SEB-induced IFN-γ production, exposure to 10 or 100 µg/ml Poly(IC) did not exert a significant effect ($p > 0.05$, Fig. 2C). No significant difference in the inhibitory effect of 100 µg/ml Poly(IC) on the production of IL-5 ($p = 0.644$), IL-13 ($p = 0.926$), IFN-γ ($p = 0.079$) and IL-17A ($p = 0.644$) was seen between asthmatic and non-asthmatic patients.

Involvement of IL-10 in the inhibitory effect of exposure of DNPCs to Poly(IC) on SEB-induced cytokine production

We next sought to determine whether the IL-10 that is produced by DNPCs following their exposure to Poly(IC) can regulate the effect of Poly(IC) on SEB-induced cytokine production. As compared with the control treatment, antibody-mediated neutralization of IL-10 significantly enhanced the production of SEB-induced IL-5 ($p = 0.012$, Fig. 3A), IL-13 ($p = 0.012$, Fig. 3B), IFN-γ ($p = 0.036$, Fig. 3C) and IL-17A ($p = 0.012$, Fig. 3D) by DNPCs exposed to Poly(IC). IL-10 neutralization also significantly enhanced SEB-induced IL-13 ($p = 0.018$) and IFN-γ ($p = 0.018$) production by

DNPCs that were not exposed to Poly(IC), whereas it only marginally enhanced SEB-induced IL-5 ($p = 0.063$) and IL-17A ($p = 0.063$) production.

Involvement of the COX pathway in the inhibitory effect of exposure of DNPCs to Poly(IC) on SEB-induced cytokine production

Since we recently reported that exposure of DNPCs to the TLR4 agonist, LPS, inhibited SEB-induced IL-5, IL-13, IFN-γ and IL-17A production by DNPCs via the COX pathway, especially through PGE2 production, we therefore investigated whether the inhibitory effect of Poly(IC) on SEB-induced cytokine production is also regulated by the COX pathway.[7] In the presence of the COX inhibitor diclofenac, the suppressive effects of Poly(IC) pretreatment of DNPCs on SEB-induced IL-5 ($p = 0.657$, Fig. 4A) and IL-13 ($p = 0.790$, Fig. 4B) production was abrogated, but Poly(IC) did not induce any significant enhancement of SEB-induction of these cytokines in the diclofenac-treated DNPCs. In addition, Poly(IC) pretreatment significantly enhanced SEB-induced IFN-γ production ($p = 0.016$, Fig. 4C) in diclofenac-treated DNPCs. In contrast, inhibition of IL-17A production was still observed following exposure of diclofenac-treated DNPCs to Poly(IC) ($p = 0.005$, Fig. 4D). 12 h exposure to Poly(IC) did not induce a significant increase in PGE2 production by DNPCs ($p = 0.060$), rather a significant decrease in PGE2 production was observed after 72 h of Poly(IC) stimulation ($p = 0.006$) (Fig. 5).

Discussion

In the present study, we investigated the regulatory effects of Poly(IC) on Th1-, Th2- and Th17-associated cytokine production in an ex vivo model of CRSwNP. Our results demonstrated that exposure of DNPCs to Poly(IC) induced a substantial suppression of SEB-induced IL-5, IL-13 and IL-17A production in a Poly(IC) dose-dependent manner, whereas there was no significant effects of 10 and 100 µg/ml Poly(IC) on SEB-induced IFN-γ production. In addition, this Poly(IC) inhibition was significantly decreased by the antibody-mediated neutralization of IL-10. These results suggest that Poly(IC)-derived IL-10 plays a substantial role in the regulatory effect of Poly(IC) on Th2- and Th17-associated cytokine production in CRSwNP.

Poly(IC) selectively induced IL-10, and did not induce IL-5, IL-13, IFN-γ or IL-17A production by nasal polyp cells. Although Poly(IC) is known to induce the production of pro-inflammatory cytokines and chemokines including IL-6, IL-8, GM-CSF, TARC, RANTES and TSLP, this is the first report to demonstrate the production of IL-10 as a result of Poly(IC) stimulation of sinonasal tissues.[10–15] IL-10 produced in the respiratory tract limits inflammation in response to pathogens and allergens.[19,20] For example, neutralization of IL-10 significantly increased allergen-specific IL-5 and IFN-γ production by nasal polyp cells sensitized to the allergens.[20] In addition, impaired production of IL-10 was seen in patients with allergic rhinitis.[21] These results suggest that signals through TLR3 might suppress airway inflammation via induction of IL-10.

Poly(IC)-induced IL-10 production has been previously reported in both humans and mice.[22,23] IL-10 is known to be produced by a variety of cells including cells of the innate immune system such as macrophages, monocytes, dendritic cells, NKT cells and NK cells and also cells of the acquired immune system such as T cells and B cells.[24] For example, mouse macrophages produce IL-10 in response to Poly(IC) in a MyD88-independent and TRIF-dependent manner.[22] In humans, Poly(IC)-treated epidermal Langerhans cells promote the differentiation of CD4+ T cells producing IFN-γ and IL-10.[23] Although nasal polyp cells are known to produce IL-10 in response to allergens and pathogens,[20,25,26] little is known about the localization of IL-10 expression in sinonasal tissues. IL-10 is
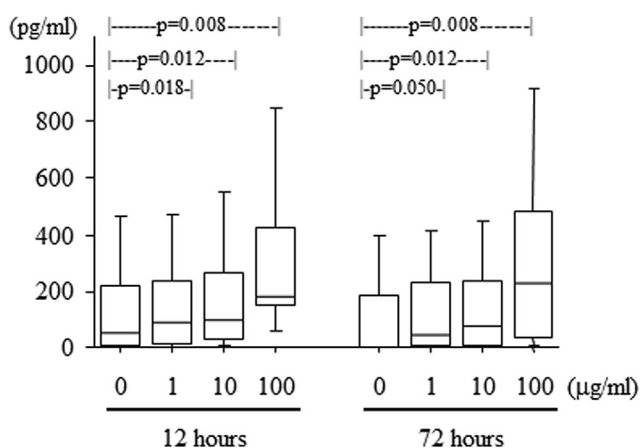

Fig. 1. Poly(IC)-induced IL-10 production by nasal polyp cells. The rectangle includes the range from the 25th to 75th percentiles; the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. *p*-values were determined using the Wilcoxon signed-rank test.
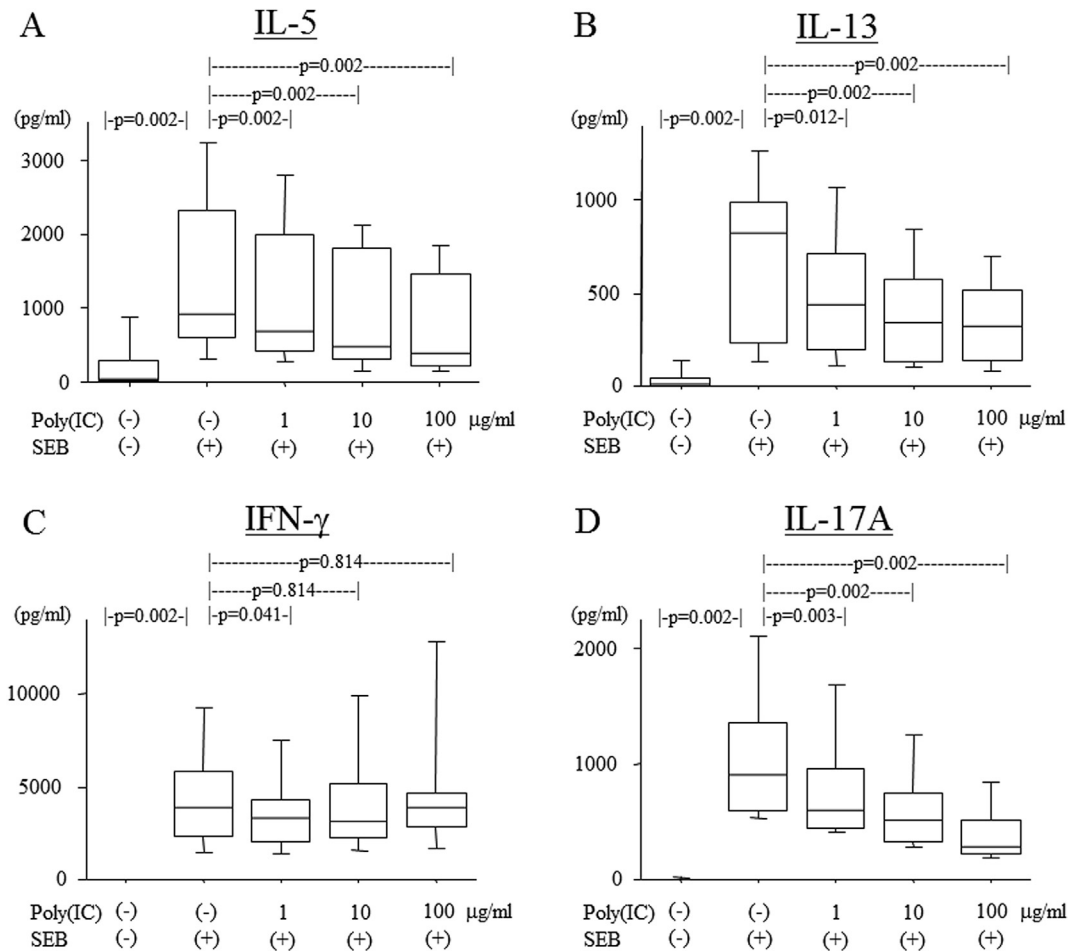
Fig. 2. Effects of pretreatment with Poly(IC) on SEB-induced cytokine production by DNPCs. DNPCs were treated with 0, 1, 10 or 100 μg/ml Poly(IC) 2 h prior to SEB stimulation. After 72 h of incubation with SEB, levels of IL-5 (A), IL-13 (B), IFN-γ (C), and IL-17A (D) within the supernatant were determined. *P*-values were determined using Wilcoxon signed-rank test.

expressed in epithelial cells and endothelial cells in turbinate mucosa from patients with allergic rhinitis.[27,28] Future experiments should investigate the cell types in nasal polyps that respond to Poly(IC) by producing IL-10.

Pre-exposure of DNPCs to Poly(IC) significantly inhibited SEB-induced IL-5, IL-13 and IL-17A production in a dose-dependent manner. These cytokines are known to be involved in the pathogenesis of CRSwNP, especially in eosinophilic inflammation.[29–32] For example, treatment with a humanized anti-IL-5 mAb significantly decreased polyp size and blood eosinophil count in patients with CRSwNP.[31] We have previously reported that the number of cells expressing IL-17A was significantly and positively correlated with the degree of eosinophilia in nasal polyps.[30] Together with the finding that pre-exposure of DNPCs to 10 and 100 μg/ml Poly(IC) had no significant effect on IFN-γ production, which is known to inhibit airway eosinophilia, these results suggest that signals mediated through TLR3 can suppress eosinophilic inflammation in CRSwNP.[33] Eosinophilic CRS is the major endotype of CRSwNP in the United States and Europe, and has been increasing in Asia.[3,34,35] For example, there was a shift from predominantly neutrophilic to eosinophilic CRSwNP in Thai patients from 1999 to 2011.[34] A crucial role of early exposure to microbial stimuli in the prevention of later chronic inflammatory conditions is known as the "hygiene (in other words microbial) hypothesis".[8] Since single-stranded RNA viruses and double-stranded DNA viruses produce double-stranded RNA during replication, which is sensed by TLR3, the present results may

reflect this hypothesis, and suggest that early exposure to viruses inhibits eosinophilia-associated cytokine production in CRSwNP.[8]

Neutralization of IL-10 significantly reversed the suppression of SEB-induced IL-5, IL-13, IL-17A and IFN-γ production by Poly(IC). This effect was particularly pronounced for IL-5 and IL-17A production since IL-10 neutralization did not significantly enhance SEB-induced IL-5 or IL-17A production in the absence of Poly(IC) exposure. These results suggest that the inhibitory effect of Poly(IC) on cytokine production by DNPCs is mediated via IL-10 production. One of the reasons why IL-10 neutralization significantly enhanced SEB-induced IL-13 and IFN-γ production by DNPCs that were not exposed to Poly(IC) may be due to the finding that DNPCs spontaneously produce IL-10, and produce significantly more IL-10 in response to SEB (data prepared for submission). IL-10 suppresses IFN-γ production under various conditions.[36]

The reason why the effect of Poly(IC) on SEB-induced IFN-γ production was not observed even though IL-10 neutralization significantly enhanced the production of SEB-induced IFN-γ by DNPCs exposed to Poly(IC) is not clear. One possibility is that Poly(IC) itself can promote IFN-γ production under various conditions.[37,38] For example, Poly(IC)-primed human dendritic cells promote IFN-γ production by CD3+ T cells and NK cells.[37] Administration of Poly(IC) increased the level of IFN-γ in *Dermatofagoides farina*-induced model of atopic dermatitis in NC/Nga mice.[38] Thus the inhibitory effect of IL-10 induced by Poly(IC) on SEB-induced IFN-γ production may be abrogated by IFN-γ-promoting effect by
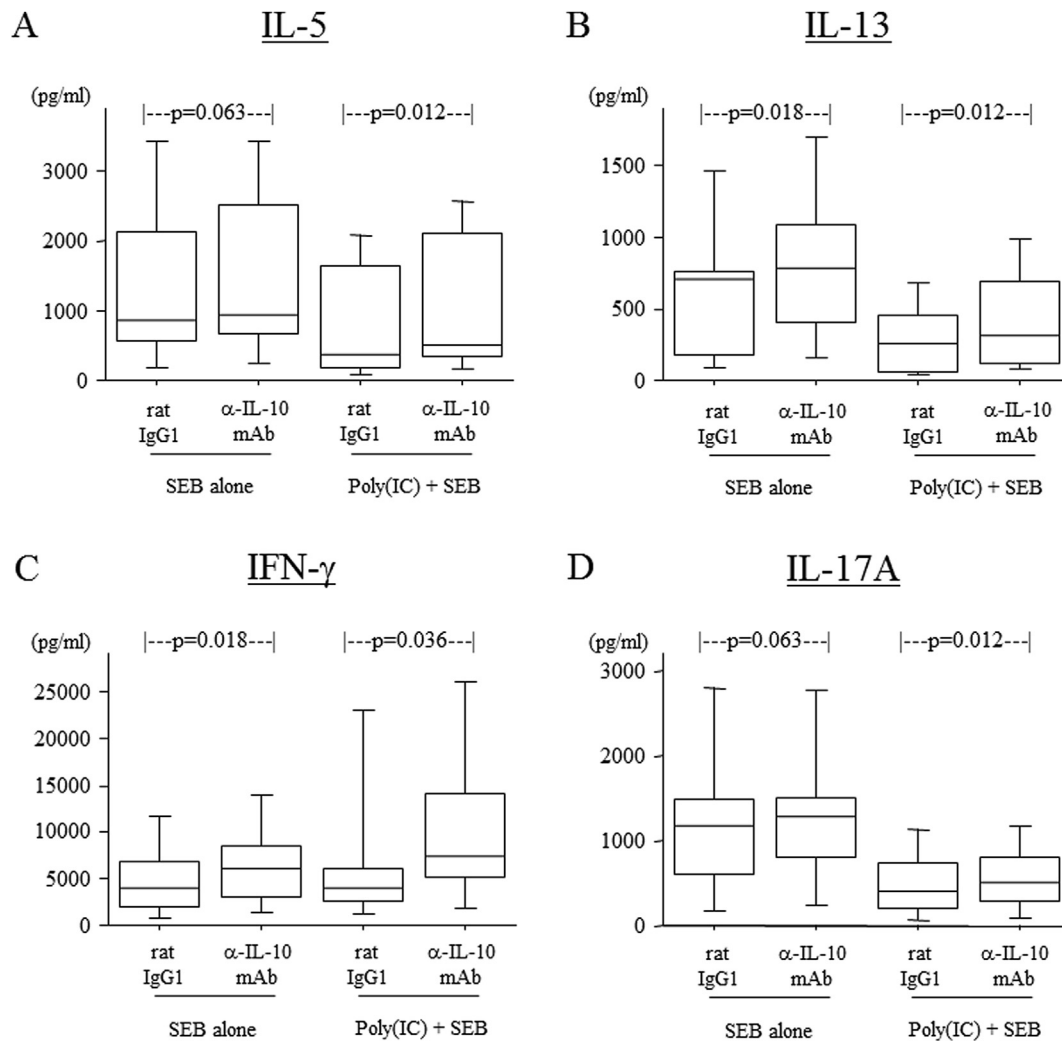
A IL-5   B IL-13
C IFN-γ   D IL-17A

Fig. 3. Effect of IL-10 neutralization on cytokine production by DNPCs. DNPCs were pretreated with or without Poly(IC) for 2 h, then stimulated with SEB in the presence of either anti-human IL-10 mAb or control rat IgG1. After 72 h incubation, levels of IL-5 (A), IL-13 (B), IFN-γ (C), and IL-17A (D) within the supernatant were determined. *p*-values were determined using the Wilcoxon signed-rank test.

Poly(IC) itself in NPs which contains various cells including T cells and NK cells. Future study should be performed whether IL-10 neutralization induces cytokine production especially IFN-γ by DNPCs in response to Poly(IC).

We have previously reported that pre-exposure of DNPCs to LPS, the TLR4 agonist, also inhibits SEB-induced IL-5, IL-13, IFN-γ and IL-17A production.[7] Although the effect on IFN-γ production was different, pre-exposure of DNPCs to both LPS and Poly(IC) resulted in an inhibitory effect on IL-5, IL-13 and IL-17A production. These results suggest that both TLR3 and TLR4 signals alleviate eosinophilia-associated cytokine production in CRSwNP. However, the mechanism by which LPS and Poly(IC) decrease cytokine production, especially the production of IL-5 and IL-13 cytokines, seems to be different. It was previously shown that pre-exposure of DNPCs to LPS significantly enhanced SEB-induced IL-5 and IL-13 production in the presence of diclofenac, and that addition of PGE2 significantly reversed this enhancement by diclofenac.[7] However, treatment with diclofenac did not lead to such a reverse by Poly(IC). Indeed, sustained production of PGE2 by DNPCs was not detected following their exposure to Poly(IC). This finding is in contrast to PGE2 production by DNPCs in response to exposure to LPS in which sustained PGE2 production as well as increased expression of COX-2 and PGE2 synthase was seen.[7] These data

suggested that the inhibitory effect of LPS on IL-5 and IL-13 production is mediated by the COX/PGE2 axis, whereas the effect of Poly(IC) is mediated by IL-10. In addition, the dose dependency of the inhibitory effects of LPS and Poly(IC) was different. Thus, the inhibitory effect of LPS on SEB-induced cytokine production was more obvious at a relatively low LPS concentration (0.2 μg/ml) as compared with a high LPS (2 μg/ml) concentration.[7] On the other hand, the inhibitory effect of Poly(IC) increased with increasing Poly(IC) concentration.

Poly(IC) pretreatment significantly enhanced SEB-induced IFN-γ production and conversely inhibited IL-17A production by diclofenac-treated DNPC. It is known that PGE2 suppresses IFN-γ production and conversely enhances IL-17A production under various conditions.[30,39] Thus cease of PGE2 release by diclofenac treatment may increase IFN-γ production and conversely decrease IL-17A production.

In conclusion, we demonstrated that pre-exposure of DNPCs to Poly(IC) dose-dependently inhibited SEB-induced Th2 and Th17-associated cytokine production in an ex vivo model of CRSwNP. The present study outlines a mechanism for the inhibitory effects of TLR3 signaling on eosinophilia-associated cytokine production, which is mediated by IL-10. These observations may provide a basis for novel therapeutic approaches targeting Poly(IC) and other
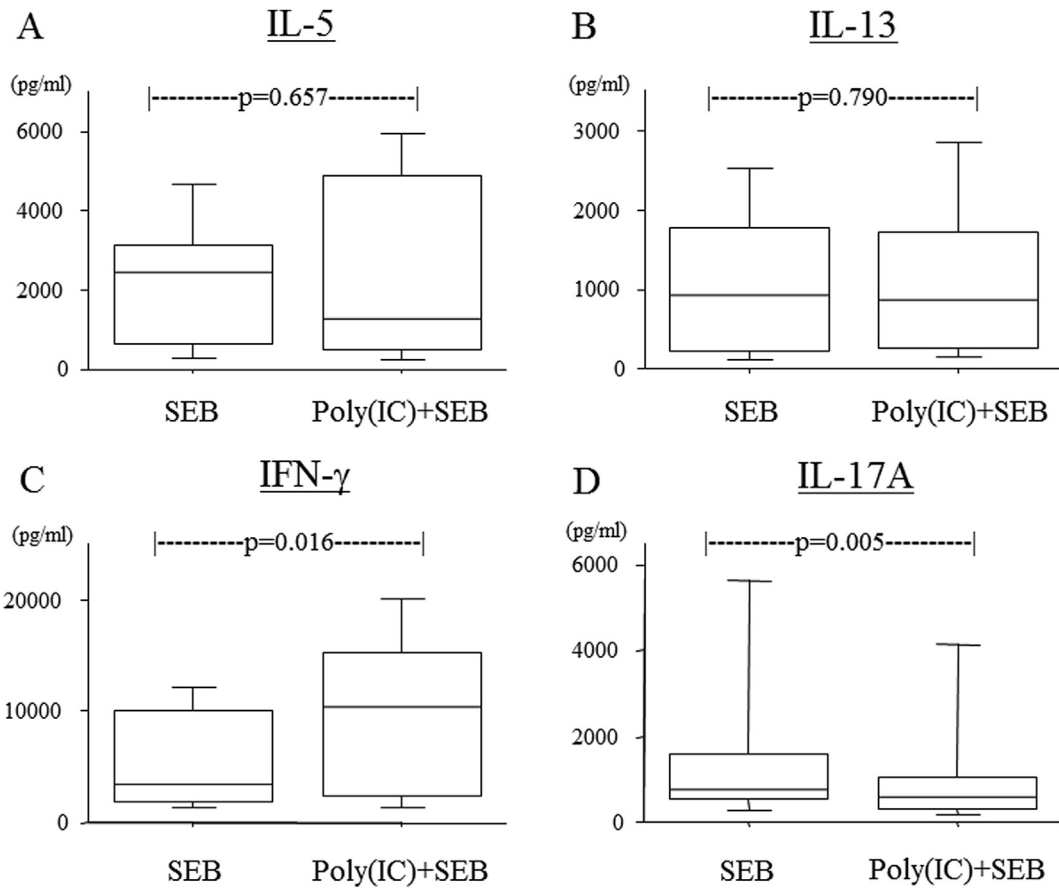
Fig. 4. Effect of COX on cytokine production by DNPCs. Diclofenac-treated DNPCs were exposed or unexposed to Poly(IC) prior to SEB stimulation. After 72 h incubation, levels of IL-5 (A), IL-13 (B), IFN-γ (C), and IL-17A (D) within the supernatant were determined. *p*-values were determined using the Wilcoxon signed-rank test.
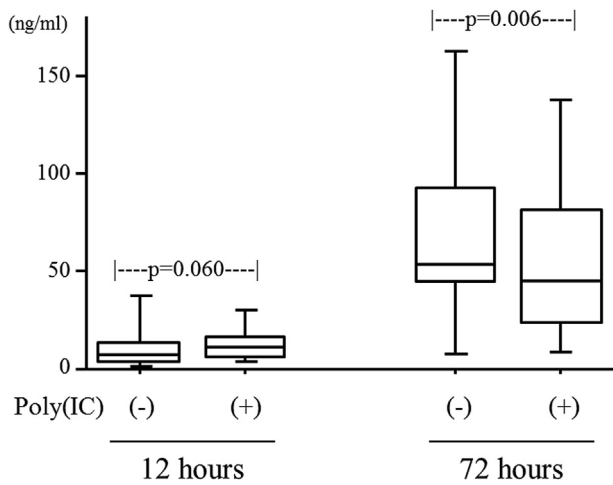

Fig. 5. Release of PGE2 by DNPCs in response to Poly(IC). DNPCs were cultured with or without 100 μg/ml Poly(IC) for 12 or 72 h, and levels of PGE2 after 72 h of incubation were measured. *p*-values were determined using the Wilcoxon signed-rank test.

components of microbes in the management of eosinophilic airway diseases such as CRSwNP, allergic rhinitis, and bronchial asthma.

Acknowledgments

Authors' contributions
MO, SK, THi and KN designed the study and wrote the manuscript. TF, SM, THa, YN, TK and KK contributed to data collection. RO, YO and KM performed the statistical analysis and interpretation of the results.

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