FACS analysis, incubated DCs were stained with Cy5-labeled CD11c and MHC class II Alexa555, and then fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 (in 50 mM NaCl, 5 mM EDTA, and 0.02% NaN_3; pH 7.5) for 10 min on ice. After blocking with 3% BSA in PBS for 15 min, the cells were washed thoroughly with PBS supplemented with 1% FCS and 0.1% NaN_3. Phagocytosis of DCs was examined by confocal microscopy (Olympus, Tokyo, Japan) and FACS analysis. We used the fluorescence intensity of immature DCs after incubation for 24 h with labeled *Lactobacillus* as an indicator of *Lactobacillus* uptake.

2.5. Cytokine production in the co-culture of DCs with Th2 cells

Th2-skewed $CD4^+$ T cells were prepared from OVA-specific T cell receptor (TCR) (DO11.10) transgenic mice in a BALB *c* background, expressing TCR α/ß specific for OVA peptide, presented in the context of I-Ad [17]. Immature DCs (5×10^5) prepared from BALB *c* mice using the method described above, were pre-incubated with 0.4 μM $OVA_{323-339}$ peptide (Loh15) with 1 μg mL of KW3110 or L-92 for 24 h, and were then co-cultured with Th2-skewed $CD4^+$ T cells (1×10^5 cells) for 48 h. After preincubation, some DCs were treated for 30 min with 10 $\mu g.mL^{-1}$ of a neutralizing rat monoclonal antibody directed against PD-L2 (TY25 [18], rat IgG2a; BioLegends, San Diego, CA, USA) or a control isotype rat IgG2a monoclonal antibody. The cells were washed 3 times to remove excess antibody before co-culture. The culture supernatants were collected for ELISA analysis of cytokine production.

2.6. Migration of DCs after sublingual administration

We administered 0.5 mg of FITC-labeled KW3110 with 100 μg of OVA in 0.01 mL PBS sublingually to BALB *c* mice, using a micropipette tip placed under the tongue, while holding the back of the mouse for 20 s. The oral mucosa and CLNs were obtained, and freshly frozen 6-μm thick sections were fixed in a cold methanol:acetone (1:1) solution for 10 min. These were examined after staining with biotin-labeled CD11c followed by Cy5-coupled streptavidin.

2.7. *In vivo* immunization study protocol

BALB *c* mice were sensitized intraperitoneally with 100 μg of OVA and 4 mg of alum (Pierce, Rockford, IL, USA) once a week for 3 weeks. From 1 week after the last sensitization, 500 μg of OVA in 20 μL PBS was administered intranasally for 7 consecutive days [19]. Sensitized mice (n=18 in each group) received 5 mg or 0.5 mg of KW3110 or L-92 in 0.1 mL PBS, administered through a gavage tube for 7 consecutive days at the same time every day. A second group of sensitized mice (n=16 in each group) received 0.5 mg of KW3110 or L-92 in 0.01 mL PBS by sublingual administration for 7 consecutive days, as described above. After the last nasal administration of OVA, the behavior of the mice was recorded by video camera, and sneezing and nasal rubbing events were counted for 5 min [19]. The mice were then sacrificed and the serum, CLNs, spleens, inguinal lymph nodes (ILNs), and BM were collected. Serum OVA-specific IgE Ab was analyzed by ELISA.

2.8. Treatment with a neutralizing anti-PD-L2 antibody

We investigated the preventive effect of treatment *in vivo* with a neutralizing anti-PD-L2 antibody. The OVA-sensitized mice were injected intravenously with 250 μg of anti-mouse PD-L2 mAb (TY25, rat IgG2a) or rat control IgG2 mAb 12 h after the last sensitization. We then administered 0.5 mg of KW3110 sublingually for 7 consecutive days.

2.9. Analysis of cytokine production from $CD4^+$ T cells in CLNs

Single-cell suspensions were prepared from CLNs and incubated with biotinylated anti-CD4 antibody (GK1.5; BioLegends, San Diego, CA, USA) at 4 °C for 30 min, followed by incubation with anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ cells were sorted using the MACS systems (Miltenyi Biotec) and suspended in complete RPMI 1640 medium. Cells were then cultured at a density of 1.5×10^5 cells per well in the presence of 1 $mg.mL^{-1}$ of OVA with $CD4^+$ T cell-depleted and irradiated splenic feeder cells (5×10^5 cells per well) in round-bottom 96-well microculture plates for 48 h. Cytokines in the supernatants were then analyzed by ELISA.

2.10. Detection of OVA-specific IgE-secreting cells

The number of OVA-specific IgE-secreting cells among CLN, ILN, spleen, and BM cells was evaluated in 96-well filtration plates (Multiscreen; Millipore Corp., Bedford, MA, USA) coated with 50 $\mu g.mL^{-1}$ OVA or BSA in PBS at 4 °C overnight, and then blocked with 10% FCS in RPMI. CLN cells (3×10^5 cells per well) were incubated on the plates at 37 °C under 5% CO_2. After incubation for 5 h, the plates were washed with PBS containing 0.1% Tween 20 (Sigma Chemical Co.) and then stained with alkaline phosphatase-conjugated anti-IgE antibodies (23 G3; Southern Biotechnology, Birmingham, AL, USA). Alkaline phosphatase activity was visualized using a BCIP/NBT phosphatase substrate (KPL, Gaithersburg, MD, USA). The plates were scanned and spots were counted automatically using an ELISPOT image analysis system (CTL Analyzers LLC, Cleveland, OH, USA).

2.11. ELISA analysis

Cytokine levels in the culture supernatant were measured by ELISA. Flat-bottom 96-well Nunc-Brand Immuno plates (Nalge Nunc International) were coated overnight at 4 °C with affinity-purified anti-IL-4 Ab (11B11), anti-IL-5 Ab (TRFK5) (all from Mabtech AB, Nacka, Sweden) or anti-IL-13 Ab (the ELISA Ready-SET-Go! Kit; eBioscience, San Diego, CA, USA). The wells were then washed and blocked with 1% BSA in PBS, and the culture supernatants were titered onto a treated plate and incubated overnight at 4 °C. Bound cytokines were detected using biotin-conjugated anti-IL-4 Ab (BVD6-24G2), anti-IL-5 Ab (TRFK4) (all Mabtech Ab), or anti-IL-13 Ab (eBioscience) followed by streptavidin–alkaline phosphatase

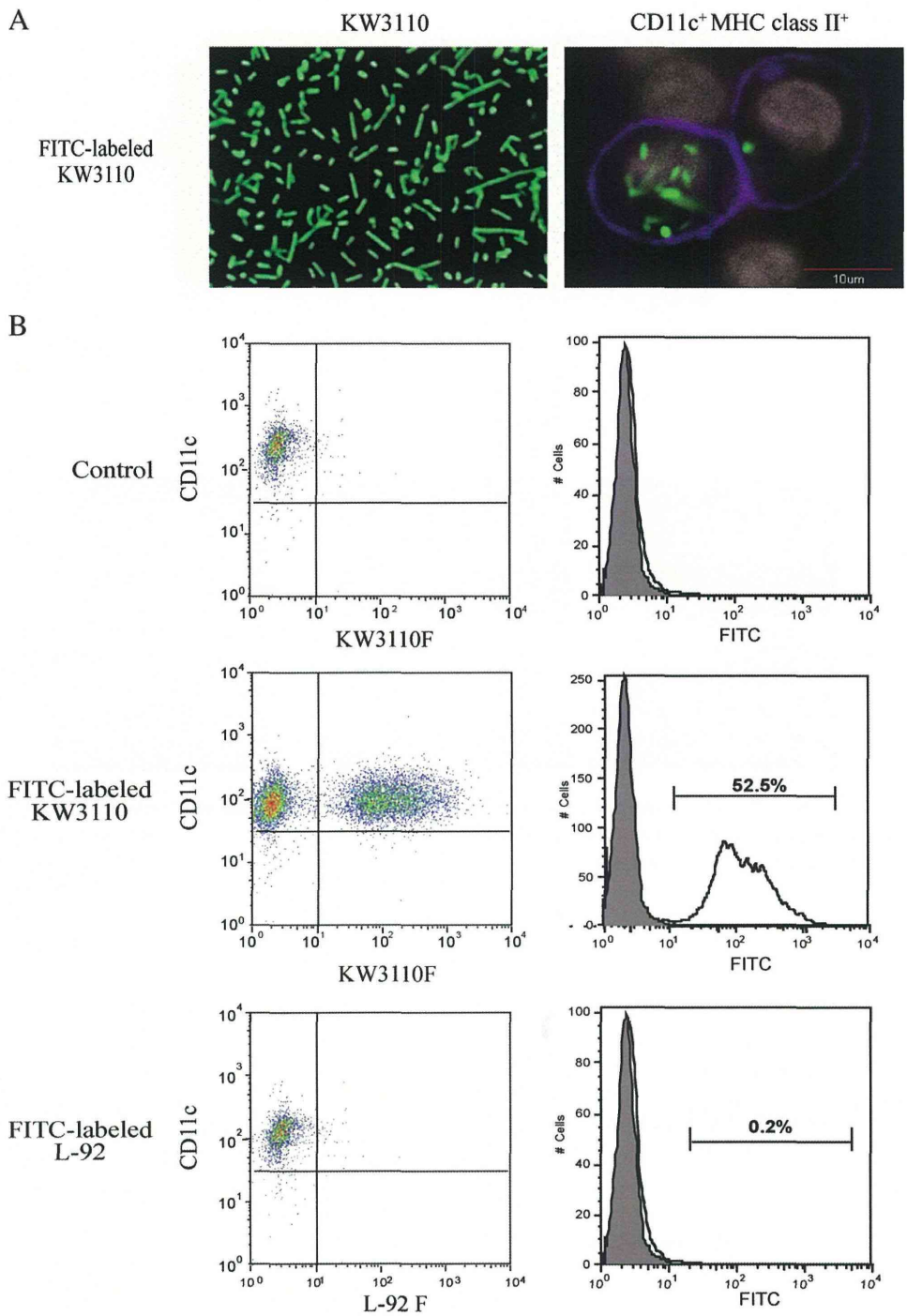

Figure 1 Phagocytosis of immature DCs analyzed by confocal microscopy (A) (green: KW3110, white: DAPI (nuclear), blue: CD11c-Cy5, red: MHC class II) and flow cytometry; (B) magnifications are 200× and 400× for the left and right images respectively. DCs phagocytosed many KW3110 bacteria in their intact form, and 52% of the cultured $CD11c^+$ DCs were found to contain FITC-labeled KW3110 after 24 h, based on FACS analysis. Only a few L-92 bacteria were phagocytosed.

(AP). The plates were developed with *p*-nitrophenyl phosphate tablets (Pierce), with the reaction terminated by the addition of 2N NaOH. Specific absorbance was measured and optical density (OD) was quantified at 410 nM using a Multiskan JX plate reader (Thermo LabSystems, Beverly, MA, USA). OVA-specific IgE was measured with a mouse anti-OVA IgE antibody assay kit (Chondrex, Redmond, WA, USA).

2.12. Flow cytometric analysis

Four-color cytometry was performed as follows. Briefly, cells were harvested from culture plates, preincubated with 10 $mg.mL^{-1}$ of unlabeled anti-CD16/32 (24G2) (BD Biosciences), and then stained on ice with a combination of Abs. We used combinations of FITC-conjugated mouse CD11c (N418)

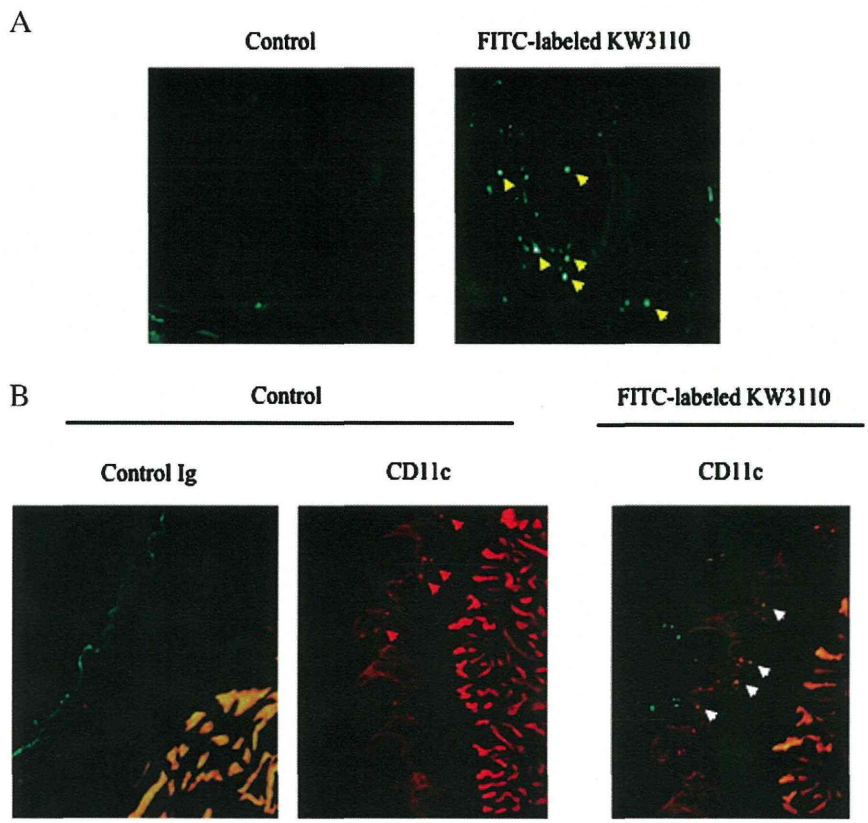

Figure 2 FITC-labeled KW3110 bacteria were detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (A, yellow arrow). Many fragmented and some intact forms of KW3110 were observed to be phagocytosed by DCs in the double staining with anti-CD11c (B, white arrow). Magnification is 100× for all images.

(eBioscience) with PE-conjugated Abs to MHC Class II IA+IE (M5/114.15), CD80 (16-10A-1), CD86 (GL-1), B7RP-1 (HK5.3), PD-L2 (122), OX40L (RM134L), 4-1BBL (TKS-1) (all eBioscience), PD-L1 (9G2), or APC-conjugated CCR-7 (4B12) (BioLegend). Data were analyzed using the Cellquest software (Becton Dickinson).

2.13. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. Data are shown as mean ± SEM or SD.

3. Results

3.1. KW3110 enhanced CCR-7 and PD-L2 expression

After incubation for 24 h with LPS, L-92, or KW3110, the expression of MHC class II^{high}, CD40, CD80, CD86, B7RP-1 and PD-L1 similarly increased; however, that of CCR-7 and PD-L2 was enhanced only by KW3110 in OVA-stimulated DCs, and not by LPS or L-92 (data not shown). Administration of *Lactobacillus* without OVA stimulation induced a low level of the surface markers, and the concentration of *Lactobacillus* and LPS required for expression of the highest levels of these markers with OVA stimulation was determined to be 10 $\mu g.mL^{-1}$ in a preliminary analysis.

3.2. KW3110 was phagocytosed in the intact form by DCs

As shown in Figure 1A, the DCs phagocytosed KW3110 bacteria in the intact form, and 52% of cultured $CD11c^+$ DCs showed uptake of FITC-labeled KW3110 over 24 h based on FACS analysis (Figure 1B). In contrast, only a few L-92 bacteria were phagocytosed, and even the uptake of FITC was very low.

3.3. KW 3110 decreased cytokine production from Th2 cells

In co-culture study of DCs and Th2-skewed $CD4^+$ T cells from DO11.10 Tg mice, KW3110 decreased IL-5 and IL-13 production (from 0.210 ± 0.13 pg/mL to 0.041 ± 0.06 pg/mL for IL-5, and from 0.206 ± 0.35 pg/mL to 0.082 ± 0.19 pg/mL for IL-13, respectively); however, L-92 did not have this influence. IL-4 production was low, with no difference between various stimulations. Treatment of KW3110-treated DCs with anti-PD-L2 antibodies abrogated the inhibition of IL-5 and IL-13 production. The level of PD-L2 on DCs was the same with stimulation by OVA alone and with L-92 in OVA-stimulated DCs. In the response provoked by OVA alone, the PD-L2 antibody did not affect IL-5 or IL-13 production.

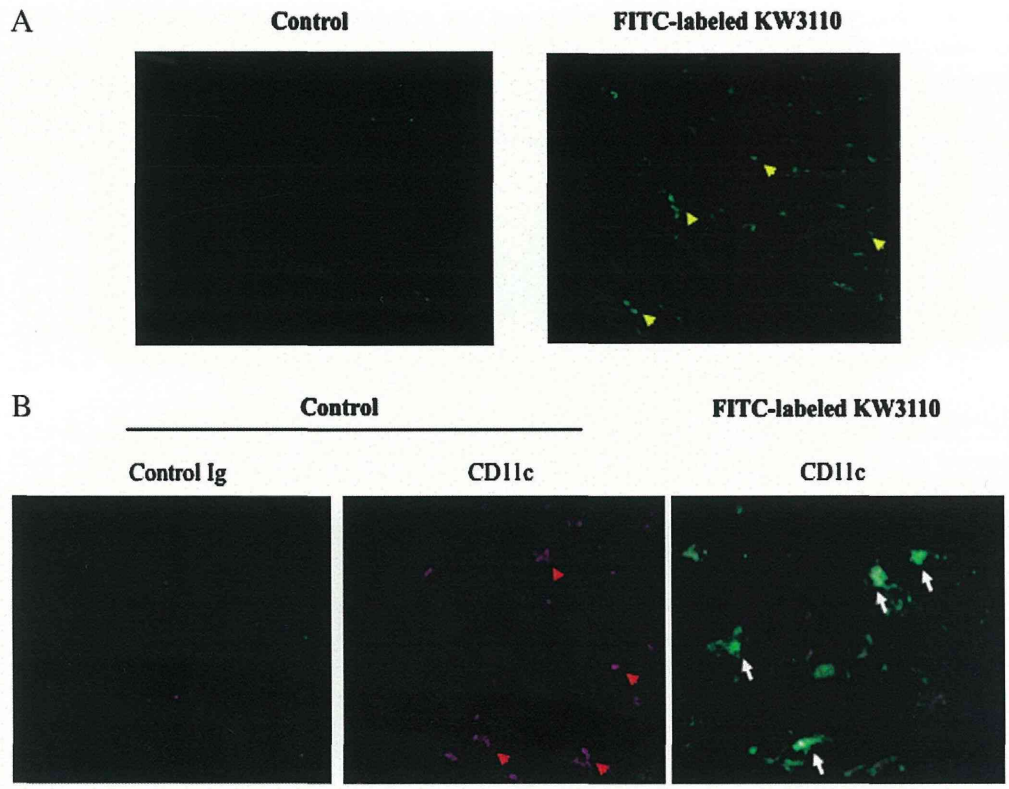

Figure 3 At 3 h after sublingual administration of FITC-labeled KW3110 stimulated with OVA, KW3110 bacteria were detected in CLNs by confocal microscopy (A, yellow arrow). CD11c-positive cells were identified by treatment with biotin-labeled anti-CD11c$^+$ (B, red arrow). Many fragmented and some intact KW3110 (CD11c$^+$ FITC$^+$ cells) bacteria were phagocytosed by DCs, as identified by double staining (B, white arrow), but were not found in the CLNs of control mice that did not receive KW3110. Magnification is 400× for all images.

3.4. Sublingually administered KW3110 migrated to CLNs

FITC-labeled KW3110 was detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (Figure 2A). The presence of CD11c-positive cells was shown by treatment with biotin-labeled anti-CD11c. Double staining with anti-CD11c showed that many fragmented and some intact KW3110 (CD11c$^+$ FITC$^+$ cells) organisms were phagocytosed by DCs (Figure 2B). Similarly fragmented and some intact forms of KW3110 were phagocytosed by DCs in CLNs 3 h after administration (Figure 3). Twelve hours later, these KW3110 were rarely detected in the oral mucosa and the CLNs.

3.5. KW3110 attenuated the nasal symptoms and serum IgE level in the OVA sensitized mice through PD-L2 expression

After OVA nasal provocation, the frequency of sneezing and nasal rubbing events decreased significantly in mice that received sublingual administration of KW3110 at 0.5 mg/day compared with those that received PBS (Table 1A). This response was dose-dependent and was stronger in mice receiving 0.5 mg.mL^{-1} KW3110 compared to those receiving a 0.05 mg.mL^{-1} dose. The response reached a plateau at a dose of 0.5 mg.mL^{-1} and there was no significant difference between mice receiving 0.5 and 5 mg.mL^{-1} KW3110 (data not shown). The frequency of sneezing and nasal rubbing events was not decreased in OVA-sensitized mice receiving sublingual L-92 or 0.5 mg of intragastric KW3110. Nasal symptoms were decreased to some extent at a ten-fold higher intragastric dose (5 mg.day^{-1}) of KW3110, but the effect was less marked than for sublingual administration at 0.5 mg/day (data not shown). Serum OVA-specific IgE was significantly reduced in mice receiving sublingual KW3110 but not in others, including those receiving intragastric KW3110 at 5 mg.day^{-1}. Treatment of OVA-sensitized mice with anti-PD-L2 mAb before KW3110 sublingual administration abrogated the effects of KW3110 (Table 1B).

3.6. Sublingually administered KW3110 decreased Th2 cytokine production and the number of IgE-secreting cells in CLNs of sensitized mice

Cytokine production from CD4$^+$ T cells in CLNs is shown in Table 2B. Of the Th2 cytokines examined, the IL-4, IL-5, and IL-13 levels were reduced in CD4$^+$ T cells from mice that received KW3110 sublingually, but not in those from mice that received intragastric KW3110. Reduction of IFN-γ or enhancement of IL-10 was not observed in CD4$^+$ T cells from any mice.

Table 1A Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4+ T cells in CLNs.

		Sneezing Counts		Nasal rubbing Counts		Serum anti-OVA IgE (ng /ml)	
Nasal symptoms and serum IgE							
Gavage tube (0.5 mg)	PBS	61±15	n.s.	48±08	n.s.	425±015	n.s.
(n=18 in each group)	KW3110	51±13	n.s.	54±08	n.s.	478±029	n.s.
	L92	68±23		44±06		468±087	
Sublingual. (0.5mg)	PBS	55±06	*	48±10	*	568±054	*
(n=16 in each group)	KW3110	23±05		20±03	n.s.	278±054	
	L92	50±15	*	35±05		457±084	*
Non immunized mice (control)		10±04 **		15±11 **		n.d.	

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

Table 1B Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4+ T cells in CLNs.

		IL-4 (ng /ml)		IL-5 (ng /ml)		IL-13 (ng /ml)		IFN-Ω (ng /ml)		IL-10 (ng /ml)	
Cytokine production in CD4+ T cells derived from cervical lymph nodes											
Gavage tube (0.5 mg)	PBS	0.48±0.07	n.s.	2.55±0.56	n.s.	3.47±0.97	n.s.	23.08±2.98	n.s.	0.23±0.04	n.s.
(n = 18 in each group)	KW3110	0.51±0.18	n.s.	2.22±0.58	n.s.	3.89±0.18	n.s.	29.25±6.24	n.s.	0.18±0.04	n.s.
	L92	0.39±0.18		2.93±0.43		4.07±0.58		12.53±3.14		0.28±0.05	
Sublingual. (0.5 mg)	PBS	0.52±0.08	*	2.47±0.48	*	4.62±0.08	*	24.62±4.08	n.s.	0.12±0.06	n.s.
(n = 16 in each group)	KW3110	0.22±0.13		1.09±0.23		1.57±0.13		22.16±0.13		0.15±0.03	
	L92	0.36±0.03	*	2.38±0.17	*	3.17±0.71	*	28.23±3.46	n.s.	0.14 ± 0.02	n.s.
Non-immunized mice (control)		n.d.		n.d.		** n.d.		n.d.		n.d.	

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable.

The number of IgE-secreting cells was higher in CLNs than in BM cells, ILNs, and the spleen from OVA-sensitized mice (Figure 4A). This number was significantly decreased in CLNs from mice that received submucosal administration of KW3110. Treatment with anti-PD-L2 antibody abrogated the reduction of Th2 cells (Tables 2A and 2B) and IgE-secreting cells (Figure 4B).

4. Discussion

DCs play a crucial role in the induction of primary T-cell-dependent immune responses [20]. Upon uptake of antigens on the mucosal surface, immature DCs in the oral mucosa are thought to migrate to the regional CLNs [21]. This suggests that the oral mucosa provides a potential route for vaccine administration and thus the interaction of oral DCs with T cells in regional lymph nodes is of interest [22,23]. In this study, we showed that *Lactobacillus* spp. administered sublingually might migrate to the CLNs and influence the maturation of DCs induced by uptake of antigen, but that the patterns differ significantly among strains.

KW3110 strongly enhanced the expression of CCR-7 and PD-L2 on DCs, compared to L-92. Sublingual administration of low doses of KW3110 in OVA-sensitized mice *in vivo* decreased IgE production and nasal symptoms induced by nasal OVA provocation, whereas the same dose of the L-92 strain or intragastric administration of KW3110 had no effect. Th2 cytokine expression and IgE synthesis in CLNs (the major area of antigen presentation and induction of effector cells in the OVA-sensitized mice) were strongly suppressed by sublingual KW3110 but not by L-92 treatment. Pre-treatment with neutralizing antibodies to PD-L2 abrogated the anti-allergic effects of sublingual KW3110. Earlier studies have shown that CCR-7 expression accelerates migration of DCs to the regional lymph nodes [24]. It is also reported that PD-L2 expression on DCs is associated with inhibition of Th2 cytokine production [18,24,25]. Matsumoto et al. showed that PD-L2 was highly expressed on pulmonary DCs and macrophages of the allergic sensitized mice, and administration of anti-PD-L2 antibody enhanced airway hyperreactivity and production of Th2 cytokines [18]. These observations suggest that DCs with high CCR-7 expression that phagocytose KW3110 migrate to the CLNs and subsequently suppress production of Th2 cytokines and IgE, mainly through expression of PD-L2.

The anti-allergic actions of L-92 observed in previous studies were brought about by induction of Treg through TLR2 [13]. However, L-92 may not stimulate DCs directly to induce CCR-7 or PD-L2, and may not effectively activate DCs in the oral submucosa when administered sublingually, due to poor

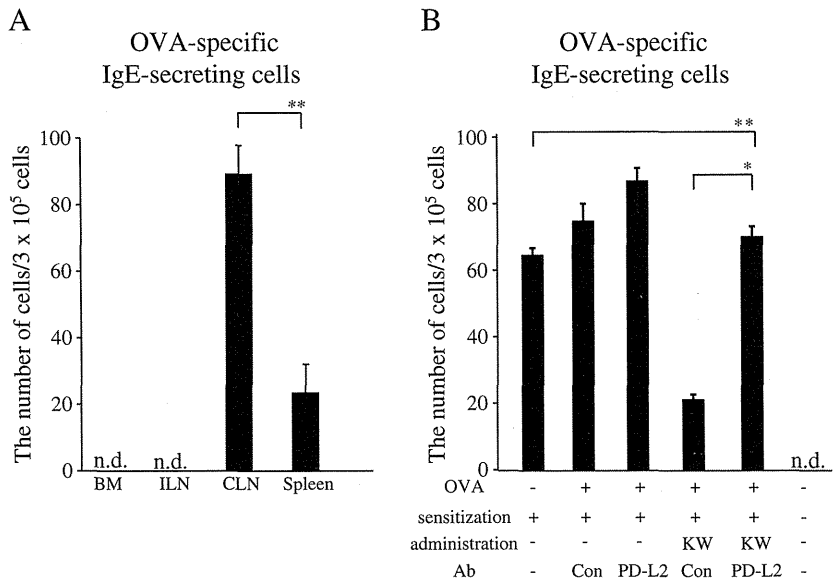

Figure 4 Inhibition of Ag-specific IgE production by KW3110. The number of OVA-specific IgE-secreting cells among cells from lymphoid organs and BM was analyzed by ELISPOT (A). Anti-PD-L2 treatment before oral submucosal administration of KW3110 blocked the reduction of IgE production in cells derived from CLNs (B).

phagocytosis. *In vitro*, DCs phagocytose a higher number of KW3110 organisms than L-92, as shown in this study. This may be because the L-92 strain is twice as large as the KW3110 strain. It is unclear if regulatory T cells were induced by sublingual administration of KW3110, because IL-10 induction or suppression of IFN-γ was not observed in the CLNs. Although the different responses among the strains of *Lactobacillus* may reflect different surface compositions and interactions with different TLRs, the roles of TLR2 or TLR9 in the immune responses induced by KW3110 are unclear [12].

KW3110 is used widely as an additive in yoghurt, beverages, and other foods, and its safety has been certified. However, in our previous study of patients with Japanese cedar pollinosis, 12 weeks intake of 50 mg day^{-1} of heat-killed KW3110, which was commenced 4 weeks before pollen dispersal, had only a limited clinical effect [26]. The KW3110 in that study was administered in capsule form; this is digested and dissolved by gastric juice or digestive enzymes, but the metabolites are unknown. In the present study, there was no benefit from intragastric administration of KW3110, but direct administration of the same dose of intact KW3110 to the oral mucosa did have an effect, with improvement in symptoms and reduced Th2 cytokine production.

5. Conclusions

Our results suggest that sublingual administration of KW3110 may reduce allergic inflammation, but the optimum dose and administration protocol require clarification. A recent review of randomized controlled studies of sublingual immunotherapy (SLIT) for allergic rhinitis suggests that this approach is safe and may be effective as an alternative route of administration [27]. Improved efficacy of SLIT could be achieved by adjuvant therapy with sublingual administration of KW3110, since the anti-allergic effects of sublingual administration were enhanced by simultaneous stimulation with an antigen in this study. A clinical study in patients with allergic rhinitis will be performed to examine this hypothesis, and may yield further information on the potential of *Lactobacillus* therapy.

Table 2A Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Administration	Neutralizing Ab	Sneezing Counts		Nasal rubbing Counts		Serum anti-OVA IgE (ng/ml)	
Nasal symptoms and serum IgE								
–	–	–	61±9	n.s.	68±11	n.s.	651±051	n.s.
OVA	–	Control Ig	52±12	n.s.	67±07	n.s.	695±037	n.s.
OVA	–	PD-L2	64±13	n.s.	70±19	n.s.	677±017	n.s.
OVA	KW3110	Control Ig	18±05	*	24±08	*	202±058	*
OVA	KW3110	PD-L2	49±07		54±17		623±015	
Non-immunized mice (control)			01±02	**	05±03	**	n.d.	

Values are presented as the mean±SD. Number of mice in each group was 18. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

Table 2B Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Stimulation	Neutralizing Ab	IL-4 (ng/ml)		IL-5 (ng/ml)		IL-13 (ng/ml)	
Cytokine production in CD4+ T cells derived from cervical lymph nodes								
–	–	–	0.41±0.13	n.s.	2.94±0.14	n.s.	4.16±0.14	n.s.
OVA	–	Control Ig	0.34±0.05	n.s.	2.83±0.58	n.s.	3.15±0.25	n.s.
OVA	–	PD-L2	0.49±0.19	n.s.	2.80±0.32	n.s.	3.49±0.36	n.s.
OVA	KW3110	Control Ig	0.14±0.04	*	0.78±0.07	*	1.08±0.09	*
OVA	KW3110	PD-L2	0.48±0.09		2.56±0.27		3.17±0.14	
Non immunized mice (control)			n.d.		n.d.		n.d.	

Values are presented as the mean±SD. Number of mice in each group was 18. *$P<0.05$; n.s., not significant; n.d., not detectable.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

[1] J. Bousquet, P. van Cauwenberge, N. Khaltaev, Allergic rhinitis and its impact on asthma, J. Allergy Clin. Immunol. 108 (2001) S147–S334.
[2] E. Valovirta, S.E. Myrseth, S. Palkonen, The voice of the patients: allergic rhinitis is not a trivial disease, Curr. Opin. Allergy Clin. Immunol. 8 (2008) 1–9.
[3] P.S. Norman, Immunotherapy: 1999–2004, J. Allergy Clin. Immunol. 113 (2004) 1013–1023.
[4] R.F. Lockey, G.L. Nicoara-Kasti, D.S. Theodoropoulos, S.C. Bukantz, Systemic reactions and fatalities associated with allergen immunotherapy, Ann. Allergy Asthma Immunol. 87 (2001) 47–55.
[5] S.L. Prescott, B. Bjorkstén, Probiotics for the prevention or treatment of allergic diseases, J. Allergy Clin. Immunol. 120 (2007) 255–262.
[6] E. Pohjavuori, M. Viljanen, R. Korpela, M. Kuitunen, M. Tiittanen, O. Vaarala, E. Savilahti, *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy, J. Allergy Clin. Immunol. 114 (2004) 131–136.
[7] G.I. Betsi, E. Papadavid, M.E. Falagas, Probiotics for the treatment or prevention of atopic dermatitis: a review of the evidence from randomized controlled trials, Am. J. Clin. Dermatol. 9 (2) (2008) 93–103.
[8] J.Z. Xiao, S. Kondo, N. Yanagisawa, N. Takahashi, T. Odamaki, N. Iwabuchi, K. Miyaji, K. Iwatsuki, H. Togashi, K. Enomoto, T. Enomoto, Probiotics in the treatment of Japanese cedar pollinosis: a double-blind placebo-controlled trial, Clin. Exp. Allergy 36 (2006) 1425–1435.
[9] H. Johannsen, S.L. Prescott, Practical prebiotics, probiotics and synbiotics for allergists: how useful are they? Clin. Exp. Allergy 39 (2009) 1801–1814.
[10] L. Van Overtvelt, H. Moussu, S. Horiot, S. Samson, V. Lombardi, L. Mascarell, A. van de Moer, R. Bourdet-Sicard, P. Moingeon, Lactic acid bacteria as adjuvants for sublingual allergy vaccines, Vaccine 28 (2010) 2986–2992.
[11] D. Fujiwara, S. Inoue, H. Wakabayashi, T. Fujii, The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance, Int. Arch. Allergy Immunol. 135 (2004) 205–215.
[12] S. Ichikawa, R. Fujii, D. Fujiwara, Y. Komiyama, T. Kaisho, M. Sakaguchi, Y. Konishi, MyD88 but not TLR2, 4 or 9 is essential for IL-12 induction by lactic acid bacteria, Biosci. Biotechnol. Biochem. 71 (2007) 3026–3032.
[13] G. Weiss, S. Rasmussen, L.H. Zeuthen, B.N. Nielsen, H. Jarmer, L. Jespersen, H. Frøkiaer, *Lactobacillus* acidophilus induces virus immune defence genes in murine dendritic cells by a Toll-like receptor-2-dependent mechanism, Immunology 131 (2010) 268–281.
[14] A. Torii, S. Torii, S. Fujiwara, H. Tanaka, N. Inagaki, H. Nagai, *Lactobacillus acidophilus* strain L-92 regulates the production of Th1 cytokine as well as Th2 cytokines, Allergol. Int. 56 (2007) 293–301.
[15] C. Scheicher, M. Mehlig, R. Zecher, K. Reske, Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor, J. Immunol. Methods 154 (1992) 253–264.
[16] K. Shida, J. Kiyoshima-Shibata, M. Nagaoka, K. Watanabe, M. Nanno, Induction of interleukin-12 by *Lactobacillus* strains having a rigid cell wall resistant to intracellular digestion, J. Dairy Sci. 89 (2006) 3306–3317.
[17] M.Y. Kimura, H. Hosokawa, M. Yamashita, A. Hasegawa, C. Iwamura, H. Watarai, M. Taniguchi, T. Takagi, S. Ishii, T. Nakayama, Regulation of T helper type 2 cell differentiation by murine Schnurri-2, J. Exp. Med. 201 (2005) 397–408.
[18] K. Matsumoto, H. Inoue, T. Nakano, M. Tsuda, Y. Yoshiura, S. Fukuyama, F. Tsushima, T. Hoshino, H. Aizawa, H. Akiba, D. Pardoll, N. Hara, H. Yagita, M. Azuma, Y. Nakanishi, B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism, J. Immunol. 172 (2004) 2530–2541.
[19] K. Takamura, S. Fukuyama, T. Nagatake, D.Y. Kim, A. Kawamura, H. Kawauchi, H. Kiyono, Regulatory role of lymphoid chemokine CCL19 and CCL21 in the control of allergic rhinitis, J. Immunol. 179 (2007) 5897–5906.
[20] B.N. Lambrecht, Dendritic cells and the regulation of the allergic immune response, Allergy 60 (2005) 271–282.
[21] J.H. Song, J.I. Kim, H.J. Kwon, D.H. Shim, N. Parajuli, N. Cuburu, C. Czerkinsky, M.N. Kweon, CCR7-CCL19/CCL21-

regulated dendritic cells are responsible for effectiveness of sublingual vaccination, J. Immunol. 182 (2009) 6851–6860.
[22] J.P. Allam, B. Niederhagen, M. Bücheler, T. Appel, H. Betten, T. Bieber, S. Bergé, N. Novak, Comparative analysis of nasal and oral mucosa dendritic cells, Allergy 61 (2006) 166–172.
[23] U.H. von Andrian, T.R. Mempel, Homing and cellular traffic in lymph nodes, Nat. Rev. Immunol. 3 (2003) 867–878.
[24] Y. Latchman, C.R. Wood, T. Chernova, et al., PD-L2 is a second ligand for PD-1 and inhibits T cell activation, Nat. Immunol. 2 (2001) 261–268.
[25] E. Oflazoglu, D.A. Swart, P. Anders-Bartholo, H.K. Jessup, A.M. Norment, W.A. Lawrence, K. Brasel, J.E. Tocker, T. Horan, A.A. Welcher, D.R. Fitzpatrick, Paradoxical role of programmed death-1 ligand 2 in Th2 immune responses in vitro and in a mouse asthma model in vivo, Eur. J. Immunol. 34 (2004) 3326–3336.
[26] S. Yonekura, Y. Okamoto, T. Okawa, M. Hisamitsu, H. Chazono, K. Kobayashi, D. Sakurai, S. Horiguchi, T. Hanazawa, Effects of daily intake of *Lactobacillus* paracasei strain KW3110 on Japanese cedar pollinosis, Allergy Asthma Proc. 30 (2009) 397–405.
[27] P. Moingeon, T. Batard, R. Fadel, F. Frati, J. Sieber, L. Van Overtvelt, Immune mechanisms of allergen-specific sublingual immunotherapy, Allergy 61 (2006) 151–165.

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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.

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,

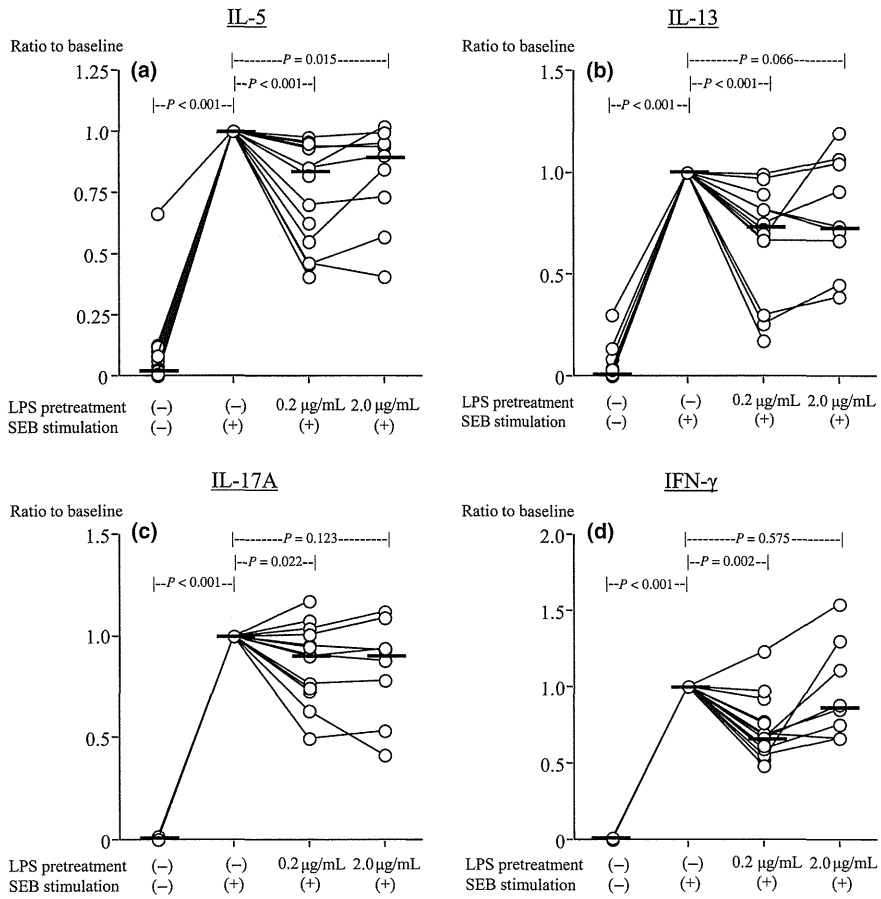

Fig. 1. Effects of pre-treatment with LPS on SEB-induced cytokine production by DNPCs. DNPCs were treated with 0, 0.2 or 2.0 μg/ml LPS 2 h prior to SEB stimulation. After 72 h of incubation with SEB, levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) within the supernatant were determined. The data 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

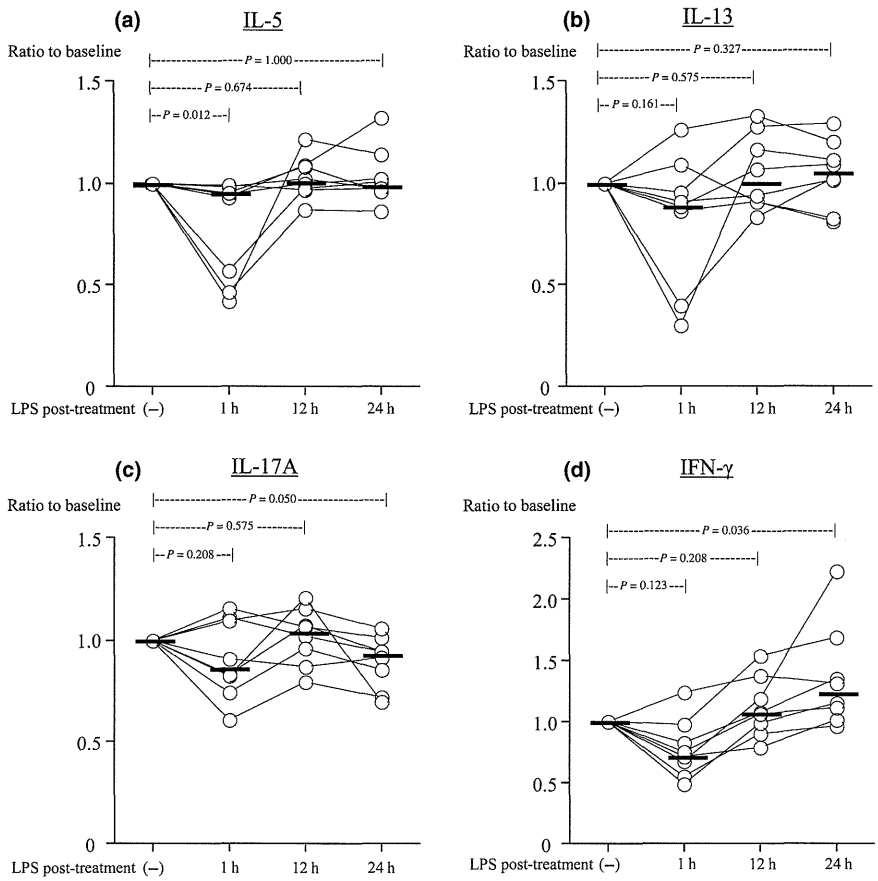

Fig. 2. Effects of post-treatment with LPS on SEB-induced cytokine production by DNPCs. At 1, 12 or 24 h after SEB stimulation, DNPCs were exposed to 0.2 μg/ml LPS. After 72 h of incubation with SEB, levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) were determined. Bars represent median values. 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

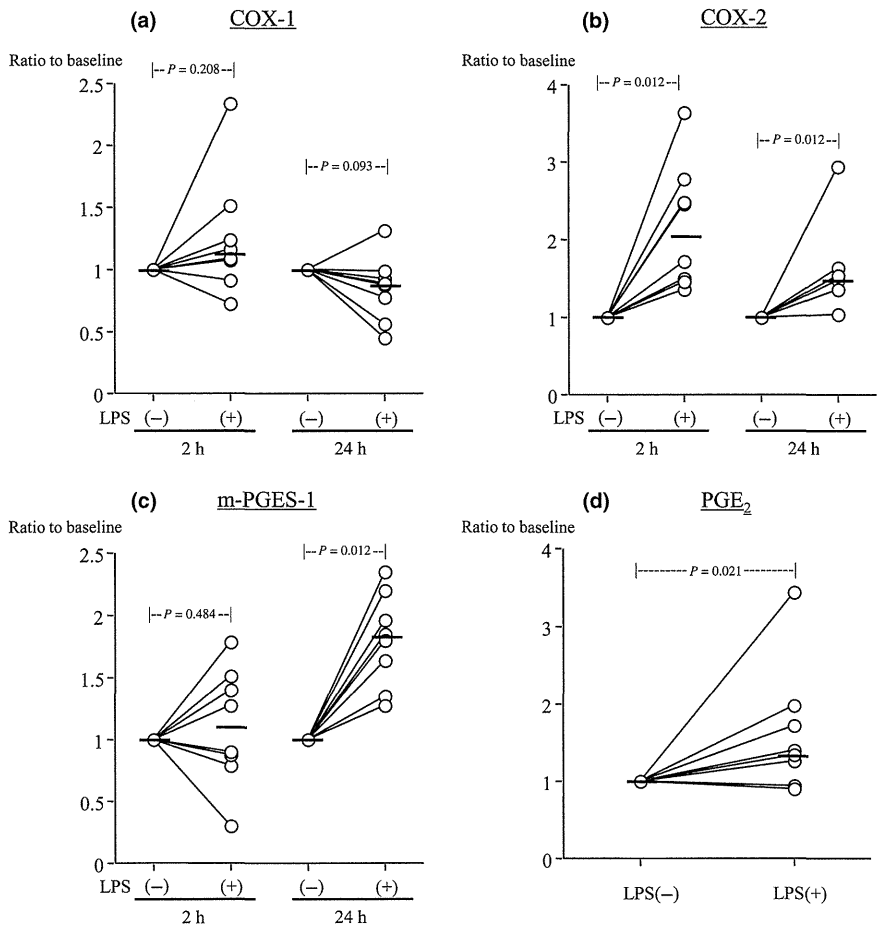

Fig. 3. Effects of LPS on expression of COX-related molecules in DNPCs. DNPCs were cultured with or without 0.2 μg/ml LPS for 2 or 24 h, and relative amounts of COX-1 (A), COX-2 (B) and m-PGES-1 (C) mRNA were determined. Alternatively, levels of PGE_2 after 72 h of incubation with LPS were measured (D). 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

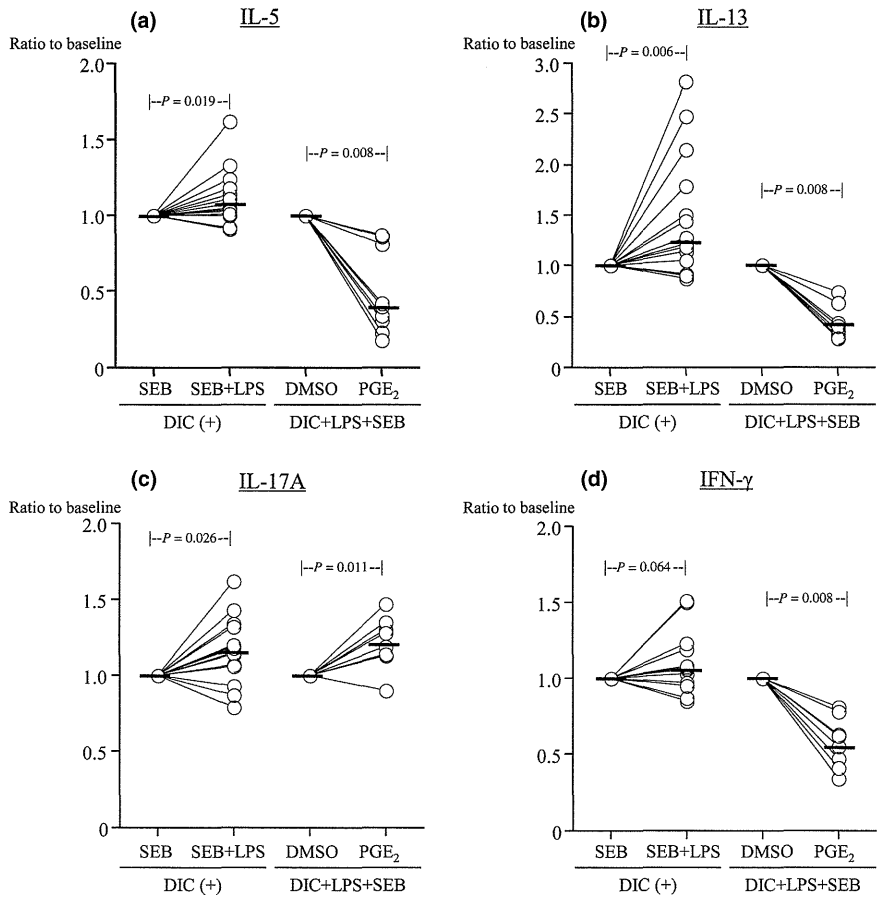

Fig. 4. Effects of LPS and PGE_2 on SEB-induced cytokine production in the presence of diclofenac. Diclofenac-treated DNPCs were exposed or unexposed to LPS prior to SEB stimulation (left side of each graph). Under these conditions, either 10^{-6} M PGE_2 or control buffer was added to the culture (right 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

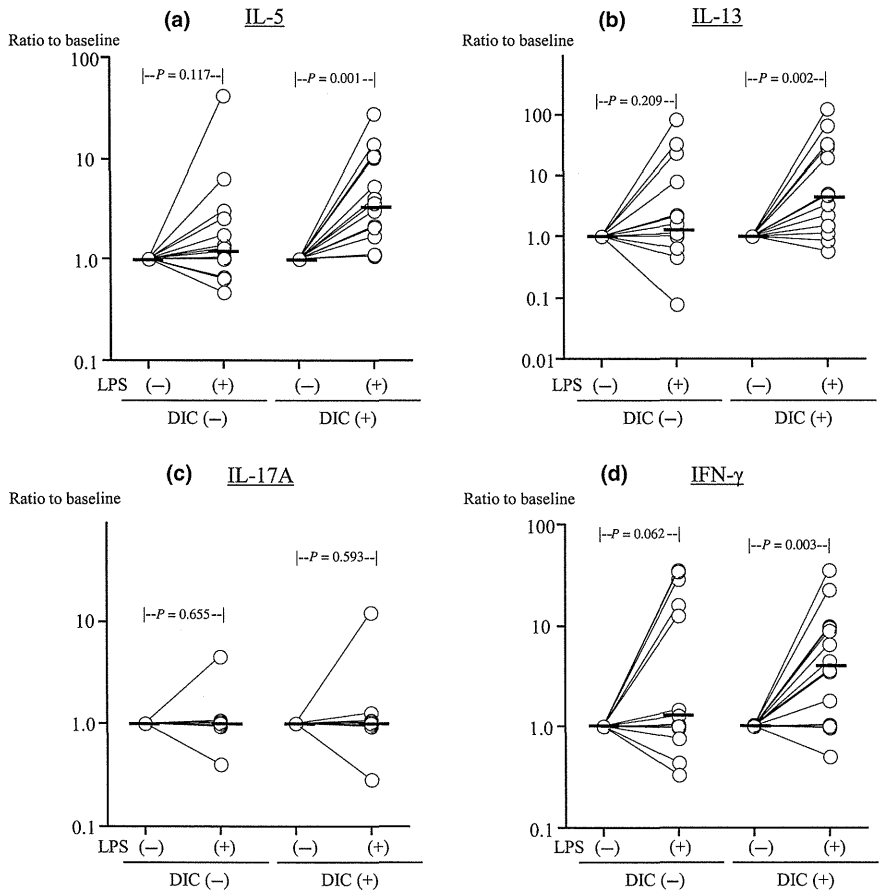

Fig. 5. Effects of diclofenac on LPS-induced cytokine production by DNPCs. Diclofenac-treated or untreated DNPCs were cultured with or without 0.2 μg/ml LPS for 72 h. Levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) were determined. The data were looked as differences from baseline. Bars represent 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.

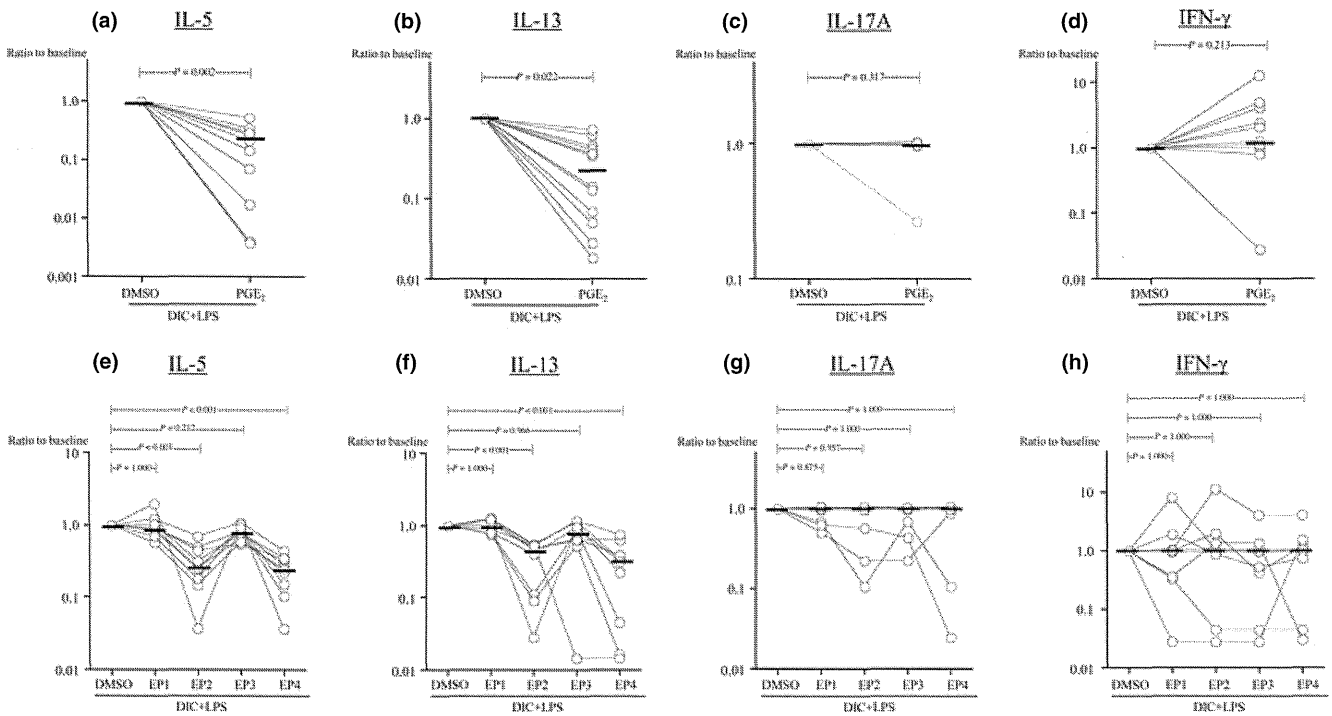

Fig. 6. Effects of PGE_2 and EP-selective agonists on LPS-induced cytokine production by diclofenac-treated DNPCs. Diclofenac-treated DNPCs were cultured with LPS in the presence of either 10^{-6} M PGE_2 (A–D), EP-selective agonists (E–H) or control buffer for 72 h. Levels of IL-5 (A, E), IL-13 (B, 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.

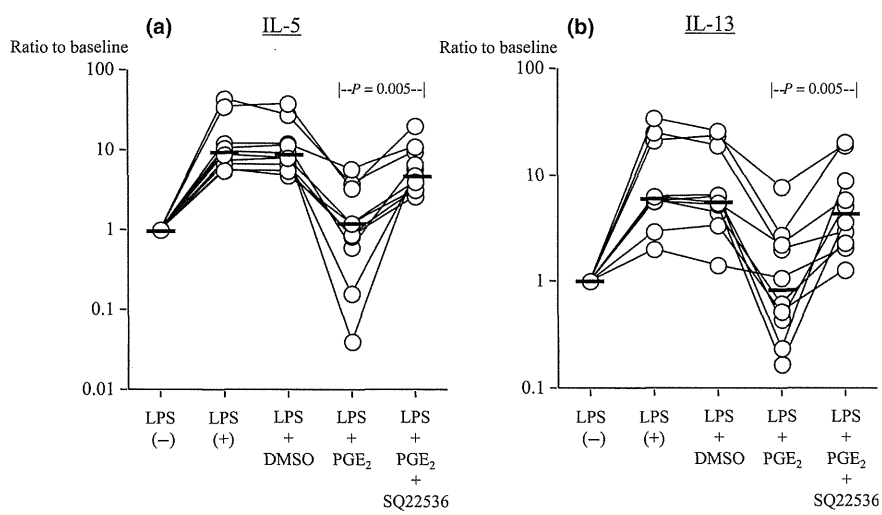

Fig. 7. Reversal of inhibitory effect of PGE_2 on LPS-induced IL-5 and IL-13 production by diclofenac-treated DNPCs with adenylate cyclase inhibitor. DNPCs were pre-treated with SQ22536 at 37°C for 1 h. Following incubation, the cells were washed with culture medium twice, after which they were treated 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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References

1 Bousquet J, Bachert C, Canonica GW *et al.* Extended global allergy and asthma European network, world allergy organization and allergic rhinitis and its impact on ashtma study group. Unmet needs in severe chronic upper airway disease (SCUAD). *J Allergy Clin Immunol* 2009; 124:428–33.

2 Zhang N, Van Zele T, Perez-Novo C *et al.* Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* 2008; 122:961–8.

3 Makihara S, Okano M, Fujiwara T *et al.* Regulation and characterization of IL-17A expression in chronic rhinosinusitis and its relationship with eosinophilic inflammation. *J Allergy Clin Immunol* 2010; 126:397–400.

4 Patou J, Gevaert P, van Zele T, Holtappels GH, van Cauwenberge P, Bachert C. Staphylocossus aureus enterotoxin B, protein, and lipoteichoic acid stimulations in nasal polyps. *J Allergy Clin Immunol* 2008; 121:110–5.

5 Okano M, Fujiwara T, Haruna T *et al.* PGE_2 suppresses staphylococcal enterotoxin-induced eosinophilia-associated cellular responses dominantly via an EP2-mediated pathway in nasal polyps. *J Allergy Clin Immunol* 2009; 123: 868–74.

6 Wang JH, Kwon HJ, Jang YJ. Rhinovirus upregulates matrix metalloproteinase-2, matrix-metalloproteinase-9, and vascular endothelial growth factor expression in nasal polyp fibroblasts. *Laryngoscope* 2009; 119:1834–8.

7 Okano M, Fujiwara T, Haruna T *et al.* Role of fungal antigens in eosinophilia-associated cellular responses in nasal polyps: comparison with enterotoxin. *Clin Exp Allergy* 2011; 41:171–8.

8 Ishinaga H, Takeuchi K, Kishioka C, Yagawa M, Majima Y. Effects of dexamethasone on mucin gene expression in cultured human nasal epithelial cells. *Laryngoscope* 2002; 112:1436–40.

9 Rodriguez D, Keller AC, Faquim-Mauro EL *et al.* Bacterial lipopolysaccharide signaling through toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. *J Immunol* 2003; 171:1001–8.

10 Bortolatto J, Borducchi E, Rodriguez D *et al.* Toll-like receptor 4 agonists adsorbed to aluminum hydroxide adjuvant attenuate ovalmun-specific allergic airway disease: role of MyD88 adaptor molecule and interleukin-12/interferon-γ axis. *Clin Exp Allergy* 2008; 38:1668–79.

11 Eldridge MW, Peden DB. Allergen provocation augments endotoxin-induced nasal inflammation on subjects with atopic asthma. *J Allergy Clin Immunol* 2000; 105:475–81.

12 Schaumann F, Muller M, Braun A *et al.* Endotoxin augments myeloid dendritic cell influx into the airways in patients with allergic asthma. *Am J Respir Crit Care Med* 2008; 177:1307–13.

13 Hofer TP, Bitterle E, Beck-Speier I *et al.* Diesel exhaust particles increase LPS-stimulated COX-2 expression and PGE_2 production in human monocytes. *J Leukc Biol* 2004; 75:856–64.

14 Wang Z, Zhang Q, Li Y, Li P, Zhang G, Li Y. Involvement of mitogen-activated protein kinases and nuclear factor kappa B pathways in signaling COX-2 expression in chronic rhinosinusitis. *Inflam Res* 2009; 58:649–58.

15 Moses T, Wagner L, Fleming SD. TLR-4-mediated COX-2 expression increases intestinal ischemia/reperfusion-induced damage. *J Leukoc Biol* 2009; 86:971–80.

16 Benninger MS, Ferguson BJ, Hadley JA *et al.* Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. *Otolaryngol Head Neck Surg* 2003; 129:S1–32.

17 Okano M, Fujiwara T, Yamamoto M *et al.* Role of prostaglandin D_2 and E_2 terminal synthases in chronic rhinosinusitis. *Clin Exp Allergy* 2006; 36:1028–38.

18 Rosenberg HF, Phipps S, Foster PS. Eosinophil trafficking in allergy and asthma. *J Allergy Clin Immunol* 2007; 119:1303–10.

19 Riechelmann H, Deutschle T, Rozsasi A, Keck T, Polzehl D, Burner H. Nasal biomarker profiles in acute and chronic rhinosinusitis. *Clin Exp Allergy* 2005; 35:1186–91.

20 Sturm EM, Shratl P, Schuligoi R *et al.* Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *J Immunol* 2008; 181: 7273–83.

21 Van Zele T, Claeys S, Gevaert P *et al.* Differentiation of chronic sinus diseases by measurement of inflammatory mediators. *Allergy* 2006; 61:1280–9.

22 Lu X, Zhang XH, Wang H *et al.* Expression of osteopontin in chronic rhinosinusitis with and without nasal polyps. *Allergy* 2009; 64:104–11.

23 Okano M, Sugata Y, Fujiwara T *et al.* EP2/EP4-mediated suppression of antigen-specific human T cell responses by prostaglandin E_2. *Immunology* 2006; 118:343–52.

24 Harris SG, Padila J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulator of immunity. *Trends Immunol* 2002; 23:144–50.

25 Saito T, Kusunoki T, Yao T *et al.* Role of interleukin-17A in the eosinophil accumulation and mucosal remodeling in chronic rhinosinusitis with nasal polyps associated with asthma. *Int Arch Allergy Immunol* 2009; 151:8–16.

26 Miyamoto M, Prause O, Sjostrand M, Laan M, Lotvall J, Linden A. Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. *J Immunol* 2003; 170:4556–72.

27 Louten J, Boniface K, de Waal Mylefyt R. Development and function of Th17 cells in health and disease. *J Allergy Clin Immunol* 2009; 123:1004–11.

28 Lee SM, Batzer G, Ng N *et al.* Regulatory T cells contribute to allergen tolerance induced by daily airway immunostimulant exposures. *Am J Respir Mol Cell Biol* 2011; 44:341–9.

29 Nonaka M, Pawankar R, Fukumoto A, Ogihara N, Skanushi A, Yagi T. Induction of eotaxin production by interleukin-4, interleukin-13 and lipopolysaccharide by nasal fibroblasts. *Clin Exp Allergy* 2004; 34:804–11.

30 Prefontaine D, Banville-Langelier AA, Fiset PO *et al.* Children with atopic histories exhibit impaired lipopolysaccharide-induced Toll-like recetpro-4 signaling in peripheral monocytes. *Clin Exp Allergy* 2010; 40:1648–57.

31 Quah PL, Kuo IC, Huang CH, Shek LP, Lee BW, Chua KY. Early onset wheeze associated with enhanced combined IL-1b, IL-6, and IL-12/IL-23p40 in LPS-stimulated cord blood mononuclear cells. *Clin Exp Allergy* 2011; 41:970–8.

32 Barr RG, Wentowski CC, Curhan GC *et al.* Prospective study of acetaminophen use and newly diagnosed asthma among women. *Am J Respir Crit Care Med* 200; 169:836–41.

33 Thomsen SF, Kyvik KO, Skadhauge LR, Steffensen I, Backer V. Intake of paracetamol and risk of asthma in adults. *J Asthma* 2008; 45:675–6.

A critical role of IL-33 in experimental allergic rhinitis

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Background: We reported previously that serum levels of IL-33 are significantly increased in patients with allergic rhinitis (AR). However, very little is known about the role of IL-33 for the development of AR.

Objective: We thought to develop a novel murine model of ragweed pollen–specific AR and examined the pathologic role for ragweed-induced IL-33 in the development of AR manifestation using IL-33–deficient ($il33^{-/-}$) mice.

Methods: Ragweed-immunized and ragweed-challenged mice were examined for early- and late-phase nasal responses. IL-33 protein expression in the nasal epithelial cells of the AR murine model and patients with AR were assessed by using confocal microscopy.

Results: After nasal challenge with ragweed pollen, ragweed-immunized wild-type mice manifested early-phase (sneezing) and late-phase (eosinophilic and basophilic accumulation) responses. In contrast, $il33^{-/-}$ and FcεRI$^{-/-}$ mice did not have both early- and late-phase AR responses. IL-33 protein was constitutively expressed in the nucleus of nasal epithelial cells and was promptly released into nasal fluids in response to nasal exposure to ragweed pollen. In human subjects we revealed constitutive expression of IL-33 protein in the nasal epithelial cells of healthy control subjects and downregulated expression of IL-33 protein in inflamed nasal epithelial cells of patients with AR. IL-33–stimulated mast cells and basophils contributed to the early- and late-phase AR manifestation through increasing histamine release and production of chemoattractants for eosinophils/basophils, respectively.

Conclusions: Ragweed pollen–driven endogenous IL-33 contributed to the development of AR responses. IL-33 might present an important therapeutic target for the prevention of AR. (J Allergy Clin Immunol 2012;130:184-94.)

Key words: *IL-33, allergic rhinitis, ragweed pollen, epithelial cells, sneezing, eosinophils, basophils, mast cells*

Allergic rhinitis (AR) is one of the most common allergic inflammatory diseases. Globally, more than 600 million persons have AR.[1] AR is divided into 2 categories: seasonal and perennial.[2] The prevalence of seasonal AR, pollinosis, is increasing in the developed world. Among allergenic weeds, ragweed (*Ambrosia* species) pollen is common and has been reported as the major source of airborne allergenic protein in the United States and many countries of central Europe.[3] At least 10% of the overall population in these countries is sensitized to ragweed, and the prevalence in atopic subjects is almost 50%.[3-5]

Nasal responses in patients with AR comprise 2 phases: IgE-dependent early-phase responses and T_H2 cytokine–dependent late-phase responses.[2,6,7] Clinical symptoms or signs, such as sneezing and rhinorrhea, occur as a result of the early-phase response within 5 to 30 minutes. Late-phase responses consist of congestion, fatigue, malaise, and irritability at 6 to 24 hours after exposure to an allergen. The major pathologic change associated with late-phase responses is influx of inflammatory cells, such as eosinophils, into the nasal mucosa.[2,6,7] The mechanisms underlying the development of bronchial asthma have been well analyzed by using a murine model. However, the precise mechanisms underlying the development of nasal responses in patients with AR have not been clearly defined.

IL-33, the latest member of the IL-1 family, is the ligand for ST2 (IL-33 receptor [IL-33R] α)[8] and shares the signaling pathway with IL-1 and IL-18.[8-10] However, unlike with IL-1 and IL-18, the protein maturation process is not necessary for IL-33 bioactivity. Full-length IL-33 has biological activity *in vivo*, and IL-33 is most likely released through cell necrosis or injury rather than cleavage by caspase.[11] Thus IL-33 has been referred to as an alarmin.[12] IL-33 was originally reported as a nuclear factor protein in endothelial cells of high endothelial venules[13]; hence it was initially called NF-HEV. Indeed, IL-33 is constitutively expressed and localized in the nucleus of epithelial and endothelial cells from various tissues.[14,15]

IL-33 has the capacity to induce T_H2 cytokine production in T_H2 cells,[8,16] mast cells,[17] basophils,[16,18] eosinophils,[19,20] and newly identified innate immune cells (natural helper cells and nuocytes),[21,22] suggesting that IL-33 has the potential to induce T_H2 cytokine–mediated allergic inflammation.[23] Indeed, IL-33

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Abbreviations used
AR: Allergic rhinitis
DNP: 2,4-Dinitrophenyl
CTMC: Connective tissue–type mast cell
FITC: Fluorescein isothiocyanate
il33$^{-/-}$: IL-33 deficient
IL-33R: IL-33 receptor
JC: Japanese cedar
MCP-1: Monocyte chemotactic protein 1
MIP-1α: Macrophage inflammatory protein 1α
MMC: Mucosal mast cell
mMCP-8: Murine mast cell protease 8
OVA: Ovalbumin
PE: Phycoerythrin
SSC: Side scatter
WT: Wild-type

is implicated in asthma,[16,18] allergic conjunctivitis,[19] and anaphylactic responses.[24] Furthermore, we showed previously that the serum IL-33 level is significantly increased in Japanese patients with seasonal AR and revealed a significant association between susceptibility to AR and IL-33 polymorphism.[25]

Given the difficulty in examining the mechanisms in human subjects, a murine model of AR is essential. However, there is no appropriate murine model of AR, especially for seasonal AR. Here we established a novel murine model of ragweed-specific AR and examined the pathologic role for endogenous IL-33 in the induction of early- and late-phase AR manifestation by using IL-33–deficient (*il33*$^{-/-}$) mice.

METHODS

For more information, see the Methods section in this article's Online Repository at www.jacionline.org.

Mice

The generation of *il33*$^{-/-}$ mice is detailed in our separate report.[26] *il33*$^{-/-}$ mice (129SvJ × C57BL/6) were backcrossed for 7 generations onto BALB/c mice, and their littermate controls (*il33*$^{+/+}$) were used for the experiments.

Human samples

A total 10 patients with AR and 5 healthy subjects were recruited from the University Hospital, Kyoto Prefectural University of Medicine; 13 patients with AR and 11 healthy subjects were recruited from the University of Fukui Hospital. Demographic and clinical characteristics of the control subjects and patients are summarized in Tables E1 and E2 in this article's Online Repository at www.jacionline.org. For more information, see the Methods section in this article's Online Repository.

Experimental AR by active immunization

Mice were immunized with a mixture of ragweed pollen (100 μg in 200 μL) and aluminum hydroxide hydrate gel (1 mg in 200 μL; Sigma-Aldrich, St Louis, Mo) by means of intraperitoneal injection on day 0 and with ragweed/PBS (100 μg in 200 μL) by means of intraperitoneal injection on day 7. A week after the boost, mice (5 mice per group) were challenged by means of nasal administration of ragweed pollen (1 mg in 20 μL of PBS) or PBS (20 μL) for 4 consecutive days. Immediately after each nasal challenge, the frequency of sneezing was counted in a blinded manner for 10 minutes. Peripheral blood was collected from the inferior vena cava 24 hours after the final nasal challenge, and then sera were prepared by using centrifugation. The mice were killed, and the nose and cervical lymph nodes were isolated for further histologic and immunologic analysis.

Flow cytometry and cell purification

Bone marrow–derived connective tissue–type mast cells (CTMCs), mucosal mast cells (MMCs), and basophils were prepared as described previously.[16,27,28] The purity of each population was greater than 97%.

Statistics

Statistical significance was calculated with the 2-tailed Student *t* test. *P* values of less than .05 were considered statistically significant.

RESULTS

Establishment of ragweed-immunized ragweed-induced AR

We first generated a murine model of ragweed-specific AR. We immunized BALB/c background *il33*$^{+/+}$ mice with ragweed pollen by means of sequential intraperitoneal injection of ragweed/alum and ragweed/PBS. Then we challenged the mice by means of nasal administration of ragweed pollen or PBS for 4 consecutive days. We counted the frequency of sneezing over a 10-minute period immediately after the last nasal challenge. Compared with PBS-challenged control mice, ragweed-challenged mice showed a significant increase in the frequency of sneezing (Fig 1, *A*), which suggests that the ragweed pollen challenge induces immediate-type AR, possibly in an IgE-dependent manner. Indeed, compared with PBS-challenged mice, ragweed-challenged mice showed significantly increased total and ragweed-specific IgE levels in their sera when measured 1 day after the final challenge ($P < .005$; Fig 1, *B*).

Histologic analysis showed a multilayered epithelium, goblet cell hyperplasia, and prominent accumulation of eosinophils in the nasal lateral mucosa, nasal turbinate, and nasal septal mucosa of ragweed-challenged mice but not of PBS-challenged mice (Fig 1, *C-E*, and see Figs E1-E3 in this article's Online Repository at www.jacionline.org). In addition, increased numbers of eosinophils in the cervical lymph nodes were observed (see Fig E4 in this article's Online Repository at www.jacionline.org). Most nasal eosinophils (Siglec-F^{+} cells) expressed ST2 (IL-33Rα; Fig E1, *C*). In all, this ragweed-specific AR murine model mimics the major features of human AR, especially ragweed-induced pollinosis, in terms of nasal symptoms and histologic changes after intranasal exposure to ragweed pollen.

il33$^{-/-}$ mice did not mount T_H2/IgE responses on ragweed challenge

To clarify the physiologic role of endogenous IL-33 in AR, we generated BALB/c background *il33*$^{-/-}$ mice.[26] Compared with *il33*$^{+/+}$ mice, ragweed-immunized *il33*$^{-/-}$ mice showed a significant reduction in the frequency of sneezing, total and ragweed-specific IgE response, and accumulation of eosinophils in the nasal mucosa and cervical lymph nodes after nasal administration of ragweed pollen. In addition, histologic analysis revealed that *il33*$^{-/-}$ mice showed a diminished degree of multilayer formation in the epithelium and goblet cell hyperplasia in the nasal mucosa (Fig 1, *A-D*, and see Figs E2-E4). Like *il33*$^{+/+}$ mice, however, ragweed-immunized and PBS-challenged *il33*$^{-/-}$ mice evidenced considerably increased total IgE levels in their sera