COX/PGE$_2$ axis critically regulates effects of LPS on eosinophilia-associated cytokine production in nasal polyps.

Short title: LPS regulates cytokine production via COX/PGE$_2$ in NP.

Takaya Higaki, Mitsuhiro Okano, Tazuko Fujiwara, Seiichiro Makihara, Shin Kariya, Yohei Noda, Takenori Haruna and Kazunori Nishizaki

Department of Otolaryngology-Head & Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

Corresponding and reprint requests to Mitsuhiro Okano, Department of Otolaryngology-Head & Neck Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikatacho, Okayama 700-8558, Japan; Tel: +81-86-235-7307, Fax: +81-86-235-7308, email: mokano@cc.okayama-u.ac.jp

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: SEB induced IL-5, IL-13, IL-17A and IFN-γ production by DNPCs. Pretreatment 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 pretreatment 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 & 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 CRSwNP.

Key words

COX, cytokine, LPS, PGE_2, Staphylococcal enterotoxin B

2

Abbreviations

COX: cyclooxygenase

CRSwNP: chronic rhinosinusitis with nasal polyps

DMSO: dimethyl sulfoxide

DNPC: dispersed nasal polyp cell

EP: E-prostanoid

m-PGES-1: microsomal PGE_2 synthase 1

LPS: lipopolysaccharide

PG: prostaglandin

SE: staphylococcal enterotoxin

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNPs) is characterized by eosinophilic inflammation, and is often associated with asthma and aspirin sensitivity [1]. While the precise etiology 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 LPS affects eosinophilia-associated cytokine production reflecting Th responses in CRSwNP.

In order to determine whether the exposure to LPS affects the

4

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]. **4 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); LPS, RPMI-1640, L-glutamine-penicillin-streptomycin solution, protease, collagenase (Type I), hyaluronidase, DNase I**, and SQ22536** (Sigma, St. Louis, MO); diclofenac sodium (Wako Pure Chemicals, Osaka, Japan); FCS (Invitrogen, Carlsbad, CA); red blood cell lysis buffer (Roche, Indianapolis, IN); and PGE_2 (Cayman, Ann Arbor, MI). 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

6

(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

DNPCs were prepared from nasal polyps by enzymatic digestion, as described previously [5]. Briefly, minced nasal polyps were incubated for 2 hours 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) in order 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). $8.5 \pm 5.3\%$, $11.7 \pm 8.9\%$, $8.9 \pm 8.2\%$, $8.5 \pm 6.8\%$, $7.8 \pm 11.1\%$, $10.9 \pm 10.5\%$, $15.5 \pm 6.7\%$, and $21.6 \pm 7.7\%$ cells in 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 hours' 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 hours 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). 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

DNPCs were pretreated with LPS at 0.2 or 2.0 μg/ml 2 hours prior to SEB stimulation in order 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 hours after SEB stimulation. To determine the role of COX and PGE_2 in the effect of LPS on SEB-induced cytokine production, DNPCs were pretreated 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

DNPCs were solely cultured with and without 0.2 μg/ml LPS for 72 hours, after which

levels of cytokines were determined. To determine whether COX and PGE_2 are involved in LPS-induced cytokine production, DNPCs were pretreated 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 hours or 72 hours 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 37^0C for 1 hour. 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

In order to determine whether 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 hours. 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

9

in the supernatants after 72 hours 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).

Results

<u>Pretreatment with LPS inhibits SEB-induced Th1/Th2/Th17 cytokine production by DNPCs</u>

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).

Pretreatment with 0.2 μg/ml LPS 2 hours 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 hour after SEB stimulation (p=0.012) (Fig. 2). Rather, addition of LPS at 24 hours 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 further analysis.

<u>LPS induces COX and PGE$_2$ expression in DNPCs</u>

In order to determine how pretreatment 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 hours after stimulation with LPS (p=0.012, Fig. 3B), and this increase

was sustained at 24 hours (p=0.012, Fig. 3B). **The amount of m-PGES-1 mRNA was**

increased at 24 hours but not at 2 hours post stimulation with LPS (p=0.012, Fig.

3C). At the functional level, DNPCs displayed a significant increase in PGE_2

production in response to LPS for 72 hours (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 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 pretreatment with LPS

on SEB-induced cytokine production were blocked. Rather, LPS pretreatment

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-pretreated DNPCs. **These results were similar to the effect when we pretreated 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 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 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=0.022, Fig. 6A) and IL-13 (p=0.022, Fig. 6B) production, but not IL-17A (p=0.109, Fig. 6C) or IFN-γ (p=0.213, 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

13

(p=0.007) production by diclofenac-treated DNPCs. Treatment with EP2 (p=0.014) and EP4 (p=0.047) receptor-selective agonist significantly suppressed the IL-5 production. Treatment with EP2 and the EP4 receptor selective agonist also showed a tendency to inhibit the IL-13 production (p=0.055 and p=0.085, respectively, Fig. 6F). **Pretreatment 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).**

14

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 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.**

IL-5 and IL-13 are major Th2 cytokines 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].

15

Together with the finding that the pretreatment 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 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 pretreatment 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 unclear.**

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

16

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.

IL-17A is a major Th17 cytokine 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 pretreatment with LPS inhibits SEB-induced IL-17A production despite PGE_2 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

17

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 analyze 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.

20

References

1. Bousquet J, Bachert C, Canonica GW, Casale TB, Cruz AA, Lockey RJ, Zuberbier T. 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, Van Bruaene N, Holtappels G, DeRuyck N, Van Cauwenberge P, Bachert C. 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, Kariya S, Noda Y, Higaki T, Nishizaki K. 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, Kariya S, Makihara S, Higaki T, Nishizaki K. 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, Kariya S, Makihara S, Higaki T, Nishizaki K. 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, de Macedo MS, Cunha FQ, Lefort J, Vargaftig BB, Russo M. 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, Keller AC, Faquim-Mauro E, Bortoluci KR, Mucida D, Gomes E, Christ A, Schnyder-Candrian S, Schnyder B, Ryffel B, Russo M. 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, Leuttig B, Peden DB, Hohfeld JM, Krug N. 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, Maier KL, Frankenberger M, Heyder J, Ziegler-Heitbrock L. 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, Hamilos DL, Jacobs M, Kennedy DW, Lanza DC, Marple BF, Osguthorpe JD, Stankiewicz JA, Anon J, Denneny J, Emanuel I, Levine H. Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngol Head Neck Surg 2003; 129: S1-32.

17. Okano M, Fujiwara T, Yamamoto M, Sugata Y, Matsumoto R, Fukushima K, Yoshino T, Shimizu K, Eguchi N, Kiniwa M, Urade Y, Nishizaki K. 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, Konya V, Sturm GJ, Lippe IT, Peskar BA, Heinemann A. Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. J Immunol 2008; 181: 7273-83.

21. Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, Bachert C. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. Allergy 2006; 61: 1280-9.

22. Lu X, Zhang XH, Wang H, Long XB, You XJ, Gao QX, Cui YH, Liu Z. Expression of osteopontin in chronic rhinosinusitis with and without nasal polyps. Allergy 2009; 64: 104-11.

23. Okano M, Sugata Y, Fujiwara T, Matsumoto R, Nishibori M, Shimizu K, Maeda M, Kimura Y, Kariya S, Hattori H, Yokoyama M, Kino K, Nishizaki K. 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, Kawano K, Kojima Y, Miyahara K, Onoda J, Yokoi H, Ikeda K. 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, Lam D, Pattar SS, Patel ND, Horner AA. 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, Guay J, An J, Mazer M, Hamid Q, Mazer BD. 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, Somers SC, Sampfer MJ, Schwartz J, Speizer FE, Camargo CA Jr. 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.**

Figure legends

Figure 1. Effects of pre-treatment with LPS on SEB-induced cytokine production by

25

DNPCs. DNPCs were treated with 0, 0.2 or 2.0 μg/ml LPS 2 hours prior to SEB stimulation. After 72 hours 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 difference from baseline.** Bars represent median values. P values were determined using Wilcoxon signed-rank test.

Figure 2. Effects of post-treatment with LPS on SEB-induced cytokine production by DNPCs. At 1, 12 **or** 24 hours after SEB stimulation, DNPCs were exposed to 0.2 μg/ml LPS. After 72 hours 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 difference from baseline.** P values were determined using Wilcoxon signed-rank test.

Figure 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 hours, 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 hours of incubation with LPS were measured (D). **The data were looked as difference from baseline.** Bars represent median values. P values were determined using Wilcoxon signed-rank test.

Figure 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 difference from baseline.** Bars represent median values. P values were determined using Wilcoxon signed-rank test.

Figure 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 hours. Levels of IL-5 (A), IL-13 (B), IL-17A (C) and IFN-γ (D) were determined. **The data were looked as difference from baseline.** Bars represent median values. P values were determined using Wilcoxon signed-rank test.

Figure 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 hours. 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 difference 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).

Figure 7. Reversal of inhibitory effect of PGE_2 on LPS-induced IL-5 and IL-13

27

production by diclofenac-treated DNPCs with adenylate cyclase inhibitor. DNPCs were pretreated with SQ22536 at 37^0C for 1 hour. 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 hours. 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.

Figure 1

A   IL-5

Ratio to baseline

|--------p=0.015--------|
|--p<0.001--|
|--p<0.001--|

1.25

1.0

0.75

0.5

0.25

0

| LPS pretreatment | (-) | (-) | 0.2μg/ml | 2.0μg/ml |
| SEB stimulation | (-) | (+) | (+) | (+) |

B   IL-13

Ratio to baseline

|--------p=0.066--------|
|--p<0.001--|
|--p<0.001--|

1.5

1.0

0.5

0

| LPS pretreatment | (-) | (-) | 0.2μg/ml | 2.0μg/ml |
| SEB stimulation | (-) | (+) | (+) | (+) |

C   IL-17A

Ratio to baseline

|--------p=0.123--------|
|--p=0.022--|
|--p<0.001--|

1.5

1.0

0.5

0

| LPS pretreatment | (-) | (-) | 0.2μg/ml | 2.0μg/ml |
| SEB stimulation | (-) | (+) | (+) | (+) |

D   IFN-γ

Ratio to baseline

|--------p=0.575--------|
|--p=0.002--|
|--p<0.001--|

2.0

1.5

1.0

0.5

0

| LPS pretreatment | (-) | (-) | 0.2μg/ml | 2.0μg/ml |
| SEB stimulation | (-) | (+) | (+) | (+) |

Figure 2

Figure 3

Figure 4

A   <u>IL-5</u>

Ratio to baseline

|--p=0.019--|

|--p=0.008--|

2.0

1.5

1.0

0.5

0

SEB   SEB+LPS   DMSO   PGE₂

DIC (+)   DIC+LPS+SEB

B   <u>IL-13</u>

Ratio to baseline

|--p=0.006--|

|--p=0.008--|

3.0

2.5

2.0

1.5

1.0

0.5

0

SEB   SEB+LPS   DMSO   PGE₂

DIC (+)   DIC+LPS+SEB

C   <u>IL-17A</u>

Ratio to baseline

|--p=0.026--|

|--p=0.011--|

2.0

1.5

1.0

0.5

0

SEB   SEB+LPS   DMSO   PGE₂

DIC (+)   DIC+LPS+SEB

D   <u>IFN-γ</u>

Ratio to baseline

|--p=0.064--|

|--p=0.008--|

2.0

1.5

1.0

0.5

0

SEB   SEB+LPS   DMSO   PGE₂

DIC (+)   DIC+LPS+SEB

Figure 5

Figure 6

Figure 7