

Induction of TARC Production by Lipopolysaccharide and Interleukin-4 in Nasal Fibroblasts

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Key Words

Fibroblasts • IL-4 • Lipopolysaccharide • TARC • Thymus and activation-regulated chemokine

Abstract

Background: Th2 cell infiltration is a characteristic feature of allergic chronic sinusitis. However, the mechanisms that cause the predominance of Th2 cells in this disease have yet to be clarified. The airway is often exposed to not only Th2 cytokines but also bacterial products such as lipopolysaccharides (LPS). A CC chemokine, TARC, is a potent chemoattractant for Th2 cells. The objectives of this study were two-fold. First, we examined whether nasal polyp fibroblasts were able to produce TARC when costimulated with LPS and IL-4. Second, we investigated whether there was any heterogeneity in TARC production among fibroblasts derived from different airway sites. **Methods:** Fibroblast lines were established from human biopsy tissue. The amount of TARC in the supernatants was measured by ELISA. The expression of TARC mRNA was quantitated by real-time PCR. **Results:** Combined stimulation with LPS and IL-4 significantly induced TARC production by nasal polyp fibroblasts in a dose- and time-dependent manner. This induction occurred in normal nasal fibroblasts, but not in normal lung fibroblasts. **Conclusions:** Via TARC production, nasal fibroblasts may play an important role in the recruitment of Th2 cells into the sinus mucosa as well as nasal polyps. The heterogeneity in TARC production may reflect functional differences between upper and lower airway fibroblasts.

Introduction

Chronic sinusitis is often associated with allergy. Histological studies demonstrated that sinus tissues from patients with allergy were extensively infiltrated with eosinophils [1]. The densities of Th2 cells and Th2 cytokines (IL-3, IL-4, IL-5 and GM-CSF) were also increased in the sinus mucosa as well as nasal polyps from patients with allergy [2–4]. Th2 cytokines are potent inflammatory mediators and thought to contribute to eosinophilic inflammation. However, the mechanisms that cause the recruitment of Th2 cells into the sinus mucosa as well as nasal polyps have yet to be clarified.

Chemokines are a group of small (8–14 kDa), structurally related molecules that induce directed migration of various types of leukocytes. The effects of chemokines are mediated by specific G-protein-coupled cell surface receptors on the target cells [5, 6]. The CC chemokine thymus and activation-regulated chemokine (TARC; CCL17) selectively attracts Th2 cells via CC chemokine receptor 4 (CCR4) [7–9]. TARC is considered to play an important role in Th2-type inflammations. Plasma TARC levels were significantly increased in patients with atopic dermatitis and correlated with the severity scoring of the atopic dermatitis index [10]. The levels of TARC were increased in both serum and sputum of asthmatic subjects [11]. Immunohistochemical studies showed higher TARC immunoreactivity in the bronchial epithelium from asthmatics [12]. It was reported that allergic sinusitis patients showed high expression of CCR4-posi-

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tive cells in the ethmoidal sinus mucosa and helper T cells and Th2 cytokines in nasal polyps [2–4]. A pathogenic relationship between sinusitis and asthma was evaluated [13]. TARC may contribute to the recruitment of Th2 cells into the sinus mucosa as well as nasal polyps. In the airway, nasal and bronchial epithelial cells [12, 14, 15], and smooth muscle cells [16] are reported to be the primary sources of TARC, but the precise mechanisms that cause the predominance of Th2 cells remain unclear.

Fibroblasts are a major cellular component of nasal polyps [17]. In addition to their role as structural cells, fibroblasts can produce various inflammatory mediators and cytokines [18, 19]. Fukuda et al. [20] reported that corneal and skin fibroblasts produced TARC. On the basis of those findings, we considered the possibility that nasal fibroblasts may also produce TARC and play a role in the recruitment of Th2 cells into the sinus mucosa and nasal polyps.

Lipopolysaccharides (LPS) are a major component of the outer membrane of gram-negative bacteria. They activate the innate immune system via Toll-like receptor 4 [21, 22]. It was reported that LPS challenge influenced allergic diseases such as nasal allergy [23] and asthma [24]. A Th2 cytokine, IL-4, has been recognized as an important inducer of TARC production [12, 15, 16, 20, 25]. Since the nasal mucosa of allergic individuals is thought to be exposed to not only bacterial products such as LPS but also Th2 cytokines including IL-4, we speculated that these two stimulants might induce TARC production by nasal fibroblasts.

In the present study, we examined whether nasal polyp fibroblasts could produce TARC in response to costimulation with LPS and IL-4. Moreover, we investigated whether there is heterogeneity in TARC production among the fibroblasts derived from the upper and lower airways.

Materials and Methods

Reagents

LPS was purchased from Sigma (St. Louis, Mo., USA). Human recombinant IL-4 was purchased from Genzyme (Cambridge, Mass., USA).

Cell Source and Culture

Primary fibroblast lines were established from human biopsy tissues and characterized as previously described [26]. Only fibroblast lines between the fourth and seventh passages were used in this study. Nasal polyp tissues were removed at polypectomy ($n = 6$). Normal nasal mucosa from the uncinate process was obtained from patients who were suffering from blowout fracture ($n = 6$). Normal lung fibroblasts were derived from surgical lung speci-

mens from patients undergoing surgical resection for cancer ($n = 6$). All the specimens were obtained from patients who had not undergone any chemotherapy or irradiation. All subjects had given written informed consent, and the study protocol was approved by the Ethics Committee of the Nippon Medical School Hospital.

Before cytokine assay, fibroblasts (1×10^5 cells) were plated in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, N.J., USA). Before real-time PCR, fibroblasts (2×10^6 cells) were plated in 60-mm culture dishes (Corning, N.Y., USA) and allowed to grow to confluence in regular growth medium (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2.5 μ g/ml amphotericin B. The same culture medium was used when fibroblasts were stimulated with various stimuli. At specified time points, supernatants were collected and centrifuged for the cytokine protein assay and cells were collected for RNA extraction.

Quantitative Real-Time RT-PCR

Total cellular RNA was extracted and purified with an RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA, 2 μ g, was reverse transcribed at 37°C for 60 min using a random primer (TaKaRa Bio, Shiga, Japan) and Omniscript reverse transcriptase (Qiagen) according to the manufacturers' protocols.

Quantitative real-time PCR was performed with a GeneAmp 5700 Sequence Detection System using TaqMan chemistry (Applied Biosystems, Foster City, Calif., USA). The primers and fluorescent probes for TARC and β -actin were purchased from Applied Biosystems. The amplification conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C.

Data analysis was performed using the software of the sequence detector system (Applied Biosystems). For quantitative analysis, we used the relative standard curve method. The standard curve was constructed using serial dilutions of cDNA containing the message for TARC and β -actin. The target quantity was calculated from the threshold cycle value by interpolation from the standard curve. The level of TARC mRNA was normalized to β -actin.

Cytokine Assay

The levels of TARC in the culture supernatants were measured by ELISA using a commercially available TARC-specific ELISA kit (R & D Systems, Minneapolis, Minn., USA). The detection limit of this assay was 7 pg/ml. The data are presented as picograms of chemokine per 1×10^6 cells.

Statistical Analysis

The paired Wilcoxon test was used for statistical analysis. A p value < 0.05 was considered statistically significant.

Results

Effect of LPS and IL-4 on TARC Production by Human Nasal Polyp Fibroblasts

We first examined whether nasal polyp fibroblasts could produce TARC upon stimulation with LPS and IL-4. The nasal polyp fibroblasts were cultured with LPS (10

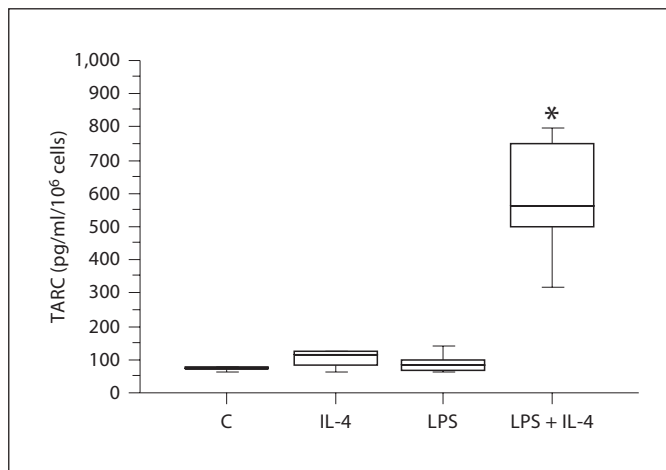
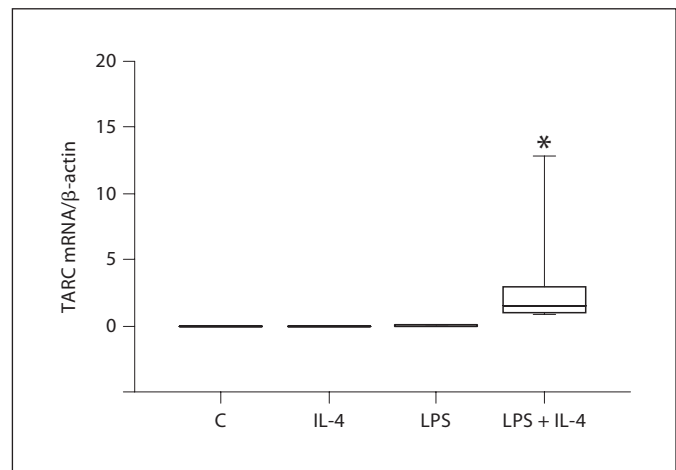


Fig. 1. Effects of LPS and IL-4 on TARC release by nasal polyp fibroblasts. Cells were stimulated with LPS (10 ng/ml) and IL-4 (1 ng/ml), alone and in combination, for 72 h. The TARC concentration in the culture supernatant was measured by ELISA. Box plots represent the median values for the 25 and 75% interquartiles; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with unstimulated cells. C = Control. $n = 6$.

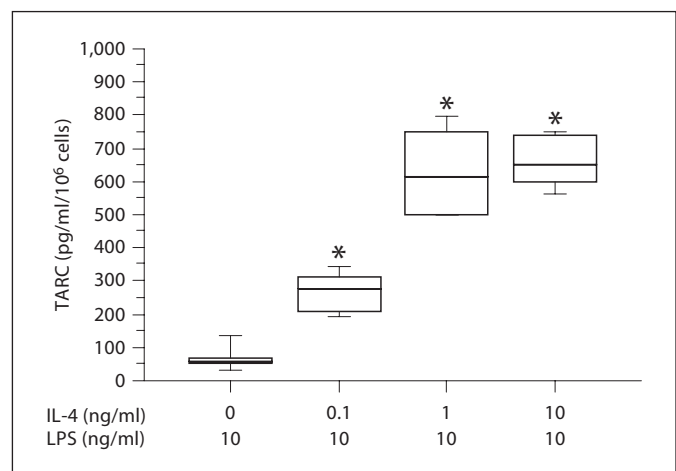
ng/ml) and IL-4 (1 ng/ml), alone and in combination. Negligible TARC was detected in the supernatants of unstimulated cells. Neither IL-4 nor LPS alone had any effect on TARC release by the cells. However, combined stimulation with LPS and IL-4 induced significant TARC release by these cells (fig. 1).

Real-time PCR was performed for quantitation of TARC mRNA levels. The expression of TARC mRNA was weak or undetectable in unstimulated cells and cells stimulated with either LPS (10 ng/ml) or IL-4 (1 ng/ml). As in the case of TARC protein, combined stimulation with LPS and IL-4 induced significant upregulation of TARC mRNA expression in the nasal polyp fibroblasts (fig. 2).

The release of TARC by nasal polyp fibroblasts occurred in a dose- and time-dependent manner. The cells were cultured with 10 ng/ml of LPS and a series of concentrations of IL-4. In the presence of the fixed LPS dose, 0.1 ng/ml IL-4 was sufficient to induce statistically significant TARC release by these cells (fig. 3). The release of TARC reached a plateau at an IL-4 concentration of 1 ng/ml. In time-course experiments, the TARC release by nasal polyp fibroblasts increased steadily for 72 h in response to the costimulation with LPS (10 ng/ml) and IL-4 (1 ng/ml; fig. 4).



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Fig. 2. Effects of LPS and IL-4 on TARC mRNA expression by nasal polyp fibroblasts. Cells were stimulated with LPS (10 ng/ml) and IL-4 (1 ng/ml), alone and in combination, for 12 h, and real-time PCR was performed. The results are normalized to β -actin expression. Box plots represent the median values for the 25 and 75% interquartiles; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with unstimulated cells. C = Control. $n = 6$.

Fig. 3. Effect of increasing the dose of IL-4 in the presence of LPS on TARC release by nasal polyp fibroblasts. Cells were stimulated with the indicated dose of IL-4 in the presence of LPS (10 ng/ml) for 72 h. Box plots represent the median values for the 25 and 75% interquartiles; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with cells stimulated with LPS alone. $n = 6$.

Comparison of LPS and IL-4-Induced TARC Production by Different Human Airway Fibroblasts

In addition to nasal polyp fibroblasts, we next examined TARC production by airway fibroblasts obtained from normal nasal tissue (mucosa of the uncinate pro-

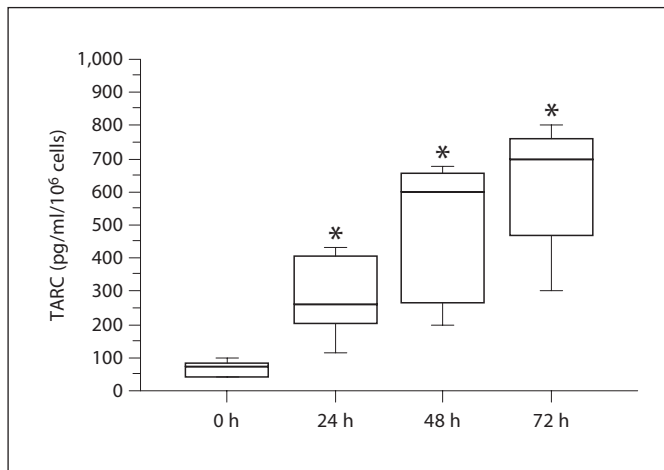


Fig. 4. Kinetics of TARC release from nasal polyp fibroblasts. Cells were cultured in the presence of both LPS (10 ng/ml) and IL-4 (1 ng/ml) for 0, 24, 48 and 72 h. TARC release by nasal polyp fibroblasts increased steadily for 72 h in response to combined stimulation with LPS and IL-4. Box plots represent the median values for the 25 and 75% interquartiles; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with 0 h. $n = 5$.

cess) and normal lung tissue (fig. 5). These cells were cultured with LPS (10 ng/ml) and IL-4 (1 ng/ml), alone and in combination. Similar to the nasal polyp fibroblasts, neither IL-4 nor LPS alone had any effect on the TARC release by these cells. Combined stimulation with LPS and IL-4 induced significant TARC release by the normal nasal fibroblasts, but not by the normal lung fibroblasts.

TARC mRNA expression was consistent with the protein data (fig. 6). After stimulation with LPS (10 ng/ml) or IL-4 (1 ng/ml), the expression of TARC mRNA was weak or undetectable in these cells. Costimulation with LPS and IL-4 induced significant TARC mRNA expression in the normal nasal fibroblasts, but not in the normal lung fibroblasts.

Discussion

In the present study, we have demonstrated that combined stimulation with LPS and IL-4 significantly induces both mRNA expression and release of TARC in nasal polyp fibroblasts. This induction occurred in a dose- and time-dependent manner. Terada et al. [15] have already reported that nasal epithelial cells showed significant TARC production in response to combined stimulation with IL-4 and TNF- α . Taken together, not

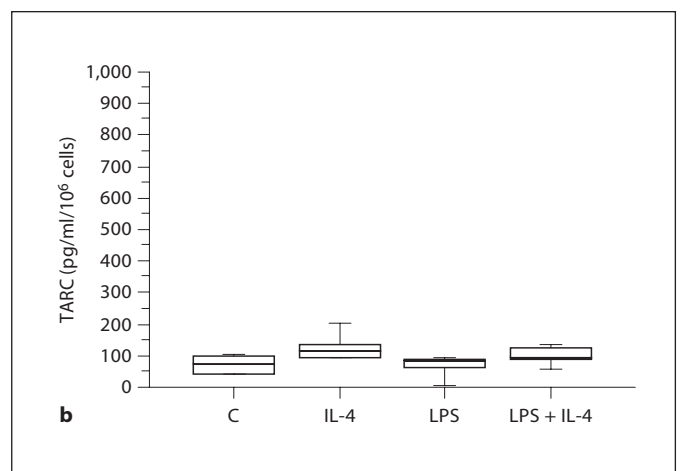
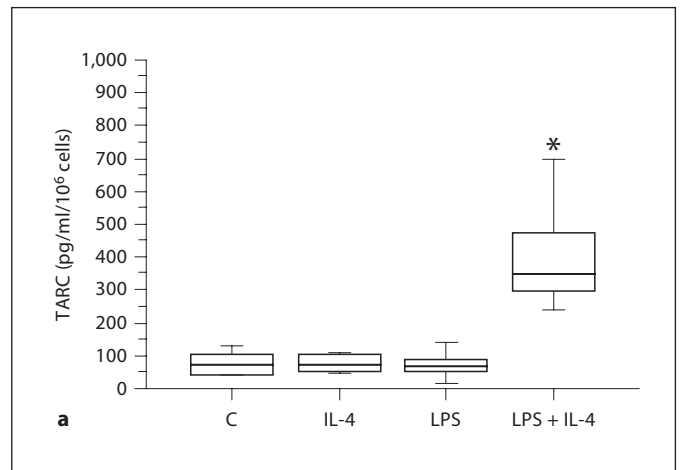


Fig. 5. Effects of LPS and IL-4 on TARC release by normal nasal fibroblasts (a) and normal lung fibroblasts (b). Cells were stimulated with LPS (10 ng/ml) and IL-4 (1 ng/ml), alone and in combination, for 72 h. TARC concentrations in the culture supernatant were measured by ELISA. Box plots represent the median values for the 25 and 75% interquartiles; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with unstimulated cells. C = Control. $n = 6$.

only epithelial cells but also nasal fibroblasts may contribute to the recruitment of Th2 cells into the upper airway.

The nasal and sinus mucosae are often affected by bacterial infections. LPS is a commonly encountered air contaminant in occupational settings [23]. The sinus fluid of patients with chronic sinusitis contains high levels of LPS [27]. In allergic individuals, the nasal mucosa is also thought to be exposed to Th2 cytokines, including IL-4. Considering our results, this LPS- and Th2 cytokine-rich milieu may promote TARC production by nasal polyp

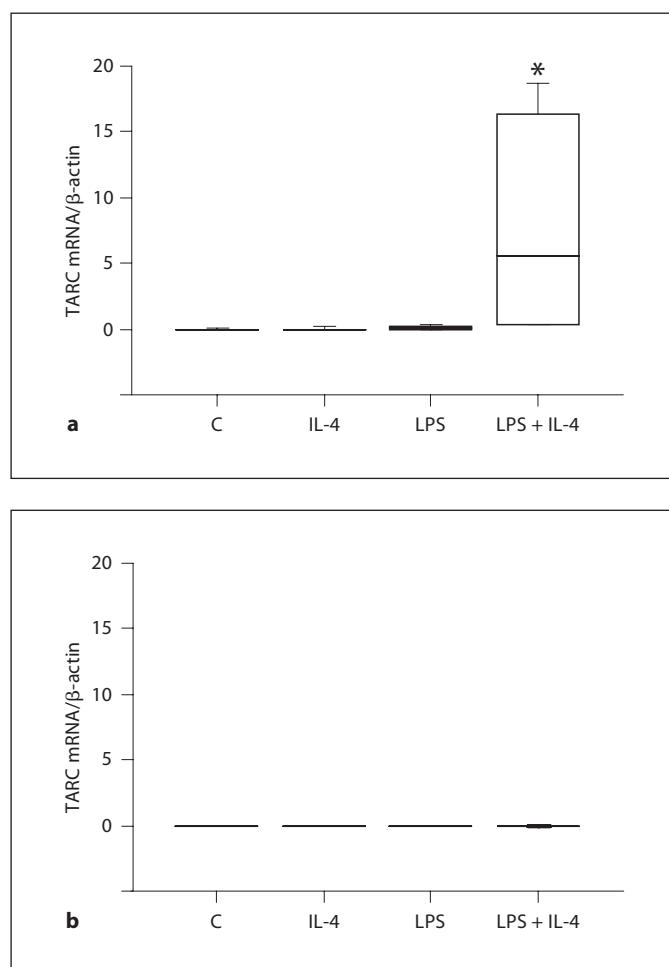


Fig. 6. Effects of LPS and IL-4 on TARC mRNA expression by normal nasal fibroblasts (**a**) and normal lung fibroblasts (**b**). Cells were stimulated with LPS (10 ng/ml) or IL-4 (1 ng/ml) alone or in combination for 12 h, and real-time PCR was performed. The results are normalized to β -actin expression. Box plots represent the median values for the 25 and 75% interquartiles; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with unstimulated cells. C = Control. $n = 6$.

fibroblasts, resulting in recruitment of Th2 cells into the upper airway.

Eotaxin, a CC chemokine, is known as a potent chemoattractant for eosinophils [28]. Nonaka et al. [29] reported that combined stimulation with LPS and IL-4 induces synergistic production of eotaxin by nasal fibroblasts. Moreover, these two stimuli synergize to induce VCAM-1 expression on human microvascular endothelial cells [30]. VCAM-1 is an important adhesion molecule for eosinophils [31]. Combined with our observation that nasal polyp fibroblasts were able to produce TARC,

a chemotactic factor for Th2 cells, it is interesting to speculate that Th2 cytokines such as IL-4 and IL-13, which are released from Th2 cells, are capable of inducing eotaxin production in nasal fibroblasts. Eotaxin, which is locally released, may in turn induce eosinophils to migrate out of the vasculature into sites of inflammation.

The mechanism by which combined stimulation with LPS and IL-4 induces TARC production by nasal polyp fibroblasts remains unclear. Current investigations indicate that LPS induces intracellular signaling cascades via activation of Toll-like receptor 4 and leads to the activation of NF- κ B [22]. NF- κ B is thought to regulate TARC expression in human airway epithelial cells [14]. IL-4 induces expression of TARC via STAT6 in human T cells [25]. Matsukura et al. [32] demonstrated that eotaxin promoter contains overlapping consensus binding sites for transcription factors, NF- κ B and STAT6, and that stimulation with these two transcription factors results in further activation of the promoter. One possible explanation for our current finding is that NF- κ B and STAT6 may coexist within the TARC promoter region and that the costimulation with LPS and IL-4 may enhance TARC gene expression via activation of both NF- κ B and STAT6.

Fibroblasts were previously thought of only as important structural cells. Recently, however, several authors have suggested new concepts concerning the functions of fibroblasts. First, fibroblasts produce various cytokines and inflammatory mediators [18]. Moreover, these cells display receptors and surface markers that are used to regulate hematopoietic cells [19]. Activation of fibroblasts by LPS, IL-1 and TNF- α , for example, results in a transient production of chemokines. Through this chemokine production, fibroblasts bring about leukocyte recruitment [33]. Therefore, fibroblasts are thought to be part of the immune system and an initiator of inflammatory responses. Moreover, compared to hematopoietic cells, fibroblasts are long-lived cells. Persistent stimulation of fibroblasts by infectious and inflammatory agents may lead to chronic inflammation [19]. Finally, fibroblasts from different anatomic regions and pathologic settings display characteristic phenotypes. The regional diversity of fibroblasts may be responsible for the localized susceptibility to disease manifestation [19, 34]. Considering these findings, we next investigated whether there was any heterogeneity in TARC production among fibroblasts derived from different airway sites (upper or lower airways).

TARC production in response to combined stimulation with LPS and IL-4 was observed in normal nasal fi-

broblasts as well as nasal polyp fibroblasts. However, in normal lung fibroblasts, even a combination of LPS and IL-4 could not induce TARC production. Several reports have already shown the heterogeneity of fibroblasts within the airways. Nonaka et al. [35] reported that upon LPS stimulation RANTES and GM-CSF protein release was only seen with nasal fibroblasts, but not with lung fibroblasts. Synergistic induction of eotaxin by IL-4 and LPS was observed for nasal fibroblasts but not lung fibroblasts [29]. Normal lung fibroblasts can produce GM-CSF, IL-8 and IL-6 when stimulated with IL-1 but not LPS. However, both LPS and IL-1 can upregulate these cytokines in nasal fibroblasts [36]. The responsiveness of nasal fibroblasts and lung fibroblasts to IL-4 was similar in terms of eotaxin production [29]. Thus, it is conceivable that the differential expression of TARC between nasal fibroblasts and lung fibroblasts upon stimulation with LPS and IL-4 depends on their LPS responsiveness. The nasal mucosa is the first barrier of the airways encountered by environmental agents. The ability of nasal fibroblasts to react directly to LPS suggests an additional defense mechanism. The differences in the response to the same stimuli reflect functional differences between upper and lower airway fibroblasts. The unique characteristics of nasal fibroblasts may be, at least in part, involved in the initiation and amplification of sinusitis.

Eotaxin is induced in lung fibroblasts by various stimuli such as TNF- α + IL-4 [37], TGF- β + IL-13 [38], IL-13 + leukotriene C4 [39], histamine and serotonin [40]. However, to our knowledge, there are no means of inducing TARC in lung fibroblasts. Fukuda et al. [20] reported that TNF- α + IL-4 induces TARC in corneal and dermal fibroblasts, but not in lung fibroblasts. TARC production in nasal fibroblasts, but not in lung fibroblasts, reflects the great regional diversity of airway fibroblasts.

In summary, we demonstrated here that combined stimulation with LPS and IL-4 induced TARC production by nasal fibroblasts. The induction of TARC in nasal fibroblasts by LPS and IL-4 suggests an important role for fibroblasts in regulating Th2 cell infiltration into the sinus mucosa as well as nasal polyps. The heterogeneity in TARC production may reflect functional differences between upper and lower airway fibroblasts.

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