

## Original article

# Interleukin (IL)-4 and to a lesser extent either IL-13 or interferon-gamma regulate the production of eotaxin-2/CCL24 in nasal polyps

**Background:** Eotaxin-2/CCL24 is a potent eosinophil attractant that has been implicated in the recruitment of eosinophils in allergic disease. We have investigated whether the cytokines interleukin (IL)-4, IL-13, and interferon (IFN)-gamma regulate eotaxin-2/CCL24 in nasal polyps.

**Methods:** Nasal polyps were cultured in the presence of the cytokines described above and the concentration of eotaxin-2/CCL24 was measured in the culture supernatant.

**Results:** IL-4 was found to be the major stimulus for eotaxin-2/CCL24 production from nasal polyps followed by IL-13 and IFN-gamma. IL-4 induced eotaxin-2/CCL24 in a dose-dependent manner with concentrations as low as 0.1 ng/ml being able to induce eotaxin-2/CCL24. By immunohistochemistry, eotaxin-2/CCL24 immunoreactivity was localized to mononuclear cells in the IL-4 stimulated nasal polyp tissue. Interestingly, nasal turbinates obtained from patients suffering from nonallergic rhinitis (vasomotor rhinitis) were also found to release eotaxin-2/CCL24 both spontaneously and following cytokine stimulation with IL-4 and IFN-gamma being major inducers of this cytokine.

**Conclusions:** All together these findings suggest that Th1 and Th2 cytokines may regulate eotaxin-2/CCL24 production in nasal polyps and nonallergic rhinitis.

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Although the mechanisms that underlie nasal polyposis are not fully understood, the clinical and morphological manifestations of this disorder have been well characterized. Nasal polyps (NPs) are transparent, pale gray edematous projections that originate from nasal ethmoid mucosa in the vicinity of the middle turbinate (1). Inflammation is a prominent feature of NPs. Histological studies have shown that about 80–90% of the polyps are characterized by abundant eosinophils, CD8+ T cells, and mast cells (1). Among these cells, eosinophils play a prominent role in the pathogenesis of NPs (2). For example, it has been shown that eosinophils promote epithelial proliferation, matrix generation, and tissue remodeling through the release of cytokines such as transforming growth factor- $\alpha$ , transforming growth factor- $\beta$ , and granulocyte-macrophage colony stimulating factor (GM-CSF) (2–4). Moreover, activated eosinophils may cause tissue damage through the release of reactive oxygen metabolites and cytotoxic granule-derived proteins such as major basic protein (5).

Eosinophil recruitment from the circulation involves a series of separate processes. Initial adhesion to endothelium and transendothelial migration is dependent upon the expression of complementary pairs of adhesion molecules on eosinophils and activated endothelial cells.

Subsequent migration across the extracellular matrix towards the inflammatory site is thought to be directed by chemotactic stimuli. A number of eosinophil chemoattractants have been described including cytokines such as interleukin (IL)-5 and GM-CSF, which are relatively weak stimuli, and small molecules such as platelet activating factor and C5a, which are potent but not selective as they also attract neutrophils (6). Members of the chemokine family including RANTES/CCL5, MCP-3/CCL7, and MCP-4/CCL13 are potent eosinophil attractants but not selective as they also attract lymphocytes and monocytes. In contrast, the eotaxins (eotaxin-1, -2 and, -3) are selective eosinophil attractants (7). These chemokines also attract a small population of lymphocytes. While eotaxin-1/CCL24 has been extensively investigated in NPs (8–10), the role of eotaxin-2/CCL24 and eotaxin-3/CCL26 in this disease has not been well defined.

Eotaxin-2/CCL24 was identified in early 1997 by random sequencing of expressed sequence tags in a cDNA library from activated human monocytes (11), and subsequently shown to be a potent eosinophil attractant (12). Increased eotaxin-2/CCL24 mRNA expression has been reported in biopsies derived from patients suffering from NPs (10). Interestingly, in this study (10), eotaxin-2/CCL24 mRNA expression showed

the highest transcript level compared with other CC chemokines such as eotaxin-1/CCL11, RANTES/CCL5, and MCP-4/CCL13. Similarly, increased eotaxin-2 mRNA expression has been found in skin biopsies obtained during the allergen-induced late-phase cutaneous response and in bronchial biopsies derived from atopic and nonatopic asthmatics (13, 14).

Both IL-4 and IL-13 are pleiotropic cytokines that play an important role in allergic inflammation (15, 16). They activate B cells inducing class switching to IgE; stimulate expression of CD23; and induce expression of class II MHC antigens (15–17). In contrast, interferon (IFN)-gamma downregulates the allergic reaction (17). It has been reported that IL-4 transgenic mice show increased levels of eotaxin-2/CCL24 mRNA compared with wild type-mice and intranasal administration of IL-4 induces accumulation of eotaxin-2/CCL24 mRNA in the lungs (18). In the present study, we have investigated whether the cytokines IL-4, IL-13, and IFN-gamma regulate the production of eotaxin-2/CCL24 in NP tissue.

## Methods

### Subjects

Eleven subjects (seven women: median age 45 years; range 22–59 years) suffering from nasal polyposis were recruited for the study. Six subjects were atopic and five were nonatopic. Their atopic status was confirmed by skin prick testing with a series of common inhalant allergens (*Dermatophagoides pteronyssinus*, mixed grass, tree pollen, feathers, cat and dog dander, and cockroach; Alk Bello, USA). Any resulting wheal, more than 2 mm or larger than that produced by the histamine control, was considered positive. For at least 2 months before the polypectomy none of the patients received any treatment with corticosteroids. One of the 11 subjects was sensitive to aspirin.

The control group included six patients (four women: median age 39 years; range 28–63 years) suffering from nonallergic rhinitis (vasomotor rhinitis). These patients had a history of chronic rhinitis characterized by nasal blockage, mucus production, and sneezing. None of the patients were either atopic (negative skin prick test) or aspirin sensitive. Similarly, none of the patients received any treatment with corticosteroids for at least 2 months before the turbinatectomy.

### Culture of nasal polyp tissue

Nasal polyps were obtained from subjects undergoing polypectomy for treatment of nasal obstruction and processed as previously described (19). After washing with culture medium (RPMI-1640), NPs were then placed on nitrocellulose paper to absorb excess fluid and weighed. After cutting NPs into 1–2-mm-large specimens, they were stimulated with different cytokines (IL-4, IL-13, TNF- $\alpha$ , TNF- $\alpha$  + INF- $\gamma$ , TNF- $\alpha$  + IL-4, and TNF- $\alpha$  + IL-13), at concentrations of 10 ng/ml and cultured at 37°C in an incubator containing 0.5% CO<sub>2</sub>. Culture medium consisted of RPMI-1640 with 5% human AB serum, 2 mmol/l mercaptoethanol, 1 mmol/l glutamine, 2 mmol/l sodium pyruvate, 100 U/ml streptomycin, and 0.5  $\mu$ g/ml fungizone. After 48-h incubation, supernatants were collected and kept at –70°C for eotaxin-2/CCL24 measurements. The release of eotaxin-2/CCL24 was also investigated in nasal turbinates obtained

from subjects suffering vasomotor rhinitis. Nasal turbinates were processed in an identical manner as described for NPs.

### Eotaxin-2 measurement

Measurement of immunoreactive eotaxin-2/CCL24 in the supernatant of cultured NPs was performed in duplicate samples using a specific sandwich ELISA following the manufacturer's protocol (R & D Systems, Minneapolis, USA). Briefly, ELISA plates (Costar, Cambridge, MA) were coated with a monoclonal mouse antihuman eotaxin-2/CCL24 antibody (5  $\mu$ g/ml, 100  $\mu$ l/well) in carbonate/bicarbonate buffer (pH 9.6). The antibody was allowed to bind overnight at 4°C, and the plates were then washed four times with PBS containing 0.05% Tween 20 before the addition of 100  $\mu$ l of either NP culture supernatant or standard, consisting of serial dilutions from 1 to 0.03 ng/ml of recombinant human eotaxin-2/CCL24 (R & D Systems). Blank wells containing no eotaxin-2/CCL24 were routinely included in each assay. After 2-h incubation at room temperature, the plates were washed four times with PBS–Tween 20, and the Biotinylated anti-eotaxin-2/CCL24 antibody was added to each well. Plates were incubated for 2 h at room temperature before washing and addition of 100  $\mu$ l/well of ExtrAvidin Peroxidase Conjugate (Sigma, St Louis, MO) at 1 : 10 000 dilution and incubation at room temperature for 30 min. Plates were washed again with PBS–Tween 20 and the substrate (TMB Sigma, St Louis) added. After 15-min incubation at room temperature, the reaction was stopped by the addition of 50  $\mu$ l of 2 M NaOH, and the absorbance of each well was measured at 450 nm in a micro-ELISA plate reader. Concentrations of eotaxin-2/CCL24 in the tissue culture supernatants were calculated from the standard curve. The lower limit of detection was 10 pg/ml, and the interassay coefficient variation was 5%.

### RNA preparation and analysis

Total RNA from nasal tissues was isolated using the TRIzol reagent (Gibco-BRL). Total cellular RNA was quantified by measuring the optical density at 260 nm and stored at –20°C after concentration was adjusted to 1 or 2  $\mu$ g/l. Then, total RNA (2  $\mu$ g) was reverse transcribed. cDNA corresponding to 1- $\mu$ g RNA served as template in a duplex-PCR reaction containing 0.8 nm of primer specific for eotaxin-2 (Forward 5'-CTCACGGGCTCTGTGGTC-3'; Reverse 5'-GGTTGGTTGCCAGGATA-3'). Amplification and analysis of the PCR products were performed as previously described (20).

### Immunohistochemistry

Either IL-4 stimulated or nonstimulated NPs were GMA-embedded and the technique of immunohistochemistry applied to 2- $\mu$ m 'semi-thin' sections of tissue as described previously (21). Briefly, endogenous peroxidase activity was inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide in Tris-buffered saline (TBS) at pH 7.6 for 10 min. After washing three times with TBS blocking medium consisting of Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 1% bovine serum albumin the technique was applied for a further 30 min. The goat anti-human eotaxin-2 polyclonal antibody (R & D systems) was then applied, and sections were incubated at 4°C overnight. Sections were washed in TBS and biotinylated antigoat IgG (R & D systems, Minneapolis, USA) applied for 2 hours. After rinsing the streptavidin-biotin-horseradish peroxidase complex (R & D systems) was applied for a further 2 h. Finally, aminoethyl carbazole in acetate buffer (pH 5.2) and hydrogen peroxide was used as a substrate to develop peroxide-

dependent red-color reaction. Sections were counterstained with Mayer's hematoxylin.

### Statistical analyses

Concentrations of eotaxin-2/CCL24 in the cytokine-stimulated NP supernatant were analyzed using the Kruskal Wallis test. To analyze differences in eotaxin-2/CCL24 concentrations between two different cytokine stimuli, the Mann-Whitney *U*-test was used. Significance was assumed at  $P < 0.05$ . Analysis was performed using SPSS 10 program.

## Results

In this study, we have used the NP explant model to investigate eotaxin-2/CCL24 production when exposed to the cytokines IL-4, IL-13, or IFN- $\gamma$ . NP tissue was obtained from subjects undergoing polypectomy for treatment of nasal obstruction. The median amount of NP tissue was 1.82 g (range 1.5–2.3 g).

IL-4 and to a lesser extent either IL-13 or IFN- $\gamma$  induce eotaxin-2/CCL24

Measurements of eotaxin-2/CCL24 in the 48-h cultured NP supernatant showed that NPs release eotaxin-2/CCL24 both spontaneously and following cytokine stimulation (Fig. 1). Interestingly, IL-4 was found to be the major stimulus for eotaxin-2/CCL24 production from NPs (Fig. 1A). Both IL-13 and IFN- $\gamma$  also stimulated eotaxin-2/CCL24 release; however, both are weak stimuli. Combination of TNF- $\alpha$  with IL-4 enhanced eotaxin-2 release (Fig. 1B),  $P < 0.05$ . In contrast, TNF-

alpha did not significantly enhance the ability of either IL-13 or IFN- $\gamma$  to release eotaxin-2/CCL24. There was no statistical difference between atopic and nonatopic polyps to release eotaxin-2/CCL24 upon any of the cytokine stimulus (data not shown).

### Immunoreactivity in nasal polyp biopsies

To investigate the potential cellular source of eotaxin-2/CCL24, the technique of immunohistochemistry was applied to either IL-4 stimulated ( $n = 3$ ) or nonstimulated NP explants ( $n = 3$ ). Scarce eotaxin-2/CCL24 immunoreactivity was observed in nonstimulated NPs in the subepithelial tissue (Fig. 2B). In contrast, intense expression was observed in IL-4-stimulated NPs (Fig. 2C and D). Eotaxin-2/CCL24 immunoreactivity was localized predominantly to mononuclear cells although some expression was seen in basal epithelial cells in the IL-4 stimulated NP explants.

Concentration-dose dependent induction of eotaxin-2/CCL24 by IL-4

To investigate the concentrations of IL-4 able to induce eotaxin-2/CCL24, NPs were stimulated with this cytokine for 24 h in a concentration range from 0.1 to 100 ng/ml. This showed that IL-4 induces eotaxin-2/CCL24 in a dose-dependent manner (Fig. 3): levels of this cytokine increased gradually achieving maximal concentrations in the culture supernatant when NPs were stimulated with 100 ng/ml. Interestingly, very low concentrations of IL-4 (0.1 ng/ml) stimulated eotaxin-2/CCL24 production.

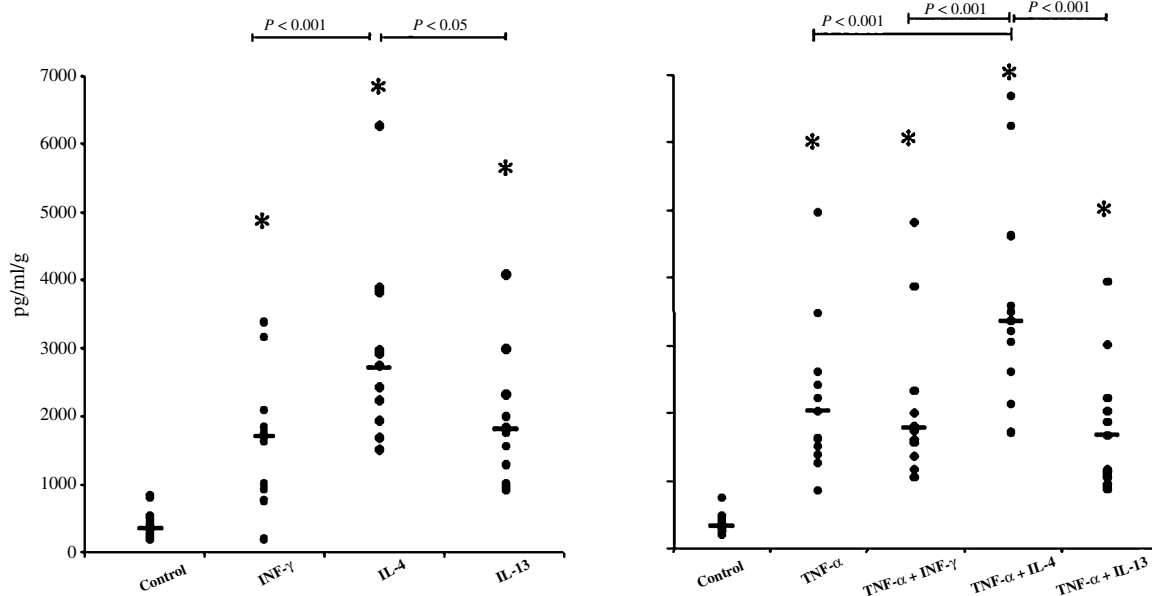


Figure 1. Levels of immunoreactive eotaxin-2 in culture supernatant derived from the cytokine-stimulated nasal polyps (48-h culture). Horizontal lines represent median values. \*Compared with control significant at  $P < 0.05$ .

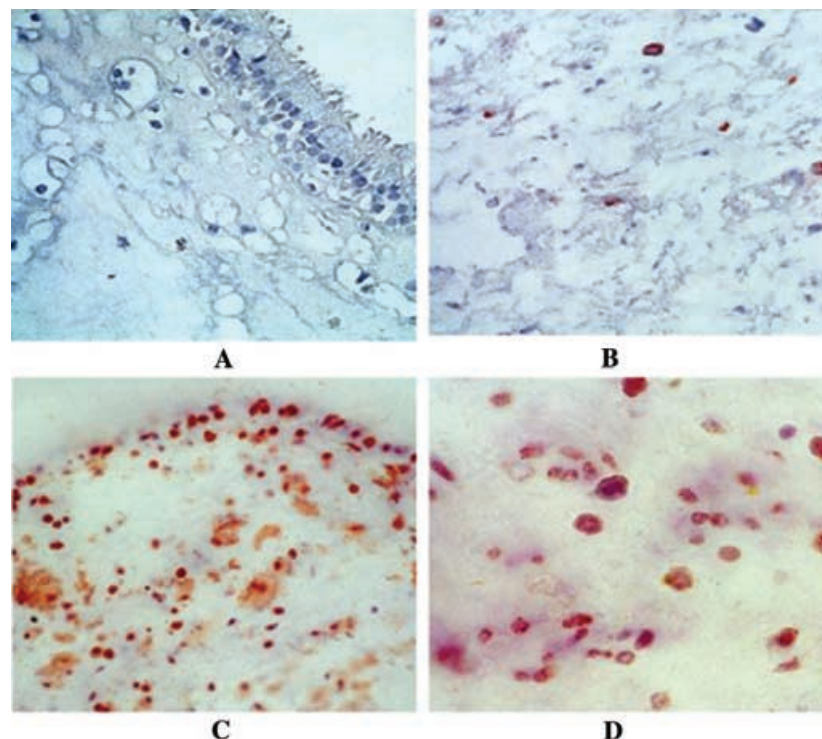


**Figure 2.** Localization of immunoreactivity for eotaxin-2 to mononuclear cells in nonstimulated (B) and IL-4-stimulated nasal polyps (C and D). Scarce eotaxin-2 immunoreactivity was observed in nonstimulated nasal polyps [B (photographs shows one out of the three NPs)]. In contrast, increased immunoreactivity was observed in IL-4-stimulated nasal polyp (C). Eotaxin-2 was localized to mononuclear cells and to a lesser extent to basal epithelium. Because of manipulation (chopping) of nasal polyps, airway epithelium was usually denuded (C). (A) A negative control nasal polyp biopsy. (Instead of the anti-eotaxin-2 antibody the isotype control antibody goat IgG 15256 was used.) Details of immunohistochemistry are given in Methods.

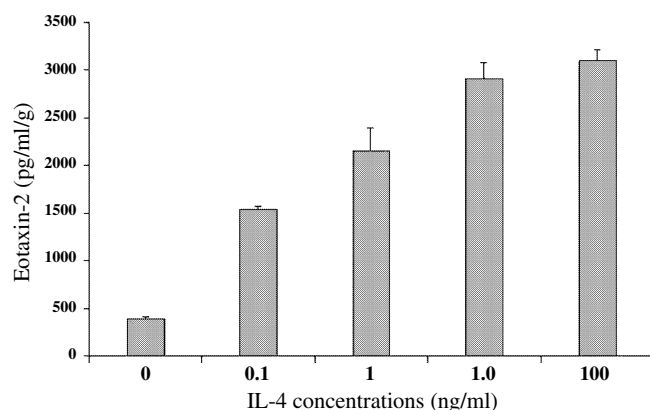


**Figure 3.** Dose-response induction of eotaxin-2 in the culture supernatant of IL-4-stimulated nasal polyps. Bars represent the mean values of four different experiments.

#### Kinetics of release of eotaxin-2/CCL24

Having shown that IL-4 was a potent stimulus, NPs were cultured in the presence of this cytokine and the kinetics of eotaxin-2/CCL24 release was studied in the culture supernatant. The IL-4 concentration used for these set of

experiments was 10 ng/ml (optimal concentration for eotaxin-2/CCL24 production, Fig. 3). There was no significant production of eotaxin-2/CCL24 in the culture supernatant within the first 12 h of stimulation. However, at 24-h levels, this chemokine increased fourfold, peaking at 48 h and gradually decreased at 96 h (Fig. 4).

#### Production of eotaxin-2/CCL24 by nasal polyps and nasal turbinates

Eotaxin-2/CCL24 production from either NPs or nasal turbinates derived from subjects suffering from nonallergic rhinitis (vasomotor rhinitis) was investigated in the 48-h culture supernatants. Figure 5A shows that NPs and nasal turbinates released comparable amounts of eotaxin-2/CCL24 spontaneously. There was no significant difference in the concentrations of eotaxin-2/CCL24 between these two groups ( $P > 0.05$ ). Similar findings were observed at eotaxin-2 mRNA expression (Fig. 5B).

The effect of Th1 and Th2 cytokines on the ability of nasal turbinates to release eotaxin-2/CCL24 was also investigated. Either IL-4 or IFN-gamma was a potent stimulus for eotaxin-2/CCL24 release (although IL-4 was a major stimulus there was no statistical difference in levels of eotaxin-2 induced by these two cytokines), see

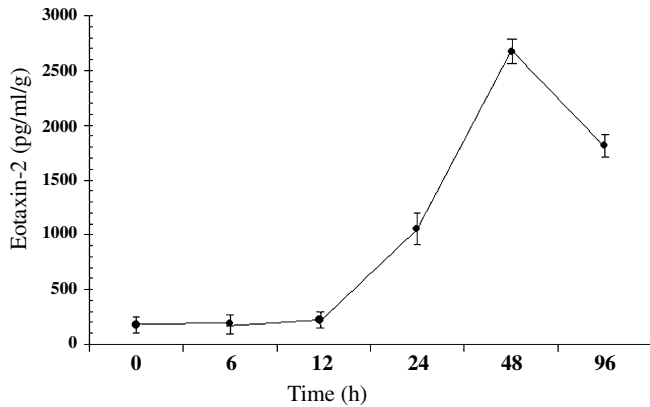


Figure 4. Kinetics of release of eotaxin-2 in IL-4-stimulated nasal polyps. Nasal polyps were stimulated with IL-4 and eotaxin-2 measured in the culture supernatant by ELISA. Bars represent mean values of four different experiments.

Fig. 6. In contrast, IL-13 was a weak stimulus for eotaxin-2 production. TNF-alpha synergized with either IL-4 or IFN-gamma but not with IL-13 to induce eotaxin-2 (data not shown).

## Discussion

The present study has demonstrated that IL-4 is a major stimulus for eotaxin-2/CCL24 release from NPs. IL-13 and IFN-gamma also stimulated eotaxin-2/CCL24; however, both are weak stimuli compared with IL-4. TNF-alpha enhanced the IL-4-induced eotaxin-2/CCL24 production, but not that induced by either IL-13 or IFN-gamma. By immunohistochemistry, eotaxin-2/CCL24 immunoreactivity was localized to mononuclear cells in NP explants. Interestingly, both IL-4 and IFN-gamma were found to be major stimuli for eotaxin-2/CCL24 release from turbinates obtained from patients suffering from nonallergic rhinitis.

Eotaxin-2/CCL24 is a potent eosinophil attractant that has been implicated in the pathogenesis of nasal polyposis (10). In a previous study, it has been reported that transgenic mice expressing IL-4 in the lung shows an increased eotaxin-2 mRNA expression (18). We have used the *in vivo* explant NP model and demonstrated that eotaxin-2/CCL24 is an inducible protein upon cytokine stimulation.

The present study has demonstrated that IL-4 is a major stimulus to induce eotaxin-2/CCL24 from NPs. Interestingly, IL-4 concentrations as low as 0.1 ng/ml were able to induce eotaxin-2/CCL24 in the culture supernatant and kinetics studies demonstrated that this cytokine is released within 24 h of stimulation, keeping its production over 96 h, which suggests a long-lasting production of eotaxin-2/CCL24 when IL-4 is released *in vivo*. Another interesting finding was the demonstration that IL-13 stimulates NPs to produce eotaxin-2/

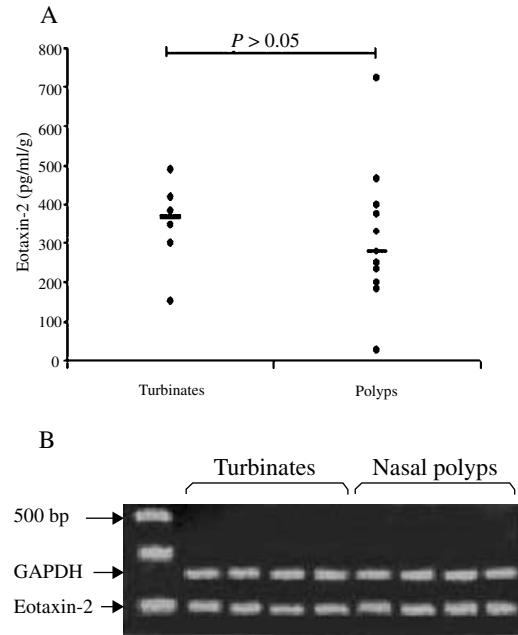


Figure 5. Spontaneous release of eotaxin-2 in the culture supernatant of nonstimulated nasal polyps and turbinates from patients suffering from vasomotor rhinitis (A). Horizontal lines represent median values. (B) Eotaxin-2 mRNA expression in both turbinates and nasal polyps.

CCL24, although compared with IL-4 this cytokine was found to be a weak stimulus. Both IL-4 and IL-13 are known to play an important role in allergic inflammation (15, 16). These cytokines activate B cells inducing class switching to IgE; stimulate expression of CD23; and induce expression of class II MHC antigens. Transfection of either IL-4 or IL-13 genes in mice causes airway inflammation, mucus hypersecretion, and subepithelial fibrosis (22, 23). In humans, increased mRNA encoding both IL-4 and IL-13 has been reported in NP biopsies (24–26), which suggests that when released they may play a major role in the pathogenesis of NPs by inducing eotaxin-2/CCL24.

We have found that IFN-gamma stimulates NPs to release eotaxin-2/CCL24. In fact, the ability of IFN-gamma to release this cytokine was equivalent to that induced by IL-13. IFN-gamma has been seen as a cytokine that downregulates the allergic response. However, this cytokine has been reported to play an important role in NPs (23–25). For example, Lee et al. (25) analyzed 14 NP biopsies derived from both atopic and nonatopic subjects and demonstrated increased IFN-gamma expression in both groups of patients, although Hamilos et al. (24) have shown that IFN-gamma expression predominates in the NPs from nonatopic subjects. Interestingly, T cell clones from NPs derived from atopic patients have been found to produce high levels of IFN-gamma compared with T cell clones derived from peripheral blood of normal subjects

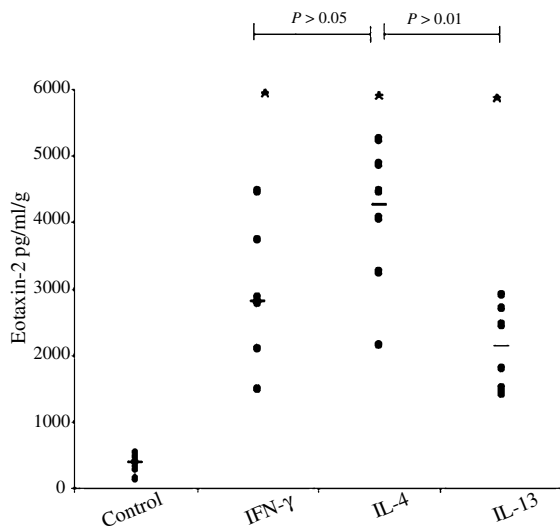


Figure 6. Levels of immunoreactive eotaxin-2 in culture supernatant derived from the cytokine-stimulated nasal turbinates (48-h culture) derived from patients suffering from vasomotor rhinitis. Horizontal lines represent median values. \*Compared with control significant at  $P < 0.05$ .

(27). The finding that IFN-gamma stimulates eotaxin-2/CCL24 from both atopic and nonatopic NPs suggests that IFN-gamma may also regulate eosinophil trafficking in nasal polyposis.

To investigate the potential cellular source of eotaxin-2/CCL24, we have analyzed NP explants by immunohistochemistry and demonstrated that eotaxin-2/CCL24 immunoreactivity was predominantly localized to mononuclear cells. Interestingly, while scarce eotaxin-2/CCL24 was seen in nonstimulated NPs, increased expression of this cytokine was observed in the IL-4 stimulated tissue. This observation further supports our finding in the IL-4-stimulated NP explant culture system and suggests that mononuclear cells may be the major cellular source for eotaxin-2/CCL24. At the time of writing, it has been reported that macrophages but not monocytes release eotaxin-2/CCL24 upon IL-4 stimulation (28). In contrast, LPS stimulated monocytes, but not macrophages, to release this cytokine, which places macrophages as major cellular source of eotaxin-2/CCL24 when IL-4 is released locally at the tissue inflammatory site.

The present study has not evaluated whether the cytokines IL-4, IL-13, and IFN-gamma stimulate turbinate explants from normal subjects to release eotaxin-2/CCL24 in culture, as it is unethical to do so. However, other groups have used different approaches and analyzed biopsies obtained from both NPs and turbin-

ates from normal subjects. For example, using RT-PCR, Jahnsen et al. (10) have reported increased eotaxin-2/CCL24 mRNA expression in NPs compared to normal turbinates. Interestingly, glucocorticoids were found to reduce eotaxin-2 mRNA in NPs (10), suggesting that this cytokine may play a prominent role in NPs. The mechanisms by which cytokines regulate eotaxin-2/CCL24 are not fully understood. However, analysis of eotaxin-2 mRNA expression in mice transgenic for IL-4, but genetically deficient in STAT-6, revealed that the IL-4-induced eotaxin-2 expression was STAT-6 dependent (18). Future studies must define the mechanisms by which the cytokines IL-4, IL-13, and IFN-gamma regulates eotaxin-2/CCL24 in humans.

Nonallergic rhinitis (vasomotor rhinitis) is a frequent cause of nasal obstruction and the etiology of this type of rhinitis remains to be shown (29). The present study has demonstrated that nasal turbinates from patients suffering this nasal disease release eotaxin-2/CCL24 spontaneously and cytokine stimulation further induces its production. Interestingly, IL-4 and IFN-gamma were found to be potent inducers of eotaxin-2/CCL24 while IL-13 was a minor stimulus. The role of IL-4 and IFN-gamma in NPs has been well investigated; however, there are no studies showing whether these cytokines may be involved in the pathogenesis of vasomotor rhinitis. It has been proposed that vasomotor rhinitis occurs as a result of an autonomic nervous system (ANS) dysfunction. Future studies must determine whether the ANS dysfunction may lead to the release of Th1- and Th2-type cytokines, which, in turn, may lead to the local production of eotaxin-2.

In summary, the present study shows that IL-4 is a major regulator of eotaxin-2/CCL24 in NPs. Both IL-13 and IFN-gamma also induce eotaxin-2/CCL24. However, both these cytokines were weak stimuli. By immunohistochemistry, mononuclear cells were identified as a major cellular source of eotaxin-2/CCL24. Interestingly, nasal turbinates from patients suffering from nonallergic rhinitis (vasomotor rhinitis) released eotaxin-2 both spontaneously and following cytokine stimulation with IL-4 and IFN-gamma being major inducers. Thus, these findings suggest that Th1- and Th2-type cytokines may differentially regulate eotaxin-2/CCL24 production in both NPs and nonallergic rhinitis.

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