

Combined Stimulation with Poly(I:C), TNF- α and Th2 Cytokines Induces TARC Production by Human Fibroblasts from the Nose, Bronchioles and Lungs

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Key Words

Fibroblasts • Thymus- and activation-regulated chemokine • IL-4 • IL-13 • TNF- α • Poly(I:C) • TARC

Abstract

Background: Th2 cells trigger allergic diseases in the respiratory tract. However, the mechanisms that cause Th2 cell infiltration remain unclear. Viral infections exacerbate allergic diseases in the respiratory tract. Thymus- and activation-regulated chemokine (TARC) recruits Th2 cells to sites of inflammation. Resident fibroblasts are thought to contribute to inflammatory cell infiltration through chemokine production. We compared the abilities of nasal, bronchiolar and lung fibroblasts to produce TARC. **Methods:** Expression of TARC mRNA was evaluated by real-time RT-PCR, while the amount of TARC in supernatants was measured by ELISA. **Results:** Costimulation with TNF- α and Th2 cytokines (IL-4, IL-13) or with poly(I:C) and Th2 cytokines (IL-4, IL-13) induced TARC production by nasal (polyp and normal) fibroblasts. Costimulation with TNF- α and Th2 cytokines (IL-4, IL-13) also induced TARC production by both bronchiolar and lung fibroblasts, but costimulation with poly(I:C) and Th2 cytokines (IL-4, IL-13) caused no induction. Combined exposure

of cells to poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) resulted in substantial production of TARC by nasal and lung fibroblasts, but much less by bronchiolar fibroblasts. **Conclusions:** TARC is directly inducible in diverse fibroblast populations from the respiratory tract (nose, bronchioles and lungs), but the mechanisms and levels of TARC production differ. Fibroblasts in the respiratory tract may contribute to Th2 cell infiltration and viral-induced exacerbation of allergic diseases, such as allergic sinusitis, asthma and allergic lung inflammation.

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Introduction

Fibroblasts are mesenchymal cells that perform many vital functions in the human body. They are responsible for much of the synthesis of cytokines and chemokines as well as the extracellular matrix in tissues and also play important roles in chronic inflammatory diseases of the respiratory tract. Many inflammatory diseases are associated with fibroblasts, either because these cells are activated in inflammatory sites or because of remodeling in which extracellular matrix accumulates in airway tissues.

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It is now clear that fibroblasts in the respiratory tract express Toll-like receptors (TLRs), and activation of TLRs on fibroblasts has been shown to induce the production of various chemokines [1, 2]. The importance of TLRs for host defense and innate immunity has been documented. In addition, TLRs play important roles in the adaptive immune system and regulation of the Th1/Th2 balance [3]. Knowledge of the regulation of chemokine synthesis by fibroblasts is thus important for understanding the pathogenesis of chronic inflammation in the respiratory tract.

Thymus- and activation-regulated chemokine (TARC) is a member of the CC chemokine family and is a potent and selective chemoattractant for Th2 cells via CC chemokine receptor 4 (CCR4). There is increasing evidence that TARC is involved in the recruitment of Th2 cells during allergic diseases of the airway [4, 5]. Th2 cells have been identified in tissues of allergic sinusitis [6]. The Th2 cytokines (IL-4 and IL-13) released by accumulated Th2 cells induce the production of other chemokines such as eotaxin, leading to eosinophil infiltration. Although epithelial cells are thought to be the main source of TARC, fibroblasts were identified as another source of this chemokine [7].

Fibroblasts constitute a heterogeneous family of cells that express different phenotypic and functional features depending on their site of origin and the state (healthy or diseased) of the tissue of origin [8]. The heterogeneity of fibroblasts has been documented [7, 9]. For instance, lipopolysaccharide, a TLR4 ligand, induced RANTES and GM-CSF [9] and synergized with IL-4 to induce TARC [7] in nasal, but not lung fibroblasts. Combined stimulation using TNF- α with Th2 cytokines (IL-4 and IL-13) markedly increased TARC expression in corneal and dermal fibroblasts but not in lung fibroblasts [10]. It was demonstrated that dsRNA, as a viral genome fragment or as a replicative intermediate of RNA virus [11], induced the production of chemokines by upper respiratory fibroblasts as well as respiratory epithelial cells [2, 12]. TLR3, a receptor for dsRNA, is expressed in respiratory epithelial cells and fibroblasts [1, 12]. However, little is known regarding differences in the responsiveness of respiratory tract fibroblasts to dsRNA alone, or in combination with TNF- α (a proinflammatory cytokine) and/or Th2 cytokines (IL-4, IL-13). Since the respiratory tract is a site of direct interaction with viruses, in addition to bacteria and their products, such as lipopolysaccharides, we designed this study to compare chemokine expression by fibroblasts derived from the nose, bronchioles and lungs.

Materials and Methods

Reagents

Human recombinant IL-4 and TNF- α were purchased from R&D Systems, Inc. (Minneapolis, Min., USA). Human recombinant IL-13 was purchased from Millipore, Co. (Billerica, Mass., USA). TLR3 ligand [synthetic analog of double-stranded RNA (dsRNA) (poly(I:C))] was purchased from InvivoGen Co. (San Diego, Calif., USA). Anti-TLR3-neutralizing antibody (Ab) (TLR3.7) was purchased from Abcam, Inc. (Cambridge, Mass., USA). Mouse IgG1 was purchased from Dako North America, Inc. (Carpinteria, Calif., USA).

Cell Sources and Culture

Primary fibroblast lines were established from human tissues and characterized as previously described [13]. Only primary fibroblast lines at the 4th passage were used in this study. All the nasal polyp specimens ($n = 5$) had been taken from patients with allergic chronic sinusitis (5 males aged 44.4 ± 9.7 years, mean $\pm$ SEM). All the patients were atopic, diagnosed on the basis of elevation of at least 1 specific IgE against 8 common aeroallergens that were tested. Normal nasal mucosa of the uncinate process ($n = 5$) was taken from patients who were suffering from blow-out fractures or isolated sphenoiditis (5 males aged 58.0 ± 8.4 years, mean $\pm$ SEM). Normal bronchiolar fibroblast lines ($n = 3$) were derived from normal bronchiolar areas (sites distal to the 7th bifurcation from a main bronchus) of surgically excised lung specimens from patients undergoing surgery (lung lobectomy) for cancer (2 males and 1 female aged 69.0 ± 4.4 years, mean $\pm$ SEM). Normal human lung fibroblasts (CCD 13Lu, CCD 25Lu) and normal lung fibroblasts (CC-2512) were obtained from the American Type Culture Collection (Rockville, Md., USA) and Lonza Walkersville, Inc. (Walkersville, Md., USA), respectively. 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 Nippon Medical School Hospital.

Before the cytokine assay, fibroblasts (1×10^4 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, Corning, N.Y., USA). The plated fibroblasts were allowed to grow to confluence in regular growth medium (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, penicillin at 100 U/ml, streptomycin at 100 μ g/ml and amphotericin B at 2.5 μ g/ml. The same culture medium was used when fibroblasts were exposed to various stimuli. At specified time points, supernatants were collected and centrifuged for the cytokine protein assay, and cells were collected for RNA extraction.

Analysis of TARC mRNA by Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

We investigated the effects of poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination, on TARC mRNA in fibroblasts. The cells were cultured for 48 h, after which the amounts of TARC mRNA in cell lysates were assayed by a quantitative real-time reverse-transcription polymerase chain reaction (quantitative real-time RT-PCR). The kinetics of TARC mRNA expression after simultaneous stimulation with poly(I:C), TNF- α and a Th2 cytokine (IL-4 or IL-13) were examined in na-

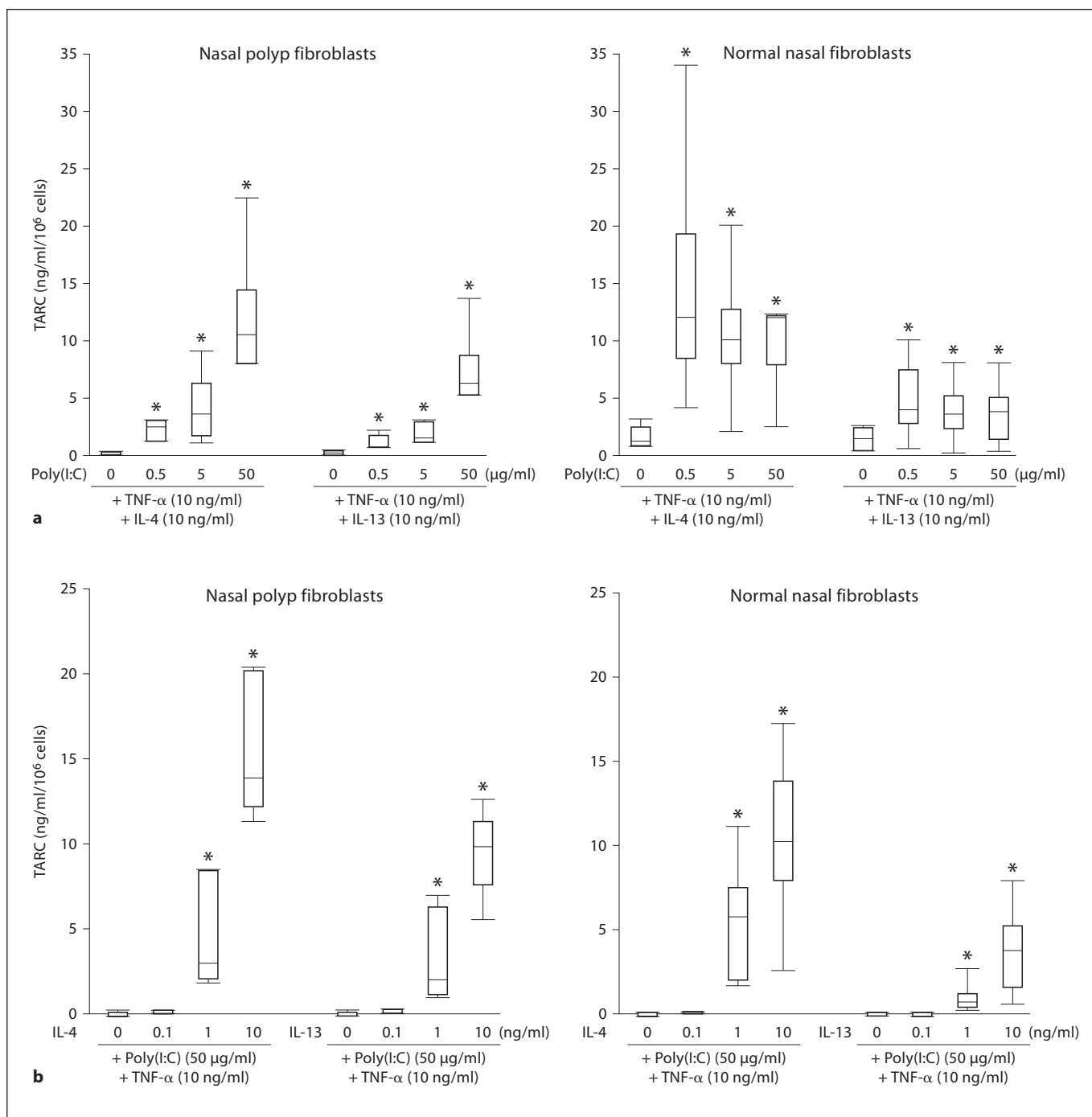


Fig. 1. Dose-effect curves of poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) on TARC release by nasal polyp and normal nasal fibroblasts. Nasal polyp and normal nasal fibroblasts were stimulated with the following: a series of concentrations of poly(I:C) with TNF- α (10 ng/ml) and Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml) [$* p < 0.05$ compared with cultures stimulated in the presence of TNF- α and Th2 cytokines but without poly(I:C)] (**a**)

or a series of concentrations of Th2 cytokine (IL-4 or IL-13) with poly(I:C) (50 μ g/ml) and TNF- α (10 ng/ml) [$* p < 0.05$ compared with cultures stimulated in the presence of poly(I:C) and TNF- α but without any Th2 cytokine] (**b**). The TARC concentrations were determined after 72 h of culture. Box plots represent the median values with 25% and 75% interquartiles; error bars represent the 10th and 90th percentiles ($n = 5$).

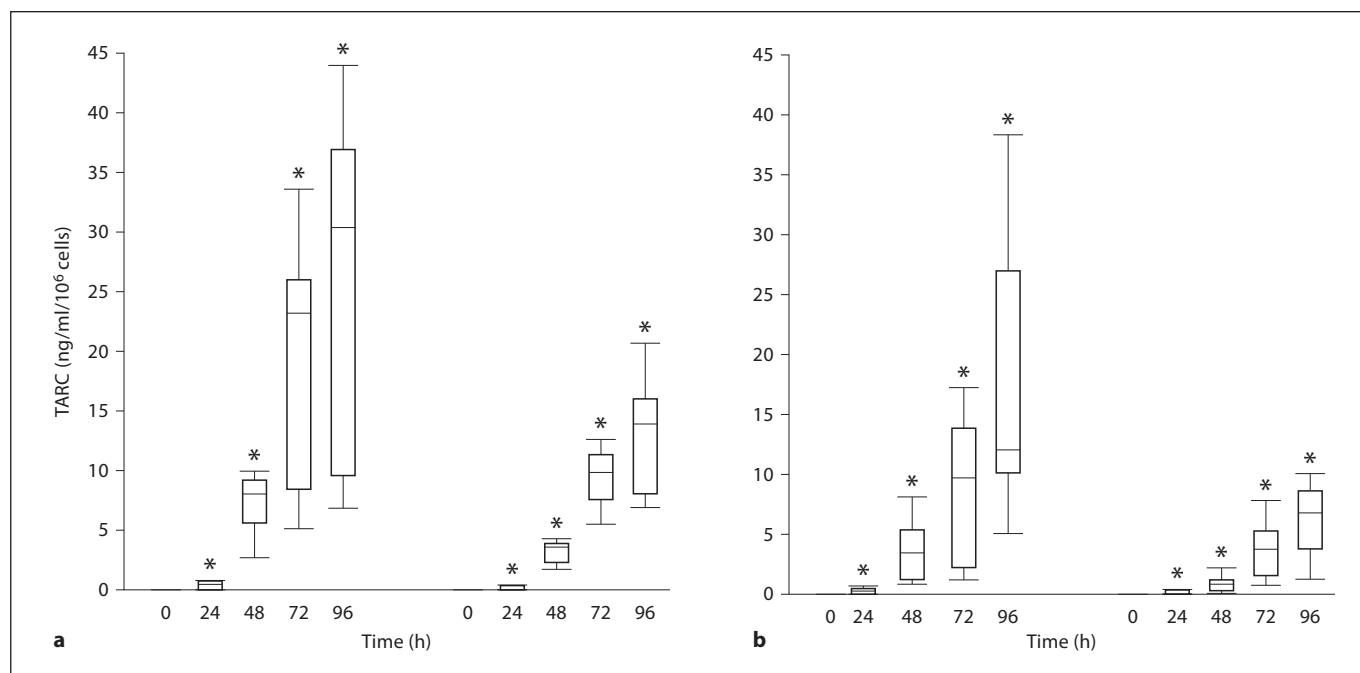


Fig. 2. Kinetics of TARC accumulation in supernatants of nasal polyp and normal nasal fibroblasts. Nasal polyp (a) and normal nasal (b) fibroblasts were treated with poly(I:C) (50 μ g/ml), TNF- α (10 ng/ml) and Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml).

Supernatants were assayed for TARC after 0, 24, 48, 72 and 96 h of culture. Box plots represent the median values with 25% and 75% interquartiles; error bars represent the 10th and 90th percentiles (n = 5). * p < 0.05 compared with 0 h.

sal (polyp and normal) fibroblasts. We next investigated the effects of poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination, on TARC mRNA in normal bronchiolar and normal lung fibroblasts after 48-hour stimulation.

Total cellular RNA was extracted and purified using an RNeasy mini kit (Qiagen GmbH, Germany). The total RNA, 2 μ g, was reverse transcribed at 37°C for 60 min using random primers (Takara) and Omniscript reverse transcriptase (Qiagen) according to the manufacturers' protocols.

Quantitative real-time RT-PCR was carried out using the TaqMan assay and ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). The primers and fluorogenic 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 sequence detector system software (Applied Biosystems). Threshold cycles were used to calculate arbitrary mRNA concentrations by the relative standard curve method. A standard curve was constructed using serial dilutions of cDNA containing the message for TARC. The level of TARC mRNA was normalized to the level of β -actin mRNA.

TARC Assay

The levels of TARC in culture supernatants were measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available human TARC ELISA kit (R&D Systems) that

detects both recombinant and natural TARC. The sensitivity of this assay system is 7.0 pg/ml and higher. The data are presented as nanograms of TARC 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

Effects of Poly(I:C), TNF- α and Th2 Cytokines (IL-4, IL-13) on TARC Production by Nasal Fibroblasts

Fibroblasts were cultured with 10 ng/ml TNF- α , a Th2 cytokine (10 ng/ml IL-4 or 10 ng/ml IL-13) and a series of concentrations of poly(I:C). The release of TARC by nasal polyp fibroblasts increased in a poly(I:C) dose-dependent manner (fig. 1a). In contrast, when normal nasal fibroblasts were cultured in the presence of the fixed concentrations of TNF- α and Th2 cytokines, stimulation with 0.5 μ g/ml poly(I:C) induced the largest TARC release, which thereafter declined in a poly(I:C) dose-dependent manner (fig. 1a). The cells were then cultured with 10 ng/

ml TNF- α , 50 μ g/ml poly(I:C) and a series of concentrations of a Th2 cytokine (IL-4 or IL-13). The release of TARC by nasal polyp and normal fibroblasts increased in a Th2 cytokine (IL-4 or IL-13) dose-dependent manner (fig. 1b). In time course experiments, the TARC release by nasal polyp and normal fibroblasts increased in a time-dependent manner in response to simultaneous stimulation with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) (fig. 2a, b).

The cells were cultured with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination. After 72 h of culture, supernatants were collected and TARC concentrations were determined. TARC was negligible in the supernatants of unstimulated control cells. No effect on TARC release was seen with poly(I:C), TNF- α , Th2 cytokines (IL-4, IL-13) or the combination of poly(I:C) and TNF- α . However, combined stimulation with TNF- α and a Th2 cytokine (IL-4 or IL-13), and also with poly(I:C) and a Th2 cytokine (IL-4 or IL-13), induced TARC release by both nasal polyp and normal fibroblasts (fig. 3a, b). Moreover, simultaneous exposure of the cells to poly(I:C), TNF- α and a Th2 cytokine (IL-4 or IL-13) resulted in substantial amounts of TARC release into the culture medium by both the nasal polyp and normal fibroblasts (fig. 3a, b).

Quantitative real-time RT-PCR was performed for quantitation of the TARC mRNA levels. No significant effect on the amount of TARC mRNA was seen with TNF- α or the Th2 cytokines (IL-4, IL-13). In contrast, stimulation with poly(I:C), poly(I:C) and TNF- α , poly(I:C) and a Th2 cytokine (IL-4 or IL-13), or TNF- α and a Th2 cytokine (IL-4 or IL-13) induced significant amounts of TARC mRNA in nasal polyp and normal fibroblasts (fig. 4a, b). Moreover, simultaneous exposure of the cells to poly(I:C), TNF- α and a Th2 cytokine (IL-4 or IL-13) induced a tremendous increase in the abundance of TARC mRNA (fig. 4a, b). The response profiles for those stimulations were similar in the nasal polyp and normal fibroblasts (fig. 4a, b).

Comparison of Poly(I:C)-, TNF- α - and Th2 Cytokine (IL-4, IL-13)-Induced TARC Production by Fibroblasts from Different Sites of the Respiratory Tract

We next examined TARC production by fibroblasts derived from normal bronchioles and normal lung tissue. The release of TARC by bronchiolar and lung fibroblasts increased in a dose-dependent manner with each of the combinations (fig. 5a, b). In time course experiments, the TARC release by bronchiolar and lung fibroblasts increased in a time-dependent manner in response to si-

multaneous stimulation with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) (fig. 6a, b).

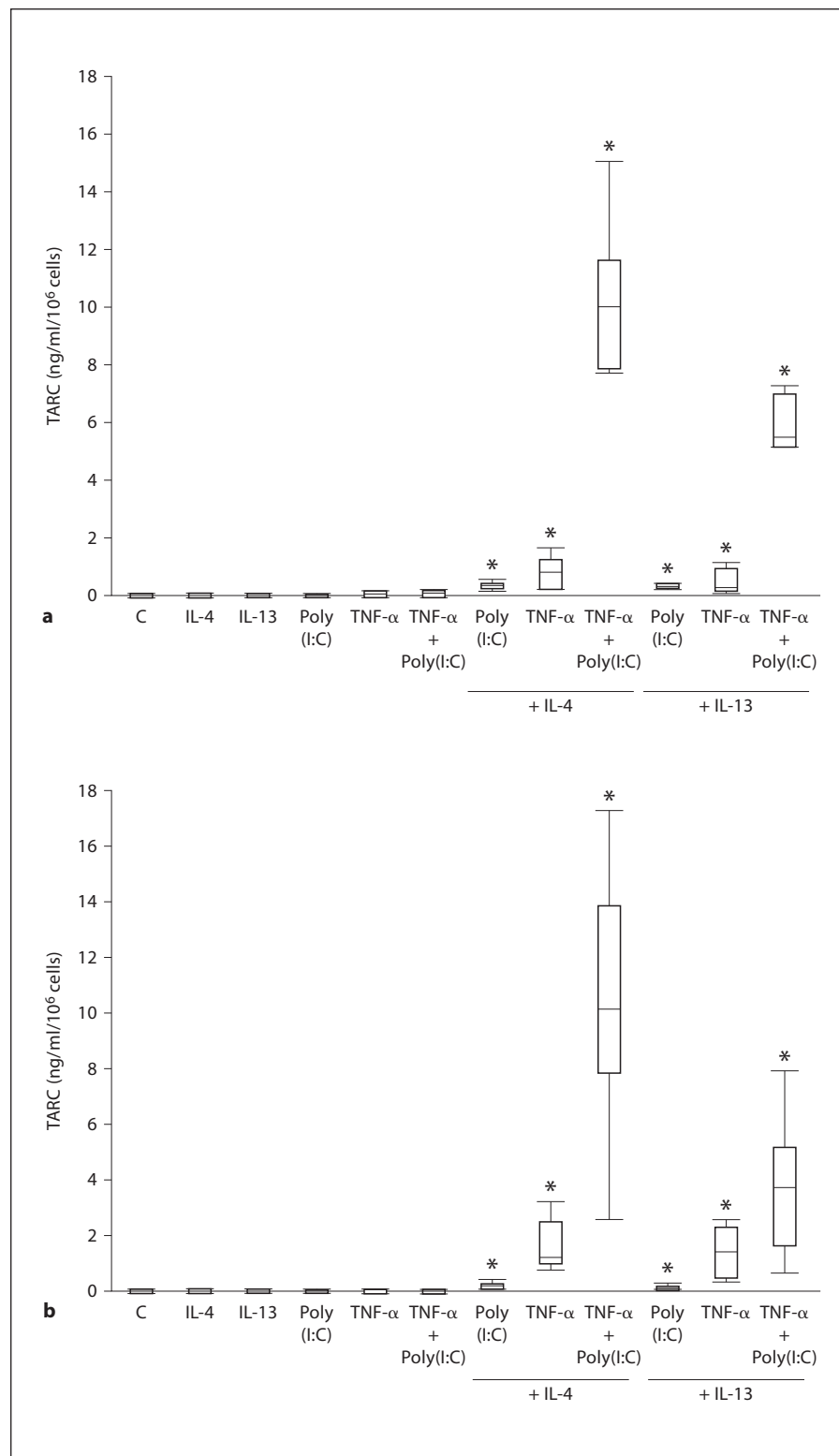
The cells were cultured with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination, for 72 h. Combined stimulation with TNF- α and Th2 cytokines (IL-4, IL-13), but not poly(I:C) and Th2 cytokines (IL-4, IL-13), induced TARC release by both bronchiolar and lung fibroblasts (fig. 7a, b). Simultaneous exposure of the cells to poly(I:C), TNF- α and a Th2 cytokine (IL-4 or IL-13) resulted in larger amounts of TARC release into the culture medium by both the bronchiolar and lung fibroblasts (fig. 7a, b), but the TARC release by bronchiolar fibroblasts was much less compared to that by the nasal and lung fibroblasts (fig. 3a, b, 7a, b). Next, we examined the TARC mRNA levels by real-time PCR. Bronchiolar and lung fibroblasts showed slight upregulation of TARC mRNA expression in response to simultaneous stimulation with TNF- α and Th2 cytokines (IL-4, IL-13), but not poly(I:C) and Th2 cytokines (IL-4, IL-13) (fig. 8a, b). Simultaneous exposure of the bronchiolar fibroblasts to poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) further upregulated TARC mRNA expression (fig. 8a). Moreover, simultaneous stimulation with all three components induced a tremendous increase in the abundance of TARC mRNA in the lung fibroblasts (fig. 8b).

To clarify the role of TLR3 in the poly(I:C)-induced production of TARC in fibroblasts, anti-TLR3-neutralizing Ab (10 μ g/ml) was added to the culture medium 1 h before stimulation with poly(I:C), TNF- α and IL-4. The Ab showed no effects on the release of TARC from poly(I:C)-, TNF- α - and IL-4-stimulated fibroblasts (fig. 9a). On the contrary, bafilomycin A1, an endosome acidification inhibitor, inhibited the release of TARC by fibroblasts stimulated with poly(I:C), TNF- α and IL-4 ($p < 0.05$, fig. 9b). Bafilomycin A1 had no effects on the TARC release induced by TNF- α and IL-4 (fig. 9b).

Discussion

Costimulation with TNF- α and Th2 cytokines (IL-4, IL-13) or with poly(I:C) and Th2 cytokines (IL-4, IL-13) induced TARC production by nasal (polyp and normal) fibroblasts. Stimulation with TNF- α and Th2 cytokines (IL-4, IL-13) also induced TARC production by both bronchiolar and lung fibroblasts, but stimulation with poly(I:C) and Th2 cytokines (IL-4, IL-13) did not. Combined exposure of cells to poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) resulted in substantial TARC production by nasal and lung fibroblasts, but much less by

Fig. 3. Effects of poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) on TARC release by nasal polyp and normal nasal fibroblasts. Nasal polyp (**a**) and normal nasal (**b**) fibroblasts were stimulated with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination. Nonstimulated cultures served as controls. After 72 h of culture, supernatants were collected, and TARC concentrations were determined by ELISA. Box plots represent the median values with 25% and 75% interquartiles; the error bars represent the 10th and 90th percentiles (n = 5). * p < 0.05 compared with control cultures. C = Control (no stimulation); poly(I:C), (50 μ g/ml); TNF- α (10 ng/ml); Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml).



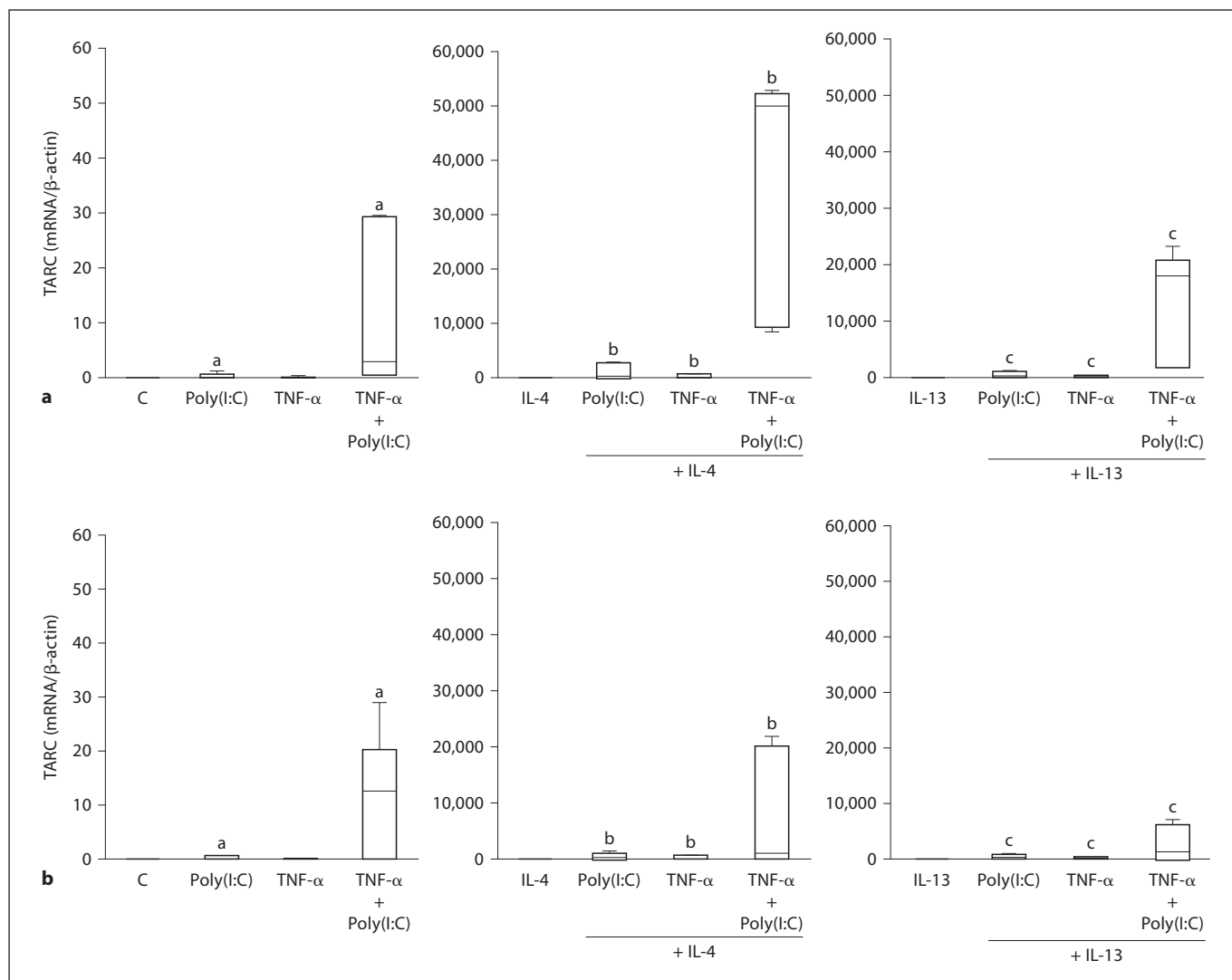


Fig. 4. TARC mRNA expression in nasal polyp and normal nasal fibroblasts. nasal polyp (**a**) and normal nasal (**b**) fibroblasts were incubated for 48 h with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination. Total RNA was isolated from the fibroblasts. After cDNA synthesis, analysis for TARC mRNA was performed. The ratio of TARC mRNA to β -actin mRNA was determined. Box plots represent the median values with 25% and

75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles. ^a $p < 0.05$ compared with control cultures. ^b $p < 0.05$ compared with cultures stimulated with IL-4. ^c $p < 0.05$ compared with cultures stimulated with IL-13. C = Control (no stimulation); poly(I:C) (50 μ g/ml); TNF- α (10 ng/ml); Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml).

bronchiolar fibroblasts. These diverse responses of fibroblasts suggest that these cells may contribute differently to the initiation, amplification and exacerbation of Th2-cell-mediated allergic conditions in the respiratory tract.

It has been demonstrated that primary cultures of human lung fibroblasts maintain their phenotypic characteristics in vitro throughout a substantial number of pas-

sages [14]. Chang et al. [15] looked at genome-wide patterns of gene expression in cultured human fibroblasts derived from different anatomical sites by using cDNA microarrays containing approximately 43,000 elements, corresponding to approximately 36,000 genes. They found that fibroblasts from each site displayed distinct and characteristic transcriptional patterns, indicating that their in vitro phenotypes are stable through several

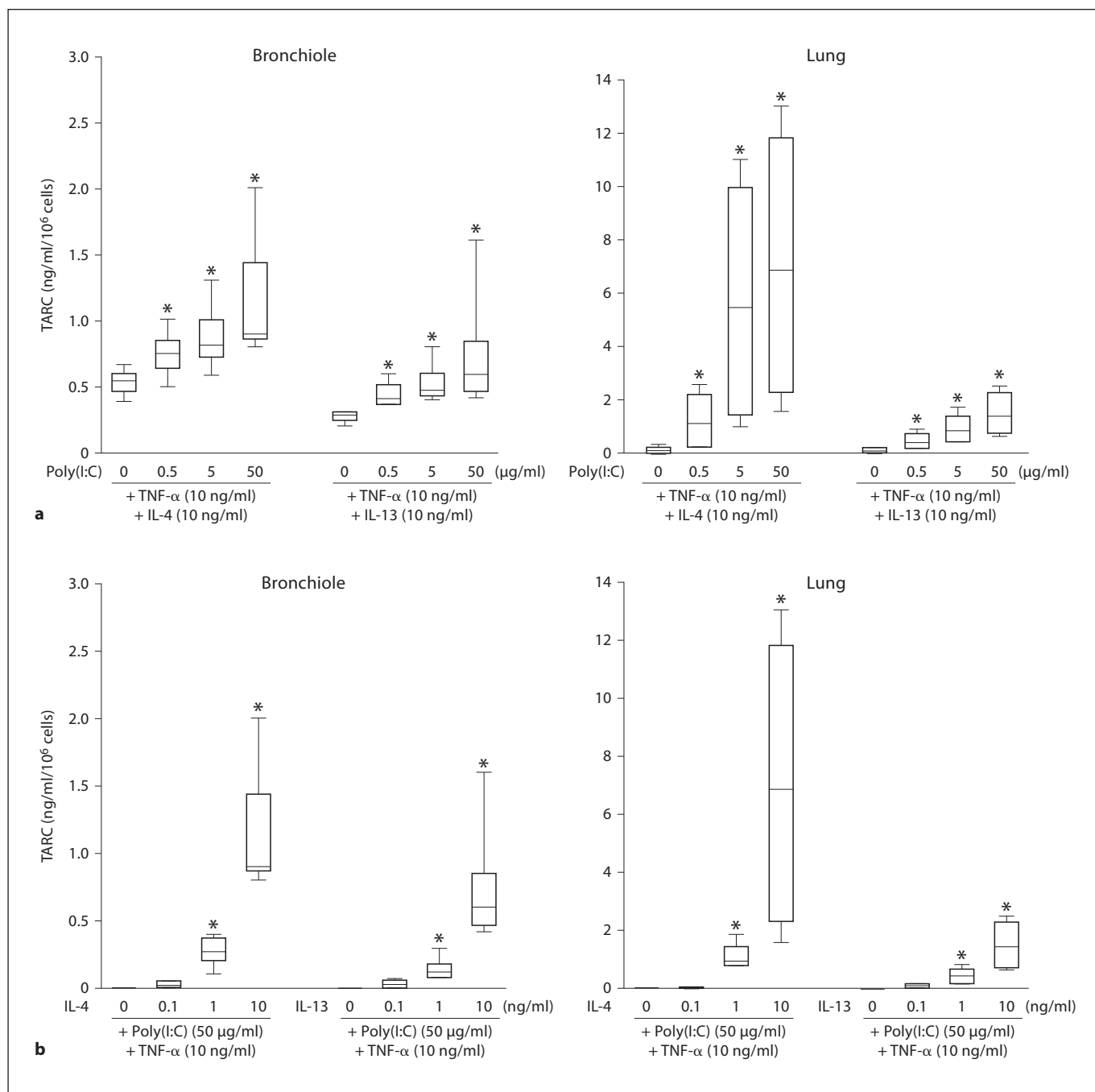


Fig. 5. Dose-effect curves of poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) on TARC release by normal bronchiolar and normal lung fibroblasts. Normal bronchiolar and normal lung fibroblasts were stimulated with the following: a series of concentrations of poly(I:C) with TNF- α (10 ng/ml) and Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml) [* $p < 0.05$ compared with cultures stimulated in the presence of TNF- α and Th2 cytokines but without poly(I:C)] (**a**) or a series of concentrations of Th2 cyto-

kine (IL-4 or IL-13) with poly(I:C) (50 μ g/ml) and TNF- α (10 ng/ml) [* $p < 0.05$ compared with cultures stimulated in the presence of poly(I:C) and TNF- α but without Th2 cytokines] (**b**). The TARC concentrations were determined after 72 h of culture. Box plots represent the median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles.

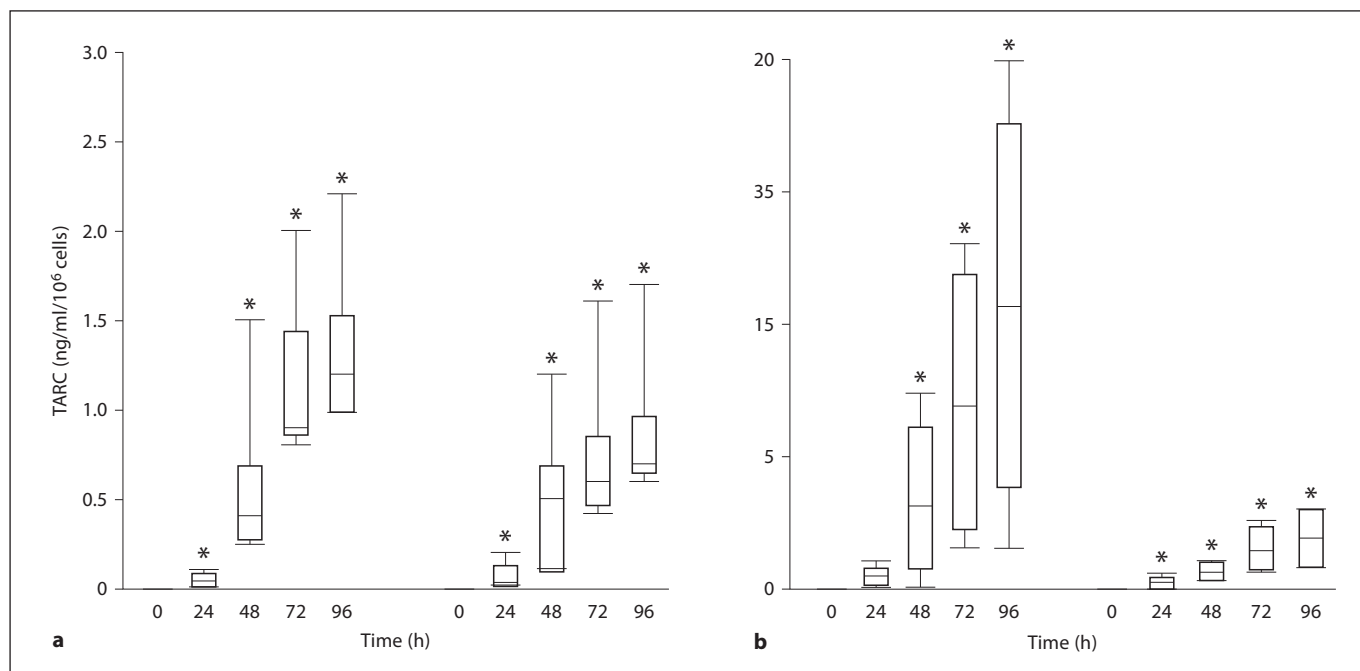


Fig. 6. Kinetics of TARC accumulation in supernatants of normal bronchiolar and normal lung fibroblasts. Normal bronchiolar (a) and normal lung (b) fibroblasts were treated with poly(I:C) (50 μ g/ml), TNF- α (10 ng/ml) and Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml). Supernatants were assayed for TARC after 0, 24, 48,

72 and 96 h of culture. Box plots represent the median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with 0 h.

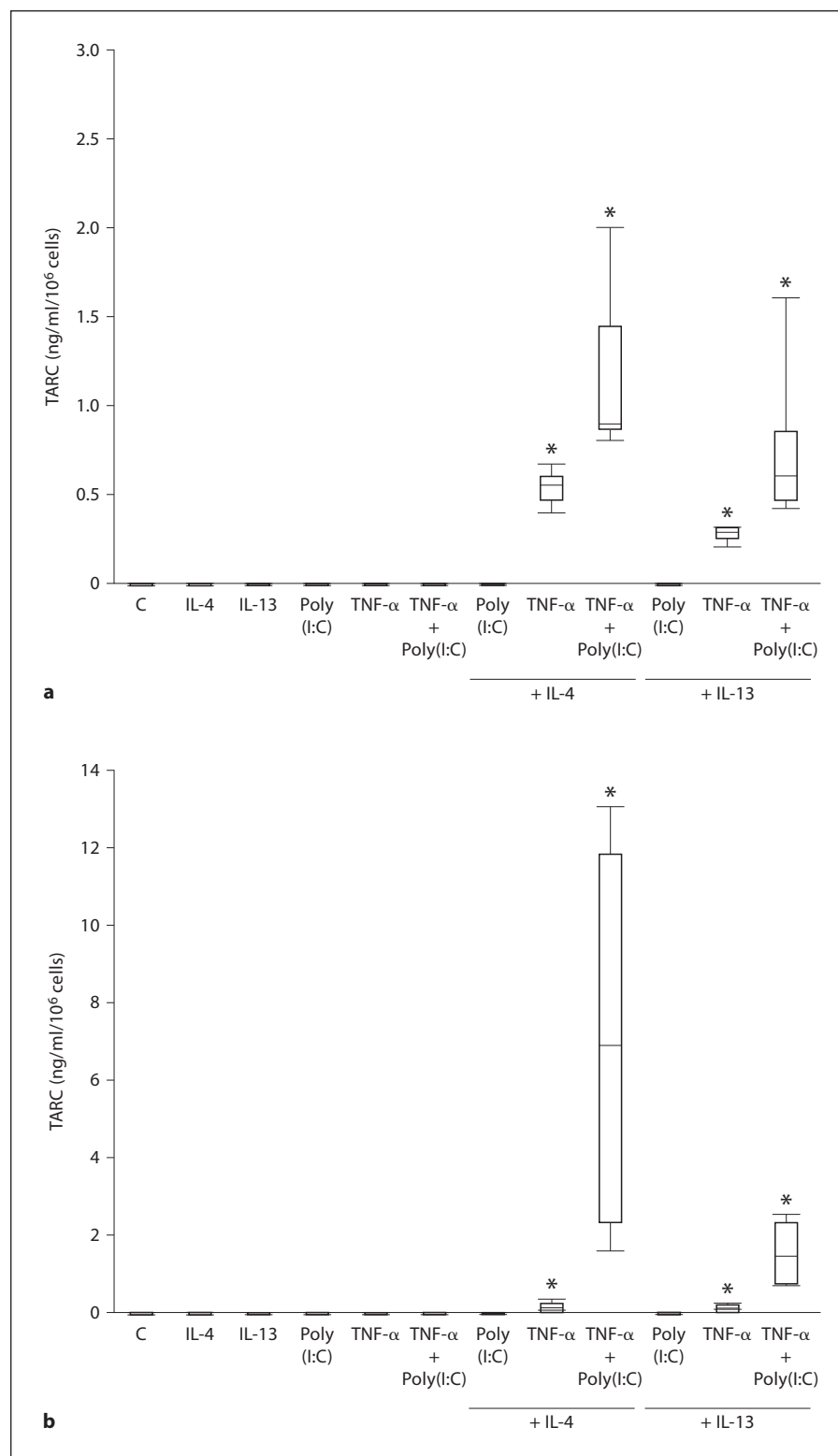
passages of culture, and that fibroblasts at different locations in the body can be considered distinct, differentiated cell types. Taken together, the use of in vitro fibroblast cell lines is an acceptable approach to studying the physiologic characteristics of these cells.

Airway inflammation develops in airway tissues in response to exposure to potentially injurious physical or biologic agents. This response includes the release of various mediators, among which cytokines and chemokines are considered to play major regulatory roles. Fibroblasts, in addition to other tissue structural cells, may actively participate in this process by releasing a variety of cytokines and chemokines [1, 9]. It is also known that proinflammatory cytokines such as TNF- α are powerful regulators of these fibroblast effector activities [10].

The family of TLRs plays a key role in pathogen recognition, and also in induction and regulation of the innate and adaptive immune responses. Fibroblasts in the respiratory tract express TLRs, and activation of TLRs on fibroblasts has been shown to induce the production of several chemokines [1, 2]. The importance of TLRs for the host defense in the respiratory tract has been demonstrat-

ed by the increased susceptibility of TLR-knockout mice to viral and bacterial infections [16]. In addition to their prominent roles in host defense and innate immunity, TLRs play important roles in the adaptive immune system and regulation of the Th1/Th2 balance [3]. Thus, TLRs are thought to exert a strong impact in relation to Th2-biased allergic diseases such as allergic sinusitis, asthma and allergic lung inflammation. Interestingly, among the TLR ligands, only poly(I:C), a synthetic analog of viral dsRNA and a well-characterized ligand for TLR3, was able to induce Th1-type cytokines and chemokines such as IFN- β and IFN-inducible genes like IP-10 and ITAC, and also induce Th2-type cytokines and chemokines such as GM-CSF and TARC in human airway epithelial cells [17]. In addition, nasal polyp fibroblasts stimulated with poly(I:C) also showed induction of TARC mRNA [18]. Our present study demonstrated that various types of fibroblasts from the respiratory tract produce TARC in response to stimulation with combinations of poly(I:C), Th2 cytokines (IL-4, IL-13) and TNF- α , and that the capacity of those fibroblasts to produce TARC differs as a function of their site of origin. Moreover, fi-

Fig. 7. Effects of poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13) on TARC release by normal bronchiolar (a) and normal lung (b) fibroblasts. Normal bronchiolar (a) and normal lung (b) fibroblasts were stimulated with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination. Nonstimulated cultures served as controls. After 72 h of culture, supernatants were collected, and TARC concentrations were determined by ELISA. Box plots represent the median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with control cultures. C = Control (no stimulation); poly(I:C) (50 μ g/ml); TNF- α (10 ng/ml); Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml).



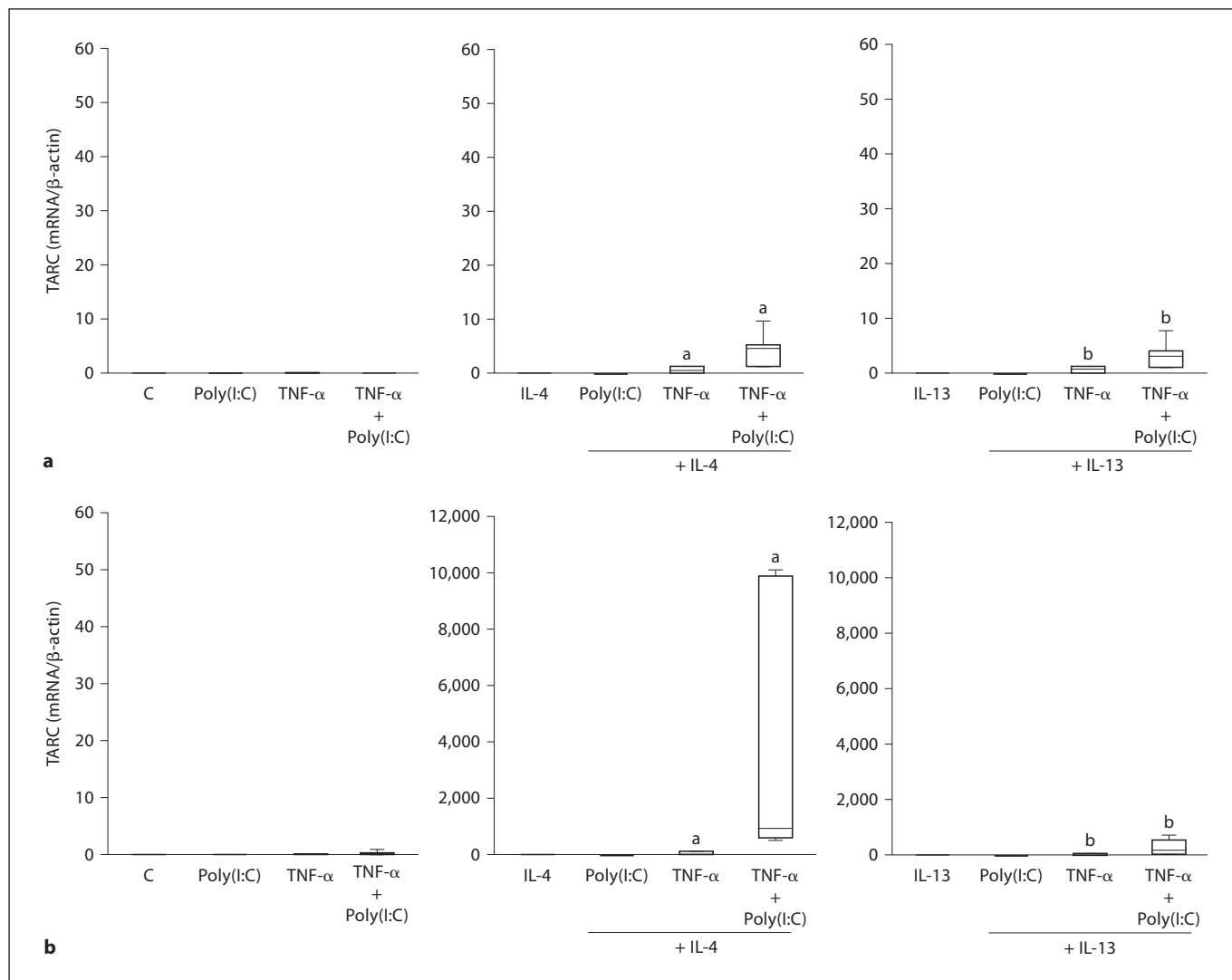


Fig. 8. TARC mRNA expression in normal bronchiolar (**a**) and normal lung (**b**) fibroblasts. Normal bronchiolar (**a**) and normal lung (**b**) fibroblasts were incubated for 48 h with poly(I:C), TNF- α and Th2 cytokines (IL-4, IL-13), alone and in combination. Total RNA was isolated from the fibroblasts. After cDNA synthesis, analysis for TARC mRNA was performed. The ratio of TARC mRNA to β -actin mRNA was determined. Box plots represent the

median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles. ^a $p < 0.05$ compared with cultures stimulated with IL-4. ^b $p < 0.05$ compared with cultures stimulated with IL-13. C = Control (no stimulation); poly(I:C) (50 μ g/ml); TNF- α (10 ng/ml); Th2 cytokines (IL-4, 10 ng/ml; IL-13, 10 ng/ml).

broblasts that were isolated from nasal polyps of patients with allergic sinusitis showed poly(I:C) dose-dependent production of TARC when stimulated in the presence of TNF- α and Th2 cytokines (IL-4, IL-13). In contrast, fibroblasts isolated from normal nasal mucosa showed peak production of TARC at 0.5 μ g/ml poly(I:C), but thereafter TARC production decreased as the poly(I:C) dose was increased (fig. 3a, b). This divergent response by

these two types of nasal fibroblasts is intriguing and warrants further study to elucidate the underlying reason.

Allergic inflammations are thought to be Th2-type immune responses, given the accumulation of Th2 cells and increased expression of Th2 cytokines such as IL-4 and IL-13 seen at mucosal sites of the airway in allergic sinusitis and asthma [5, 6]. These cytokines are known to synergize with TNF- α or poly(I:C) to induce chemokines

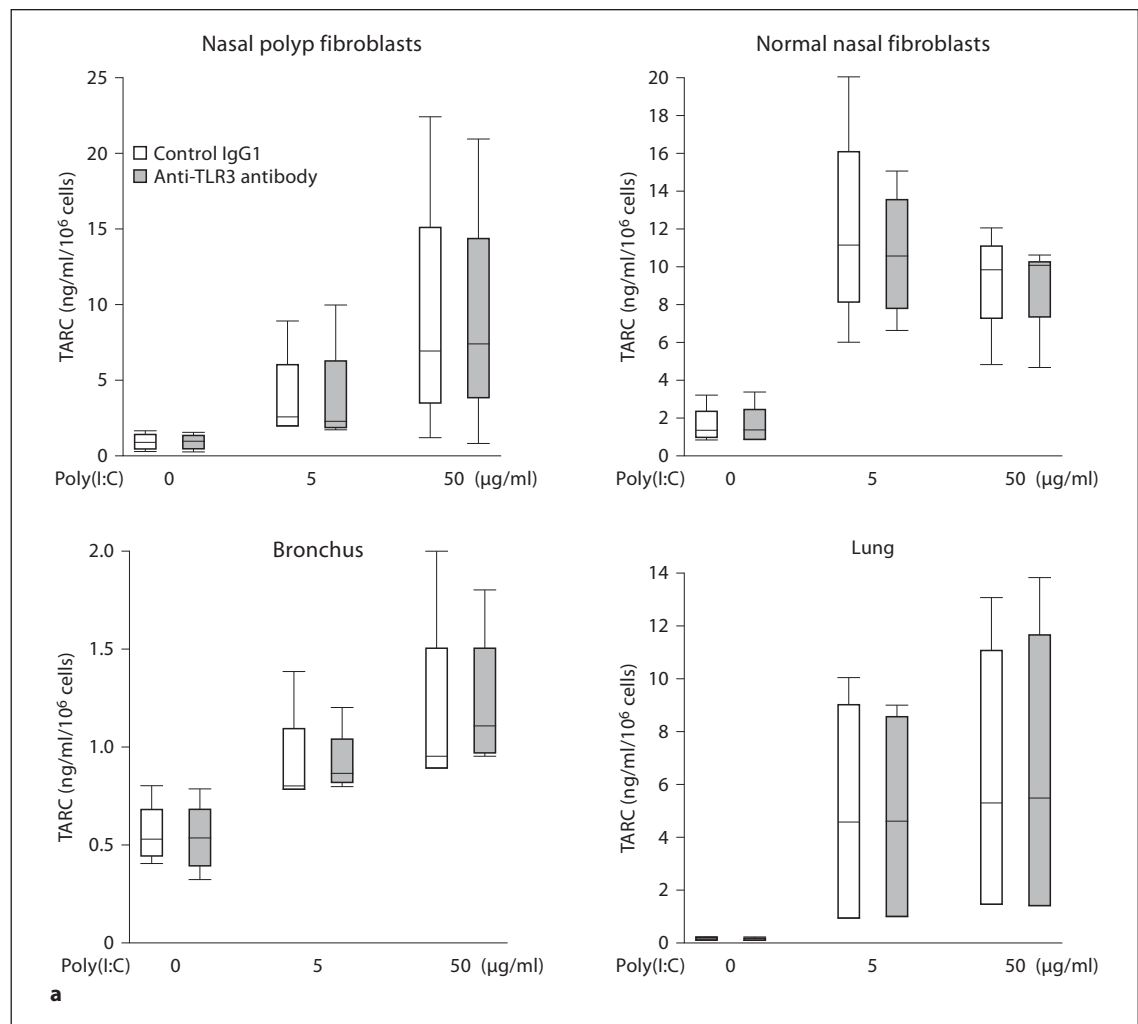


Fig. 9. a Effects of an anti-TLR3-neutralizing Ab on poly(I:C)-, TNF- α - and IL-4-induced release of TARC from fibroblasts. Anti-TLR3-neutralizing Ab or control mouse IgG1 was added 1 h before 72 h stimulation with 50 μ g/ml poly(I:C), 10 ng/ml TNF- α and 10 ng/ml IL-4. Box plots represent the median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles.

such as eotaxin, which attract eosinophils, and TARC, which recruits Th2 cells [10, 19–21]. Local production of TARC by nasal fibroblasts in response to stimulation by TNF- α and Th2 cytokines (IL-4, IL-13) or poly(I:C) and Th2 cytokines (IL-4, IL-13) might play an important role in the induction, maintenance and virus-induced exacerbation of upper airway allergic inflammation as a result of further promotion of Th2 cell infiltration. The inability of bronchiolar and lung fibroblasts to release TARC in response to poly(I:C) and Th2 cytokines (IL-4, IL-13) and their ability to respond to combined exposure to TNF- α and Th2 cytokines (IL-4, IL-13) or poly(I:C), TNF- α and

Th2 cytokines (IL-4, IL-13) suggest that the effector contributions of these cells to TARC production in the inflammatory response are limited compared to nasal fibroblasts. Fukuda et al. [10] used RT-PCR and ELISA and reported that TNF- α and Th2 cytokines (IL-4, IL-13) failed to induce TARC production in lung fibroblasts. In our present study, we found that lung fibroblasts in fact produced trace amounts of TARC in response to stimulation with TNF- α and Th2 cytokines (IL-4, IL-13). We think that this discrepancy between the two findings may be explained as follows. Fukuda et al. stimulated their lung fibroblasts for 24 h and then used an ELISA tech-

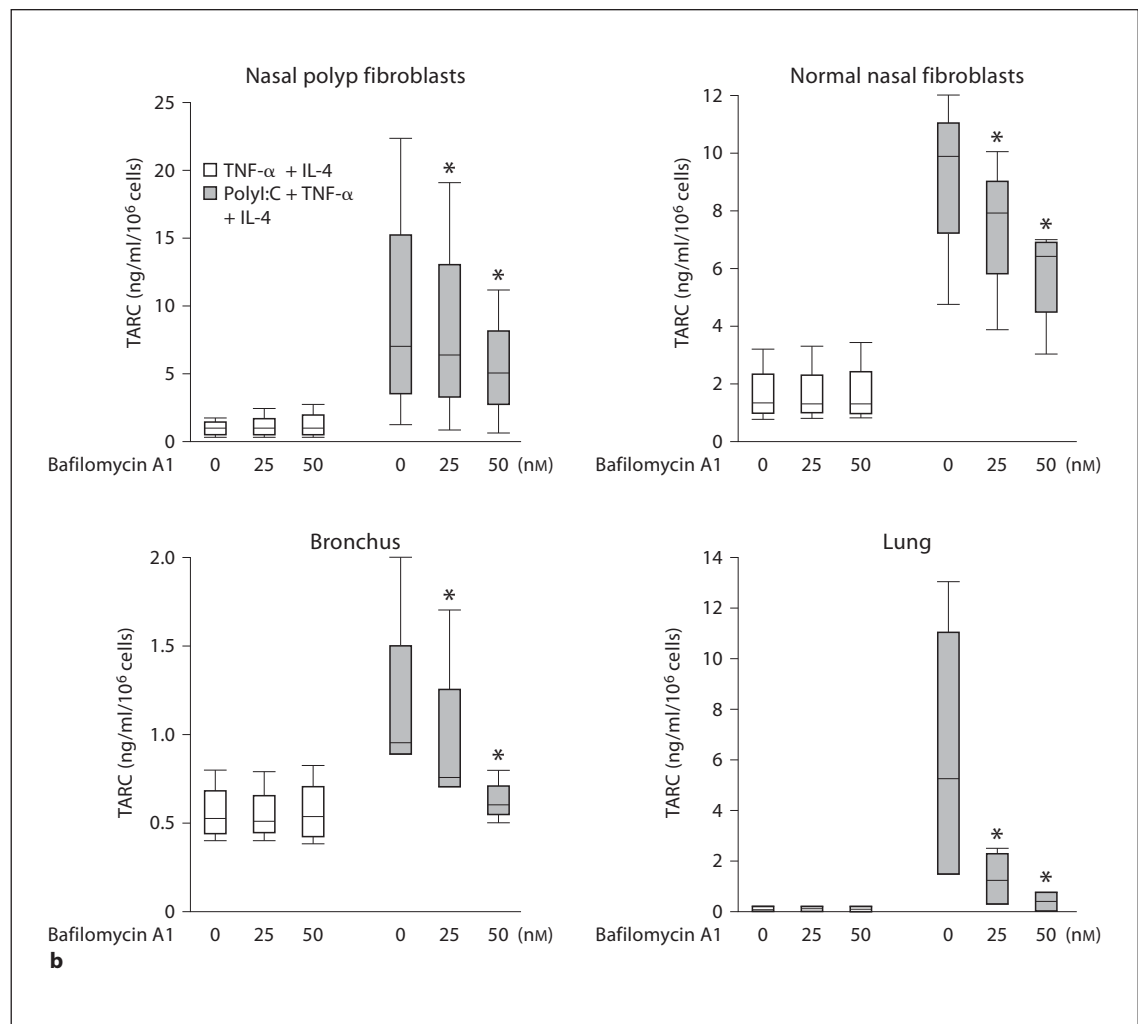


Fig. 9. b Effects of bafilomycin A1 on poly(I:C)-, TNF- α - and IL-4-induced release of TARC from fibroblasts. Bafilomycin A1 inhibited the release of TARC from fibroblasts stimulated with 50 μ g/ml poly(I:C), 10 ng/ml TNF- α and 10 ng/ml IL-4, but not when poly(I:C) was omitted. Box plots represent the median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles. * $p < 0.05$ compared with the same stimulation but without bafilomycin.

nique to look for TARC in the culture supernatant. We, on the other hand, stimulated our lung fibroblasts for 72 h. Accordingly, there is a possibility that our longer period of stimulation resulted in a higher – and thus detectable – level of TARC in the culture supernatant. Moreover, whereas we used real-time RT-PCR to detect TARC mRNA, Fukuda et al. employed RT-PCR. Real-time RT-PCR is more sensitive than RT-PCR, and it can thus be surmised that by using the more sensitive technique we were able to confirm induction of trace levels of TARC mRNA. Taken together, given that Th2 cytokines and TNF- α are expressed and CCR4-positive T cells infiltrate

the mucosa in asthma and allergic lung diseases such as eosinophilic pneumonia [5, 22, 23], TARC production by bronchiolar and lung fibroblasts might play a role in allergic diseases of the lower respiratory tract by promoting infiltration of Th2 cells, leading to eosinophilic inflammation.

Respiratory viruses such as rhinoviruses are well recognized as major triggers of acute exacerbations of asthma. It has been suggested that viral-triggered exacerbation is an impaired Th1 response and may represent a relevant mechanism in regard to IFN deficiency [24, 25]. Decreased generation of IFNs has been documented in

peripheral blood mononuclear cells as well as epithelial cells from asthmatics compared to in normal subjects [24, 25]. Additionally, Stephens et al. [26] recently showed that a respiratory viral infection increases recruitment of circulating Th2 cells specific for other allergens into the lungs, suggesting that chemokines such as TARC are generated in the lungs after a viral infection. Although epithelial cells are the major target of respiratory viruses in the airway, they are also able to infect airway fibroblasts and induce chemokine expression [27]. Viral infection might also damage infected epithelial cells, causing leakage of virus-derived dsRNA that stimulates surrounding cells, including fibroblasts and smooth muscle cells. Bardin et al. [28] used in situ hybridization to explore localization of virus in the nasal mucosa after rhinovirus infection, and they detected signals not only in the epithelium but also in the submucosa, where fibroblasts and smooth muscle cells are located. This is further supported by the finding that leakage of rhinovirus-derived RNA into the blood during upper respiratory rhinovirus infections is more prominent in asthmatics than nonasthmatics [29]. Thus, in asthmatics, the resident cell types responsible for TARC production at mucosal sites are thought to be epithelial cells, smooth muscle cells and fibroblasts [4, 30].

It has been reported that TLR3 is expressed on the cell surface in lung fibroblast lines, and can be blocked with

anti-TLR3 Ab [31] although the surface and/or intracellular locations of TLR3 differ among the various cell types. We were unable to suppress TARC release from nasal (polyp and normal), bronchiolar and lung fibroblasts by adding an anti-TLR3-neutralizing Ab at a concentration that blocked the synthesis of IFN in poly(I:C)-, TNF- α - and IL-4-stimulated fibroblasts, as reported by Matsumoto et al. [31]. In contrast, bafilomycin A1, an endosome acidification inhibitor, inhibited TARC release from all the fibroblasts we used. These results suggest that the poly(I:C)-induced TARC release is not mediated via TLR3 on the cell surface but via intracellular TLR3 or other receptors such as retinoic acid-inducible gene I, melanoma differentiation-associated gene 5 or double-stranded RNA-dependent protein kinase [11, 31–33].

In conclusion, we have demonstrated for the first time that bronchiolar and lung fibroblasts are able to produce TARC. Our observations suggest that fibroblasts in the respiratory tract may actively participate in the pathogenesis of initiation, amplification and exacerbation of Th2-cell-mediated allergic conditions.

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