

Differential expression of thymus- and activation-regulated chemokine (CCL17) and macrophage-derived chemokine (CCL22) by human fibroblasts from cornea, skin, and lung

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Background: Allergic diseases of the ocular surface, skin, and lung are triggered by T_H2 cells, which are recruited by thymus- and activation-regulated chemokine (TARC; CCL17) and macrophage-derived chemokine (MDC; CCL22). Resident fibroblasts are thought to contribute to inflammatory cell infiltration through chemokine production.

Objective: We sought to provide insight into the clinical differences apparent among these allergic diseases of the eye, skin, and lung, and we compared the abilities of corneal, dermal, and lung fibroblasts to produce TARC and MDC.

Methods: The amounts of chemokines released into the culture supernatant were determined by means of ELISA, and the intracellular abundance of chemokine mRNAs was quantitated by means of reverse transcription and real-time PCR analysis. **Results:** Neither TNF- α , IFN- γ , IL-4, nor IL-13 alone induced the release of TARC from or affected the amount of TARC mRNA in corneal, dermal, or lung fibroblasts. The combination of TNF- α with either IL-4 or IL-13, however, markedly increased both TARC release and the abundance of TARC mRNA in corneal and dermal fibroblasts, but not in lung fibroblasts. Neither MDC release nor MDC mRNA was detected in any of the 3 types of fibroblasts stimulated with any of the cytokines examined.

Conclusion: These results indicate that cytokine regulation of TARC expression differs among fibroblasts derived from the cornea, skin, or lung. Corneal and dermal fibroblasts might thus be important sources of TARC during allergic inflammation. (J Allergy Clin Immunol 2003;111:520-6.)

Key words: Fibroblast, thymus- and activation-regulated chemokine, macrophage-derived chemokine, IL-4, IL-13, T_H2 cell

Fibroblasts are abundant in connective tissue, where they confer mechanical strength by providing a supporting framework of extracellular matrix. These cells con-

Abbreviations used

CRTH2: Chemoattractant receptor for T_H2 cells
MDC: Macrophage-derived chemokine
MEM: Minimum essential medium
TARC: Thymus- and activation-regulated chemokine

tribute to maintenance of normal tissue structure not only through the synthesis and degradation of extracellular matrix proteins but also by secreting various bioactive substances. Tissue-resident fibroblasts thus function as sentinel or effector cells during allergic inflammation, modulating the activities of immune cells by producing cytokines and chemokines.¹⁻⁴ Although fibroblasts play an important role as a source of biologic mediators in the initiation and amplification of inflammation, the overproduction of these factors by fibroblasts might prevent resolution of this condition and lead to chronic persistent inflammation.⁵ Knowledge of the regulation of chemokine synthesis by fibroblasts is thus important for an understanding of the pathogenesis of inflammation.

Fibroblasts are not a homogeneous population of cells, however. They express different structural and functional features depending on their location within the body and local stimuli.⁶⁻¹¹ In addition, the different phenotypes of fibroblasts from different sites are maintained even after prolonged culture in vitro.^{4,12,13} The clinical manifestations of allergic diseases differ among various organs, including the eye (ocular surface), skin, and lungs, possibly, at least in part, as a result of differences in the resident fibroblasts. For example, unlike skin and lung fibroblasts, which are of mesenchymal origin, corneal fibroblasts are derived from neural ectoderm. However, the specific roles of fibroblasts in different organs during allergic inflammation remain poorly understood.

Thymus- and activation-regulated chemokine (TARC; CCL17) and macrophage-derived chemokine (MDC; CCL22) are members of the CC chemokine family and are potent and selective chemoattractants for T_H2 cells,^{14,15} which express the corresponding chemokine receptor (CCR4) on their surfaces.^{16,17} Allergic inflammation is thought to be a T_H2 cell–dominant immune response, given the pronounced accumulation of T_H2

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cells apparent at sites of such inflammation in individuals with vernal keratoconjunctivitis, atopic dermatitis, or asthma.¹⁸⁻²⁰ The secretion of cytokines, including IL-4, IL-5, IL-10, and IL-13, by the accumulated T_H2 cells results in immunoglobulin class switching in B cells and the release of IgE from B cells, as well as the activation of eosinophils, leading to tissue destruction. The local production of TARC and MDC might therefore play an important role in the induction and maintenance of allergic inflammation^{21,22} as a result of the consequent promotion of T_H2 cell infiltration.

To characterize the differential roles of fibroblasts from different organs in the pathogenesis of allergic diseases, such as vernal keratoconjunctivitis, atopic dermatitis, and asthma, we have now compared the effects of various cytokines on the production of TARC and MDC by human fibroblasts derived from the cornea, skin, and lung.

METHODS

Materials

Minimum essential medium (MEM) and trypsin (0.05%)–EDTA (0.53 mmol/L) were obtained from Gibco-BRL (Grand Island, NY), FBS was from Dai-ichi Pharmaceutical (Osaka, Japan), and cell-culture dishes (Falcon) were from Becton Dickinson (Franklin Lakes, NJ). Human recombinant TNF- α , IFN- γ , IL-4, and IL-13 were obtained from Genzyme (Cambridge, Mass). Paired antibodies to TARC and MDC and human TARC and MDC protein standards for ELISAs were from R&D Systems (Minneapolis, Minn). An RNeasy kit for isolation of total RNA was from Qiagen (Hilden, Germany), and a TaKaRa RNA PCR kit AMV version 2.1 was from Takara Shuzo (Shiga, Japan). LightCycler-DNA Master SYBR Green I was obtained from Roche Molecular Biochemicals (Mannheim, Germany), ethidium bromide and DNA molecular size standards (Marker 11) were from Nippongene (Toyama, Japan), and agarose (NuSieve 3:1) was from FMC Bioproducts (Rockland, Me). All reagents used for cell culture were endotoxin minimized.

Cell culture

Human corneal fibroblasts were isolated, as described previously.^{1,23,24} Normal human dermal fibroblasts were obtained from Cascade Biologics (Portland, Ore) and Clonetics (Walkersville, Md), and normal human lung fibroblasts were from Cascade Biologics and the American Type Culture Collection (Rockville, Md). Cells were seeded in 6-well culture plates and grown until confluent in MEM supplemented with 10% FBS. They were then stimulated with cytokines, as described previously.²³ In brief, the culture medium was replaced with serum-free MEM, and the cells were incubated for 24 hours, after which the medium was changed to serum-free MEM supplemented with cytokines. After incubation for the indicated times, the medium was collected from each culture dish and centrifuged at 120g for 5 minutes. The resulting supernatant was stored at -80°C for subsequent assay of TARC or MDC. The fibroblasts remaining in the dish were exposed to trypsin-EDTA, and their number was determined with a hemocytometer.

Determination of TARC and MDC concentration by means of ELISA

The concentration of TARC or MDC in culture supernatants was determined in quadruplicate with solid-phase ELISAs. The limits of detection for TARC and MDC were 3.9 and 15.6 pg/mL, respec-

tively, and data are presented as picograms or nanograms of chemokine per 1×10^6 cells.

Quantitative RT-PCR analysis of TARC and MDC mRNAs

The abundance of TARC, MDC, eotaxin (CCL11), and IL-8 (CXCL8) mRNAs was determined by means of reverse transcription and quantitative real-time PCR analysis, as described previously.²³ In brief, total RNA was extracted from fibroblasts and subjected to reverse transcription, and the resulting cDNA was then subjected to real-time PCR with a LightCycler instrument (Roche Molecular Biochemicals). Transcripts of the constitutively expressed gene for glyceraldehyde-3-phosphate dehydrogenase served to normalize the amounts of TARC, MDC, eotaxin, and IL-8 mRNAs in each sample. The sequences of the PCR primers for TARC, MDC, and eotaxin were as described previously.^{1,23,25} PCR primers for IL-8 were obtained from R&D systems. Real-time PCR data were analyzed with LightCycler software 3.01 (Roche Molecular Biochemicals). To verify the specificity of amplification, we also subjected PCR products to electrophoresis on a 3% agarose gel, which was then stained with ethidium bromide (1 μ g/mL) and examined with a Nighthawk system (pdi, Huntington Station, NY). The PCR primers yielded amplification products of the expected sizes of 198, 115, 322, 158, and 79 bp for TARC, MDC, eotaxin, IL-8, and glyceraldehyde-3-phosphate dehydrogenase mRNAs, respectively.

Statistical analysis

Data are presented as means $\pm$ SEM. Results were compared between 2 groups with the unpaired Student *t* test and among 3 or more groups with the Dunnett test. Statistical analyses were performed with StatView for Windows software (version 5.0; SAS Institute, Cary, NC). A *P* value of less than .05 was considered statistically significant.

RESULTS

Effects of cytokines on TARC release by human corneal, dermal, and lung fibroblasts

We first examined whether cultured corneal, dermal, or lung fibroblasts produce TARC in the absence or presence of stimulation with various cytokines by using cells obtained from 3 different donors. Fibroblasts derived from neither cornea, skin, nor lung tissue released detectable amounts of TARC when incubated for 24 hours in the absence or presence of TNF- α , IFN- γ , IL-4, or IL-13, each at a concentration of 10 ng/mL (Fig 1). However, stimulation with the combination of TNF- α and either IL-4 or IL-13 (but not IFN- γ) resulted in a synergistic increase in the amount of TARC in the culture supernatants of corneal and dermal fibroblasts. TARC production by lung fibroblasts remained unaffected by these cytokine combinations. Given that the responses of the cells from the 3 different donors were almost identical, we performed subsequent experiments with fibroblasts from one donor.

We next examined the dose dependence of the effects of TNF- α and either IL-4 or IL-13 on the release of TARC by corneal and dermal fibroblasts. The cells were cultured for 24 hours with various concentrations of TNF- α in the absence or presence of IL-4 (10 ng/mL) or IL-13 (10 ng/mL, Fig 2) or with various concentrations of IL-4 or IL-13 in the absence or presence of TNF- α (10

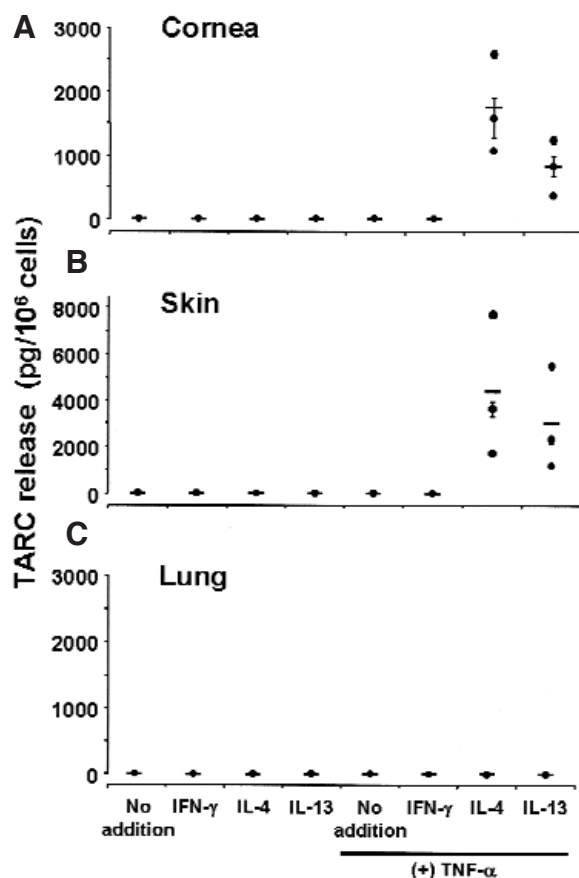


FIG 1. Effects of cytokines on TARC release by human fibroblasts derived from different donors. Corneal (A), dermal (B), or lung (C) fibroblasts derived from 3 different donors were incubated for 24 hours in the absence or presence of the indicated cytokines (10 ng/mL), after which the concentration of TARC in the culture supernatant was measured by means of ELISA. Circles represent the mean $\pm$ SEM values obtained from 4 separate experiments with cells from a single donor; horizontal bars represent the overall mean values for cells from all 3 donors.

ng/mL, Fig 3). In the absence of IL-4 or IL-13, neither corneal nor dermal fibroblasts released detectable amounts of TARC at TNF- α concentrations of up to 100 ng/mL. However, in the presence of IL-4 or IL-13, TNF- α induced a dose-dependent increase in TARC release that was statistically significant at concentrations of 0.1 ng/mL in corneal fibroblasts and 1.0 ng/mL in dermal fibroblasts (Fig 2). TARC release was more pronounced in corneal fibroblasts than in dermal fibroblasts in the presence of lower concentrations (≤ 1.0 ng/mL) of TNF- α and either IL-4 or IL-13 (10 ng/mL); at higher concentrations of TNF- α (>1.0 ng/mL), the 2 types of cells released similar amounts of TARC. In the absence of TNF- α , neither IL-4 nor IL-13 induced detectable TARC release by fibroblasts. However, in the presence of TNF- α , IL-4 and IL-13 each induced a dose-dependent increase in TARC release that was statistically significant at concentrations of 0.1 ng/mL for IL-4 and 10 ng/mL for IL-13 in both corneal and dermal fibroblasts (Fig 3). The dose-response relationships for IL-4 and IL-13 were thus

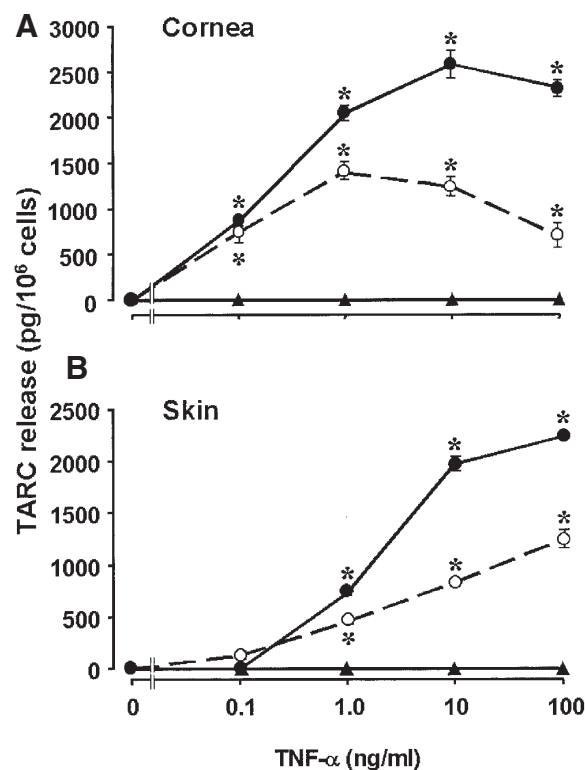


FIG 2. Dose dependence of the effects of TNF- α in the presence of IL-4 or IL-13 on TARC release by corneal (A) or dermal (B) fibroblasts. Cells were cultured for 24 hours with the indicated doses of TNF- α in the presence of IL-4 (filled circles), IL-13 (open circles), or IFN- γ (triangles), each at a concentration of 10 ng/mL. Culture supernatants were then assayed for TARC by means of ELISA. Data are means $\pm$ SEM of values from 4 separate experiments. * P < .01 (Dunnett test) versus the corresponding value for cells incubated in the absence of cytokines.

similar in corneal and dermal fibroblasts, with IL-4 being more potent in its effect on TARC release than IL-13. Again, IFN- γ , either at a concentration of 10 ng/mL in the presence of various concentrations of TNF- α (Fig 2) or at concentrations of up to 100 ng/mL in the presence of TNF- α at 10 ng/mL (Fig 3), had no effect on TARC release from corneal or dermal fibroblasts.

We examined the time course of TARC release by corneal and dermal fibroblasts incubated with the combination of TNF- α (10 ng/mL) and either IL-4 (10 ng/mL) or IL-13 (10 ng/mL). After a delay of approximately 12 hours, the release of TARC into the culture medium increased for up to 72 hours in both types of fibroblasts (Fig 4). The amounts of TARC released by these 2 types of cells were similar up to 24 hours, the amount of TARC released by corneal fibroblasts was greater than that released by dermal fibroblasts at 48 hours, and the amounts of released TARC were again similar for the 2 cell types at 72 hours. Lung fibroblasts incubated with the same combinations of TNF- α and either IL-4 or IL-13 for 72 hours again did not release detectable amounts of TARC (data not shown).

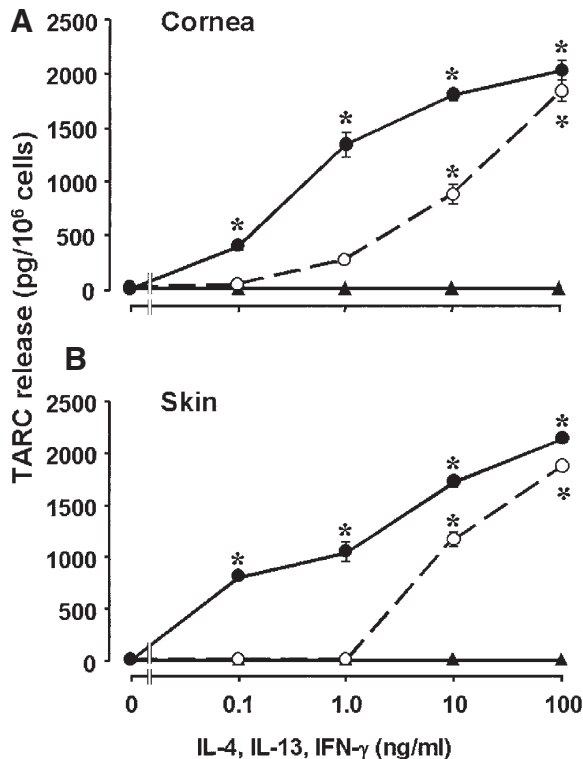


FIG 3. Dose dependence of the effects of IL-4 or IL-13 in the presence of TNF- α on TARC release by corneal (A) or dermal (B) fibroblasts. Cells were cultured for 24 hours with the indicated doses of IL-4 (filled circles), IL-13 (open circles), or IFN- γ (triangles) in the presence of TNF- α (10 ng/mL). Culture supernatants were then assayed for TARC by means of ELISA. Data are means $\pm$ SEM of values from 4 separate experiments. * P < .01 (Dunnett test) versus the corresponding value for cells incubated in the absence of cytokines.

Effects of cytokines on the abundance of TARC mRNA in corneal, dermal, and lung fibroblasts

The effects of cytokines on the abundance of TARC mRNA in corneal, dermal, and lung fibroblasts were determined by means of reverse transcription and quantitative real-time PCR analysis. Exposure of any of the 3 types of fibroblasts for 24 hours to TNF- α , IFN- γ , IL-4, or IL-13 alone, each at a concentration of 10 ng/mL, had no effect on the amount of TARC mRNA (Fig 5). In corneal and dermal fibroblasts, stimulation with the combination of TNF- α and either IL-4 or IL-13 induced a marked increase in the abundance of TARC mRNA compared with that apparent in unstimulated cells (42- and 12-fold increases in corneal fibroblasts and 53- and 19-fold increases in dermal fibroblasts, respectively). TARC mRNA was not detected in lung fibroblasts incubated in the absence or presence of these cytokine combinations, which is consistent with the results obtained for TARC release.

Effects of cytokines on MDC synthesis by human corneal, dermal, and lung fibroblasts

Fibroblasts derived from the cornea, skin, or lung did

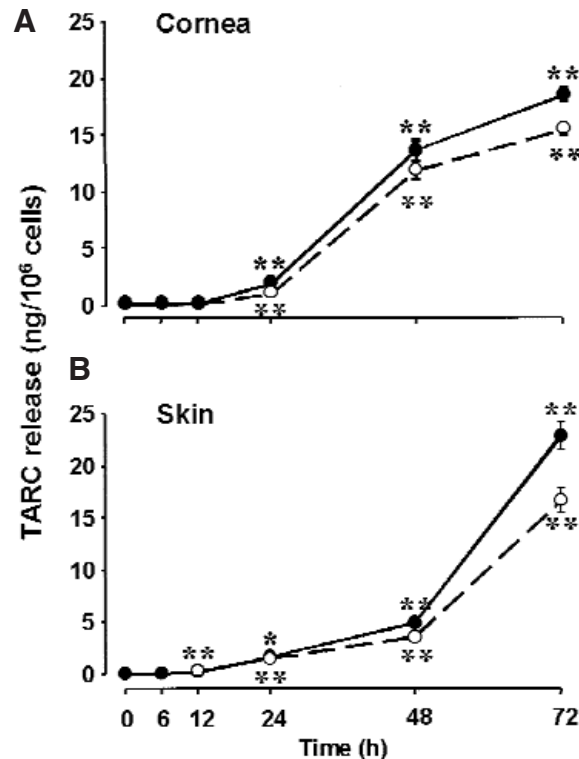


FIG 4. Time course of TARC release from corneal (A) or dermal (B) fibroblasts stimulated with the combination of TNF- α and either IL-4 or IL-13. Cells were cultured for the indicated times in the presence of TNF- α (10 ng/mL) together with either IL-4 (filled circles) or IL-13 (open circles), each at a concentration of 10 ng/mL. The culture supernatants were then assayed for TARC by means of ELISA. Data are means $\pm$ SEM of values from 4 separate experiments. * P < .05 and ** P < .001 (Dunnett test) versus the corresponding time point for cells incubated in the absence of cytokines.

not release detectable amounts of MDC into the culture supernatant during incubation for 24 hours in the absence or presence of TNF- α , IFN- γ , IL-4, or IL-13 alone, each at a concentration of 10 ng/mL. In contrast to TARC release, MDC release from any of the 3 types of fibroblasts was also not induced by means of incubation with the combination of TNF- α and either IL-4, IL-13, or IFN- γ . Corneal, dermal, or lung fibroblasts incubated for 24 hours in the absence or presence of TNF- α , IFN- γ , IL-4, or IL-13 alone, each at a concentration of 10 ng/mL, did not contain detectable amounts of MDC mRNA. The combination of TNF- α and either IL-4, IL-13, or IFN- γ also did not increase the abundance of MDC mRNA in these cells (data not shown).

Response of lung fibroblasts to cytokines

Finally, given the lack of response of human lung fibroblasts in the preceding experiments, we confirmed the sensitivity of these cells to the cytokines studied by determining the effects of the latter on the abundance of eotaxin mRNA. Consistent with previous observations,³ the abundance of eotaxin mRNA in lung fibroblasts was increased in response to stimulation with TNF- α , IL-4, or IL-13, each at a concentration of 10 ng/mL (13.3-, 9.0-,

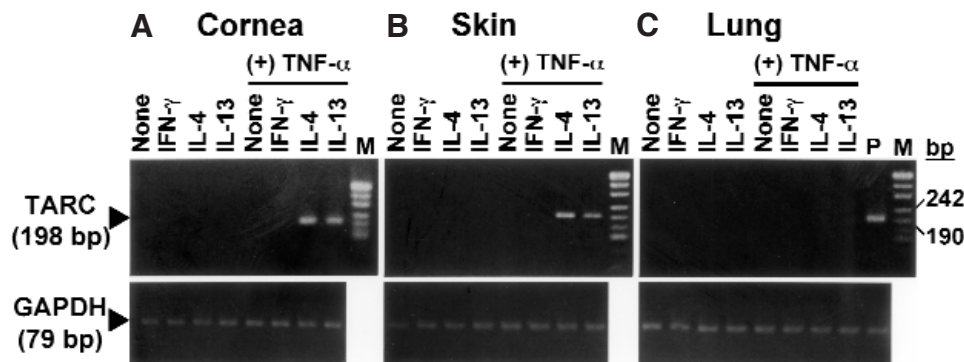


FIG 5. Effects of cytokines on the abundance of TARC mRNA in human fibroblasts. Fibroblasts derived from the cornea (A), skin (B), or lung (C) were cultured for 24 hours in the absence or presence of the indicated cytokines, each at a concentration of 10 ng/mL. Total RNA was then isolated, and the amount of TARC mRNA was determined by means of reverse transcription and real-time quantitative PCR analysis. The positions and sizes of amplification products derived from TARC and glyceraldehyde-3-phosphate dehydrogenase cDNAs are indicated. Data are from a representative experiment that was repeated 3 times with similar results. Lanes M, DNA molecular size standards; lane P, PCR analysis of human thymus cDNA as a positive control.

and 10.5-fold increases, respectively). Furthermore, exposure of the cells to the combination of TNF- α and either IL-4 or IL-13 resulted in a synergistic increase in the amount of eotaxin mRNA (44- and 51-fold increases, respectively). IFN- γ , which has previously been shown to inhibit eotaxin synthesis in fibroblasts,²⁶ completely inhibited (at a concentration of 10 ng/mL) the stimulatory effect of TNF- α on the abundance of eotaxin mRNA in human lung fibroblasts. We also confirmed that the amount of IL-8 mRNA in lung fibroblasts was increased (155-fold) by TNF- α (10 ng/mL), as described previously.²⁷ Lung fibroblasts thus responded to all of the cytokines used in this study.

DISCUSSION

Our results demonstrate that TARC expression is regulated differentially by cytokines in fibroblasts derived from human cornea, skin, and lung. We have previously shown that TNF- α and IL-4 synergistically increase TARC expression in human corneal fibroblasts.²³ We have now shown that corneal and dermal fibroblasts represent a potentially important source of TARC and that stimulation of these cells with the combination of TNF- α and either of the T_H2 cytokines IL-4 or IL-13 induces marked increases in both the release of TARC protein and the abundance of TARC mRNA. The cytokine-induced increases in TARC release by these fibroblasts are dependent both on the concentration of TNF- α , IL-4, and IL-13, as well as on the duration of incubation. In contrast, lung fibroblasts did not release detectable amounts of TARC in response to any of the cytokines examined. Neither the release of MDC protein nor the presence of MDC mRNA was detected in corneal, dermal, or lung fibroblasts. The differential responses of corneal and dermal fibroblasts on the one hand and lung fibroblasts on the other in terms of cytokine-induced TARC expression suggest that these cells play different

roles in the initiation of corresponding T_H2 cell-mediated allergic conditions (ie, vernal keratoconjunctivitis, atopic dermatitis, and asthma, respectively).

The release of various mediators contributes to the migration of inflammatory cells. Fibroblasts and other structural cells participate in this process by releasing a variety of chemokines.^{4,5,28} The release of eotaxin, a potent and selective chemoattractant for eosinophils, by fibroblasts is thus thought to play an important role in allergic inflammation in various organs.¹⁻⁴ In contrast to TARC expression, however, the production of eotaxin appears to be similarly regulated by cytokines in fibroblasts from the cornea, skin, and lung. Eotaxin expression is thus stimulated by TNF- α , IL-4, or IL-13, with the combination of TNF- α and either IL-4 or IL-13 having a synergistic effect, in all 3 types of fibroblasts.¹⁻³ In addition, IFN- γ inhibits the release of eotaxin induced by TNF- α in these cells.²⁶

Studies of TARC expression and the effects of neutralizing antibodies to TARC *in vivo* have revealed an important role for this chemokine in T_H2 cell-mediated inflammation in various mouse models.^{21,29,30} In addition to immune cells, such as CD4⁺ T cells and dendritic cells,³¹ tissue-resident epithelial cells in nasal mucosa, lung, and skin produce TARC in response to cytokines.³²⁻³⁴ We have also investigated whether corneal epithelial cells produce TARC; we were unable to detect either TARC release or intracellular TARC mRNA in simian virus 40-transformed human corneal epithelial cells incubated in the absence or presence of various cytokines (unpublished data). These observations thus reinforce the notion that the resident cell types responsible for TARC production during inflammation (only fibroblasts in the cornea, both epithelial cells and fibroblasts in the skin, and only epithelial cells in the nasal mucosa and lung) differ among organs and tissues. The cornea is an avascular tissue that lacks hematopoietic cells and immune cells under normal conditions, being composed only of

epithelial cells, fibroblasts, and endothelial cells. TARC produced by corneal fibroblasts thus might be responsible for T_H2 cell infiltration into the cornea.

The chemokine MDC also plays an important role in T_H2 cell–dominant inflammation, given that neutralization of MDC results in attenuation of pulmonary allergic inflammation in mice.²² In the present study MDC was not produced by either corneal, dermal, or lung fibroblasts in response to any of the cytokines examined. Similarly, MDC expression at either the protein or mRNA level was not detected in simian virus 40–transformed human corneal epithelial cells incubated in the absence or presence of cytokines (unpublished data) or in bronchial epithelial cells.³³ In vitro studies have suggested that MDC expression is restricted to immune cells, such as dendritic cells, B lymphocytes, monocytes, macrophages, natural killer cells, and CD4⁺ T lymphocytes.²² In addition, MDC has been shown to be expressed in vivo in dendritic cells and T lymphocytes both in biopsy specimens of human atopic dermatitis lesions and in a mouse model of this condition.^{21,35} Together, these observations indicate that the source of MDC during inflammation is likely infiltrated inflammatory cells rather than tissue-resident cells, such as fibroblasts and epithelial cells.

In addition to CCR4, CCR8 and the chemoattractant receptor for T_H2 cells (CRTH2) are also expressed on the surface of human T_H2 cells, and corresponding ligands, such as I-309 (CCL1) and PGD₂, induce chemotaxis of these cells.^{36–39} The interactions of CCR8 with I-309 and of CRTH2 with PGD₂ might thus also play important roles in the recruitment of T_H2 cells during allergic inflammation. The synthesis of I-309 in human fibroblasts in response to allergy-associated cytokines has not been described, however. Although human embryonic lung fibroblasts produce PGD₂,⁴⁰ the production of this agent by adult human lung fibroblasts in response to cytokines has not been demonstrated. Nevertheless, it remains possible that lung fibroblasts contribute to T_H2 cell infiltration during inflammation by producing agonists for CCR8 or CRTH2 rather than by synthesizing CCR4 agonists.

In summary, we have shown that human corneal and dermal fibroblasts, but not lung fibroblasts, release large amounts of TARC in response to cytokines, suggesting that fibroblasts resident in different organs play different roles in allergic inflammation. Given that TARC is a potent chemoattractant for T_H2 cells, fibroblasts in the cornea and skin likely contribute to the initiation and persistence of T_H2 cell–dominant allergic inflammation at these sites.

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