TGF-β and IL-13 Synergistically Increase Eotaxin-1 Production in Human Airway Fibroblasts

Sally E. Wenzel, John B. Trudeau, Steve Barnes, XiuXia Zhou, Meghan Cundall, Jay Y. Westcott, Kelly McCord and Hong Wei Chu

*J Immunol* 2002; 169:4613-4619; doi: 10.4049/jimmunol.169.8.4613

http://www.jimmunol.org/content/169/8/4613

Why *The JI*?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References

This article cites 23 articles, 4 of which you can access for free at:
http://www.jimmunol.org/content/169/8/4613.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
TGF-β and IL-13 Synergistically Increase Eotaxin-1 Production in Human Airway Fibroblasts

Sally E. Wenzel, John B. Trudeau, Steve Barnes, Xiuxia Zhou, Meghan Cundall, Jay Y. Westcott, Kelly Mc Cord, and Hong Wei Chu

Chronic diseases may involve an “innate” response followed by an adaptive immune response, of a Th1 or Th2 variety. Little is known regarding the interactions of these responses. We hypothesized that TGF-β1 (innate response factor associated with wound repair) in combination with IL-13 (Th2 factor) might augment inflammatory processes associated with asthma. Airway fibroblasts were cultured from asthmatic subjects and normal controls. These fibroblasts were exposed to TGF-β1 and IL-13 alone or in combination, and eotaxin-1 expression and production were evaluated. At 48 h, eotaxin-1 production was markedly increased with the combination of TGF-β1 and IL-13 (p < 0.0001) compared with either stimulus alone. mRNA increased slightly at 1 h with IL-13 or TGF-β1 plus IL-13, peaked, and became significantly increased over IL-13 alone at 24 h. Protein was measurable from 6 h with IL-13 and TGF-β1 plus IL-13, but greater levels were measured over time with the combination. Actinomycin ablated the increase in mRNA and protein seen with IL-13 alone and with TGF-β1 plus IL-13. Cycloheximide blocked the increase in mRNA at 6 h in both conditions, but also blocked the increase at 24 h with TGF-β1 plus IL-13. STAT-6 was rapidly activated with both IL-13 and the combination, without difference. Finally, eotaxin-1-positive fibroblasts were identified in severe asthma biopsies in greater numbers than in normals. These results support the concept that interactions of innate and adaptive immune systems may be important in promoting the tissue eosinophilia of asthma, particularly in those with more severe disease. The Journal of Immunology, 2002, 169: 4613–4619.

The Th2 immune system, involving cytokines such as IL-4 and IL-13, is believed to be important to asthma pathogenesis (1, 2). More recently, asthma has also been described as a chronic injury and repair process, involving activation of the nonspecific (innate) immune system. Although a variety of factors contribute to innate responses, considerable evidence exists to suggest a role for TGF-β1 or TGF-β2 in asthma and the eosinophilia associated with it (3, 4). Whether these two systems interact in any way in asthma, or in other diseases, is not clear.

Although tissue eosinophilia is a hallmark of asthmatic inflammation, the precise mechanisms behind the eosinophilia remain unclear. Th2 cytokines such as IL-4 and/or IL-13 have been associated with eosinophilia in murine and human models (2, 5). However, because these cytokines do not directly attract eosinophils, indirect stimulation of a second eosinophil chemoattractant may be required (6). In a manner similar to Th2 cytokines, the innate factors TGF-β1 and TGF-β2 have been associated with airway eosinophils, especially in subjects with more severe disease (3, 4, 7). Like IL-4 and IL-13, TGF-β is not known to have direct chemotactic effects on eosinophils, and the mechanism behind the associated eosinophil increases remains unclear.

Eotaxin-1, a C-C chemokine with potent direct chemotactic effects on eosinophils, is known to be regulated by Th2 factors such as IL-4 and IL-13, likely involving a STAT-6 pathway (8). Eotaxin-1 can be produced by resident cells including fibroblasts and smooth muscle cells, and this resident cell production has been suggested to contribute directly to tissue eosinophilia (9, 10). Interestingly, eotaxin-1 production from fibroblasts, smooth muscle cells, and epithelial cells has been shown to synergistically increase after stimulation with IL-4/IL-13 and other innate response factors, namely TNF-α and IL-1 (11–13).

Given the previously reported increases in TGF-β1/2 and IL-13 in asthma and the association of each with eosinophils, we hypothesized that these innate and adaptive immune factors could synergistically activate resident cells such as fibroblasts. To explore this relationship, primary fibroblasts were obtained from asthmatic and normal subjects and exposed to TGF-β1 in combination with IL-13. Eotaxin-1 protein and mRNA were measured and activation of STAT-6 was determined. For comparison, IL-8 protein was also measured. Finally, human airway tissue was evaluated for the presence of eotaxin-1+ fibroblasts.

Materials and Methods

Fibroblast culture

Endobronchial biopsies were obtained from asthmatics ranging in severity from mild to severe, as well as from normal control subjects as previously described (4). Briefly, severe asthmatics were all on high-dose inhaled or oral glucocorticoids and had continual symptoms and airflow obstruction. Mild-moderate asthmatics were stable asthmatics on no or low-moderate doses of inhaled glucocorticoids, but with chronic airflow limitation. Normal controls had no evidence of any respiratory disease. All subjects were nonsmokers and none had a history of smoking >5 pack years (4). The study was approved by the National Jewish Institutional Review Board and all subjects gave informed consent. Biopsies were obtained from third to fifth order subcarinae, placed in chilled medium (DMEM (Life Technologies, Rockville, MD) with 10% FBS (Gemini, Woodland, CA) and penicillin/streptomycin/gentamicin) and minced into small pieces using a scalpel. The pieces were placed into plastic 60-mm dishes (BD Biosciences, Franklin Lakes, NJ) and incubated at 37°C/5% CO2 for 5–7 days until fibroblasts advanced out from the tissue. The tissue was then removed.
Fibroblasts were passed during the proliferative state and were all studied at the third passage. In disease group comparison and dose response experiments, where the primary outcome was eotaxin-1 protein, the fibroblasts were plated in 24-well dishes and serum starved. After 24 h, FBS (0.5%) and the stimuli were then added in the described concentrations. Time course studies required adequate numbers of fibroblasts at the early time periods and were done on three- to fourth-passage fibroblasts grown to near confluency (80–90%) in 60-mm dishes (with 10% FBS) before being serum starved for 24 h. Medium was replaced and replaced with DMEM/0.5% FBS and the appropriate stimuli. Supernatants and cells were harvested at the described time points and stored for analysis of eotaxin-1 protein and mRNA, STAT-6, and phosphorylated STAT (pSTAT)-3 protein.

**Enzyme immunoassay for eotaxin-1 and IL-8**

Sandwich ELISAs were used to quantify these chemokines. Eotaxin-1 Abs and standard were purchased from R&D Systems (Minneapolis, MN), with Ab MAB320 used to coat plates and biotinylated BAF320 used as a detection Ab. A matched Ab pair (MAB208 and BAF208) was used to measure IL-8 (R&D Systems). Avidin-HRP was purchased from BD Pharmingen (San Diego, CA) and tetramethylbenzidine substrate was from KPL Laboratories (Gaithersburg, MD). Assay sensitivity ranged from 10 to 20 pg/ml for all assays and cell culture supernatants were assayed without any purification or concentration.

**Northern blot for eotaxin-1**

Total RNA was extracted from fibroblasts by TRIzol reagent (Life Technologies). RNA samples (18 μg) were separated on a 1% agarose/formaldehyde gel and transferred to a Hybond-N membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in 10× SSC overnight at room temperature. The membrane was fixed using an UV autocrosslinker and then prehybridized at 42°C for 6–8 h. The blot was hybridized with a 32P-labeled cDNA probe for human eotaxin-1 (corresponding to bases 274–773; GenBank accession no. D49372) at 42°C overnight, followed by washing with 0.5% SDS in 2× SSC twice (10 min each wash) at room temperature, and then 0.1% SDS in 0.1× SSC at 65°C for 30 min. The membrane was then exposed to Hyperfilm MP (Amersham Pharmacia Biotech) for 24 h. After removing the eotaxin-1 probe, the membrane was rehybridized with a 32P-labeled ubiquitin cDNA probe (Clontech Laboratories, Palo Alto, CA).

**Real-time PCR for measurement of eotaxin-1 gene expression**

Eotaxin-1 mRNA expression in fibroblasts was determined by reverse transcription (RT), followed by real-time quantitative PCR. Total RNA of fibroblasts was extracted using TRIzol reagent (Life Technologies). RT was performed using 1 μg of total RNA and random hexamers in a 50-μl reaction according to the manufacturer’s protocol (PE Applied Biosystems, Foster City, CA). The eotaxin-1 primers and the probe, labeled with a 5′-reporter dye FAM and a 3′-reporter dye TAMRA, were designed using Primer Express software (PE Applied Biosystems). The following are sequences for eotaxin-1 primers and probe: forward primer, 5′-AGGAGATCCAGCAGTGTCAAAT-3′; reverse primer, 5′-GCACAGATCTTCTTTGGCCAGTT-3′; probe, 5′-TCCCCAGGAAGCTGTTACCTTCAAGACC-3′. Real-time PCR was performed on the ABI Prism 7700 sequence detection system (PE Applied Biosystems). The 50-μl PCR contained 30 ng cDNA, 100 nM fluorgenic probe, and 50 nM primers and other components from the TaqMan RT-PCR kit. GAPDH was evaluated using the same PCR protocol as eotaxin-1. The specificity of PCR for both eotaxin-1 and GAPDH was verified by no signal in no-template controls or RT (−) fibroblast samples. The threshold cycle (CT) was recorded for each sample to reflect the mRNA expression levels. A validation experiment proved the linear dependence of the CT value for both eotaxin-1 and GAPDH on the template RNA concentrations and the consistency of ΔCT (eotaxin-1 average CT − GAPDH average CT) in a given sample at different RNA concentrations. Therefore, ΔCT was used to reflect the relative eotaxin-1 mRNA expression levels. To determine the effects of different stimuli on eotaxin-1 gene expression as compared with nonstimulated cells, ΔΔCT was calculated (ΔΔCT = ΔCT stimulus − ΔCT nonstimulated cells). Eotaxin-1 mRNA was indexed to the GAPDH using the formula 1/(2^ΔΔCT) × 100%. 2^ΔΔCT was calculated to demonstrate the fold change of eotaxin-1 gene expression in stimulated cells as compared with the nonstimulated cells.

Abbreviations used in this paper: pSTAT, phosphorylated STAT; CHX, cycloheximide; RT, reverse transcription; CT, threshold cycle.

**Western blot STAT-6 and pSTAT-6**

Fibroblasts (1 × 10^5) were lysed in buffer (0.1% SDS, 1.0% Nonidet P-40 in 50 mM Tris-HCl and 150 mM NaCl, and 10 mM EDTA with 1% protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO)). Samples were bath-sonicated on ice and boiled, and the whole cell lysate protein was electrophoresed on a 7.5% acrylamide-bis-SDS gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham, Arlington Heights, IL). The blots were blocked with 5% nonfat milk/Tween (1/10,000), followed by overnight 4°C exposure in pSTAT-6 polyclonal Ab (1/1000 in 5% BSA in TBS/Tween (1/1000)) (Tyro6®; Cell Signaling Technology, St. Louis, MO). After extensive washing, the HRP-conjugated secondary Ab (Fab′2) was added (1/10,000), and the blot was washed again, developed using the ECL Western blot detection reagents, and immediately exposed to Hyperfilm (Amersham). The membrane was then stripped, checkered for complete Ab removal, then rebloked and reprobed with STAT-6 polyclonal Ab (1/750; Santa Cruz Biotechnology, Santa Cruz, CA) followed by the same washings and development. Specific bands were scanned and semiquantified by densitometry (Scion Image Analysis software; National Institutes of Health, Bethesda, MD). Quantification was performed by evaluating the ratio of the density of the STAT-6 bands to that of the pSTAT-6 bands for all conditions.

**Tissue double immunofluorescent staining for eotaxin-1/fibroblasts**

To confirm the eotaxin-1 expression by fibroblasts, double immunofluorescent staining was performed in endobronchial biopsy tissue sections from seven severe asthmatics and six normal controls. The acetone-fixed and glycol methacrylate-embedded 2-μm tissue sections were incubated with a rabbit polyclonal Ab against human eotaxin-1 (BioSource International, Camarillo, CA) and a mouse mAb against human fibroblast Ag (Ab1; 60-biochem, San Diego, CA). These sections were then rinsed and incubated with FITC-conjugated anti-rabbit and rhodamine-conjugated anti-mouse Abs (DAKO, Carpinteria, CA). The nuclei were counterstained with DAPI. The slides were mounted and observed with an Olympus BX51 fluorescence microscope (Olympus, Melville, NY). The tissue triple-colored fluorescent images were processed and overlaid using the Adobe Photoshop 6.0 software (Adobe Systems, Mountain View, CA). Negative control slides were similarly processed but with the primary Abs omitted or replaced with nonimmune serum. No staining was seen in the negative control slides.

**Statistics**

Data were checked for normality of distribution. Data that were normally distributed were represented as means ± SEM. Log-transformation was done on variables that were heavily right skewed, producing variables that were roughly normally distributed. Graphic representation of log-transformed data was done by recovering the mean − SEM, the mean, and the mean + SEM back to the original scale. This produces SEs that are not equal, with the larger SE being that described in the positive direction. Differences between conditions and subject groups were analyzed by ANOVA on both normal and log-transformed data, because the log-transformation produced data that were roughly normally distributed when there were significant differences between the conditions, specific inter-condition differences were assessed by Tukey-Kramer testing. A p value of ≤0.05 was considered statistically significant.

**Results**

**Eotaxin-1 protein levels from cultured fibroblasts of asthmatic and normal subjects**

Eotaxin-1 protein was measured in the supernatants of primary fibroblasts cultured for 48 h in the presence of TGF-β1 (0.5 ng/ml) and IL-13 (30 ng/ml), alone and in combination. Thirty separate primary cultures were performed, all on third-passage fibroblasts. The 30 primary cultures were obtained from 12 normal subjects, 6 mild-moderate asthmatics, and 12 severe asthmatics. The combination of TGF-β1 and IL-13 produced a synergistic and significant increase in eotaxin-1 protein from cultures in all groups as compared with unstimulated TGF-β1 alone or IL-13 alone (overall p < 0.0001; combination significantly greater than any other condition) (Fig. 1). When the three subject groups were analyzed separately, there was no difference in the eotaxin protein response to TGF-β1 plus IL-13 among the groups (normal =
TGF- 

eotaxin-1 produced. IL-4 (10 ng/ml) was used in combination with 

variability from subject to subject in the absolute amount of 

pattern was consistent among the groups, there was considerable 

and IL-13 (30 ng/ml). The combination of TGF- 

synergistically increased eotaxin-1 protein levels in the supernatants (p < 0.0001). Data are ex- 

pressed as means ± SEM.

FIGURE 1. Primary human airway fibroblasts (n = 30 different cultures from the three subject groups) cultured for 48 h with medium, TGF-β1 (0.5 

ng/ml), IL-13 (30 ng/ml), and both TGF-β1 (0.5 ng/ml) and IL-13 (30 ng/ml). The combination of TGF-β1 and IL-13 synergistically increased 

eotaxin-1 protein levels in the supernatants. Overall p < 0.0001. *p < 0.05 compared to all other conditions.

2253 ± 559 pg/ml, mild-moderate = 4457 ± 1262 pg/ml, se- 

vere = 3411 ± 987 pg/ml, p = 0.30). Although the response 

pattern was consistent among the groups, there was considerable 

variability from subject to subject in the absolute amount of 

eotaxin-1 produced. IL-4 (10 ng/ml) was used in combination with 

TGF-β1 (0.5 ng/ml) in three cultures with similar results (data not 

shown) (12). Likewise, TGF-β2 (at similar doses to TGF-β1) 
could also be substituted for TGF-β1 with nearly identical results 

(data not shown). Because the results were similar and the initial 

studies had been done with IL-13 and TGF-β1 (data not 

shown), TGF-β1 was used here. The results of the combination of 

TGF-β1 and IL-13 were similar to those seen for the single cytokines. Moreover, the combination 

synergistically increased the levels of 

Eotaxin-1 mRNA.

FIGURE 2. Primary human airway fibroblasts (n = 25 different cultures from the three subject groups) cultured for 48 h with medium, TGF-β1 (0.5 

ng/ml), IL-13 (30 ng/ml), and both TGF-β1 (0.5 ng/ml) and IL-13 (30 ng/ml). IL-8 protein levels increased at significant but low levels in the 

TGF-β-stimulated wells and in the presence of the combination (overall 

p < 0.001). Data are expressed as mean ± SEM.

Northern blot and real-time PCR for eotaxin-1 mRNA

Northern analysis was performed on four fibroblast cultures (two severe asthmatics and two normal controls). In each case, mRNA for 
eotaxin-1 was only marginally detected at 48 h in unstimulated 

wells, or with TGF-β1 (0.5 ng/ml) or IL-13 (30 ng/ml) alone. In a 

similar manner as to what was seen for protein, there was a large 
increase in eotaxin-1 mRNA after the addition of both, without 
difference between normal controls and asthmatics (Fig. 3). Real- 
time PCR was then used to quantify eotaxin-1 mRNA from 10 
normal controls, 5 mild-moderate, and 10 severe asthmatic pri- 
mary fibroblast cultures at 48 h. Eotaxin-1 mRNA was present at 

low levels in most fibroblasts at baseline. There was little effect of 

TGF-β1 (0.5 ng/ml). IL-13 (30 ng/ml) generated a small increase 
in eotaxin-1 mRNA levels, while the combination significantly and 
synergistically increased the levels of fibroblast eotaxin-1 mRNA 

(p < 0.0001) (Fig. 4). Similar to protein levels, there were no 
differences in response among the asthmatic or normal subject 

groups.

Dose response for TGF-β1 and IL-13

Dose-response curves evaluating eotaxin-1 protein were constructed in six separate primary third-passage cultures. The doses 

used ranged from 0.05 to 50 ng/ml TGF-β1 and from 0.3 to 30 ng/ml IL-13. The dose-response range chosen was larger for 

TGF-β1 because of the possibility of a bimodal effect for TGF-β1. 

As shown in Fig. 5, the minimum TGF-β1 dose required was 0.5 

ng/ml, while the minimum IL-13 dose required was 3 ng/ml. Be-

tween 0.05 and 0.5 ng/ml TGF-β1 and between 0.3 and 3 ng/ml IL-13 there was a large increase in the total response. The effect 
appeared to plateau at 5 ng/ml TGF-β1 and 30 ng/ml IL-13. 

TGF-β1 at 50 ng/ml with IL-13 at 30 ng/ml produced less 
eotaxin-1 than the 5 ng/ml dose in two of the three subjects studied 
at that dose (data not shown). The maximum response appears to 

occur with the combination of 5 ng/ml TGF-β1 and 30 ng/ml IL-

13. However, due to the small, nonsignificant differences between 

0.5 and 5 ng/ml TGF-β1 and the number of studies done with the 

lower dose, 0.5 ng/ml TGF-β1 and 30 ng/ml IL-13 were chosen for 

the time course studies and for analysis of the STAT-6 pathway.

Time course

Responses to medium alone, TGF-β1 (0.5 ng/ml), IL-13 (30 ng/ 

ml), or the combination were evaluated at 0 min, 15 min, 1 h, 6 h, 

24 h, and 48 h. Four separate fibroblasts cultures (one normal 

and three asthmatic) were used. Eotaxin-1 protein was not present 

consistently in the medium above the detection limit of the assay until 

6 h. At 6 h, a low level of eotaxin-1 could be measured in the cells 

stimulated with both IL-13 alone and TGF-β1 plus IL-13 in similar 

amounts (119 ± 61 and 78 ± 53 pg/ml, respectively). However, by 

24 h, differences between IL-13 alone and TGF-β1 plus IL-13

FIGURE 3. Northern blot from two separate primary human airway fi- 

broblast cultures stimulated with medium TGF-β1 (0.5 ng/ml), IL-13 (30 

ng/ml), and both TGF-β1 (0.5 ng/ml) and IL-13 (30 ng/ml). The combi-

nation synergistically increased the amount of eotaxin-1 mRNA.
appeared (750 ± 176 and 2303 ± 221 pg/ml, respectively) which were maintained at 48 h (Fig. 6A).

There were no significant increases in eotaxin-1 mRNA at any time point with medium alone (overall p > 0.1). With TGF-β1 there was a slight but consistent decline in eotaxin-1 mRNA through 6 h. At 24 and 48 h there were small but significant increases in mRNA over that of medium alone (Fig. 6B). With either IL-13 or TGF-β1 plus IL13, increases in eotaxin-1 mRNA were seen as early as 1 h after stimulation (Fig. 6C; note the marked increase in range of the y-axis over that in Fig. 6B). At 6, 24, and 48 h, the increase in eotaxin mRNA was significantly greater than that seen with medium or TGF-β1 alone. The increase in mRNA with IL-13 slowly increased out to 48 h. In contrast, the increase in mRNA with TGF-β1 plus IL-13 increased significantly over that of IL-13 alone at 24 h. By 48 h, the mRNA level with the combination declined by ~80%, no longer different from that of IL-13 alone.

Effects of actinomycin and cycloheximide (CHX) on protein and mRNA
Actinomycin (100 nM) inhibited (>90%) the increase in mRNA and protein at all time points for IL-13 alone and for the combination. It also significantly blocked the small increase in mRNA with TGF-β1 at 24 and 48 h. CHX blocked the protein production

**FIGURE 4.** Quantitative real-time PCR assessment of the fold changes in eotaxin-1 mRNA after 48 h of stimulation with TGF-β1 (0.5 ng/ml), IL-13 (30 ng/ml), or the combination over unstimulated values (n = 25 total assessments from the three subject groups). Only the combination of TGF-β1 and IL-13 significantly increased eotaxin mRNA (overall p < 0.0001). The boxes represent the mean ± SEM, while the lines represent the mean. All the data have been reconverted from the log-transformed data back to the original scale.

**FIGURE 5.** Evaluation of the dose response in eotaxin-1 protein to increasing doses of TGF-β1 and IL-13 (n = 6 primary cultures from three normal controls and three asthmatics). Threshold doses of 3 ng/ml IL-13 and 0.5 ng/ml TGF-β1 were required before eotaxin-1 production was observed. Data are expressed as mean ± SEM.

**FIGURE 6.** A. Time course for eotaxin-1 protein in unstimulated fibroblasts and fibroblasts exposed to medium, TGF-β1 (0.5 ng/ml), IL-13 (30 ng/ml), or TGF-β1 plus IL-13 from one normal and three asthmatic cell cultures. Data presented are means ± SEM. B, Time course for eotaxin-1 mRNA from cells treated with medium or TGF-β1 (0.5 ng/ml) alone from one normal and three asthmatic fibroblast cultures. The effect of CHX (100 nM) on eotaxin-1 mRNA from these two conditions is also shown. CHX significantly blunted the TGF-β1-induced increase in eotaxin mRNA at 24 and 48 h. The boxes represent the mean ± SEM, while the lines represent the mean. All the data have been reconverted from the log-transformed data back to the original scale. C. Time course for eotaxin-1 mRNA from IL-13 alone and TGF-β1 plus IL-13-stimulated cells from one normal and three asthmatic cell cultures. The increase in eotaxin mRNA at 24 h was significantly greater than all other conditions and time points. The effect of CHX (100 nM) on eotaxin-1 mRNA from these two conditions is also shown. CHX blunted the response at 6 h under both conditions but also significantly blocked the significant increase in eotaxin mRNA at 24 h in cells stimulated with the combination. The boxes represent the mean ± SEM, while the lines represent the mean. All the data have been reconverted from the log-transformed data back to the original scale.
from all conditions by >90%. Additionally, CHX (100 nM) completely inhibited the increase in mRNA at 6 h under both IL-13 and TGF-β1 plus IL-13 conditions. CHX had no effect on the IL-13-stimulated mRNA at later time points (Fig. 6C). In contrast, CHX significantly blunted the peak increase in mRNA after TGF-β1 plus IL-13 at 24 h, while having no effect on the mRNA at 48 h. Of interest, the small increase in mRNA at 24 and 48 h with TGF-β1 alone was blocked by CHX (Fig. 6C). These data suggest that the increase in eotaxin-1 is at least partially transcriptionally regulated and that one or more proteins produced between 6 and 24 h after stimulation with TGF-β1 is responsible for the synergistic increase in eotaxin-1 mRNA and protein that occurs with TGF-β1 plus IL-13.

Activation of STAT-6

Western blots of pSTAT-6 (Fig. 7) evaluated the time course of activation at 15 min, 1 h, 2 h, 6 h, and 24 h post-stimulation with medium, TGF-β1 (0.5 ng/ml), IL-13 (30 ng/ml), or the combination. Neither TGF-β1 alone nor the unstimulated control demonstrated phosphorylation of STAT-6. Both IL-13 and TGF-β1 plus IL-13 induced phosphorylation of STAT-6 within 15 min of stimulation. The increase in pSTAT-6 was partially maintained through 2 h and decreased substantially (but was still present) at 6 h. There were no differences in the amount of pSTAT-6 between those cells treated with IL-13 alone or in combination with TGF-β, although there was a tendency for the phosphorylation to be greater with IL-13 alone. Nonphosphorylated STAT-6 was present at baseline for 15-hydroxyeicosatetraenoic (15-lipoxygenase-1), thus indirectly suggesting the presence of TGF-β2 (15). Th2 cytokines such as IL-4/-13 up-regulate the enzymatic source for 15-hydroxyeicosatetraenoic (15-lipoxygenase-1), thus indirectly suggesting the presence of TGF-β and Th2 cytokines in the same subjects. Other groups have directly reported increases in eotaxin in asthmatics, especially those with more severe disease, it is conceivable this combination of factors contributed to the significant increase in eotaxin-1+ fibroblasts observed in the severe asthmatic tissue studied here.

In earlier studies, TGF-β (pan isoforms) from multiple cell sources was found to be significantly elevated in a subgroup of severe steroid-dependent asthmatics with persistent eosinophilia (3, 4, 14). A parallel study found that levels of the eicosanoid 15-hydroxyeicosatetraenoic acid were elevated in bronchoalveolar lavage fluid in the same group of severe eosinophilic asthmatics (15). Th2 cytokines such as IL-4/-13 up-regulate the enzymatic source for 15-hydroxyeicosatetraenoic (15-lipoxygenase-1), thus indirectly suggesting the presence of TGF-β and Th2 cytokines in the same subjects. Other groups have directly reported increases in IL-13 and IL-4 (1, 16). Interestingly, a recent report suggested that IL-13 can up-regulate tissue TGF-β1 in a murine model (17). Combined, these data suggest that both TGF-β1 and IL-13 are present in the submucosa of asthma patients with access to airway fibroblasts. Dose-response studies demonstrate that the effect of the combination to increase eotaxin-1 follows a dose-response paradigm, although the large increase from 0.05 to 0.5 ng/ml TGF-β1 and from 0.3 to 3 ng/ml IL-13 suggests that some element of a “threshold” response exists. These responses occurred without difference in asthmatics (of all groups) and normal controls. Additionally, the doses required for the effect were low and within physiologic ranges.

The increase in human airway fibroblast eotaxin-1 production to TGF-β and IL-13 occurred at both the protein and mRNA levels. The up-regulation of the mRNA occurred quickly (within 1 h of stimulation) in both IL-13 alone and TGF-β plus IL-13-stimulated cells. However, by 24 h the magnitude of the increase in mRNA was significantly greater in the cells treated with both TGF-β1 and IL-13. Actinomycin suppressed both eotaxin-1
mRNA and the protein response to 90% of the untreated response, supporting a transcriptional level regulation of these increases. Interestingly, CHX also inhibited the increase in mRNA at 6 h (for both IL-13 and TGF-β1 plus IL-13 stimulation). In cells treated with IL-13 alone, this effect was lost by 24 h. In contrast, the peak increase in mRNA at 24 h with TGF-β1 and IL-13 was also significantly blocked by CHX. Interestingly, the smaller but significant increase in eotaxin-1 mRNA at 24 and 48 h in fibroblasts treated with TGF-β1 alone was also blocked by CHX. These results suggest that synthesis of specific proteins between 6 and 24 h may be responsible for the synergistic increase in mRNA, either by increasing transcription at later time points or by stabilizing the mRNA. A similar effect of TGF-β1 to stabilize mRNA (for elastin) has been previously described in fibroblasts (18).

Because IL-13 (and IL-4) can signal through an IL-4/IL-13R complex/STAT-6 pathway and eotaxin-1 has been described to have a STAT-6 response element in its promoter, the activation of the STAT-6 pathway was evaluated (19, 20). Not surprisingly, IL-13 alone produced marked phosphorylation of STAT-6. The addition of TGF-β1 to the system did not appear to enhance this activation and, if anything, may have decreased it slightly. These findings suggest that, although STAT-6 may be involved, the mechanism behind the synergistic effect of TGF-β1 on eotaxin-1 production is not due to augmented phosphorylation of STAT-6. Further studies of the promoter region of eotaxin-1 for involvement of other transcription factors, including those newly synthesized by the combination, should help elucidate the mechanisms of activation and production.

The combination of IL-13 with TNF-α or IL-1 has also been reported to synergistically increase eotaxin-1 production in fibroblasts, epithelial cells, and smooth muscle cells (11–13, 20). Studies in our laboratory with IL-13 and TNF-α demonstrated that the production of eotaxin-1 was similar to that seen with TGF-β1 and IL-13, although variability existed from cell culture to cell culture (data not shown). Because the signaling mechanisms for these factors (TGF-β compared with TNF-α or IL-1) are quite disparate, the similar eotaxin-1 responses in the presence of IL-4 or IL-13 are surprising. TNF-α and IL-1 signal primarily through protein-protein interactions and serine/threonine kinases leading to activation of NF-κB. In contrast, TGF-β signals primarily through Smads and AP-1 transcription factors. Synergy with neither TNF-α/IL-1 nor TGF-β1 appears to be completely dependent on enhanced activation of STAT-6 (12, 20). It is also not clear whether there are further additive or synergistic effects when all three stimuli are used. Studies to evaluate the commonalities in the signaling and protein responses to TNF-α/IL-1 vs TGF-β should prove helpful.

The increased eotaxin-1 levels seen with combinations of IL-1 or TNF-α with IL-13 were associated with high levels of IL-8 in the supernatants (12, 13). In contrast, the combination of TGF-β and IL-13 did not lead to high levels of IL-8, but rather to increased procollagen I (21). It is possible that an early inflammatory reaction, commonly involving IL-1 and TNF-α, in the presence of IL-4/13 could induce a fibroblast phenotype augmenting both eosinophilia and neutrophilia. In contrast, as the situation becomes more chronic (in the presence of TGF-β), eosinophils could become dominant with associated increases in profibrotic factors.

In an IL-13-driven murine system, eotaxin-1 appears to contribute to the maintenance of airway tissue eosinophilia (10, 22). The cellular source for eotaxin-1 was not determined, but the tissue location of the eosinophils and the findings described here strongly implicate the fibroblast. These findings may also explain why an Ab to IL-5, a cytokine believed to be important in eosinophilia and asthma-like reactions in mice, failed to improve asthmatic responses in humans, even though it profoundly decreased sputum (luminal) eosinophils (23). A recently reported follow-up study suggested that the IL-5 Ab did not completely reverse tissue (in contrast to luminal and blood) eosinophilia, supporting additional tissue factors (24). It is conceivable that eotaxin-1-positive airway

fibroblasts, confirmed in the current study in severe asthmatic subjects, could have contributed to the persistent tissue eosinophilia seen in those patients. However, studies in humans specifically designed to evaluate that question, including the use of CCR3 antagonists currently in development, will be required before the relationship of eotaxin to eosinophilia can be confirmed.

Finally, this study supports a specific and substantial interaction of the innate immune system and the TH2 immune system. This study suggests that the addition of an atopic background to the repair of a wound, specifically a chronically healing one as seen in asthma, may substantially alter the repair process from that seen in the absence of a TH2 background. It is likely that up-regulation of eotaxin-1 is only one example. If the interaction proves to involve some modification of the STAT-6 response, then multiple other STAT-6 responsive genes, such as VCAM-1, could also be involved. The relevance of these findings to human disease is suggested by the confirmation of eotaxin-1 in fibroblasts in a group of subjects with known increases in TGF-β1 and probable increases in IL-4/IL-13. Uncovering the mechanisms driving the interactions should prove beneficial to understanding the relationships between the innate and the TH2 immune systems, which may eventually lead to improvement in the understanding of diseases such as asthma.

Acknowledgments

We thank Dr. Dave Riches and Dr. William Schiemann for their thoughtful critique of the manuscript as well as Barbara Schoen for her expert technical support.

References