

# Tumor Necrosis Factor- $\alpha$ Enhances mRNA Expression and Secretion of Interleukin-6 in Cultured Human Airway Smooth Muscle Cells

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Airway smooth muscle (ASM) is considered to be an end-target cell for the effects of mediators released during airway wall inflammation. Several reports suggest that activated ASM may be capable of generating various proinflammatory cytokines. We investigated the effects of tumor necrosis factor (TNF)- $\alpha$ , a potent proinflammatory cytokine, on cultured human ASM cells by examining the expression and release of the cytokine interleukin (IL)-6, cell proliferation, and the expression pattern of *c-fos* and *c-jun*, two nuclear proto-oncogenes constituting the activator protein-1 transcription factor. Growth-arrested cell monolayers were stimulated with human recombinant TNF- $\alpha$  in a concentration- and time-dependent manner. TNF- $\alpha$  stimulated the expression of IL-6 messenger RNA (mRNA), which was detected after 15 min, reaching a maximum at 1 h. IL-6 protein was readily detected in ASM cell-conditioned medium after 2 h of TNF- $\alpha$  stimulation. Protein levels increased in a time- and concentration-dependent manner. Release of IL-6 elicited by TNF- $\alpha$  was significantly inhibited by dexamethasone, cycloheximide, and nordihydroguaiaretic acid (NDGA). TNF- $\alpha$  did not alter DNA biosynthesis up to 48 h or cell numbers up to 120 h. Northern blot analysis of proto-oncogene expression revealed that *c-fos* and *c-jun* mRNA levels were elevated after 30 min of TNF- $\alpha$  incubation with maximum levels at 1 h and 45 min, respectively. Expression of *c-fos* mRNA was downregulated by NDGA. Four hours of TNF- $\alpha$  treatment resulted in translocation of *c-jun* immunofluorescence from the cytoplasm to the nucleus in human ASM cells. Our results suggest that despite the lack of a mitogenic response to TNF- $\alpha$ , upregulation of primary response genes in human ASM cells may account for the induction of proinflammatory cytokines, such as IL-6, in human airways.

Asthma is a chronic disease of the airways characterized by reversible airway obstruction and airway hyperresponsiveness. Important pathologic features of asthmatic airways include inflammatory cell infiltration, epithelial shed-

ding, basement membrane thickening, and increased mass of airway smooth muscle (ASM) (1-3). Infiltration of lymphocytes and granulocytes, especially eosinophils, and their concomitant release of cytokines appear to play a central role in mediating the airway inflammatory response (4). Several inflammatory cell-derived cytokines have also been implicated in ASM cell division and growth (5-7), and may therefore be linked to the observed hyperplasia and hypertrophy of smooth muscle in asthmatic airways (1, 2). In addition to their contractile and proliferative properties, ASM cells may modulate airway inflammation by the synthesis and secretion of proinflammatory secondary mediators, thereby acting as "immune effector" cells in the perpetuation of the airway inflammatory reaction (8-12).

Tumor necrosis factor (TNF)- $\alpha$  is a potent proinflammatory cytokine and its role as a potential mediator in asthma has been well described (13, 14). It has been shown in humans that inhaled TNF- $\alpha$  increases bronchial responsiveness (15). TNF- $\alpha$  can also modulate cultured ASM cells to proliferate (6, 7). Another important biologic action of TNF- $\alpha$  is its ability to induce an influx of inflammatory cells into tissues through either chemotactic mechanisms or increased expression of adhesion molecules (16-18).

Interleukin (IL)-6, a pleiotropic cytokine, has proinflammatory effects relevant to airway wall inflammation (19), including mucus hypersecretion (20), as well as stimulation of hyperplasia and hypertrophy of cultured guinea pig ASM cells (5). Expression of the IL-6 gene can be induced in many different cell types after stimulation with TNF- $\alpha$  (21) and decreased by treatment with corticosteroids (22-24). The gene contains sequences for the serum responsive element and the consensus sequences for transcription factors such as activator protein (AP)-1 and nuclear factor kappa-B (NF- $\kappa$ B). These regulatory sequences play an important role in transcriptional activation of the IL-6 gene (25). TNF- $\alpha$  can induce NF- $\kappa$ B and AP-1 in human lung, which could be of relevance for the expression of IL-6 (18, 26, 27).

Several investigators have shown that the levels of both TNF- $\alpha$  and IL-6 are upregulated in patients with acute severe asthma (28-30). However, the molecular mechanisms involved in the relationship between TNF- $\alpha$  and IL-6 gene expression have not been investigated in airway inflammation. In this study, we investigated the effects of TNF- $\alpha$  on the expression and release of IL-6 by human ASM cells. We also examined the messenger RNA (mRNA) expression pattern of the constituent proteins of the AP-1 transcription factor complex, *c-jun* and *c-fos*. ASM cell proliferation was also assessed in relation to TNF- $\alpha$  and/or IL-6. Furthermore, we determined whether the secretion of IL-6

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**Abbreviations:** activator protein, AP; airway smooth muscle, ASM; bovine serum albumin, BSA; complementary DNA, cDNA; cycloheximide, CHX; Dulbecco's modified Eagle's medium, DMEM; enzyme-linked immunosorbent assay, ELISA; fetal bovine serum, FBS; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; granulocyte-macrophage colony-stimulating factor, GM-CSF; interleukin, IL; mitogen-activated protein, MAP; messenger RNA, mRNA; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; nordihydroguaiaretic acid, NDGA; optical density, OD; phosphate-buffered saline, PBS; regulated on activation, normal T cells expressed and secreted, RANTES; reverse transcriptase/polymerase chain reaction, RT-PCR; standard error of the mean, SEM; tumor necrosis factor, TNF.

by human ASM cells was sensitive to inhibition by the glucocorticosteroid dexamethasone, as well as the protein synthesis inhibitor cycloheximide (CHX) and the lipoxygenase pathway inhibitor nordihydroguaiaretic acid (NDGA).

## Materials and Methods

### Human ASM Cell Isolation and Culture

Human ASM cells were isolated and cultured as described previously (31, 32). Briefly, bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus obtained from patients who underwent surgery for lung carcinoma. After removal of the epithelium, pieces of smooth muscle were dissected free of adherent connective and parenchymal tissue under aseptic conditions. Smooth muscle pieces were incubated in Hanks' balanced salt solution (Life Technologies BV, Breda, The Netherlands) containing bovine serum albumin (BSA; 10 mg/ml), collagenase (type XI, 1 mg/ml), and elastase (3.3 U/ml) (Sigma BV, Zwijndrecht, The Netherlands) at 37°C in a humidified incubator (ASSAB, model T154; Clean Air Techniek BV, Woerden, The Netherlands) containing 5% CO<sub>2</sub> in air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM; Life Technologies BV) containing 10% (vol/vol) fetal bovine serum (FBS; Bio-Whitaker BV, Verviers, Belgium) supplemented with sodium pyruvate (1 mM), nonessential amino-acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (1.5 µg/ml) (Life Technologies BV). Cells were subsequently seeded at  $2 \times 10^5$  cells per 35-mm dish and maintained in culture by replacing the medium every 72 h. After 10 to 14 d in culture, ASM cells grew to confluence and they were then removed by trypsinization (0.5% trypsin; 0.02% ethylenediaminetetraacetic acid [EDTA]; Life Technologies BV) and subcultured into 25-cm<sup>2</sup> tissue culture flasks. Cells were further passaged into 75-cm<sup>2</sup> tissue culture flasks. Confluent cells in the fourth to sixth passages were used for experiments.

Immunocytochemical staining of confluent serum-deprived primary cultures of human ASM cells, using monoclonal antibodies to smooth muscle  $\alpha$ -actin and smooth muscle-myosin heavy chain (Sigma BV) (31, 33), demonstrated that the cultures were essentially free (> 95%) of other contaminating cell types.

### Stimulation of ASM Cells

Human ASM cells were harvested from 75-cm<sup>2</sup> flasks by treatment with trypsin during passages 4 to 6. Cells were seeded into 24-well plastic tissue culture plates at a density of  $3 \times 10^4$  cells/well and allowed to adhere for 24 h. To synchronize cellular growth, human ASM cells were washed twice in phosphate buffered saline (PBS; 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, pH 7.4) and cultured in serum-free DMEM containing 1 µM insulin, 5 µg/ml transferrin, and 100 µM ascorbate (Sigma BV) for 72 h. Using flow cytometric analysis of human ASM cells stained with propidium iodide, we previously found that 72 h of serum deprivation resulted in approximately 85% of human ASM cells remaining in the G<sub>0</sub>/G<sub>1</sub> phase (S. McKay and H. S. Sharma, unpublished observations). Growth-arrested cell monolayers were stimulated with TNF- $\alpha$  (Knoll AG, Ludwigshaven, Germany) in fresh FBS-free DMEM in a concentration-dependent (0, 1, 5, 10, 25, 50, 100, and 500 U TNF- $\alpha$ /ml; 24 h) and time-dependent (15, 30, 45 min, 1, 2, 4, 8, 16, 24, 48, 72, 96, 120 h; 50 U/ml TNF- $\alpha$ ) manner. In a separate set of experiments, serum-deprived cells were stimulated with human recombinant IL-6 (Promega, Leiden, The Netherlands) in fresh FBS-free DMEM in a concentration-dependent (0, 1, 5, 10, 50 ng IL-6/ml; 72 h) manner.

An appropriate concentration of TNF- $\alpha$  (50 U/ml) was selected for further experiments. Where inhibitors were used, cells

were preincubated for 1 h with either dexamethasone (1 nM to 1 µM) (US Biochemicals and Amersham Nederland BV, 's-Hertogenbosch, The Netherlands), CHX (1 nM to 1,000 nM), or NDGA (1 µM to 10 µM) (Sigma BV) before the addition of TNF- $\alpha$  for 1 h (c-fos mRNA expression), 2 and 4 h (IL-6 mRNA expression), or 16 and 24 h (IL-6 protein assay). Cell-conditioned medium (0.5 ml) was collected and stored at -20°C until assayed for IL-6 levels by enzyme-linked immunosorbent assay (ELISA).

To measure cell-associated IL-6 levels, human ASM cells were lysed in single detergent lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride) after 4 h incubation in either serum-deprived medium or with TNF- $\alpha$ . The lysate was stored at -80°C until assayed for IL-6 levels by ELISA.

ASM cell viability was determined immediately by mitochondrial-dependent reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma BV) to formazan as described previously (12, 32, 34). Cell monolayers were washed in 0.5 ml PBS, MTT (200 µl in DMEM; final concentration, 0.5 µg/ml) was added to each well, and the cells were incubated for 5 h before overnight solubilization in an additional 200 µl 10% sodium dodecyl sulfate in 0.1 M HCl. A 200-µl aliquot from each duplicate well was transferred to a 96-well microplate, and the optical density (OD) was determined using an automated dual wavelength spectrophotometer (3550 microplate reader; Bio-Rad, Veenendaal, The Netherlands) at a test wavelength of 595 nm and a reference of 690 nm. Cell viability was expressed as arbitrary OD units.

### Isolation of Total Cellular RNA

TNF- $\alpha$ -treated and -untreated human ASM cells were washed in PBS and directly lysed in guanidinium thiocyanate buffer. The lysate was repeatedly passed through a 23-gauge needle in order to shear the genomic DNA. Total cellular RNA was isolated using the method described previously (31, 35). The RNA concentration was estimated by OD measurements and a DNA/protein ratio of  $\geq 1.8$  was accepted. Furthermore, the quality of RNA was tested on a denaturing formaldehyde agarose gel. RNA samples were stored at -20°C until processed for reverse transcriptase/polymerase chain reaction (RT-PCR) and Northern blot analysis.

### RT-PCR

RT-PCR was performed to detect IL-6 mRNA expression in cultured human ASM cells and subsequently to prepare a human-specific complementary DNA (cDNA) probe. Reverse transcription of 5 µg of total RNA was performed using avian myeloblastosis virus (AMV) reverse transcriptase (20 U) (HT Biotechnologies, Cambridge, UK), 1 mM of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP), 2 µg oligo(dT)<sub>12-18</sub> primer (Pharmacia Biotechnologies, Woerden, The Netherlands), 1 U/µl RNase inhibitor (Promega), 5.0 mM MgCl<sub>2</sub>, 50 mM KCl, 25 mM Tris-HCl (pH 8.3), and 2.0 mM dithiothreitol in a total volume of 50 µl. Oligo(dT) and dissolved RNA were incubated at 70°C for 10 min and placed on ice. Subsequently, the remaining ingredients were added and samples were incubated at 42°C for 40 min.

Five microliters of the cDNA samples were amplified. PCR was performed using 0.5 µM concentration of forward and reverse primers (Life Technologies BV); dATP, dGTP, dCTP, and dTTP at a final concentration of 0.25 mM each; 0.5 U *Taq* polymerase (Perkin Elmer, Gouda, The Netherlands); 1.5 mM MgCl<sub>2</sub>; 50 mM KCl; 10 mM Tris-HCl (pH 8.3) in a final volume of 20 µl. Oligonucleotide primers for human IL-6 were sense primer, 5'-TACATCCTCGACGGCATCTCA-3', and anti-sense primer, 5'-AGTTGTCATGTCCTGCAGCCA-3', giving rise to a PCR

product of 398 bp. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 5'-GGCCATCCACAGTCT-CTGGGT-3' and 5'-CCGAGCCACATCGCTCAGAC-AC-3' primers, giving rise to a product of 594 bp, was used as reference. The PCR was carried out in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) at 94°C for an initial 10 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 90 s. Final extension was for 10 min at 72°C. Products were resolved by electrophoresis on a 1% agarose ethidium bromide-stained gel and then visualized using ultraviolet luminescence and photographed. The PCR product was purified using a Wizard PCR purification system (Promega) before sequencing in an automated Applied Biosystems Prism 310 genetic analyzer (Perkin Elmer, Nieuwekerk a/d IJssel, The Netherlands). Cycle sequencing reactions using an ABI prism dye terminator kit were performed according to the manufacturer's instructions (Perkin Elmer). The IL-6 PCR product was cloned into the pGEM-T easy plasmid vector and transformed into JM109 cells (Promega). The insert positive clones were processed for plasmid DNA isolation using a Wizard DNA purification kit (Promega).

### Northern Blot Analysis

For Northern hybridization, samples of total RNA (10  $\mu$ g) were denatured at 65°C in a formaldehyde containing loading buffer and size-fractionated on a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide-stained gels were photographed, and RNA was transferred onto hybond-N membrane (Amersham Nederland BV) by the alkaline downward capillary transfer method (36). The filters were air-dried and UV cross-linked in a gene linker (Bio-Rad Laboratories BV). Blots were hybridized as described previously (31). The cDNA probes used for hybridization were human IL-6 (360-bp fragment), mouse *c-fos* (2.1-kb fragment), and mouse *c-jun* (2.6-kb fragment). A GAPDH cDNA probe (American Type Culture Collection, Rockville, MD) was used to rehybridize membranes for reference purposes. Hybridization signals were quantified by scanning laser densitometry using the Ultrascan XL enhanced laser densitometer (LKB, Bromma, Sweden). Signals were normalized with respect to GAPDH mRNA values and expressed as relative OD in TNF- $\alpha$ -stimulated cells versus control cells.

### Measurement of IL-6 Protein Levels by ELISA

IL-6 protein levels in ASM cell lysates and cell-conditioned medium were determined using a solid-phase sandwich ELISA (Medgenix, Breda, The Netherlands). Samples of cell-conditioned medium were diluted until the level of IL-6 was within the linearity limits of the standard curve of the assay. Subsequently, the samples were preincubated with IL-6 capture antibody followed by biotinylated IL-6 detecting antibody. After addition of the streptavidin-peroxidase conjugate (Central Laboratory for Blood Transfusion, Amsterdam, The Netherlands), tetramethylbenzidine (ICN Biomedicals Inc., Costa Mesa, CA) was added and the absorbance of the resulting colored product was measured at 450 nm using an automated spectrophotometer (Bio-Rad Laboratories BV). The concentration of IL-6 was expressed in nanograms per milliliter. The detection limit of the IL-6 ELISA method was 50 pg of IL-6/ml. No correction for cell number variation was made because the cells were serum deprived 16 to 24 h after plating out, allowing insufficient time for proliferation.

### Evaluation of Cellular Proliferation

**<sup>3</sup>H]Thymidine incorporation assay.** Effects of recombinant TNF- $\alpha$  or IL-6 on DNA biosynthesis was evaluated by incorporation of [methyl-<sup>3</sup>H]thymidine (Amersham Nederland BV). Confluent cells in the fourth passage were washed in PBS, detached

by trypsinization, and transferred into 24-well plates at a seeding density of  $6 \times 10^4$  cells/well. After 24 h, the subconfluent cell monolayers were growth arrested as described previously. Cells were incubated with [methyl-<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) in either fresh FBS-free DMEM (control) or DMEM containing TNF- $\alpha$  (50 U/ml) for 16, 24, or 48 h, or IL-6 (0, 1, 5, 10, 50 ng/ml) for 72 h in order to assess DNA synthesis. After stimulation, the cells were washed in PBS, fixed with ice-cold methanol, and exposed to ice-cold trichloroacetic acid (5% wt/vol). The acid-insoluble fraction was lysed in 0.3 M NaOH, and the incorporated radioactivity was determined by liquid scintillation spectrometry in a Packard 1500 Tri-carb liquid scintillation analyzer (Packard-Becker BV, Delft, The Netherlands).

**Cell counting.** Cells were incubated in either fresh FBS-free DMEM, FBS-free DMEM containing TNF- $\alpha$  (50 U/ml) for 24, 48, 72, 96, or 120 h, or FBS-free DMEM containing IL-6 (0, 1, 5, 10, or 50 ng/ml; 7 d). After stimulation, the cell-conditioned medium was removed, and the cells were dispersed in 50  $\mu$ l of trypsin-EDTA by incubating at room temperature for 10 min. Cells were counted in a Bürker hemocytometer (Bürker, Marienfeld, Germany) using the trypan blue dye (Sigma BV) exclusion method.

### Immunocytochemistry

An affinity-purified rabbit polyclonal antibody against human *c-jun* (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to demonstrate the cellular localization of the AP-1 transcription factor in human ASM cells stimulated with TNF- $\alpha$ . Cells were allowed to attach to multiwell slides for 24 h in FBS-containing medium and were subsequently synchronized before stimulation with TNF- $\alpha$  (50 U/ml) for 2 or 4 h, or FBS-free medium. After two washes in ice-cold PBS, the cells were fixed in ice-cold methanol and permeabilized in PBS containing 0.5% Tween-20. Non-specific binding was blocked by incubating the cells in PBS containing 10% serum and 5% BSA, the cells were then washed and subsequently incubated with *c-jun* antibody in a dilution of 1:100; the negative control cells were not incubated with the primary antibody. After incubation, the cells were washed twice in PBS/Tween and further incubated with a fluorescein isothiocyanate (FITC)-conjugated antirabbit antibody (Santa Cruz Biotechnology). Unbound antibody was washed away using PBS and the sections were mounted in glycerol. Specimens were visualized under a microscope equipped with fluorescence ultraviolet optics (Carl Zeiss BV, Weesp, The Netherlands) and photographed.

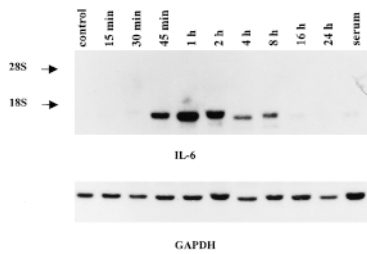
### Statistical Analysis

Data in the text and figures are expressed as mean  $\pm$  standard error of the mean (SEM) of observations from *n* patients. Statistical analysis was performed by using the two-tailed, independent sample *t* test. Significance was accepted at  $P < 0.05$ .

## Results

### IL-6 mRNA Expression in Relation to TNF- $\alpha$

To examine IL-6 mRNA expression in human ASM cells treated with TNF- $\alpha$  (50 U/ml) for 1, 2, or 4 h, we performed RT-PCR employing human-specific IL-6 oligonucleotide primers. Agarose gel electrophoresis revealed PCR products of 398 bp in size. The PCR product was purified and subjected to DNA sequencing, which confirmed that the amplified PCR product was indeed IL-6, with a 100% sequence homology to the human IL-6 cDNA, as reported previously by Tonouchi and coworkers (37). The IL-6 PCR product was subsequently cloned into a pGEM-T easy vector, and the recombinant plasmid DNA encoding



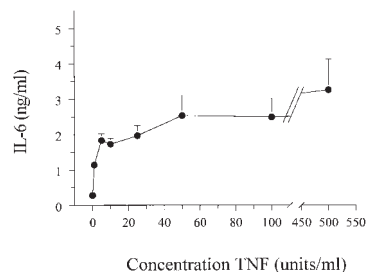
**Figure 1.** Northern blot analysis of IL-6 mRNA expression. Human ASM cells were treated with TNF- $\alpha$  for the times indicated at the top of each lane, and total cellular RNA was extracted and subjected to Northern hybridization using human IL-6 cDNA probes as described in MATERIALS AND METHODS. Rehybridization with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes.

IL-6 was used as a probe to detect and quantify IL-6 mRNA by Northern blot analysis. Densitometric analysis of Northern blots showed that TNF- $\alpha$  induced the expression of mRNA encoding IL-6 after 15 min of stimulation with a maximal induction at 1 h and then the expression decreased gradually (Figure 1). The gene remained slightly upregulated for up to 16 h.

#### Release of IL-6 Protein in Response to TNF- $\alpha$

A concentration-dependent increase in the release of IL-6 protein from human ASM cells was found after stimulation with TNF- $\alpha$  for 24 h (Figure 2). The highest IL-6 production occurred in response to 500 U/ml of TNF- $\alpha$ . Conditioned media derived from serum-deprived, untreated ASM cells contained very low ( $\pm 0.25$  ng/ml) levels of IL-6 protein. To verify that TNF- $\alpha$ -stimulated human ASM cells release the *de novo* synthesized IL-6, we also measured its levels in the cell lysates of stimulated and unstimulated control cells. IL-6 levels in cell lysates of unstimulated ASM cells were negligible ( $0.10 \pm 0.01$  ng/ml). MTT reduction assays showed that ASM cell viability was not affected by TNF- $\alpha$  (up to 500 U/ml; untreated cells,  $0.24 \pm 0.02$  versus TNF- $\alpha$ -treated cells,  $0.26 \pm 0.03$  OD units,  $n = 4$ ,  $P < 0.05$ ).

In a second set of experiments, we found that TNF- $\alpha$  (50 U/ml) caused a time-dependent increase in IL-6 protein in



**Figure 2.** TNF- $\alpha$  concentration-dependent production of IL-6 by human ASM cells. Growth-arrested ASM cells were stimulated in the absence (control) or presence of varying concentrations of TNF- $\alpha$  for 24 h. Data represent the mean  $\pm$  SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from four different patients.

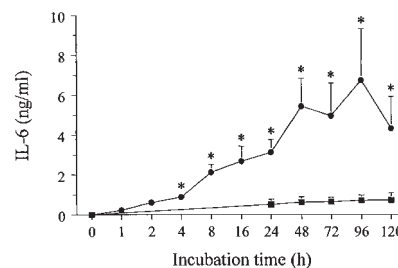
the ASM cell-conditioned media (Figure 3). IL-6 could be detected after just 2 h of stimulation with TNF- $\alpha$  and continued to increase before reaching a plateau around 48 h. The levels remained elevated for up to 120 h of stimulation. IL-6 levels were significantly higher in the conditioned media derived from TNF- $\alpha$ -treated ASM cells compared with untreated cells at all the time points studied.

#### Influence of Inhibitors on IL-6 Expression and Secretion

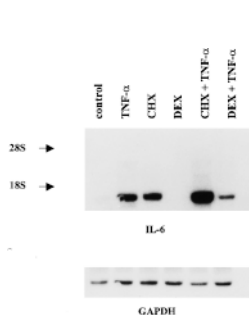
In Figure 4, the effects of dexamethasone and CHX on the expression of IL-6 mRNA from untreated and TNF- $\alpha$ -treated (50 U/ml) cells are shown. Interestingly, treatment with CHX (1  $\mu$ M) resulted in increased IL-6 mRNA levels comparable to those present during TNF- $\alpha$  treatment. When cells were incubated with both TNF- $\alpha$  and CHX, IL-6 mRNA levels were further increased by 2.8-fold. However, dexamethasone significantly inhibited the TNF- $\alpha$ -induced expression of IL-6 mRNA by more than 50%.

Addition of TNF- $\alpha$  (50 U/ml) to ASM cell cultures preincubated with dexamethasone (1 nM to 1  $\mu$ M) showed a concentration-dependent inhibition in the production of IL-6 from these cells measured by ELISA (Figure 5A). Dexamethasone, at a concentration of 100 nM, significantly decreased the TNF- $\alpha$ -induced IL-6 protein levels by 83% from  $3.98 \pm 0.81$  to  $1.17 \pm 0.18$  ng/ml. Levels of IL-6 in cell-conditioned medium after treatment with dexamethasone at 1  $\mu$ M were not significantly different than IL-6 levels in conditioned medium from unstimulated cells ( $n = 4$ ,  $P < 0.05$ ). Preincubation of human ASM cells with CHX (1 nM to 1  $\mu$ M) also showed a concentration-dependent inhibition in the production of IL-6 protein by cells stimulated with an optimal concentration (50 U/ml) of TNF- $\alpha$  for 24 h (Figure 5B). A total of 1  $\mu$ M of CHX reduced the release of IL-6 by 25% from  $3.08 \pm 0.86$  to  $2.28 \pm 0.62$  ng/ml ( $n = 4$ ,  $P < 0.05$ ) (Figure 5B).

The lipoxygenase pathway inhibitor NDGA reduced the secretion of IL-6 protein at a concentration of 1  $\mu$ M. As shown in Figure 6, NDGA significantly inhibited the TNF- $\alpha$ -induced production of IL-6 at 4, 8, and 16 h by  $60.9 \pm 13.3$ ,  $42.3 \pm 7.3$ , and  $41.9 \pm 6.8\%$ , respectively ( $n = 3$ ,  $P < 0.05$ ), whereas IL-6 levels in cell-conditioned medium af-



**Figure 3.** Time-dependent production of IL-6 protein by TNF- $\alpha$ -stimulated human ASM cells. Growth-arrested human ASM cells were stimulated with 50 U/ml of TNF- $\alpha$  (circles) for various time points. Control cells (squares) received only serum-free medium. Data represent the mean  $\pm$  SEM of triplicate values from independent experiments using conditioned medium from ASM cells cultured from four patients. \* $P \leq 0.05$  as compared with respective controls.



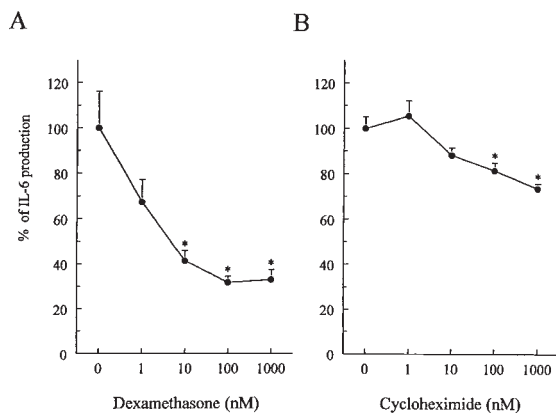
**Figure 4.** Effects of CHX and dexamethasone on IL-6 mRNA expression. Human ASM cells were pretreated with 1  $\mu$ M of either CHX or dexamethasone (DEX) for 1 h before incubation with or without TNF- $\alpha$  for 2 h. Total cellular RNA was extracted and subjected to Northern hybridization using human IL-6 cDNA probe as described in MATERIALS AND METHODS. Rehybridization with a GAPDH cDNA probe (*lower panel*) was performed for reference purposes.

ter treatment with NDGA alone were not significantly different from unstimulated control cells.

We also examined the effects of dexamethasone and CHX on cell viability by using the MTT reduction assay. Performing treatment with the previously mentioned reagents, cell viability was not significantly affected up to 24 h of stimulation. For instance, in TNF- $\alpha$  + dexamethasone and TNF- $\alpha$  + CHX-treated cells, the MTT reduction assay values were  $0.28 \pm 0.01$  and  $0.28 \pm 0.01$  versus  $0.27 \pm 0.01$  ODU ( $n = 4$ ).

#### Effects of TNF- $\alpha$ and IL-6 on ASM Cell Proliferation

To ascertain whether human recombinant TNF- $\alpha$  induces cell proliferation, [ $^3$ H]thymidine incorporation up to 48 h and changes in cell number during 120 h of incubation were determined. Treatment of serum-deprived human ASM cells with TNF- $\alpha$  (50 U/ml) up to 48 h of incubation did not induce DNA biosynthesis as compared with untreated control cells (data not shown). Furthermore, TNF- $\alpha$  incubation did not significantly increase ASM cell number as compared with controls (Figure 7).



**Figure 5.** Effects of dexamethasone and cycloheximide on IL-6 release from TNF- $\alpha$ -treated human ASM cells. Growth-arrested human ASM cells were incubated with 50 U/ml of TNF- $\alpha$  in the presence of increasing concentrations of dexamethasone (A) or cycloheximide (B) as described in MATERIALS AND METHODS. Data represent the mean  $\pm$  SEM of triplicate values from independent experiments using conditioned medium from ASM cells from four patients. \* $P \leq 0.05$  compared with TNF- $\alpha$ -stimulated cells in the absence of an inhibitor.

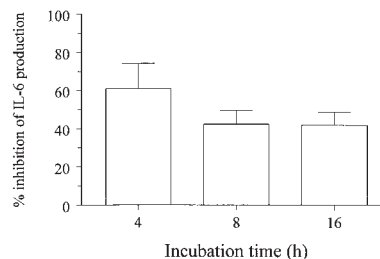
To establish whether human recombinant IL-6 induces ASM cell proliferation, [ $^3$ H]thymidine incorporation and changes in cell number were determined using varying concentrations of IL-6 (including the concentration found to be secreted by TNF- $\alpha$ -stimulated ASM cells). Data shown in Figure 8 depict that IL-6 did not significantly alter the DNA biosynthesis as well as cell number in human ASM cells.

#### Expression of *c-fos* and *c-jun* Proto-oncogenes in Relation to TNF- $\alpha$

Representative Northern blots showing the expression pattern of the proto-oncogenes *c-fos* and *c-jun* in TNF- $\alpha$ -stimulated human ASM cells are shown in Figure 9. Densitometric analysis of the Northern blots revealed that TNF- $\alpha$  induced the expression of mRNAs encoding *c-fos* and *c-jun* after 30 min of stimulation. The induction of *c-fos* mRNA was transient and reached a maximum at 60 min followed by an abrupt decline. However, the expression of *c-jun* reached a maximum level at 45 min of TNF- $\alpha$  stimulation and levels remained elevated. FBS (10%) also induced the expression of *c-jun* mRNA. Furthermore, in an attempt to examine whether NDGA could block the *c-fos* expression, we found that at 1 and 10  $\mu$ M concentrations, TNF- $\alpha$ -induced expression levels of *c-fos* mRNA were decreased by  $60.8 \pm 12.1$  and  $69 \pm 9\%$ , respectively (Figure 10).

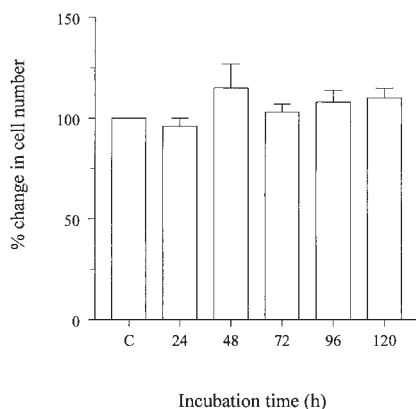
#### Immunocytochemical Localization of C-JUN in Human ASM Cells

Figure 11 illustrates representative microscopic fields of either serum-deprived or TNF- $\alpha$ -treated human ASM cells stained with antibodies recognizing C-JUN protein. At 2 h of TNF- $\alpha$  (50 U/ml) stimulation, C-JUN was localized in the cytoplasm of human ASM cells (Figure 11, *middle panel*). After 4 h of TNF- $\alpha$  stimulation, the C-JUN was translocated to the nuclei, giving rise to prominent staining in the nuclei rather than in the cytoplasm (Figure 11, *right panel*). Untreated cells showed a faint background immunofluorescence in their cytoplasm and nucleus (Figure 11, *left panel*). Additionally, the negative controls, prepared by omitting the primary antibody C-JUN, showed no immunofluorescence.



**Figure 6.** Effect of NDGA on TNF- $\alpha$ -induced release of IL-6. Growth-arrested human ASM cells were incubated with 50 U/ml of TNF- $\alpha$  in the presence of 1  $\mu$ M NDGA as described in MATERIALS AND METHODS. Data represent the percent mean  $\pm$  SEM inhibition of TNF- $\alpha$ -induced IL-6 production by NDGA. IL-6 production in NDGA-treated cells was significantly ( $P \leq 0.05$ ) inhibited at 4, 8, and 16 h of incubation. Data were calculated from quadruplicate values from three independent experiments (three different patients).



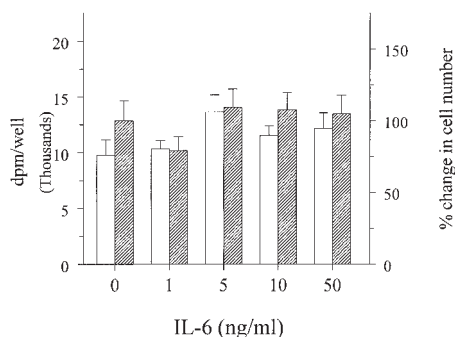


**Figure 7.** Proliferation of human ASM cells in relation to TNF- $\alpha$ . Growth-arrested human ASM cells were stimulated with 50 U/ml TNF- $\alpha$  for 24, 48, 72, 96, and 120 h, and cellular proliferation was assessed by cell counting in relation to nonstimulated cells using a hemocytometer. Values are represented as mean percent change in cell numbers relative to controls  $\pm$  SEM of four measurements from four separate experiments.

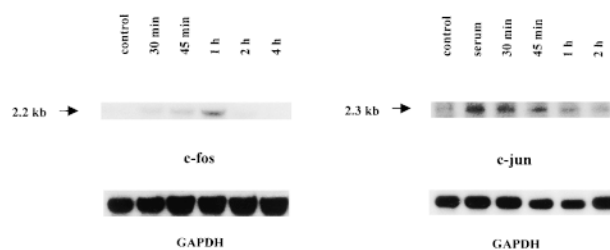
## Discussion

Our study shows that human ASM cells in culture can be stimulated by TNF- $\alpha$  to express and release a proinflammatory cytokine, IL-6, and that this effect is concentration and time dependent. IL-6 protein and mRNA levels remained very low in unstimulated ASM cells, suggesting that an inducible rather than a constitutive mechanism is involved. The concentrations of TNF- $\alpha$  at which significant IL-6 production by human ASM cells were observed were within the range likely to be present during airway inflammation (30, 38).

Recently, several groups have shown that, in addition to contractile responses, ASM cells are potentially capable of modulating airway inflammation by synthesizing and secreting proinflammatory secondary mediators like IL-8, eotaxin, regulated on activation, normal T cells expressed



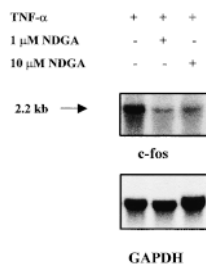
**Figure 8.** Proliferation of human ASM cells in relation to IL-6. Growth-arrested human ASM cells were stimulated with IL-6 (0, 1, 5, 10, or 50 ng/ml) for 72 h or 7 d, and cellular proliferation was assessed by thymidine incorporation (open bars) or cell counting (hatched bars), respectively. Values are represented as dpm/well (left y-axis) or mean percent change in cell numbers (right y-axis) relative to controls  $\pm$  SEM of four measurements from two separate experiments.



**Figure 9.** Northern blot analysis of c-fos and c-jun expression in human ASM cells treated with TNF- $\alpha$ . Total RNA samples from control and TNF- $\alpha$ -treated human ASM cells at various time points were subjected to Northern hybridization with radiolabeled cDNA probes as described in MATERIALS AND METHODS. Representative Northern blots for c-fos (left panel, 2.2 kb mRNA) and c-jun (right panel, 2.3 kb mRNA) are shown. Serum-deprived ASM cells were incubated with TNF- $\alpha$  (50 U/ml) for the times indicated at the top of each panel. Rehybridization of each filter with a GAPDH cDNA probe (lower bands) was performed for reference purposes. Each experiment was repeated at least four times using human ASM cells originating from different individuals.

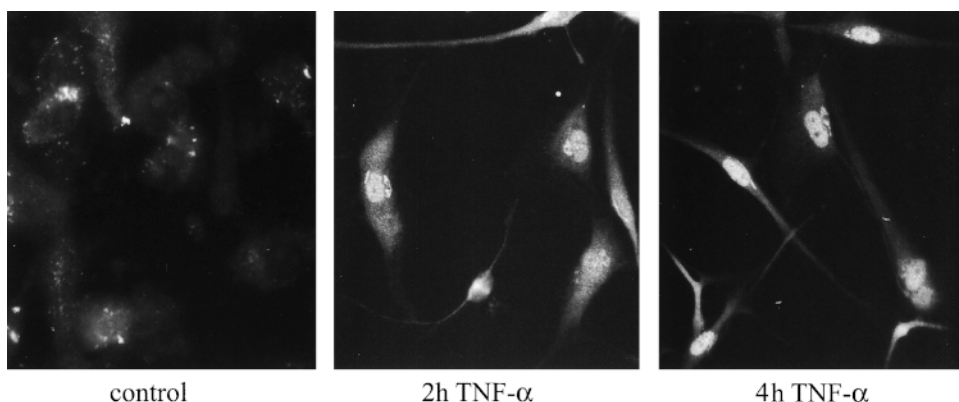
and secreted (RANTES), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (8–12). Thus, ASM cells may also act as “immune effector” cells in perpetuating airway inflammation. Similarly, our results suggest that human ASM may contribute directly to airway inflammation by interacting with TNF- $\alpha$ , a proinflammatory cytokine found to be upregulated in symptomatic asthmatic airways, by the production and release of the cytokine IL-6. Stimulation of human ASM cells with IL-1 $\alpha$ , transforming growth factor- $\beta_1$ , and CD40 ligand also leads to the production of IL-6 (18, 39). IL-6 is a 21-kD pleiotropic cytokine that is produced by a variety of cells, including airway epithelial cells (40), lung fibroblasts (41), macrophages and monocytes (42), and vascular smooth muscle cells (43). It has a number of proinflammatory properties that could be relevant in the development and perpetuation of airway inflammation during asthma, including the terminal differentiation of B cells into antibody-producing cells (44), upregulation of IL-4-dependent immunoglobulin E production (45), and stimulation of cytotoxic T-cell differentiation (46).

A potential mechanism for the induction of IL-6 gene expression could involve events such as the activation of



**Figure 10.** Northern blot analysis of c-fos expression in human ASM cells treated with TNF- $\alpha$  in the presence or absence of NDGA. Total RNA samples from TNF- $\alpha$ -treated (1 h) human ASM cells in the presence or absence of NDGA were subjected to Northern hybridization with radiolabeled c-fos probes as described in MATERIALS AND METHODS. A representative Northern blot for c-fos (2.2 kb mRNA, lower panel) is shown. Incubation conditions are indicated at the top of the panel. Rehybridization with a GAPDH cDNA probe (lower panel) was performed for reference purposes. Each experiment was repeated at least three times using human ASM cells originating from different individuals.

tions are indicated at the top of the panel. Rehybridization with a GAPDH cDNA probe (lower panel) was performed for reference purposes. Each experiment was repeated at least three times using human ASM cells originating from different individuals.



*Figure 11.* Immunocytochemical localization of C-JUN in TNF- $\alpha$ -treated human ASM cells. Immunoreactive C-JUN was localized in TNF- $\alpha$ -treated ASM cells using an affinity purified human C-JUN polyclonal antirabbit antibody and FITC-conjugated secondary antibody as described in MATERIALS AND METHODS. Translocation of C-JUN to the nucleus of human ASM cells after 4 h of stimulation with TNF- $\alpha$  was observed. Cells were fixed after treatment with serum-depleted medium (*left panel*,  $t = 0$ , control) or TNF- $\alpha$  (50 U/ml) for 2 h (*middle panel*) or 4 h (*right panel*), and then subjected to immunofluorescence staining.

the AP-1 transcription factor binding site on the IL-6 gene. The promoter region of the IL-6 gene has been shown to contain two AP-1 binding sites in addition to a number of other functionally important elements (25). The transcription factor AP-1 is a heterodimer constituting the oncoproteins resulting from *c-fos* and *c-jun* gene translation. AP-1 may be activated by various cytokines, including TNF- $\alpha$ , via different types of protein tyrosine kinases and mitogen-activated protein (MAP) kinases, which subsequently activate a cascade of intracellular kinases (47), leading to the upregulation of the IL-6 gene. We have shown in this study that the mRNAs for the *c-fos* and *c-jun* proto-oncogenes are upregulated by TNF- $\alpha$  stimulation of human ASM cells *in vitro*. Additionally, we observed that TNF- $\alpha$ -induced *c-fos* mRNA expression could be inhibited by NDGA. The subsequent induction of IL-6 mRNA and secretion of IL-6 protein were also inhibited. Furthermore, immunoreactive C-JUN was localized in the cytoplasm of cells treated with TNF- $\alpha$  for 2 h, and mainly in the nucleus of cells after 4 h of treatment, suggesting that the induced expression and translocation of AP-1 could play a role in the observed gene expression for IL-6 in human ASM cells.

As corticosteroids are known to have potent anti-inflammatory effects and are clinically effective in suppressing airway inflammation, we also determined whether dexamethasone was capable of inhibiting TNF- $\alpha$ -mediated expression and secretion of IL-6 by human ASM cells. We found that IL-6 gene expression and protein secretion by human ASM cells was significantly decreased but not completely abolished by treatment with dexamethasone. The precise mechanism of this downregulation has not been determined in ASM cells, but it has been demonstrated in HeLa cells to be due to the binding of ligand-activated glucocorticoid receptor to the transcriptional regulatory regions of the IL-6 promoter (22). Zitnik and coworkers (24) and Tobler and colleagues (48) suggested that dexamethasone exerts its action mainly by decreasing the stability of IL-6 mRNA through AUUUA-rich motifs in the 3' untranslated region. Dexamethasone has also been shown to inhibit the production of several other proinflammatory

cytokines, including GM-CSF, IL-8, and RANTES in human ASM cells (8, 10, 12). This suggests that ASM could be another important therapeutic target for the anti-inflammatory effects of steroids (49). Surprisingly, the inhibition by dexamethasone is variable for the previously mentioned cytokines. GM-CSF production by human ASM cells is completely abolished by 100 nM of dexamethasone, whereas the inhibition of RANTES and IL-8 production did not exceed 50% (8, 10, 12). In our experiments, we could decrease the effect of TNF- $\alpha$  on IL-6 production by 83% with 100 nM of dexamethasone.

The molecular basis of dexamethasone-mediated inhibition of GM-CSF gene expression has been reported to involve post-transcriptional destabilization of GM-CSF mRNA (48). On the other hand, inhibition of IL-6 and IL-8 gene expression and protein synthesis have been shown to involve both transcriptional inhibition and post-transcriptional destabilization mechanisms by dexamethasone (22, 24, 48, 50, 51). Kwon and associates (52) reported that dexamethasone did not affect the TNF- $\alpha$ -induced RANTES mRNA half-life in eosinophils, suggesting that purely transcriptional inhibition mechanisms are responsible. The degree of involvement of transcriptional inhibition and/or post-transcriptional destabilization mechanisms may account for the variation in the observed dexamethasone-induced inhibition in cytokine expression and secretion by human ASM cells.

CHX (100 nM), a protein synthesis inhibitor, significantly reduced, but did not abolish, IL-6 protein production by TNF- $\alpha$ -stimulated human ASM cells. It is very likely that 1  $\mu$ M of CHX was insufficient to completely inhibit *de novo* synthesis of IL-6. Also, we found that the increased release of IL-6 into the conditioned medium was due to the *de novo* synthesized IL-6 and not due to the release of preformed IL-6. This is evident from the ELISA data, which show that ASM cells store a negligible amount of IL-6.

To determine if the remaining protein production was a result of an inhibited mRNA degradation pathway, ASM cells were incubated with either TNF- $\alpha$ , CHX, or a combination of TNF- $\alpha$  and CHX, and IL-6 mRNA expression was assessed by Northern blot analysis. We found that in

CHX-treated (1  $\mu$ M) human ASM cells, IL-6 mRNA was expressed to the same extent as in TNF- $\alpha$ -treated cells. The combined treatment with CHX and TNF- $\alpha$  led to an almost threefold increase in mRNA for IL-6 compared with treatment with either CHX or TNF- $\alpha$  alone. Our results suggest that the inhibition of protein synthesis by CHX may also involve the inhibition of mRNA degradation pathway via a limited synthesis of RNase activity, as advocated previously (40). This can lead to an accumulation of IL-6 mRNA in human ASM cells, which can subsequently lead to higher levels of IL-6 protein in the presence of CHX.

Radiolabeled thymidine incorporation demonstrated that TNF- $\alpha$  (50 U/ml) had no immediate mitogenic effect on human ASM cells in this study, in agreement with Belvisi and coworkers (53) but in contrast to work published by Amrani and colleagues (7). This inconsistency may be explained by taking into account that Amrani and coworkers (7) included 0.3 or 3% serum in their culture medium, whereas our culture medium was completely free of serum. TNF- $\alpha$  and growth factors (present in serum) activate MAP kinases, MAP kinases being key transducers of extracellular signals in proliferation and differentiation pathways. Signals activated by these varying stimuli may converge to induce proliferation in a synergistic manner, possibly explaining the proliferative response to TNF- $\alpha$ . Also, Stewart and colleagues (6) found a mitogenic response to TNF- $\alpha$  at very low concentrations (0.3 to 30 pM) in human ASM cells, whereas at a higher concentration (300 pM), this mitogenic effect was abolished. De and coworkers (5) have reported that IL-6 causes hyperplasia and hypertrophy of cultured guinea pig ASM cells at concentrations comparable to those present in the conditioned medium of TNF- $\alpha$ -treated cells in this study. We found, however, that human recombinant IL-6 at concentrations ranging between 1 and 50 ng/ml was not mitogenic for human ASM cells. Therefore, it could be stated here that the continuing time-dependent accumulation of IL-6 in the cell-conditioned media in this study did not exhibit an autocrine mitogenic effect on the cells. Apparently, the upregulation of c-fos and c-jun, proteins forming the AP-1 transcription factor that is normally associated with cellular proliferation, is not sufficient to cause human ASM cells to proliferate but is probably involved in other processes, such as regulating the inflammatory response through upregulation of proinflammatory cytokines such as IL-6 (47).

A number of cells involved in the inflammatory response in asthma are important sources of proinflammatory cytokines such as TNF- $\alpha$  and come into close proximity to the smooth muscle layer. They may be capable of releasing concentrations of TNF- $\alpha$  that can stimulate AP-1 regulated expression and release of IL-6 from cells. Our data suggest a potentially important role for TNF- $\alpha$  and smooth muscle-derived IL-6 in airway inflammation in asthma. The precise role of IL-6 released from human ASM in the pathogenesis of asthma and/or other pulmonary disorders is unclear, but the capacity of ASM to produce IL-6 suggests that the cells could participate in perpetuating or regulating local inflammatory events in the airways.

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