

Chronic exposure to TNF- α increases airway mucus gene expression *in vivo*

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Background: Hypersecretion of mucus plays an important role in the pathogenesis and severity of asthma. The primary proteins in mucus are mucin glycoproteins; MUC-5AC is the primary airway mucin gene. The calcium chloride-activated channel gene hCLCA1 (gob-5 in the mouse) has been suggested to increase MUC-5AC gene expression, and both are increased in asthmatic patients and murine models. TNF- α increases the expression of these genes *in vitro* but has not been investigated *in vivo*.

Objective: We sought to determine whether TNF- α increases gene expression of gob-5 and MUC-5AC and induces mucus cell metaplasia *in vivo*.

Methods: Naive BALB/c mice received 50 ng of recombinant murine TNF- α (rmTNF- α) intratracheally daily for 1, 2, or 3 weeks; another group received the same dose of intratracheal rmTNF- α daily for 3 weeks and then alternate-day treatment for 3 additional weeks (total of 6 weeks). AKR mice received 50 ng of rmTNF- α intratracheally for 3 or 6 weeks daily. Naive nontreated mice were used as control animals. Airway gene products for gob-5 and MUC-5AC were determined by means of real-time PCR. Lung tissue sections were stained with periodic acid-Schiff/Alcian blue to assess mucus cell metaplasia.

Results: rmTNF- α significantly increased gene expression of airway gob-5 and MUC-5AC after 2 weeks in the BALB/c mice. There was noticeable mucus staining in all mice treated for at least 3 weeks with TNF- α and in 80% of the mice receiving 2 weeks of treatment. After 3 weeks of treatment, the AKR mice also showed increased gob-5 expression.

Conclusions: This study demonstrates for the first time that TNF- α alone *in vivo* is sufficient to increase airway mucus gene expression in 2 murine strains. (J Allergy Clin Immunol 2005;116:1256-63.)

Key words: Asthma, mucus, MUC-5AC, gob-5, TNF- α

Abbreviations used

AB:	Alcian blue
APTI:	Airway pressure time index
BALF:	Bronchoalveolar lavage fluid
EGF:	Epidermal growth factor
GAPDH:	Reduced glyceraldehyde-phosphate dehydrogenase
PAS:	Periodic acid-Schiff
rmTNF- α :	Recombinant murine TNF- α

Hypersecretion of mucus plays an important role in the pathogenesis of many asthma phenotypes and has been linked with disease fatality.^{1,2} Pathologic examination of patients dying from acute asthma frequently reveals mucosal plugging of airway lumens.³ In addition, decreasing FEV₁ has been shown to be independently associated with a history of sputum production, suggesting that increased mucus production increases asthma severity.⁴ The primary proteins in mucus are the mucin glycoproteins (mucins) and are produced by goblet cells and submucosal glands.⁵ Mucins are encoded by specific genes (MUC genes), of which several have been identified to date and are named numerically (ie, MUC-1, MUC-2, and MUC-4). MUC-5AC is the predominant MUC gene in the airway. Animal models of asthma and tissue samples from asthmatic patients have demonstrated that airway MUC-5AC is increased compared with levels seen in control animals.⁶⁻⁹ An additional gene, hCLCA1 (also known as gob-5 in the mouse), which regulates calcium-activated chloride conductance, has been shown to induce mucin gene expression (MUC-5AC) in the human mucopidermoid cell line NCI-H292 and in airway explant tissue,^{10,11} thereby potentially acting as a preliminary step in mucus production. Several studies have demonstrated increased expression of hCLCA1 or gob-5 in asthmatic patients and animal models of asthma.^{10,12}

The cytokines IL-4, IL-5, IL-9, and IL-13 have been shown to induce mucus gene expression and mucus cell hypersecretion *in vitro* and *in vivo*.¹³⁻¹⁷ However, the role of TNF- α alone in mucus cell metaplasia has not been extensively studied and has never been investigated *in vivo*. Furthermore, most work has focused on the study of MUC-5AC and not gob-5. Some groups have demonstrated that TNF- α increases MUC-5AC and, more recently, gob-5 expression in cell culture.^{11,18} The purpose of this study was to examine the role of

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TNF- α on airway mucus cell metaplasia and the expression of gob-5 and MUC-5AC *in vivo* by using 2 different murine strains.

METHODS

Mice and reagents

Six week-old BALB/c and AKR mice were purchased from Jackson Laboratory (Bar Harbor, Me) and maintained in the animal facility at Mount Sinai School of Medicine. Standard guidelines for laboratory animal care were followed,¹⁹ and institutional permission for animal handling was approved through our animal facilities. The mice were housed in plastic cages and maintained on a 12-hour diurnal cycle. Food and water were provided *ad libitum*. Mouse rectal temperatures were determined by using BAT-12 (Physitemp Instruments, Clifton, NJ). Murine recombinant TNF- α was purchased from R&D Systems (Minneapolis, Minn). A rapid endotoxin detection kit (Pyrostat) was purchased from Associates of Cape Cod, Inc (Falmouth, Mass). Antibodies for ELISA were purchased from R&D Systems.

TNF- α administration

Mice were administered intratracheal recombinant murine TNF- α (rmTNF- α) by using different protocols. We first determined the kinetics of gob-5 and MUC-5AC increased expression after intratracheal rmTNF- α to determine the optimal time for death after the last rmTNF- α dose. Six-week-old BALB/c mice were given a 1-time intratracheal instillation of 500 ng of rmTNF- α and killed 6, 12, 24, or 48 hours after administration ($n = 5$ per group). Five naive mice were killed to determine baseline gene expression. RT-PCR was then performed to determine the expression of these gene products in lung tissues. In a subsequent protocol, 6-week-old BALB/c mice received 50 ng of rmTNF- α intratracheally daily for 1 week ($n = 5$), 2 weeks ($n = 5$), or 3 weeks ($n = 6$); another group received 50 ng of intratracheal rmTNF- α for 3 weeks daily, with subsequent same-dose alternate-day treatments for an additional 3 weeks ($n = 6$; total of 6 weeks of rmTNF- α treatment). In the final protocol, 6-week-old AKR mice were given 50 ng of intratracheal rmTNF- α daily for 3 ($n = 6$) or 6 ($n = 5$) weeks. Intratracheal administration was performed by anesthetizing the mice with a mixture of ketamine and xylazine and suspending them on a 60° incline board by their upper incisors. Treatment was delivered through a soft cannula placed in the distal part of the oropharynx, with the animal's tongue extended by a flat tweezers. Age- and species-matched naive mice served as control animals for all protocols ($n = 5$ per group). We had previously performed intratracheal PBS challenge in naive mice and found no significant difference in bronchoalveolar lavage fluid (BALF) cell numbers or differential cell counts (unpublished data) when compared with those of naive mice. The rmTNF- α preparation was made in sterile PBS, and each mouse received preparations in a total volume of 50 μ L, which was determined to be optimal for distribution to both lobes of the lung.²⁰⁻²² The level of endotoxin in the rmTNF- α stock (100 ng/ μ L) and working concentration (1 ng/1 μ L) were determined by using the rapid endotoxin kit (Associates of Cap Cod, Inc), which detects endotoxin levels of greater than 0.25 EU/mL.

Airway pressure recording

Twelve hours after the last rmTNF- α intratracheal challenge (optimal time determined on the basis of kinetic studies), airway responsiveness to acetylcholine was measured as previously described.²³ Mice were anesthetized intraperitoneally with pentobarbital (80 mg/kg), and a 16-gauge cannula was placed in the trachea. Mice were ventilated with a constant tidal volume of 0.2 mL at 120 breaths/min. Decamethonium bromide (25 mg/kg) was administered

into the inferior vena cava to eliminate muscle movements. Airway pressure was measured with a pressure transducer through a port in the tracheal cannula. A stable baseline was recorded for at least 2 minutes, and acetylcholine (50 μ g/kg) was injected into the inferior vena cava; the recording was continued for at least 4 minutes. Airway responsiveness was estimated as the time-integrated change in peak airway pressure, referred to as airway pressure time index (APTI).

BALF preparation and cell differential counts

After airway pressure recording, the chest was opened. The right main-stem bronchi was occluded by a forceps, the left lung underwent lavage with 0.5 mL of cold HBSS (without calcium or magnesium), and the BALF was collected into chilled tubes. The BALF was centrifuged, and the supernatant was transferred and stored at -80°C for cytokine measurement by means of ELISA. The cell pellet was resuspended in HBSS, and slides were prepared by means of cytocentrifugation of samples at 700 rpm for 7 minutes. Total cell numbers were determined with a hemocytometer. The slides were then stained with Diff-Quick (Dade Diagnostics of PR, Aguada, Puerto Rico), and cell differential counts were obtained by counting at least 500 cells per slide by means of light microscopy. Cytokine ELISA was performed according to the manufacturer's directions (R&D systems).

Lung histology

Lung tissue was surgically removed and separated from the trachea after BALF collection. The left lower lobe was fixed in neutral-buffered formaldehyde, and 5-mm paraffin sections were stained with periodic acid-Schiff (PAS)/Alcian blue (AB) for evaluation of goblet cells. (AKR mice lung samples were stained with PAS alone.) PAS/AB-positive mucosubstances were graded by using the semiquantitative analysis developed by Finotto et al²⁴: 0, no mucosubstances; 1, mucosubstances detectable; 2, high amount of mucosubstances. Slides were examined in a blinded fashion in regard to length of TNF- α treatment. This grading system was used to calculate a mean mucus index score for each group. In addition, lung sections were stained with hematoxylin and eosin.

RNA extraction and reverse transcription

After BALF collection, the left upper and middle lobes were placed in TRIZOL (Invitrogen, Carlsbad, Calif) and frozen at -80°C until RNA extraction. To extract RNA, samples were thawed at 4°C , homogenized, and then extracted according to the manufacturer's directions.

Semiquantitation of PCR products

RT-PCR was performed on lung specimens obtained from the kinetic studies to determine the optimal time for death after TNF- α administration. Optimal time was defined as the time in which the maximal increase in gob-5 and MUC-5AC gene expression developed. RT-PCR was performed in a total volume of 50 μ L by using either 6 μ g of total cDNA as a template (gob-5 and MUC-5AC) or 4 μ g of total cDNA (reduced glyceraldehyde-phosphate dehydrogenase [GAPDH]). Primers used were as follows: gob-5 (sense 5'-GGAGCTGCAGGATGGAATC-3' and antisense 5'-GACTGGTTGATTTCTGCGCTG-3', 2779 bp, NCBI accession number NP 059502²⁵); MUC-5AC (sense 5'-GTGCAGGGCTCAGTTCCTTC-3' and antisense 5'-TGGTCTCTGTTTCGTGCTG-3', 224 bp, NCBI accession no. L42292); and GAPDH (sense 5'-GGTGAAGGTCGGTGTGAACGGATTTT-3' and antisense 5'-TGGACCTGTGGTTGTGACCTCAAACTTGGC-3', 500 bp). PCR products were examined by means of electrophoresis on a 1.5% agarose gel. Data were processed with the Gel Doc 2000 (Bio-Rad, Hercules, Calif) and Quantity One 4.2.1 image software (Bio-Rad, Hercules,

Calif), and relative quantification of gob-5 and MUC-5AC was determined by means of optical density readings and compared with the optical density of the control gene GAPDH.

Quantitative real-time PCR

Real-time reverse transcription quantitative PCR was performed by using a previously published SYBR green protocol with ABI7900 HT (Applied Biosystems, Foster City, Calif).²⁶ Each transcript in each sample was assayed 3 times, and the median threshold cycle was used to calculate the fold change values and control changes for each gene. Three housekeeping genes were used for global normalization in each experiment (β -actin, RPS11, and α -tubulin). Data validity by means of modeling of reaction efficiency and analysis of measurement precision was described previously.²⁷ Primer sequences for these reactions were as follows: gob-5 (sense 5'-TCCAGGCTGTGGATAAGTCC-3' and antisense 5'-TATCTTCA GCACGTGGATGC-3'); MUC-5AC (sense 5'-CATGGAGGGGA CCTGGAAC-3' and antisense 5'-CCACATGGGGTCACACCTTC-3'); IL-5 (sense 5'-GGGCTTCCTGCTCTATCTA-3' and antisense 5'-CAGTCATGGCACAGTCTGAT-3'); IL-13 (sense 5'-GTCCACACAGGGCAACTGAG-3' and antisense 5'-GGGCA TAGGCAGCAAACCAT-3'); RPS11 (sense 5'-CGTGACGAAGA TGAAGATGC-3' and antisense 5'-GCACATTGAATCGCACAG TC-3'); α -tubulin (sense 5'-TGCCTTTGTGCACTGGTATG-3' and antisense 5'-CTGGAGGAGTTTGACGACAC-3'); and β -actin (5'-AGGTGACAGCATTTGCTTCTG-3' and antisense 5'-GCTG CCTCAACACCTCAAC-3'). The quantitative expression of IL-5 and IL-13 was determined to investigate whether TNF- α instillation induced expression of these genes, which have also been demonstrated to induce mucin gene expression.^{16,17}

Statistical analysis

Statistical analyses were performed with the Student *t* test for comparison between 2 groups. Pearson correlation coefficients were used to determine correlation values. A *P* value of less than .05 was considered statistically significant. All statistical analyses were performed with SigmaPlot/Stat software (Systat Software, Inc, Point Richmond, Calif).

RESULTS

Gob-5 and MUC-5AC expression peaks at 12 hours after rmTNF- α administration

A single 500-ng dose of rmTNF- α was instilled intratracheally in naive BALB/c mice. After death of mice 6, 12, 24, or 48 hours later, RT-PCR was performed to determine lung gob-5 and MUC-5AC expression. (Lung samples were also collected from naive mice to determine baseline gene expression.) Data were normalized to GAPDH. Baseline mRNA expression was 0 and 0.29 for gob-5 and MUC-5AC, respectively. The mRNA expression of these genes peaked 12 hours after TNF- α instillation (gob-5 = 0.46 [*P* < .001] compared with naive mice; MUC-5AC = 0.852 [*P* < .05] compared with naive mice). By 48 hours, the mRNA levels decreased to 0.21 for gob-5 (*P* < .05 compared with the 12-hour sample and *P* < .001 compared with baseline) and 0.29 for MUC-5AC (*P* < .05 compared with the 12-hour sample but not significantly different from baseline). In our subsequent protocols, we killed the mice 12 hours after the last rmTNF- α instillation.

Goblet cell metaplasia in airways of mice treated with rmTNF- α

After 1, 2, 3, and 6 weeks of treatment with intratracheal rmTNF- α in the BALB/c mice, goblet cell metaplasia was observed in 2 of 5, 4 of 5, 5 of 5, and 5 of 6 mice, respectively. The PAS/AB staining was most pronounced in the mice receiving 3 weeks of daily rmTNF- α (Fig 1, A). Airways in 2 of the 3-week-treated and 3 of the 5 six-week-treated AKR mice contained PAS-positive mucus cells. Fig 1, B through D, shows PAS/AB-stained lung sections from 3-week and 6-week rmTNF- α -treated and control BALB/c mice, and Fig 1, E through G, shows 3-week-treated and 6-week-treated and control AKR mice. Examples of different grades of PAS/AB staining are shown in the figures and noted in the legends. No PAS/AB-positive cells were detected in either the BALB/c or AKR control lungs. (We did not detect endotoxin at a concentration >0.25 EU/mL in either our stock or working rmTNF- α preparations.)

Cellular inflammation increased in rmTNF- α -treated lungs

BALB/c mice treated with at least 2 weeks of rmTNF- α had significantly increased BALF total cell counts (Fig 2, A). Similarly, after 3 weeks of treatment, AKR mice had increased BALF total cell counts (Fig 2, B). The differential cell counts did not significantly differ between the TNF- α -treated BALB/c groups and the naive mice (Fig 2, C); however, the percentage of neutrophils increased in the lungs of treated AKR mice (Fig 2, D). Because of increased total cell numbers in the BALB/c mice treated with longer courses of TNF- α , the total neutrophil count increased (ie, from 1.4×10^3 in the control mice to 5.4×10^3 in the 6-week-treated mice). No eosinophils were present in the BALF from either strain. In accord with BALF data, lungs from the BALB/c mice treated with at least 2 weeks of rmTNF- α had increased inflammatory cells on hematoxylin and eosin staining (data not shown).

gob-5 and MUC-5AC expression in rmTNF- α -treated mice

Intratracheal administration of rmTNF- α significantly increased gob-5 and MUC-5AC expression in the BALB/c mice after at least 2 weeks of treatment, which continued to increase with length of treatment (Fig 3, A). In the AKR mice gob-5 gene expression increased 23.2- and 28.2-fold, and MUC-5AC expression increased 2.57- and 3.44-fold compared with that seen in the nontreated mice for the 3- and 6-week-treated groups, respectively (Fig 3, B).

Effect of rmTNF- α administration on airway hyperresponsiveness

Twelve hours after the last rmTNF- α administration, airway hyperresponsiveness was determined by measuring the airway pressure changes over time after acetylcholine challenge; the results are expressed as APTI. APTI levels of BALB/c untreated mice compared with those of 6-week rmTNF- α -treated mice were 266.44 ± 97.1 and

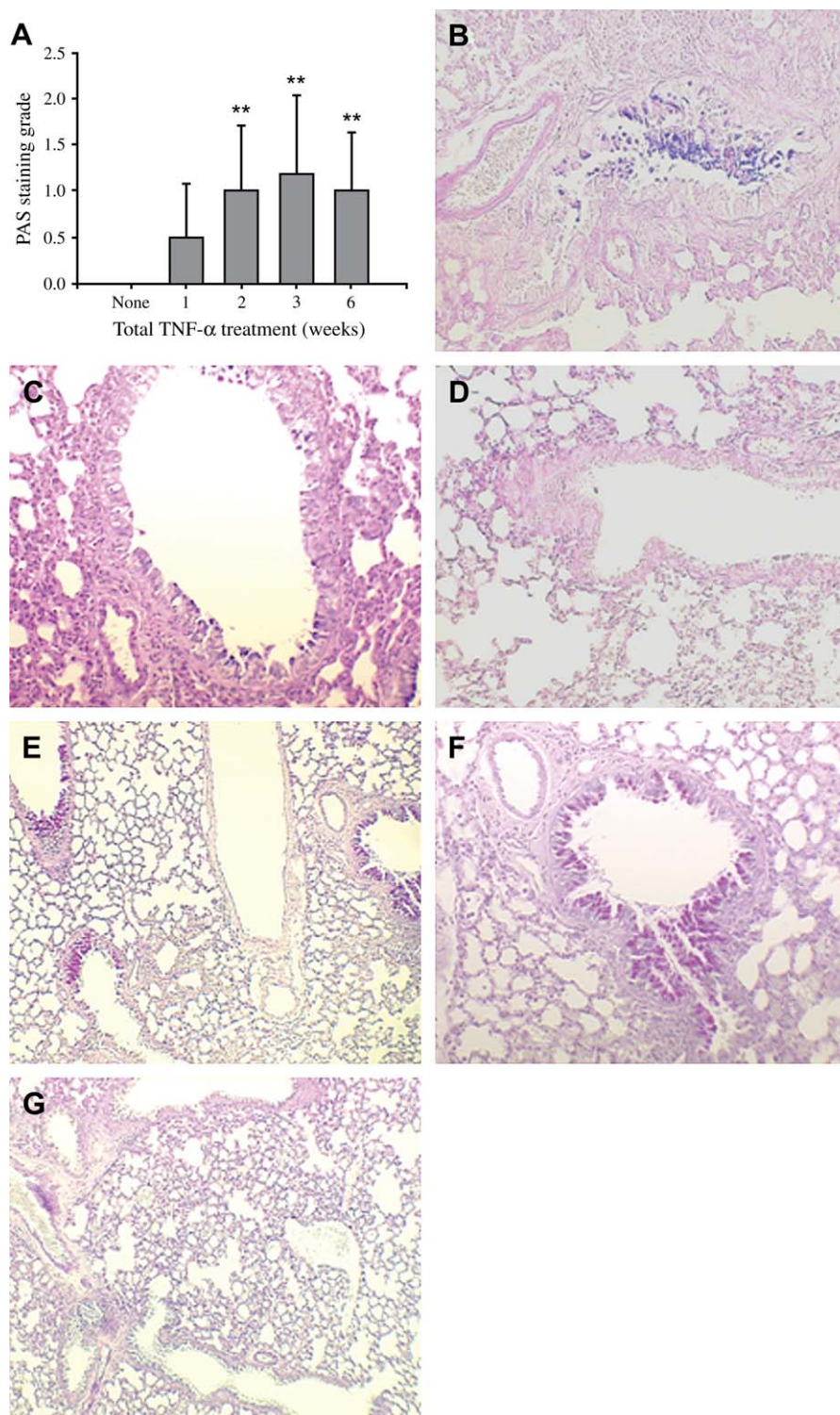


FIG 1. Goblet cell metaplasia. **A**, Right upper lung PAS/AB staining was measured semiquantitatively for the BALB/c mice (described in the Methods section, mean $\pm$ SD). **B-G**, Mucus (deep purple) for 3-week-treated (Fig 1, **B**, grade 1) and 6-week-treated (Fig 1, **C**, grade 2) BALB/c mice, 3-week-treated (Fig 1, **E**, grade 2) and 6-week-treated (Fig 1, **F**, grade 2) AKR mice, and control BALB/c (Fig 1, **D**) and AKR (Fig 1, **G**) mice. Magnification: 20 $\times$ for Fig 1, **B**, **C**, **D**, and **F**, and 10 $\times$ for Fig 1, **E** and **G**. ** $P < .01$ compared with control mice.

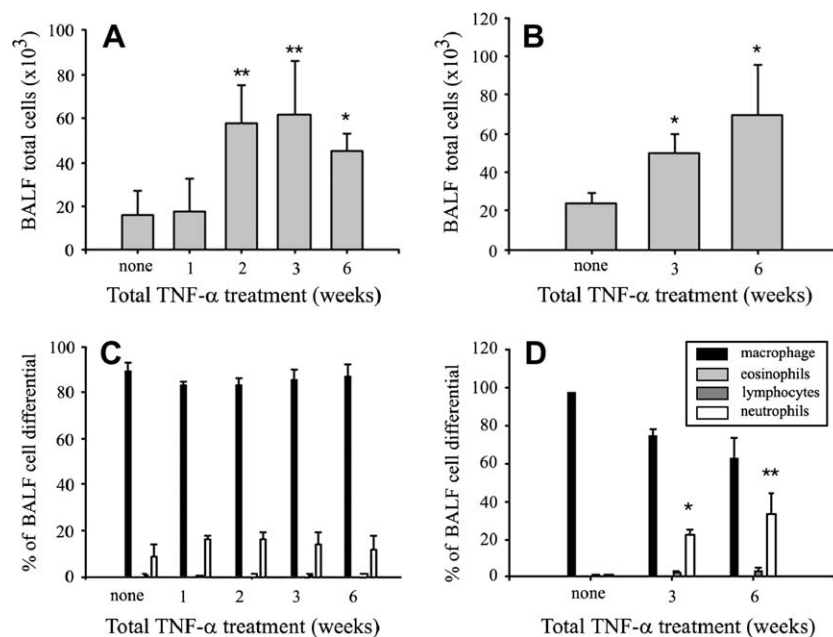


FIG 2. Effect of rmTNF- α on pulmonary inflammation. After death, lungs underwent lavage, total leukocytes were counted, and BALF was cytocentrifuged on slides, fixed, and stained for leukocyte differential. **A** and **B**, Total BALF cell numbers for BALB/c (**Fig 2, A**) and AKR (**Fig 2, B**) mice. **C** and **D**, Leukocyte BALF differential from BALB/c (**Fig 2, C**) and AKR (**Fig 2, D**) mice. Data are presented as means $\pm$ SD. * P < .05 and ** P < .01 compared with control mice.

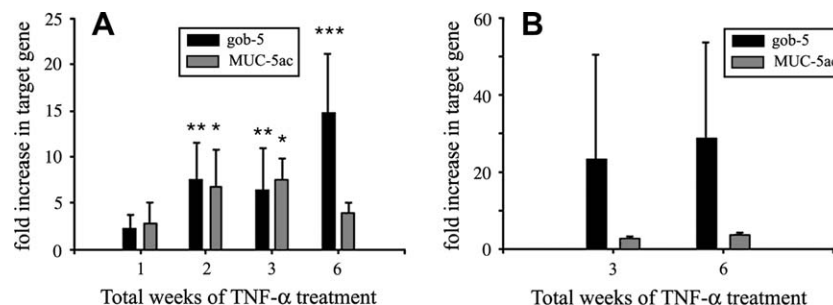


FIG 3. Real-time quantitative PCR for mucus genes. Quantitative PCR was performed by using primers specific for gob-5 and MUC-5AC and normalized to control genes (β -actin, α -tubulin, and RPS11). Specific mRNA levels are expressed as a fold increase in copy number compared with that seen in control nontreated BALB/c (**A**) and AKR (**B**) mice. Results are expressed as means $\pm$ SD. * P < .05, ** P < .01, and *** P < .001 compared with 1 week of treatment.

415.73 $\pm$ 76.9 cm H₂O/s, respectively (P = .05). In the AKR mice who were not treated or received 3 or 6 weeks of total rmTNF- α instillation, the APTI levels were 383.3 $\pm$ 14.6, 400.9 $\pm$ 37.4, and 536.56 $\pm$ 27.07 cm H₂O/s, respectively (P < .01 in the nontreated versus 6-week-treated rmTNF- α mice).

rmTNF- α does not increase BALF supernatant and lung tissue IL-4, IL-5, or IL-13 levels

Because TNF- α might increase expression of other cytokines important in mucus gene expression, we measured IL-4, IL-5, and IL-13 levels in the BALF supernatant from the BALB/c rmTNF- α -treated and control mice. Quantification of IL-4, IL-5, and IL-13 from the BALF supernatant by means of enzyme immunoassay revealed

that rmTNF- α did not significantly increase any of these cytokines in the BALB/c mice treated with TNF- α compared with levels seen in nontreated control animals. To look at lung tissue gene expression, we measured IL-5 and IL-13 levels in the AKR mice by means of real-time PCR. There was no significant change of these cytokines from the nontreated AKR mice compared with the TNF- α -treated mice (data not shown).

rmTNF- α administered at a dose of 50 ng daily was tolerated without significant temperature changes or weight loss

Because high concentrations of TNF- α might produce fever and cachexia, we followed these parameters in our BALB/c mice. Rectal temperatures at baseline; 30, 60, and

90 minutes; and 3, 8, 12, and 24 hours after the first dose of 50 ng of intratracheal rmTNF- α in 5 BALB/c mice demonstrated no significant difference compared with those in mice that had received a 1-time intratracheal PBS treatment. The temperature initially decreased after anesthesia administration but returned to baseline by the 4-hour time point. A group of BALB/c mice ($n = 4$) was followed for body weight after a total of 6 weeks of rmTNF- α treatment. Compared with age-matched naive nontreated mice, there was no significant difference in body weight by the end of the time period.

DISCUSSION

Mucus hypersecretion increases asthma morbidity and mortality. Reducing mucus production could therefore be an important adjunctive therapy for asthma. However, the mechanisms of initiation and regulation of mucus production have not been fully defined. The cytokines IL-4, IL-5, IL-9, and IL-13 increase the expression of hCLCA1 (or gob-5 in the murine system) and MUC-5AC.¹³⁻¹⁶ Inhibition of hCLCA1 *in vitro* suppressed the expression of MUC-5AC, suggesting that the former gene might be one of the first steps in mucus cell metaplasia-hyperplasia, inducing mucin gene expression.¹⁰ Previous *in vitro* studies showed that TNF- α can induce mucin-producing genes and mucus cell development.^{11,18,28} Antigen challenge of sensitized mice or human asthmatic subjects results in increased TNF- α expression in BALF, peripheral blood, and tissues biopsy specimens.²⁹⁻³³ In this study we have, for the first time, demonstrated that TNF- α induces mucus cell metaplasia and increases expression of genes important for this *in vivo*.

This study demonstrated that TNF- α alone increases mucus gene expression. Our study design is unique in that we examined the chronic exposure of rmTNF- α in the murine system. We chose this approach because asthma is a chronic disease, and even in asymptomatic periods there is evidence of ongoing airway inflammation and cytokine production. Our results in the BALB/c mice demonstrated that the expression of gob-5 and MUC-5AC increased after 2 weeks of daily rmTNF- α administration and remained increased at 3 weeks. Of note, the gob-5 expression increased after the daily 3-week and additional 3-week alternating intratracheal instillation time period, even though the MUC-5AC expression decreased. This might be a result of our protocol, in which during the last 3 weeks of this time period, rmTNF- α was given on alternating days. Other studies have demonstrated that after removal of antigen challenge in a sensitized mouse, mucus cell staining can decrease.³⁴ Furthermore, the half-life of rmTNF- α is short. Although our primary end point was mucus gene expression, we also noted that the mucus cell grade decreased at the 6-week time point. These results might suggest that although gob-5 is important in mucus gene expression, the expression of MUC-5AC might correlate better with histologic findings, perhaps because gob-5 is expressed before MUC-5AC.

Administration of rmTNF- α to AKR mice increased gob-5 and MUC-5AC expression. We chose to study an additional murine strain because inbred mice differ in their ability to mount physiologic, pathologic, and inflammatory airway changes. BALB/c and AKR strains experience many of the pathologic airway features of asthma (BALF eosinophilia, mucus cell metaplasia, and airway hyperresponsiveness) after antigen sensitization and challenge and are frequently used as models in asthma.³⁵⁻³⁷ Demonstrating the effect of TNF- α in 2 different strains strengthens our observations. The MUC-5AC expression increased more in the BALB/c mice than in the AKR mice after rmTNF- α treatment. This might also be consistent with MUC-5AC correlating better with mucus cell metaplasia (as determined on the basis of histology) than gob-5. The histologic evaluation of these AKR mice demonstrated that fewer of the mice had positive mucus staining compared with the BALB/c mice but that rmTNF- α alone was still able to induce mucus cell metaplasia. (We wish to emphasize that our primary end point was gene expression.) That there were differences in MUC-5AC expression and mucus histology in the AKR and BALB/c mice after rmTNF- α treatment is not entirely clear, but this might be strain related.

The mechanisms of TNF- α regulation of mucus cell metaplasia might be through direct upregulation of gob-5, MUC-5AC, or both. Borchers et al³⁸ demonstrated that TNF- α exposure increased MUC-5AC mRNA half-life through transcript stabilization. TNF- α has also been shown *in vitro* to increase the expression of inducible nitric oxide synthase, which in turn produces nitric oxide.^{39,40} Nagaki et al⁴¹ demonstrated that the methacholine-induced increase in mucus glycoprotein secretion from feline and human airway submucosal glands was significantly inhibited by the nonselective nitric oxide synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME).

There could also be indirect effects of TNF- α on mucus gene expression. TNF- α might increase cellular recruitment to the airways through its ability to induce intercellular adhesion molecule 1 expression.³² Our data showed that the total BALF cell count was increased in both of the murine strains treated with TNF- α and that the total percentage of polymorphonuclear cells increased in the AKR mice. TNF- α has also been shown to activate neutrophils⁴² and eosinophils,⁴³ which could induce mucus gene metaplasia. We found no BALF eosinophils, but there was an increase in neutrophil number in both the BALB/c and AKR strains. Neutrophils might produce mucus metaplasia through several mechanisms, including release of neutrophil elastase, which has been shown in mucus induction.⁴⁴ TNF- α might induce or act synergistically with other proinflammatory cytokines involved in the pathogenesis of asthma, including IL-4, IL-5, and IL-13,⁴⁵ which have also been demonstrated to upregulate mucin gene expression.^{13,16,17} We found in our protocol that administration of TNF- α to naive AKR mice did not increase IL-5 or IL-13 expression from lung tissue or IL-4, IL-5, or IL-13 levels in BALF supernatants from the BALB/c mice. However, to exclude the possibility

of TNF- α inducing production of other cytokines, it might be necessary to perform our protocols in IL-5 or IL-13 knockout mice.

Finally, TNF- α might indirectly increase mucus gene expression through its actions with the epidermal growth factor (EGF) and its receptor.^{46,47} TNF- α increased expression of epidermal growth factor receptor, which colocalized with MUC-5AC protein (on the basis of immunohistochemistry) and areas positive for PAS/AB staining in human bronchi.⁴⁸ Nadel⁴⁶ instilled a 1-time dose of TNF- α into naive rats along with EGF and noted increased PAS/AB staining in the animals compared with that seen in those given EGF alone. This group did not comment on the effects of TNF- α alone, and this study differs from our study in that it was administered once and not chronically. Our study also measured airway hyperresponsiveness and noted that with rmTNF- α treatment, the APTI measurements increased. This AHR could be explained by several factors, including direct muscle airway contraction by the rmTNF- α ,⁴⁹ recruitment of neutrophils into the airway, or increased mucus production occluding the airways.

A relationship between TNF- α and mucus cell metaplasia has potential clinical importance in the treatment of asthma, especially as an adjunctive therapy. Anti-TNF- α antibody treatment has been successfully used in other inflammatory diseases, including inflammatory bowel disease and rheumatoid arthritis, and there is currently a study to determine the effects of anti-TNF- α on the treatment of asthma.

In conclusion, the cytokine TNF- α upregulates important genes in mucus cell regulation, gob-5 and MUC-5AC, and induces mucus cell metaplasia in a murine system. The mechanisms are not entirely clear, and further studies are necessary to investigate the role of TNF- α in direct and indirect induction of mucus metaplasia. Furthermore, our results also suggest the importance of work looking at its regulation as a new treatment modality in asthma, in particular interfering with TNF- α receptor binding.

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