

Downregulation of a disintegrin and metalloproteinase 33 by IFN- γ in human airway smooth muscle cells

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Background: A disintegrin and metalloproteinase 33 (ADAM33) has been identified as a susceptibility gene for asthma. ADAM33 is expressed in airway smooth muscle (ASM) cells and is suggested to play a role in the function of these cells. However, there is little information on the regulation of ADAM33.

Objective: To investigate whether ADAM33 is more highly expressed in ASM cells of patients with asthma than in those of normal subjects, and whether there is any inflammatory mediator (asthma-related cytokine/chemokine) that could modulate the expression of ADAM33 in ASM cells.

Method: smRNA and protein expression of ADAM33 in bronchial biopsy specimens was investigated (*in situ* hybridization and immunohistochemistry). Effects of cytokines on expression of ADAM33 in cultured human ASM cells were evaluated by measuring mRNA (real-time RT-PCR) and protein (Western blotting).

Results: ADAM33 mRNA and protein in biopsied specimens were more highly expressed in ASM cells of patients with asthma than in cells of normal subjects. Cultured ASM cells expressed ADAM33 at both the mRNA and the protein levels. IFN- γ reduced the mRNA expression dose-dependently and time-dependently, whereas IL-4 and IL-13 or chemokines did not affect the expression. The reduction by IFN- γ was partially restored by U0126, inhibitor for mitogen-activated protein kinase kinase 1/2, suggesting a role for extracellular signal-regulated kinase pathway. Further studies using cycloheximide and actinomycin-D suggested that the downregulation was at the transcriptional level.

Conclusion: The expression of ADAM33 by ASM cells is increased in patients with asthma, and its expression may be regulated by IFN- γ .

Clinical implications: IFN- γ might have a role in suppressing ADAM33 in ASM cells. (J Allergy Clin Immunol 2006;117:1016-1022.)

Key words: Asthma, airway remodeling, airway smooth muscle, a disintegrin and metalloproteinase 33, IFN- γ , extracellular signal-regulated kinase pathway

Bronchial asthma is a chronic airway disease characterized by excessive contraction of airway smooth muscle, inflammation of the airway mucosa, and bronchial hyper-responsiveness (BHR). BHR is likely related to damage of airway epithelial cells, hyperplasia of airway smooth muscle (ASM) cells, and deposition of fibrous tissue.¹ Activation of the epithelial-mesenchymal tropic unit in the airway, by interacting with cells which produce T_H2 cytokines, is proposed to lead to proliferation of ASM cells, BHR, and airway remodeling.² However, the mechanisms and the type of mediators that regulate the increased mass of ASM are not fully understood.

A disintegrin and metalloproteinase (ADAM)-33, a recently discovered ADAM family member, has been identified as an asthma susceptibility gene on chromosome 20p13 through a genetic linkage and association study of families with asthma and BHR.³ ADAM33 is a highly polymorphic gene containing more than 70 single nucleotide polymorphisms,^{3,4} and some of them are shown to be associated with BHR and asthma.^{3,5-7} Some of the significant single nucleotide polymorphisms were located in noncoding regions and are suggested to affect alternative splicing, splicing efficiency, or mRNA turnover.³

ADAM proteins are a family of type I transmembrane proteins with a domain containing an N-terminal secretion signal sequence and the following domains: pro-, metalloprotease, disintegrin-like, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane, and cytoplasmic domains.⁸ In ADAM33, 2 isoforms of ADAM33, α and β isoforms, have been first described.⁹ The β isoform lacks 26 amino acids between a cysteine-rich domain and an EGF-like domain. The cysteine-rich domain links ADAM proteins to cell surface molecules,¹⁰ which may facilitate cell-cell and cell-matrix interactions, and the EGF-like domain supposedly plays a role in membrane fusion.¹¹ ADAM33 is preferentially expressed in smooth muscle cells, myofibroblasts, and fibroblasts, but not in epithelial cells, T cells, or inflammatory cells.³ The selective expression of ADAM33 in these structural cells suggests that alterations in the activity of ADAM33 may cause

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Abbreviations used

ADAM:	A disintegrin and metalloproteinase
ASM:	Airway smooth muscle
BHR:	Bronchial hyperresponsiveness
COL1A1:	α 1, Type I, collagen
DMSO:	Dimethyl sulfoxide
EGF:	Epidermal growth factor
ERK:	Extracellular signal-regulated kinase
JAK2:	Janus kinase
JNK:	c-Jun N-terminal kinase
LCM:	Laser capture microdissection
MAPK:	Mitogen-activated protein kinase
MEK:	Mitogen-activated protein kinase kinase
S9:	Ribosomal protein S9
STAT:	Signal transducer and activator of transcription
TIMP:	Tissue inhibitor of metalloproteinase

abnormalities in the function of ASM cells and fibroblasts.^{2,12} The lack of maturation of the β isoform and its probable localization to the endoplasmic reticulum suggest that β isoform may have dominant-negative activity.¹³ Molecular functions of α and β forms of ADAM33 might be different in inducing smooth muscle proliferation and hypertrophy.

Recent understanding of asthma pathogenesis has shown that a balance in expression of T_H1 – T_H2 cytokines is important in allergic airway diseases and that change in this balance acts directly on ASM cells to modify their biological function.^{14–17} In addition to these cytokines, certain chemokines that are implicated in asthmatic inflammation have been shown to induce chemotaxis or proliferation of smooth muscle cells.^{18–20} Among the various members of the ADAM family, ADAM8 was shown to be induced by allergen challenge, by IL-4, and by IL-13 in a murine model.²¹ To date, there are few *in vitro* or *in vivo* data on the relationship between the expression of ADAM33 in ASM cells and the allergic inflammation microenvironment.²² We aimed to investigate in this study (1) whether expression of ADAM33 mRNA was increased in the smooth muscle layer of bronchial biopsy samples from patients with asthma compared with normal subjects, and (2) whether T_H2 or T_H1 cytokines (IL-4, IL-13, or IFN- γ) or asthma-related chemokines could influence the expression of α or β isoforms of ADAM33 on human ASM cells.

METHODS

Study subjects

Patients with asthma ($n = 10$) and normal control subjects ($n = 10$) were recruited for the study of tissue expression of ADAM33 from the asthma clinics of Montreal Chest Institute, Quebec, Canada. The diagnosis of asthma was based on American Thoracic Society criteria.²³ All of the subjects were nonsmokers. All patients with asthma were atopic on the basis of a skin test. Patients had not been treated with inhaled or systemic corticosteroids and had experienced no exacerbation during the 12 weeks preceding the study. None of the control subjects had any lung disease. None of the subjects in either

group had experienced respiratory tract infections for at least 12 weeks. An institutional ethics committee approved the study, and written informed consent was obtained from subjects with and without asthma before undergoing fiberoptic bronchoscopy.

Fiberoptic bronchoscopy and biopsy processing

Fiberoptic bronchoscopy was performed according to published guidelines.²⁴ Biopsy samples were processed and stored for *in situ* hybridization and immunohistochemistry as described previously²⁵ (see Methods in this article's Online Repository at www.jacionline.org).

In situ hybridization

cDNA coding for ADAM33 in plasmid pT7T3D-pac (American Type Culture Collection #1027875) was kindly provided by Dr M. E. Rothenberg and linearized by *Not* I or *Eco*R I digestion. Antisense and sense RNA probes were prepared by transcription in the presence of ³⁵S-labeled uridine triphosphate and either T7 or T3 RNA polymerase (Promega, Madison, Wis). This probe spans from exon R to V, which recognizes both ADAM33 α and β isoforms. The percentage of incorporation of the radiolabel was between 40% to 50%. *In situ* hybridization was performed as previously described^{25,26} with slight modification that EM-1 Hypercoat emulsion RPN41 (Amersham Biosciences, Buckinghamshire, United Kingdom) was used for emulsion. For quantification, the number of positive cells in the smooth muscle layers and the number of smooth muscle cells were counted by dark field microscopy.

Cell culture

Primary ASM cells were purchased from Clonetics (Cambrex Bio Science, Walkersville, Md). Cells between the 3rd and 6th passage were used. ASM cells were grown, as recommended, in their optimal medium (SmGM-2; Cambrex Bio Science) containing 5% FBS at 37°C in a humidified incubator with 5% CO₂. ASM cells were serum-deprived for 24 hours before treatment with cytokines (recombinant human [rh]IL-4, rhIL-13, rhIFN- β , and rhIFN- γ ; R&D, Minneapolis, Minn) or chemokines (rhIL-309/CC chemokine ligand [CCL]1, rh monocyte chemoattractant protein-1/CCL2, rh macrophage inflammatory protein-1 α /CCL3, rh-eotaxin/CCL11; R&D). In inhibition experiments, inhibitors of signal transduction pathways were added 30 minutes before the addition of IFN- γ . Inhibitors (AG490 [Janus kinase (JAK)–2 inhibitor], SP600125 [c-Jun N-terminal kinase (JNK) inhibitor], SB203580 (stress-activated protein kinase-2 [SAPK2] inhibitor, p38 pathway), and U0126 (mitogen-activated protein kinase kinase [MEK]–1/2 inhibitor [inhibitor of MEK1/2, the upstream kinases of extracellular signal-regulated kinase [ERK]]) were purchased from Sigma-Aldrich (St Louis, Mo). These inhibitors were dissolved in dimethyl sulfoxide (DMSO; final concentration of 0.1%, vol/vol) and added to the medium. Vehicle controls contained the same amount of DMSO.

Quantitative RT-PCR

Total RNA was extracted from ASM cells using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada). cDNA was synthesized from 1 μ g RNA by using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, Calif) according to the manufacturer's protocol. Specific primer sets were designed for ADAM33 α and ADAM33 β from the reported sequence (GenBank accession #NM_153202; see this article's Fig E1 in the Online Repository at www.jacionline.org) and for α 1, type I, collagen (COL1A1; NM_000088). Ribosomal protein S9 (S9; BC020462) was used as an internal control (housekeeping gene). Sequences of the primers are shown in Table I. Real-time PCR was performed by using LightCycler (Roche Applied Sciences,

TABLE I. Sequences of the primers used for PCR and predicted sizes of amplicons

Product	Primers	Predicted size of amplicon (Base pairs)
<i>ADAM33</i> α	Sense: 5'-GACCTAGAATGGTGTGCCAGA-3' Antisense: 5'-AGCCTGGCTTGTTCACAGAAG-3'	159
<i>ADAM33</i> β	Sense: 5'-ACCTAGATGGCCAGGAAGTGA-3' Antisense: 5'-TGCAAACCATTCTAGGTCCAC-3'	120
<i>S9</i>	Sense: 5'-TGCTGACGCTTGATGAGAAG-3' Antisense: 5'-CGCAGAGAGAAGTCGATGTG-3'	307
<i>COL1A1</i>	Sense: 5'-CTGGTCCCAAGGCTTCCAAGG-3' Antisense: 5'-CTTCACCCTTAGCACCAACAGC-3'	477

Laval, Quebec, Canada) with SYBR Green Master Mix (Qiagen) according to the manufacturer's protocol (see cycling details in this article's Methods section in the Online Repository at www.jacionline.org). The value obtained for *ADAM33* was standardized to the value for *S9* to produce an expression ratio for each gene of interest.

Laser capture microdissection of ASM cell bundles

Laser capture microdissection (LCM) was performed to confirm *ADAM33* mRNA expression in bronchial smooth muscle cells from patients with asthma by using the PixCell II System (Arcturus, Mountain View, Calif) as described.²⁷ Briefly, frozen tissues were sectioned at 8-μm thickness. Smooth muscle bundles were captured by laser of 15-μm diameter. Total laser bursts were 3000 to 5000 per subject. After samples were captured on CapSure HS LCM caps (Arcturus), mRNA was extracted by using RNeasy Micro Kit (Qiagen).

Immunohistochemistry and immunofluorescent staining

To detect *ADAM33* protein in bronchial biopsy tissue, immunohistochemistry was performed. Formalin-fixed, paraffin-embedded tissues of 5-μm thickness were used. After deparaffinization and blocking with blocking solution (DakoCytomation, Glostrup, Denmark), sections were incubated with antibody to cytoplasmic domain of *ADAM33* (1:3000; Triple Point Biologics, Forest Grove, Ore) at 4°C overnight. Then, slides were incubated with biotinylated swine antirabbit antibody (1:100; DakoCytomation) for 1 hour at room temperature and avidin-biotin complex (StreptABCComplex; DakoCytomation). The reaction was visualized by using diaminobenzidine (DakoCytomation) as the chromogen.

For detection of *ADAM33* protein in ASM cells, immunofluorescent staining was used (see this article's Methods section in the Online Repository at www.jacionline.org). Cells were cultured to 80% to 90% confluence on 8-well glass slides. Slides were incubated with anti-SP621 antibody²⁸ (rabbit polyclonal antibody to the cytoplasmic domain of *ADAM33*, courtesy of Dr S. P. Umland; 1:200 dilution) or preimmune rabbit serum overnight at 4°C. Then, goat antirabbit antibody conjugated with AlexaFluor 488 (1:750 dilution; Molecular Probes, Eugene, Ore) was applied. For nuclear staining, Hoechst 33342 (Molecular Probes) was used. Slides were examined by fluorescence microscopy (BX51; Olympus, Tokyo, Japan). Images were recorded with a digital camera (CoolSNAP-Pro; MediaCybernetics, Silver Spring, Md).

Western blot analysis

Protein lysates from cells were prepared as previously described.²⁷ For detection of *ADAM33*, 50 μg protein was heat-denatured in SDS, reduced with β-mercaptoethanol, and separated by 1-dimensional

10% SDS-PAGE followed by transfer to polyvinylidene fluoride membranes (Hybond-P, Amersham). Membranes were blocked by 5% dry nonfat milk for 1 hour at room temperature and incubated with SP-621 antibody (1:30,000) for 48 hours at 4°C, and then donkey antirabbit IgG conjugated to horseradish peroxidase (Amersham) at 1:10,000 for 1 hour at room temperature. For detection, ECL plus Western-blotting Detection Reagent and Hyperfilm (Amersham) was used, and the blots were digitally scanned. Densitometric quantification of the protein bands was performed with ImagePro software version 4 (Media Cybernetics, Silver Spring, Md).

For detection of phosphorylated- or total-signal transducer and activator of transduction (STAT)-1, ERK, JNK, and p38, 20 μg protein was loaded into a gel. Rabbit phosphorylated- or total-STAT1, ERK1/2, JNK, and p38 antibodies (Cell Signaling Technology, Beverly, Mass) were used at 1:1000, and membranes were incubated overnight at 4°C.

Statistical analysis

Differences in *ADAM33*-positive cells in bronchial biopsies between subjects with asthma and control subjects were analyzed by the nonparametric Mann-Whitney *U* test. Data from quantitative PCR were expressed as means ± SEMs and analyzed by using the Student *t* test for comparison between 2 preparations. For multiple comparisons, ANOVA followed by Scheffé test was employed. A *P* value < .05 was considered significant.

RESULTS

Expression of *ADAM33* mRNA and protein in bronchial biopsy specimens

Biopsy specimens from 2 subjects with asthma did not contain enough smooth muscle for adequate evaluation, and they were removed from analysis of *ADAM33* mRNA expression. Therefore, biopsies from 10 normal subjects and 8 subjects with mild asthma were used for the *in situ* hybridization study, and their characteristics are shown in this article's Table E1 in the Online Repository at www.jacionline.org. Using *in situ* hybridization, cells positive for *ADAM33* mRNA were detected among the smooth muscle cells from subjects with asthma and control subjects (Fig 1, A and B). No significant hybridization signal was present with the antisense RNA probe (not shown). *ADAM33* mRNA-positive smooth muscle cells were counted and expressed as a percentage of the total number of smooth muscle cells on the whole slide. The positive/total smooth muscle cell ratio was higher in subjects with asthma than in control subjects (*P* = .002; Fig 1, C). To confirm expression of

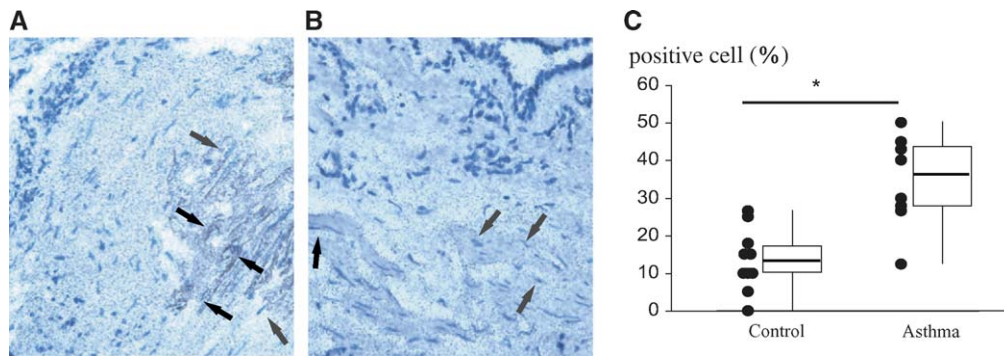


FIG 1. *In situ* hybridization study of *ADAM33* using bronchial biopsy specimens from patients with asthma (**A**) and normal subjects (**B**; $\times 400$). Positive signal is indicated as black granules. Black arrows, positive cells; gray arrows, negative cells. **C**, The ratio of SM cells with positive signals to total SM cells. ASM cells from patients with asthma showed more positive cells compared with controls ($*P = .002$). Box-bar graph indicates ranges, first/third quarters, and medians.

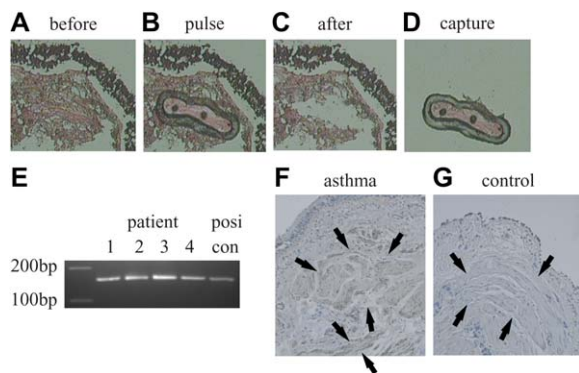


FIG 2. **A-D**, LCM for SM cells. **A**, Before dissection; **B**, after shooting; **C**, after dissection; and **D**, captured SM cells ($\times 200$). **E**, RT-PCR amplicons from laser-captured SM cells were loaded to 3% agarose gel. *ADAM33* protein detected as brown by immunohistochemistry was stronger in patients with asthma (**F**) than control subjects (**G**). SM layers are shown between arrows. Note increased SM mass in subjects with asthma (**F**). *posi con*, Positive control.

ADAM33 mRNA in ASM bundles, we employed LCM (Fig 2, A-D). cDNA of the microdissected samples from suitable biopsies of patients with asthma ($n = 4$) was analyzed by real-time PCR, and *ADAM33* α amplicon (~ 160 bp) was shown to be present (Fig 2, E). Further, immunohistochemistry using antibody to *ADAM33* was performed in 4 normal subjects and 4 subjects with asthma. *ADAM33* protein was more strongly expressed in ASM in subjects with asthma compared with normal subjects (Fig 2, F and G). Eosinophilic inflammation and increased smooth muscle mass were observed in patients with asthma compared with control subjects (not shown).

Effect of cytokines and chemokines on *ADAM33* mRNA expression in ASM cells

Using quantitative RT-PCR, mRNA of the splice isoforms *ADAM33* α and β was identified in ASM cells. PCR product of *ADAM33* α was ~ 160 bp, whereas that of *ADAM33* β was ~ 120 bp. Both of the products are

compatible with the length of products estimated from the sequence (not shown). ASM cells were treated for 24 hours with IL-4, IL-13, or IFN- γ in various concentrations and various lengths of time. IL-4 and IL-13 treatment did not result in any significant modification of mRNA expression of either isoform (Fig 3, A). In contrast, there was a dose-dependent downregulation of mRNA of both isoforms with increasing concentrations of IFN- γ at 24 hours (Fig 3, B). Both isoforms of *ADAM33* were inhibited to the same extent in response to IFN- γ ($\sim 60\%$ by 10 ng/mL IFN- γ ; Fig 3, B). The decreased expression of *ADAM33* was observed by 8 hours after treatment with 10 ng/mL IFN- γ (Fig 3, B). We also investigated whether any asthma-related chemokines (I-309/CCL1, monocyte chemoattractant protein-1/CCL2, macrophage inflammatory protein-1 α /CCL3, or eotaxin/CCL11) or IFN- β modulated the expression of *ADAM33* α and β . None of the treatments affected the expression of *ADAM33* (not shown).

MEK1/2 inhibitor U0126 restored IFN- γ -induced downregulation of *ADAM33* mRNA

IFN- γ acts via the JAK/STAT1 signal transduction pathway for the regulation of many genes. To show the activation of signaling pathways activated by IFN- γ in ASM cells, cells were stimulated with 10 ng/mL IFN- γ for 1, 5, 10, 15, 30, and 60 minutes, and cell lysates were analyzed by Western blotting. Phosphorylation of STAT1 at Tyr701 was observed after 1 minute of stimulation, peaked at 30 minutes, and was sustained as long as 60 minutes (not shown). Recent reports suggest that mitogen-activated protein kinase (MAPK) pathways are also activated by IFN- γ and cause biological effects in various types of cells. To confirm activation of ERK, JNK, and p38 MAPK by IFN- γ in ASM cells, Western blot analysis was performed under the same conditions as phospho-STAT1. Maximal phosphorylation of ERK (Fig 4, A) and JNK (not shown) was achieved at 30 minutes and 60 minutes, respectively, and was sustained as long as 120 minutes, whereas phosphorylation of p38 was not evident

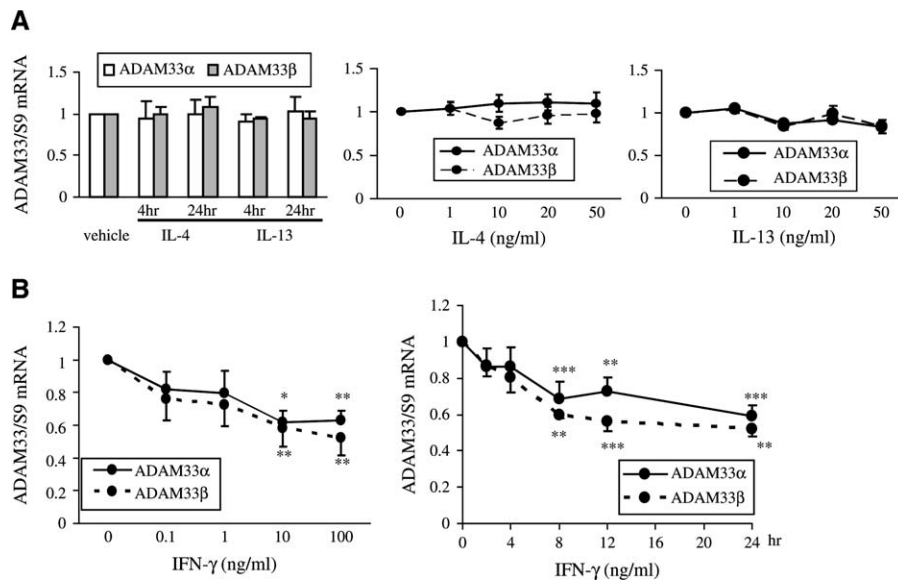


FIG 3. Effect of IL-4 and IL-13 (**A**) and IFN-γ (**B**) on *ADAM33* mRNA expression by quantitative RT-PCR. Ratio of *ADAM33* to *S9* of ASM cells treated with vehicle in each experiment was set as 1, and relative values of cytokine-stimulated/vehicle-treated are expressed. Means $\pm$ SEMs of 3 experiments performed in duplicate are shown (same in Figs 3-6). * $P < .05$; ** $P < .01$; *** $P < .05$.

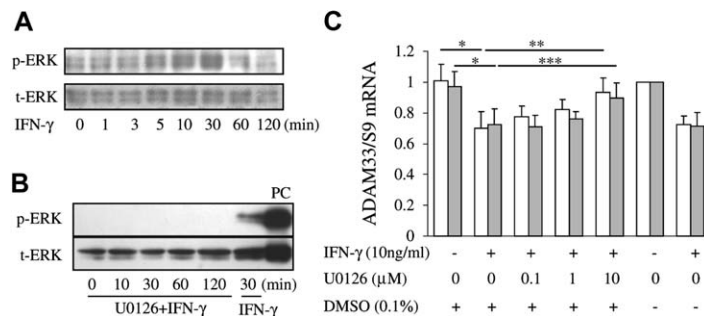


FIG 4. Western blot analysis showing activation of ERK1/2 by IFN-γ (10 ng/mL; **A**) and its abrogation by MEK inhibitor U0126 (10 μmol/L; **B**). PC, Positive control. **C**, Effect of U0126 on the downregulation of *ADAM33* by IFN-γ. Vehicle contains $1 \times 10^{-5}\%$ BSA for IFN-γ and 0.1% DMSO for U0126. White and grey bars express *ADAM33α* and *ADAM33β*. * $P < .05$; ** $P < .01$; *** $P < .07$. p-ERK, Phosphorylated-ERK; t-ERK, total-ERK.

(not shown). IFN-β did not activate ERK pathway in ASM cells (not shown).

To investigate which signal transduction pathway is involved in IFN-γ-induced downregulation of *ADAM33*, we treated ASM cells with various concentrations of AG490, U0126, SP600125, or SB203580 thirty minutes before treatment with IFN-γ. Treatment of the cells with 10 μmol/L U0126 restored levels of *ADAM33* to ~90% of the gene expressed in vehicle-treated, IFN-γ-untreated cells (Fig 4, C), suggesting that the ERK pathway is involved in the observed downregulation of *ADAM33*. AG490, SP600125, and SB203580 did not affect the downregulation of *ADAM33* by IFN-γ (not shown). To confirm the efficiency of the inhibitor in blocking the ERK pathway, cell lysates treated with 10 μmol/L U0126 thirty minutes before stimulation with IFN-γ were analyzed for phosphorylation of ERK1/2. Abrogation of ERK1/2

phosphorylation was confirmed by Western blot (Fig 4, B). The downregulation of *ADAM33* by IFN-γ was not totally restored by MEK-MAPK inhibition by U0126, suggesting that other pathways are also involved in the downregulation.

IFN-γ downregulates *ADAM33* at the transcriptional level

To determine further at which step IFN-γ modulates the mRNA expression of *ADAM33*, *de novo* synthesis of protein was blocked by cycloheximide. Pretreatment of ASM cells with cycloheximide (10 μg/mL) for 1 hour did not restore levels of *ADAM33α* by IFN-γ (not shown), which suggests that *de novo* synthesis of protein is not likely to be involved in the downregulation by IFN-γ. As a control for this experiment, *S9*, which is used as a housekeeping gene, and *COL1A1*, which was recently shown to be

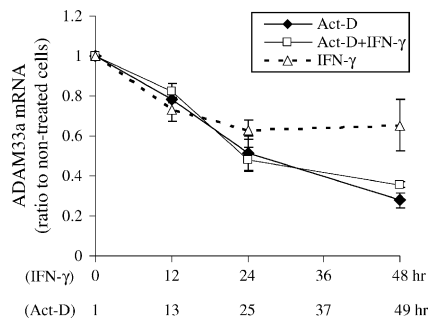


FIG 5. Effect of IFN- γ on decay kinetics of *ADAM33* mRNA in ASM cells quantified by real-time RT-PCR. After 24-hour starvation, cells were treated with actinomycin D (Act-D; 2.5 μ g/mL), IFN- γ (10 ng/mL), or both for the indicated time. Act-D was given 1 hour before IFN- γ . Values are indicated as the ratio to the baseline.

downregulated by IFN- γ induction of regulatory factor for X box family protein,²⁹ were used. mRNA for *S9* was not affected by IFN- γ or cycloheximide, whereas that of *COL1A1* was downregulated by IFN- γ to $\sim 70\%$, and the levels were restored by cycloheximide (not shown).

To block mRNA transcription, actinomycin-D (2.5 μ g/mL; 1 hour) was used before treatment with or without IFN- γ (Fig 5). After 48 hours, *ADAM33* α mRNA in actinomycin D-treated cells was reduced to $\sim 30\%$ of non-treated cells. However, the reduction of mRNA was not enhanced by IFN- γ . This means that IFN- γ did not promote degradation of mRNA already transcribed, suggesting that IFN- γ does not regulate *ADAM33* at the posttranscriptional level. Taken together, it is likely that IFN- γ downregulates *ADAM33* mRNA mainly at the transcriptional level.

Expression of ADAM33 protein by cultured ASM cells

We used both immunofluorescent microscopy and Western blot analysis to detect ADAM33 protein in cultured ASM cells. Immunoreactivity was detected in the cytoplasm of ASM cells (Fig 6, A and B). For control staining, preimmune serum of the rabbit that produced SP621 antibody was used instead of the antibody. There was no significant immunofluorescent signal in the control staining (Fig 6, C and D).

Cell lysates were used to detect ADAM33 protein by Western blot analysis. Membranes were blotted for 48 hours with anti-ADAM33 antibody (SP621) or the preimmune serum. Incubation with anti-ADAM33 antibody resulted in the detection of a clear band at ~ 110 kD, which was absent on incubation with the preimmune serum (Fig 6, E). The band at ~ 110 kD is consistent with unprocessed ADAM33.³⁰ Next, lysates from ASM cells treated for 48 hours with 10 ng/mL IFN- γ or vehicle (control) were investigated for ADAM33 expression (Fig 6, F). Quantity of total protein did not change by treatment with IFN- γ compared with vehicle. OD of the band at ~ 110 kD was decreased for IFN- γ -treated samples compared with that of vehicle-treated samples ($65.8\% \pm 9.4\%$ of the vehicle-treated samples; $P = .01$).

DISCUSSION

In the current study, we have demonstrated that ADAM33 is expressed in the smooth muscle layer of airway biopsies to a greater extent in subjects with asthma than in subjects without asthma. We have also shown that IFN- γ but not IL-4, IL-13, or IFN- β can modulate *ADAM33* mRNA and protein expression in cultured ASM cells and that the ERK signaling pathway is involved in the downregulation of transcription by IFN- γ .

ADAM33 has been shown to be expressed in ASM cells and in airway fibroblasts,^{3,28} suggesting the role of this gene in modifying the functions of these cells such as proliferation, differentiation, and migration.^{2,12} Therefore, quantitative histological studies in asthmatic samples are essential to prove regulation of *ADAM33* mRNA, and to show the relationship of ADAM33 with BHR.²⁸ Haitchi et al²² have investigated mRNA expression of this gene quantified by RT-PCR using total RNA extracted from bronchial biopsy samples of subjects with asthma and normal subjects and have reported that the expression was not different between the 2 groups. We compared *ADAM33* expression in ASM cells and found that *ADAM33* mRNA in these cells is expressed more highly in subjects with asthma than in normal controls. Our finding is consistent with a recent study by Lee et al.³¹ In this study, increased levels of ADAM33 protein were detected in bronchoalveolar lavage fluid from subjects with asthma compared with normal subjects.³¹ In agreement with their results, ADAM33 protein was also more highly expressed in bronchial tissues from subjects with asthma compared with normal subjects. Considering their findings together with ours, ADAM33 is highly expressed in asthmatic airways, and the expression may be regulated at the mRNA level.

The roles of T_H2 cytokines such as IL-4 and IL-13 in allergic inflammation and their reciprocal relationship with T_H1 cytokines such as IFN- γ in asthma have been extensively explored.^{32,33} T_H2 cytokines have also been implicated in airway remodeling. Laporte et al¹⁶ have described constitutive expression for IL-4R α , IL-13R $\alpha 1$, and IL-13R $\alpha 2$ receptors on ASM cells. It has been shown that IL-4 and IL-13 can affect the ability of smooth muscle cells to produce a number of mediators and molecules including matrix metalloproteinases.³⁴ These cytokines are known to induce ADAM8, another member of ADAM family, in a murine model.²¹ We thus hypothesized that T_H2 cytokines could modify the expression of ADAM33 in ASM cells. However, our study failed to show any modification of ADAM33 expression by these cytokines.

We also examined the effect of IFN- γ , the prototypical T_H1 cytokine, on the expression of ADAM33 in ASM cells. We found a downregulation of the gene by IFN- γ in ASM cells. IFN- γ is involved in repair of bronchial epithelial cell damage as shown by Trautmann et al³⁵ and Leir et al.³⁶ Its expression is usually low in asthma. Robinson et al³⁷ have shown that corticosteroid therapy in patients with asthma is accompanied by an increase of IFN- γ -positive cells. The sustained IFN- γ knockout

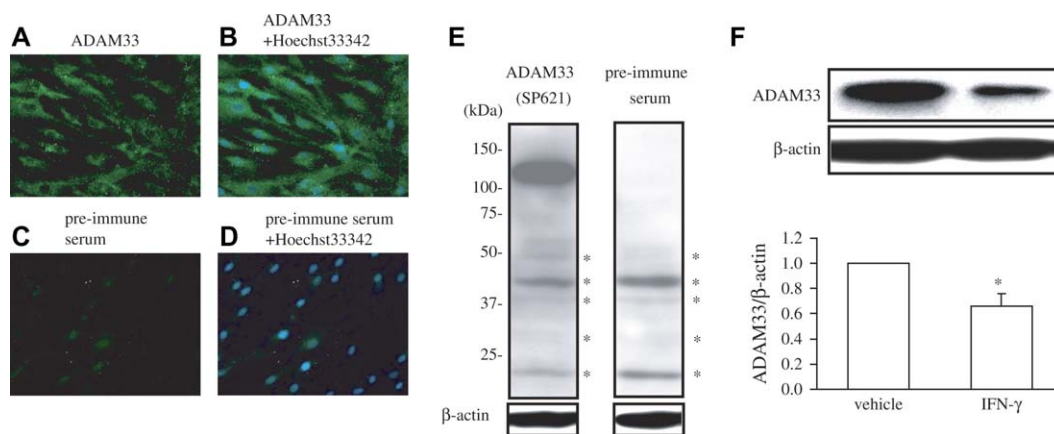


FIG 6. A-D, Immunofluorescent study of ADAM33 in cultured ASM cells ($\times 200$). ADAM33 antibody (A and B) and preimmune serum (negative control; C and D). Nuclear staining with Hoechst33342 (B and D). E and F, Western blot analysis of ADAM33 expression in ASM cells. *Nonspecific bands (E). Treatment with IFN- γ for 48 hours decreased the amount of ADAM33 protein compared with the vehicle-treated sample (* $P < .05$; F).

mouse shows an exaggerated increase of airway hyperresponsiveness after ovalbumin challenge,³⁸ whereas recombinant IFN- γ administered in the knockout model³⁸ or IFN- γ gene therapy in an ovalbumin model³⁹ attenuates airway hyperresponsiveness. Our data suggest that the level of IFN- γ might have an important role in regulating the level of ADAM33 in asthmatic airways.

Our *in vitro* study showed a 30% to 40% inhibition of ADAM33 expression by IFN- γ . Expression of IFN- γ in subjects with mild asthma in this study showed a tendency to be lower compared with normal subjects (Q. Hamid, unpublished data, 2005). Although our results suggest a possible interaction between ADAM33 expression and cytokine milieu in the bronchus, the biological relevance of the effect of IFN- γ on the expression of ADAM33 is not clear. Catalytic function of this protein to cleave substrates such as β -amyloid precursor protein and Kit-ligand-1 was found recently.⁴⁰ On the other hand, disintegrin domain of ADAM33 was shown to have interaction with integrins.⁴¹ However, more efficient biological substrates or ligands *in vivo* are to be identified in relation to the pathogenesis of asthma. Therefore, it is still difficult to interpret our results on the effect of IFN- γ into the biological effect of ADAM33. ADAM33 is inhibited by a group of natural inhibitors of matrix metalloproteinases: tissue inhibitors of metalloproteinases (TIMPs).⁴⁰ Indeed, matrix metalloproteinase/TIMP balance was increased in acute asthma,^{42,43} and corticosteroid therapy in asthma increased TIMP expression.^{44,45} Decreased TIMPs could further sustain catalytic function of ADAM33. Thus, a small difference of ADAM33 expression for a long period could have a role in asthmatic pathogenesis.

Yoshinaka et al⁹ have identified a splice variant of *ADAM33*, produced through the deletion of 78 bp in exon 17 of the native gene. This alternate transcript is known as *ADAM33 β* , whereas the native mRNA is known as *ADAM33 α* . In addition to the 2 isoforms, Powell et al⁴⁶ showed the existence of several splice variants of

ADAM33 at mRNA and protein levels. There is little information regarding the modulation of the transcription of these splice variants. We identified the 2 spliced variants in human ASM cells and focused on the differences in the expression between α and β isoforms. When the cells were treated with IFN- γ , both isoforms of *ADAM33* were downregulated to a similar extent ($\sim 60\%$ to 70% of the control, at 10 ng/mL). In addition, the expression of metalloproteinase domain mRNA was decreased by IFN- γ to the same extent as α and β isoforms (data not shown). These observations suggest that mRNA expression of *ADAM33* in response to IFN- γ may be regulated in the promoter region. Regulation of *ADAM33* gene by IFN- γ may differ between subjects with and without asthma because of polymorphisms present in the upstream region of the gene.⁴

We next explored the role that the ERK pathway plays in the downregulation of *ADAM33* induced by IFN- γ . The main intracellular signaling induced by IFN- γ is the JAK/STAT pathway, but JAK inhibitor had no effect on the downregulation by IFN- γ . Recent studies have provided evidence that activation of MAPK pathways, including the ERK pathway, also plays an important role in the signal transduction of IFN- γ in a STAT1-dependent or independent manner.⁴⁷⁻⁵⁵ Nguyen et al⁵⁴ suggested that activation of the ERK pathway may antagonize IFN- γ -induced binding activity of STAT1. ERK is linked to the negative regulation of STAT3 activity in response to growth factors through phosphorylation of Ser 727, resulting in inhibition of DNA binding of STAT3.^{56,57} It is not clear whether ERK downregulated *ADAM33* by inhibition of the activation of STAT1 or by activation of inhibitory transcription factor independently of STAT1. An elucidation of the mechanism of how the ERK pathway inhibits transcription of *ADAM33* is needed.

In conclusion, our finding that IFN- γ downregulates ADAM33 suggests that abnormality in migration and proliferation of ASM cells proposed in airway remodeling

may be partly a result of loss of the inhibitory effect of IFN- γ on ADAM33. It is necessary to study transcription factors and the targeted promoter region, which could have a key role in the regulation of ADAM33 expression. It is also important to study the effect of ADAM33 on physiological functions of ASM cells.

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REFERENCES

- Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicombe SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol* 2000;105:193-204.
- Davies DE, Wicks J, Powell RM, Puddicombe SM, Holgate ST. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol* 2003;111:215-25.
- Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, et al. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 2002;418:426-30.
- Chae SC, Yoon KH, Chung HT. Identification of novel polymorphisms in the Adam33 gene. *J Hum Genet* 2003;48:278-81.
- Howard TD, Postma DS, Jongepier H, Moore WC, Koppelman GH, Zheng SL, et al. Association of a disintegrin and metalloprotease 33 (ADAM33) gene with asthma in ethnically diverse populations. *J Allergy Clin Immunol* 2003;112:717-22.
- Werner M, Herbon N, Gohlke H, Altmüller J, Knapp M, Heinrich J, et al. Asthma is associated with single-nucleotide polymorphisms in ADAM33. *Clin Exp Allergy* 2004;34:26-31.
- Jongepier H, Boezen HM, Dijkstra A, Howard TD, Vonk JM, Koppelman GH, et al. Polymorphisms of the ADAM33 gene are associated with accelerated lung function decline in asthma. *Clin Exp Allergy* 2004;34:757-60.
- Blobel CP. Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNF alpha and Notch. *Cell* 1997;90:589-92.
- Yoshinaka T, Nishii K, Yamada K, Sawada H, Nishiwaki E, Smith K, et al. Identification and characterization of novel mouse and human ADAM33s with potential metalloprotease activity. *Gene* 2002;282:227-36.
- Iba K, Albrechtsen R, Gilpin B, Fröhlich C, Loechel F, Zolkiewska A, et al. The cysteine-rich domain of human ADAM 12 supports cell adhesion through syndecans and triggers signaling events that lead to beta1 integrin-dependent cell spreading. *J Cell Biol* 2000;149:1143-56.
- Chen H, Pyluck AL, Janik M, Sampson NS. Peptides corresponding to the epidermal growth factor-like domain of mouse fertilin: synthesis and biological activity. *Biopolymers* 1998;47:299-307.
- Shapiro SD, Owen CA. ADAM-33 surfaces as an asthma gene. *N Engl J Med* 2002;347:936-8.
- Umland SP, Wan Y, Shah H, Garlisi CG, Devito KE, Braunschweiger K, et al. Mouse ADAM33: two splice variants differ in protein maturation and localization. *Am J Respir Cell Mol Biol* 2004;30:530-9.
- Hirst SJ, Hallsworth MP, Peng Q, Lee TH. Selective induction of eotaxin release by interleukin-13 or interleukin-4 in human airway smooth muscle cells is synergistic with interleukin-1beta and is mediated by the interleukin-4 receptor alpha-chain. *Am J Respir Crit Care Med* 2002;165:1161-71.
- Moore PE, Church TL, Chism DD, Panettieri RA Jr, Shore SA. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L847-53.
- Laporte JC, Moore PE, Baraldo S, Jouvin MH, Church TL, Schwartzman IN, et al. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. *Am J Respir Crit Care Med* 2001;164:141-8.
- Wen FQ, Liu X, Manda W, Terasaki Y, Kobayashi T, Abe S, et al. TH2 Cytokine-enhanced and TGF-beta-enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN- γ and corticosteroids. *J Allergy Clin Immunol* 2003;111:1307-18.
- Haque NS, Fallon JT, Pan JJ, Taubman MB, Harpel PC. Chemokine receptor-8 (CCR8) mediates human vascular smooth muscle cell chemotaxis and metalloproteinase-2 secretion. *Blood* 2004;103:1296-304.
- Selzman CH, Miller SA, Zimmerman MA, Gamboni-Robertson F, Harken AH, Banerjee A. Monocyte chemotactic protein-1 directly induces human vascular smooth muscle proliferation. *Am J Physiol Heart Circ Physiol* 2002;283:H1455-61.
- Kodali RB, Kim WJ, Galaria II, Miller C, Schecter AD, Lira SA, et al. CCL11 (Eotaxin) induces CCR3-dependent smooth muscle cell migration. *Arterioscler Thromb Vasc Biol* 2004;24:1211-6.
- King NE, Zimmermann N, Pope SM, Fulkerson PC, Nikolaidis NM, Mishra A, et al. Expression and regulation of a disintegrin and metalloproteinase (ADAM) 8 in experimental asthma. *Am J Respir Cell Mol Biol* 2004;31:257-65.
- Haitchi HM, Powell RM, Shaw TJ, Howarth PH, Wilson SJ, Wilson DI, et al. A disintegrin and metalloprotease 33 expression in asthmatic airways and human embryonic lungs. *Am J Respir Crit Care Med* 2005;171:958-65.
- Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. *Am Rev Respir Dis* 1987;136:225-44.
- Workshop summary and guidelines: investigative use of bronchoscopy, lavage, and bronchial biopsies in asthma and other airway diseases. *J Allergy Clin Immunol* 1991;88:808-14.
- Muro S, Taha R, Tscopoulos A, Olivenstein R, Tonnel AB, Christodoulou P, et al. Expression of IL-15 in inflammatory pulmonary diseases. *J Allergy Clin Immunol* 2001;108:970-5.
- Hamid Q, Azzawi M, Ying S, Moqbel R, Wardlaw AJ, Corrigan CJ, et al. Expression of mRNA for interleukin-5 in mucosa bronchial biopsies from asthma. *J Clin Invest* 1991;87:1541-6.
- Lajoie-Kadoch S, Joubert P, Letuve S, Halayko AJ, Martin JG, Soussi-Gounni A, et al. TNF- α and IFN- γ inversely modulate expression of the IL-17E receptor in airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2006;290:1238-46.
- Umland SP, Garlisi CG, Shah H, Wan Y, Zou J, Devito KE, et al. Human ADAM33 messenger RNA expression profile and post-transcriptional regulation. *Am J Respir Cell Mol Biol* 2003;29:571-82.
- Sengupta P, Xu Y, Wang L, Widom R, Smith BD. Collagen alpha1(I) gene (COL1A1) is repressed by RFX family. *J Biol Chem* 2005;280:21004-14.
- Garlisi CG, Zou J, Devito KE, Tian F, Zhu FX, Liu J, et al. Human ADAM33: protein maturation and localization. *Biochem Biophys Res Commun* 2003;301:35-43.
- Lee JY, Park SW, Chang HK, Kim HY, Rhim T, Lee JH, et al. A disintegrin and metalloproteinase 33 protein in asthmatics: relevance to airflow limitation. *Am J Respir Crit Care Med* 2006;173:729-35.
- Robinson D, Hamid Q, Ying S, Bentley A, Assoufi B, Durham S, et al. Prednisolone treatment in asthma is associated with modulation of bronchoalveolar lavage cell interleukin-4, interleukin-5, and interferon- γ cytokine gene expression. *Am Rev Respir Dis* 1993;148:401-6.
- Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001;344:350-62.
- Sasaguri T, Arima N, Tanimoto A, Shimajiri S, Hamada T, Sasaguri Y. A role for interleukin 4 in production of matrix metalloproteinase 1 by human aortic smooth muscle cells. *Atherosclerosis* 1998;138:247-53.
- Trautmann A, Schmid-Grendelmeier P, Kruger K, Cramer R, Akdis M, Akkaya A, et al. T cells and eosinophils cooperate in the induction of bronchial epithelial cell apoptosis in asthma. *J Allergy Clin Immunol* 2002;109:329-37.
- Leir SH, Holgate ST, Lackie PM. Inflammatory cytokines can enhance CD44-mediated airway epithelial cell adhesion independently of CD44 expression. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L1305-11.
- Robinson DS, Hamid Q, Ying S, Tscopoulos A, Barkans J, Bentley AM, et al. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.

38. Yoshida M, Leigh R, Matsumoto K, Wattie J, Ellis R, O'Byrne PM, et al. Effect of interferon- γ on allergic airway responses in interferon- γ -deficient mice. *Am J Respir Crit Care Med* 2002;166:451-6.
39. Behera AK, Kumar M, Lockey RF, Mohapatra SS. Adenovirus-mediated interferon γ gene therapy for allergic asthma: involvement of interleukin 12 and STAT4 signaling. *Hum Gene Ther* 2002;13:1697-709.
40. Huang J, Bridges LC, White JM. Selective modulation of integrin-mediated cell migration by distinct ADAM family members. *Mol Biol Cell* 2005;16:4982-91.
41. Zou J, Zhu F, Liu J, Wang W, Zhang R, Garlisi CG, et al. Catalytic activity of human ADAM33. *J Biol Chem* 2004;279:9818-30.
42. Tonnel AB, Gosset P, Tillie-Leblond I. Characteristics of the inflammatory response in bronchial lavage fluids from patients with status asthmaticus. *Int Arch Allergy Immunol* 2001;124:267-71.
43. Lemjabbar H, Gosset P, Lamblin C, Tillie L, Hartmann D, Wallaert B, et al. Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *Am J Respir Crit Care Med* 1999;159:1298-307.
44. Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999;104:356-63.
45. Lee YC, Lee HB, Rhee YK, Song CH. The involvement of matrix metalloproteinase-9 in airway inflammation of patients with acute asthma. *Clin Exp Allergy* 2001;31:1623-30.
46. Powell RM, Wicks J, Holloway JW, Holgate ST, Davies DE. The splicing and fate of ADAM33 transcripts in primary human airways fibroblasts. *Am J Respir Cell Mol Biol* 2004;31:13-21.
47. Song JH, Wang CX, Song DK, Wang P, Shuaib A, Hao C. Interferon- γ induces neurite outgrowth by upregulation of p35 neuron-specific cyclin-dependent kinase 5 activator via activation ERK1/2 pathway. *J Biol Chem* 2005;280:12896-901.
48. Choudhury GG. A linear signal transduction pathway involving phosphatidylinositol 3-kinase, protein kinase C epsilon, and MAPK in mesangial cells regulates interferon- γ -induced STAT1alpha transcriptional activation. *J Biol Chem* 2004;279:27399-409.
49. Wu T, Han C, Shelhamer JH. Involvement of p38 and p42/44 MAP kinases and protein kinase C in the interferon- γ and interleukin-1alpha-induced phosphorylation of 85-kDa cytosolic phospholipase A(2) in primary human bronchial epithelial cells. *Cytokine* 2004;25:11-20.
50. Shibuya Y, Hirasawa N, Sakai T, Togashi Y, Muramatsu R, Ishii K, et al. Negative regulation of the protein kinase C activator-induced ICAM-1 expression in the human bronchial epithelial cell line NCI-H292 by p44/42 mitogen-activated protein kinase. *Life Sci* 2004;75:435-46.
51. Gendron FP, Chalimoniuk M, Strosznajder J, Shen S, Gonzalez FA, Weisman GA, et al. P2X7 nucleotide receptor activation enhances IFN γ -induced type II nitric oxide synthase activity in BV-2 microglial cells. *J Neurochem* 2003;87:344-52.
52. Chung HK, Lee IK, Kang H, Suh JM, Kim H, Park KC, et al. Statin inhibits interferon- γ -induced expression of intercellular adhesion molecule-1 (ICAM-1) in vascular endothelial and smooth muscle cells. *Exp Mol Med* 2002;34:451-61.
53. Hu J, Roy SK, Shapiro PS, Rodig SR, Reddy SP, Platanias LC, et al. ERK1 and ERK2 activate CCAAAT/enhancer-binding protein-beta-dependent gene transcription in response to interferon- γ . *J Biol Chem* 2001;276:287-97.
54. Nguyen VA, Chen J, Hong F, Ishac EJ, Gao B. Interferons activate the p42/44 mitogen-activated protein kinase and JAK-STAT (Janus kinase-signal transducer and activator transcription factor) signaling pathways in hepatocytes: differential regulation by acute ethanol via a protein kinase C-dependent mechanism. *Biochem J* 2000;349:427-34.
55. Takaoka A, Tanaka N, Mitani Y, Miyazaki T, Fujii H, Sato M, et al. Protein tyrosine kinase Pyk2 mediates the Jak-dependent activation of MAPK and Stat1 in IFN- γ , but not IFN-alpha, signaling. *EMBO J* 1999;18:2480-8.
56. Chung J, Uchida E, Grammer TC, Blenis J. STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol* 1997;17:6508-16.
57. Sengupta TK, Talbot ES, Scherle PA, Ivashkiv LB. Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. *Proc Natl Acad Sci U S A* 1998;95:11107-12.