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Down-Regulated NF-E2–Related Factor 2 in Pulmonary Macrophages of Aged Smokers and Patients with Chronic Obstructive Pulmonary Disease

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Pulmonary macrophages are one of the sources of various antioxidant and detoxification enzymes for which NF-E2–related factor 2 (Nrf2) is a key transcriptional factor. Although Nrf2 deficiency reportedly induces severe emphysema in mice exposed to cigarette smoke (CS), no reports have studied Nrf2 regulation in chronic obstructive pulmonary disease (COPD). In this study, Nrf2 activation in response to CS was evaluated in human alveolar macrophages, and age-related differences in CS-induced Nrf2 regulation in mouse alveolar macrophages were determined. Furthermore, Nrf2 mRNA levels in human macrophages harvested by bronchoalveolar lavage or laser capture microdissection were measured. CS induced nuclear Nrf2 accumulation and up-regulation of Nrf2 target genes without substantial changes in Nrf2 mRNA levels in human alveolar macrophages. In humans, the Nrf2 mRNA level in lavaged macrophages of young subjects ($n = 14$) was independent of smoking status; however, the Nrf2 mRNA level was down-regulated in the lavaged macrophages of older current smokers ($n = 14$) compared with older nonsmokers ($n = 9$) ($P < 0.001$). Among older subjects, the macrophage Nrf2 mRNA level was inversely correlated with oxidized glutathione and carbonylated albumin levels in bronchoalveolar lavage fluid. In mice, aging suppressed the CS-induced up-regulation of Nrf2 target genes, as well as Nrf2, in alveolar macrophages. Furthermore, the Nrf2 mRNA level was decreased in laser capture microdissection–retrieved macrophages obtained from subjects with COPD ($n = 10$) compared with control subjects ($n = 10$) ($P = 0.001$). In conclusion, CS induces Nrf2 activation in macrophages, and Nrf2 expression is decreased in the macrophages of older current smokers and patients with COPD.

Keywords: cigarette smoking; bronchoalveolar lavage; laser capture microdissection

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is not fully reversible (1). COPD is a major public health problem and is the fifth leading cause of death worldwide; its prevalence is expected to increase in the next few decades (2). Cigarette smoking is the major risk factor for COPD, yet the molecular and cellular mechanisms that are responsible for the development of COPD are not fully understood.

NF-E2–related factor 2 (Nrf2), a member of the cap'n'collar family of the basic leucine zipper transcription factors, is a key transcription factor that regulates the expression of several antioxidant and detoxification genes (3, 4). Nrf2 heterodimerizes with another basic leucine zipper protein, such as small Maf (MafF, MafG, and MafK) or Jun (c-Jun, Jun-D, and Jun-B) and then binds to the antioxidant response element (ARE) in target gene promoters (5). In normal mouse lungs, immunohistochemical studies have shown that Nrf2 protein is localized in airway epithelium, in type II alveolar epithelial cells, and in resident macrophages (6). Nrf2-deficient mice are highly susceptible to oxidative stress and reactive electrophiles; they develop severe emphysema when exposed to cigarette smoke or elastase (7–9).

There is a marked increase in the number of macrophages in the lungs of smokers and in patients with COPD. Macrophages play an important role in the pathogenesis of COPD because they release many compounds, such as reactive oxygen species, chemotactic factors, inflammatory cytokines, smooth muscle constrictors, mucus gland activators, extracellular matrix proteins, and matrix metalloproteinases (10). On the other hand, pulmonary macrophages are the primary defense against inhaled particles; they phagocytose deposited particles and detoxify inhaled organic fractions through metabolic activation by phase I and II enzymes (11). It has been reported that macrophages produce many Nrf2-regulated antioxidants (4, 8, 12–17). Indeed, intratracheal elastase treatment has been shown to up-regulate some Nrf2 target genes, primarily in alveolar macrophages in mice. Moreover, transplantation of bone marrow derived from wild-type mice into Nrf2-deficient mice has been shown to rescue severe elastase-induced emphysema; Nrf2-positive macrophages appear, which suggests that macrophages play a crucial role via the Nrf2-dependent system in protecting the lungs against the development of emphysema (9).

There have been no studies dealing with Nrf2 regulation in pulmonary macrophages exposed to cigarette smoke (CS) or with the relationship between Nrf2 regulation in human pulmonary macrophages and COPD. In the present study, Nrf2 regulation in response to CS exposure was assessed *in vitro* using human alveolar macrophages. Second, the effects of aging on CS-induced regulation of Nrf2 and its target genes in human and mouse alveolar macrophages were determined. Finally, the association between Nrf2 mRNA levels in the pulmonary macrophages, bronchiolar epithelium, and alveolar septa and the presence of COPD was investigated using site-specific gene expression analysis using dissection techniques.

CLINICAL RELEVANCE

There have been no studies on the relationship between Nrf2 and chronic obstructive pulmonary disease (COPD). Acute cigarette smoke exposure leads to Nrf2 activation in human macrophages, and Nrf2 expression is decreased in pulmonary macrophages in current smokers and patients with COPD.

MATERIALS AND METHODS

Collection of Human Alveolar Macrophages

Human alveolar macrophages were collected from healthy volunteers as described previously (18). The bronchoalveolar lavage fluid (BALF) was centrifuged, and the cell-free supernatants were stored at −80°C

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until use. Cell pellets were counted in a hematocytometer, and smears stained with Diff-Quik (Sysmex International Reagents, Kobe, Japan) were used to identify differential profiles after cytospin preparation. Differential counts were performed by examining at least 300 cells using a standard light microscope. BAL cells were adjusted to 1×10^6 cells/ml and plated on plastic plates for 1 hour at 37°C in RPMI 1640 medium. After two washes in PBS, the adherent cells were used as alveolar macrophages for the following procedures. The alveolar macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml penicillin/streptomycin.

Collection of Mouse Alveolar Macrophages

Mouse alveolar macrophages were collected from male C57BL/6 mice (young and older groups; 3 mo and 20 mo of age, respectively) and male Imprinting Control Region (ICR) mice (adult and older groups; 8–10 mo and 19–20 mo of age, respectively) (CLEA Japan, Tokyo, Japan). The mice were killed by CO_2 narcosis. The lungs were lavaged with 0.6 ml of saline five times through a tracheal cannula. The BAL cells were adjusted to 2×10^4 cells/ml (pooled from three mice for mRNA analysis) or 5×10^5 cells/ml (pooled from eight mice for protein analysis) and plated on plastic plates for 1 hour at 37°C in RPMI 1640 medium. After two washes in PBS, the adherent cells were used as alveolar macrophages for the following procedures. The alveolar macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS and 100 U/ml penicillin/streptomycin.

Preparation and Exposure of Cigarette Smoke Extract

The smoke of two cigarettes (12 mg of tar and 1.0 mg of nicotine) (Marlboro; Philip Morris, Richmond, VA) was bubbled through 15 ml of culture media using a 60-ml plastic syringe. Each cigarette was completely burned after six draws of the syringe, with each individual draw taking approximately 10 seconds to complete, and the smoke was bubbled into media at a speed of 1 ml/s. The resulting suspension was defined as 100% cigarette smoke extract (CSE) and was filtered through a 0.22-μm filter to remove bacteria and large particles. The pH of the CSE solution was between pH 7.1 and 7.4. Subsequently, the CSE was diluted to appropriate concentration with culture media. CSE concentrations of 10% for human macrophages and 2.5% for mouse macrophages were chosen to maintain cell viabilities at 80% after 24 hours of exposure to CSE. The cells were exposed to CSE within 15 minutes after the CSE was prepared. After incubation, the cells were harvested for RNA isolation or protein extraction.

Nrf2 Immunocytochemistry and Immunohistochemistry

For immunocytochemistry, the cells were grown on four-well Lab-TEK chamber slides (Nalge Nunc International Corp., Rochester, NY) and fixed in 4% paraformaldehyde for 10 minutes. After washing in PBS, the slides were processed using a catalyzed signal amplification kit (DakoCytomation, Glostrup, Denmark) according to the manufacturer's protocol. The slides were incubated for 15 minutes at room temperature with anti-Nrf2 rabbit polyclonal antibody (sc-13032; Santa Cruz Biotechnology Inc., Santa Cruz, CA) (19) diluted to 1:500 with PBS. The slides were counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan).

For immunohistochemistry, the lung tissue was obtained from three lifelong nonsmokers, two Global Initiative for Obstructive Lung Disease (GOLD) stage I patients, and one GOLD patient with stage IV COPD who had lung resections to remove lung tumors. The tissue specimens were fixed in 10% phosphate-buffered formalin and embedded in paraffin; 5-μm sections were deparaffinized in xylene and rehydrated with graded alcohols. Antigenic activity was retrieved by incubating the specimens in 10 mM of citrate buffer (pH 6.0) in a microwave for 10 minutes. After washing in PBS, the sections were processed using a catalyzed signal amplification kit and prepared for immunocytochemistry. These sections were incubated for 15 minutes at room temperature with 1:500 anti-Nrf2 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.) and counterstained with Mayer's hematoxylin. Normal rabbit immunoglobulin fraction (DakoCytomation) was used as a negative control. To avoid run-to-run variations in the immunoreaction, all specimens were stained in the same run.

Western Blotting

Nuclear extracts of human alveolar macrophages were made using ice-cold NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Whole-cell lysates of mouse alveolar macrophages were obtained with T-PER Tissue Extraction Reagent (Pierce). The protein concentrations were determined using the BCA Protein Assay (Pierce). For the Western blotting analysis, 3.5 μg of nuclear extracts or 10 μg of whole-cell lysates were resolved on 8% SDS-PAGE gels and transferred onto Immun-Blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA). After blocking for 1 hour with 7.5% nonfat dried milk in TBS containing 0.5% Tween 20 at room temperature, the membranes were incubated overnight with 1:100 to 1:1,000 anti-Nrf2 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.) at 4°C, followed by incubation for 1 hour with 1:10,000 horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (DakoCytomation). After washing, immunoreactive proteins were detected by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and subsequent autoradiography.

Subjects

Two sets of subjects were recruited: one set for the BAL study and the other set for the surgical tissue study. Some patients in each group had been subjects in our previous studies (18, 20). In all subjects, steady-state measurements of vital capacity and FEV_1 were obtained (CHESTAC-55V; Chest Co., Tokyo, Japan). Current smokers were arbitrarily defined as individuals who had smoked up to at least 24 hours before the procedures. To objectively confirm the current smoking status, all subjects were examined for exhaled CO levels using a CO analyzer (Micro Smokerlyser; Bedfont, Rochester, UK). Written informed consent was obtained from each subject, and the Ethics Committee of Hokkaido University School of Medicine approved the study protocols.

Sampling of Pulmonary Macrophages, Bronchiolar Epithelial Cells, and Alveolar Septa from Human Lung Tissue

Six or more blocks of peripheral lung tissue ($1.0 \times 1.0 \times 0.5$ cm) were collected and frozen as soon as possible after lung resection. The lung tissue was placed in the base of a cryomold (Sakura Finetek U.S.A., Torrance, CA), carefully overlaid with additional Tissue-Tek OCT (Sakura Finetek U.S.A.), and immediately frozen with dry ice. Sampling of pulmonary macrophages and bronchiolar epithelial cells was performed by laser capture microdissection (LCM) using a PixCell II System (Arcturus Engineering, Mountain View, CA) as described previously (20). Macrophages localized in the alveolar space and in the alveolar walls were harvested by immuno-LCM using CD-68 antibodies (DakoCytomation) as described previously (20). A total of 30,000 laser bursts was used to collect the macrophages from each subject. LCM of bronchiolar epithelial cells was performed on hematoxylin-stained peripheral lung sections; a total of 40,000 laser bursts was used to collect cells from each subject. Sampling of alveolar septa was performed using a manual dissection technique. Alveolar septa were identified, and adjacent unwanted tissues, such as airways, vessels, and pleurae, were manually dissected out under visual control using an 18-gauge, fine sterile needle, and the remaining tissue was harvested from hematoxylin-stained lung tissues. Four serial nonstained sections adjacent to the sections used for LCM were used for analysis as whole lung tissue specimens.

RNA Purification and Quantitative RT-PCR

RNA extraction, reverse transcription, and PCR were performed as described previously (18, 20). Briefly, total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany). The quantity and quality of the RNA were determined using a LabChip kit (Agilent Technologies, Palo Alto, CA). The RNA was reverse transcribed using the TaqMan Reverse Transcription Reagents and the RT Reaction Mix (Applied Biosystems, Foster City, CA) on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The resulting first-strand cDNA was used as a template for RT-PCR. 5′-exonuclease–based fluorogenic PCR was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Taqman Gene Expression Assays probes (Applied Biosystems) were used for human glyceraldehyde-3-phosphatase-dehydrogenase (GAPDH) (assay ID: Hs99999905_m1); human Nrf2 (Hs00232352_m1); human heme oxygenase-1 (HO-1) (Hs00157965_m1); human NAD(P)H:quinone oxidoreductase-1 (NQO1) 1 (Hs00168547_m1); human glutamate-cysteine ligase, modifier subunit (GCLM) (Hs00157694_m1);

human glutathione reductase (GSR) (Hs00167317_m1); mouse β2-microglobulin (Mm00437764_m1); mouse Nrf2 (Mm00477784_m1); mouse HO-1 (Mm00516007_m1), mouse NQO1 (Mm00500821_m1); mouse GCLM (Mm00514996_m1); and mouse GSR (Mm00833903_m1). The relative amount of each gene mRNA in the samples was assessed by interpolation of their threshold cycles from a standard curve, which was then normalized against human GAPDH mRNA or mouse β2-microglobulin mRNA.

Measurement of Total Glutathione and Oxidized Glutathione in BALF

Total glutathione and oxidized glutathione (glutathione disulfide [GSSG]) levels in BALF were measured using a glutathione assay kit (Cayman Chemical Co., Ann Arbor, MI), as described previously (21).

Assessment of Carbonylated Albumin in BALF

Oxidation of individual BALF proteins was measured using Western blotting analysis as described previously (21). BALF was derivatized with dinitrophenylhydrazine (DNP) using the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Temecula, CA) with slight modification (21). Blots were performed using the anti-DNP antibody and scanned with a GT-9500 scanner (Epson, Nagano, Japan); the intensity of the bands was calculated using NIH Image software (version 1.62). Because a major carbonyl protein band for all of the subjects was a 68-kD protein corresponding to albumin (21), the DNP units of the 68-kD band were quantified by dividing by the result of a standard sample from a representative young nonsmoker. The value was normalized based on the BALF albumin concentration.

Data Presentation and Statistical Analysis

Data are expressed as the mean ± SE or the median and range. For the demographic data, differences between two groups were analyzed using an unpaired *t* test; more than two groups were compared using single-factor ANOVA followed by a *post hoc* Tukey-Kramer test. For other data, differences between the two groups were analyzed using the Mann-Whitney U test; more than two groups were compared using the Kruskal-Wallis test followed by the Mann-Whitney U test. Correlations were analyzed using Spearman's rank method. All tests were done using the StatView J 5.0 System (SAS Institute Inc., Cary, NC). Statistical significance was set at $P < 0.05$.

RESULTS

Nrf2 Regulation in Response to Acute CSE Exposure in Human Alveolar Macrophages

To examine whether Nrf2 is activated in response to CSE, nuclear accumulation of Nrf2 was evaluated using immunocytochemistry and Western blotting. On immunocytochemistry, Nrf2 was constitutively present in the cytoplasm of human alveolar macrophages isolated from young healthy volunteers (Figure 1A). In contrast, Nrf2 was strongly detected in the nuclei of human alveolar macrophages after exposure to 10% CSE for 1 hour (Figure 1A). Western blotting of nuclear extracts obtained from human alveolar macrophages after exposure to CSE for up to 2 hours confirmed the nuclear accumulation of Nrf2 protein (~110 kD) (Figure 1B).

Next, CSE-induced transcriptional regulation of Nrf2 and several Nrf2 target genes in human alveolar macrophages was investigated. We selected HO-1, NQO1, GCLM, and GSR genes because we recently found that these genes were primarily regulated by Nrf2 in response to CS in the immortalized mouse Clara cell line (C22 cells) (22) and because these genes were reported to be down-regulated in the lungs of CS-exposed, Nrf2-deficient mice (7). CSE exposure did not change Nrf2 expression over a 24-hour period, except for slight up-regulation at 2 hours (Figure 1C). On the other hand, Nrf2 target genes HO-1, NQO1, GCLM, and GSR were significantly up-regulated after CSE exposure (Figure 2). The results of these *in vitro* studies indicate

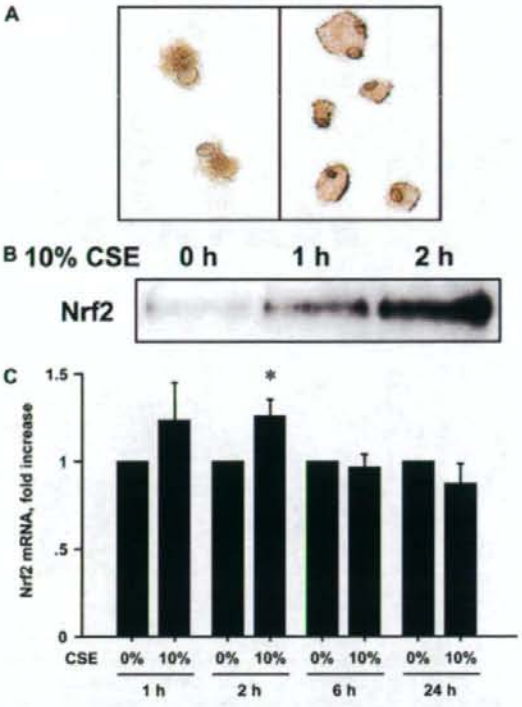

Figure 1. Regulation of Nrf2 in response to cigarette smoke extract (CSE) in human alveolar macrophages (*A*) Nrf2 immunocytochemistry. *Left panel*: Untreated human alveolar macrophages. *Right panel*: Human alveolar macrophages stimulated with 10% CSE for 1 hour. Positive immunostaining appears brown. Original magnification: ×400. HO-1 = human heme oxygenase-1. (*B*) Immunoblot analysis of nuclear extracts isolated from human alveolar macrophages with anti-Nrf2 antibody. Exposure to 10% CSE increased Nrf2 (~110 kD) in the nuclear extracts. NQO1 = human NAD(P)H:quinone oxidoreductase-1. (*C*) Nrf2 mRNA expression in human alveolar macrophages in response to CSE. Alveolar macrophages were harvested by bronchoalveolar lavage (BAL) from three healthy volunteers. The data are shown as means ± SE of three experiments for each volunteer ($n = 3$). Values are corrected for GAPDH and expressed as fold increases against the value of the nontreatment control subjects at each time point. $^*P < 0.05$ compared with nontreatment control subjects at each time point. GCLM = human glutamate-cysteine ligase, modifier subunit.

that, in human alveolar macrophages, acute CSE exposure activates Nrf2 mainly via accumulation of its protein in the nucleus and then up-regulates several Nrf2 target genes.

Expression of Nrf2 and Nrf2 Target Genes in Alveolar Macrophages Harvested by BAL

The effects of aging and chronic smoking on Nrf2 expression levels in human pulmonary macrophages were determined. In this BAL study, 37 healthy volunteers were enrolled; the volunteers included seven young and seven older lifelong nonsmokers as well as nine young and 14 older current smokers (Table 1). All current smokers had stopped smoking cigarettes for at least 12 hours before to the BAL procedure. None of the subjects in the BAL study had a history of asthma or had a respiratory infection within the month before enrollment. Among the young

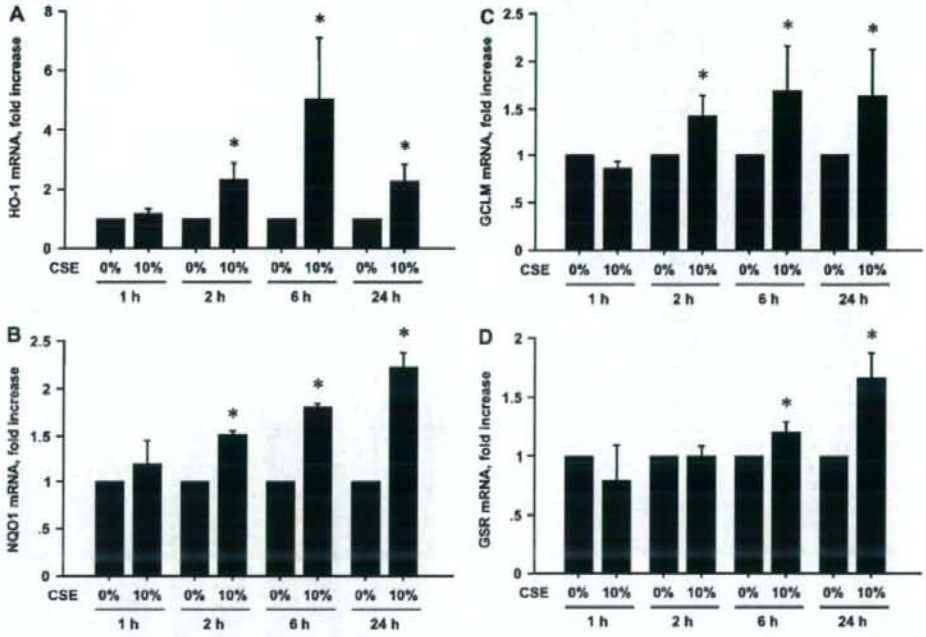

Figure 2. mRNA expression of Nrf2 target genes in response to CSE in human alveolar macrophages (*A*) HO-1 mRNA. (*B*) NQO1 mRNA. (*C*) GCLM mRNA. (*D*) Human glutathione reductase (GSR) mRNA. Alveolar macrophages were harvested by BAL from three healthy volunteers. The data are shown as means ± SE of three experiments for each volunteer ($n = 3$). Values are corrected for GAPDH and expressed as fold increases against the value of the nontreatment control subjects at each time point. *$P < 0.05$ compared with nontreatment control subjects at each time point.

volunteers, FEV_1/FVC was significantly lower in current smokers than in lifelong nonsmokers. Among the older volunteers, the FEV_1% predicted was significantly lower in current smokers than in lifelong nonsmokers, although none of the subjects had COPD. Regardless of age, the percentage recovery of instilled fluid was significantly lower in current smokers than in lifelong nonsmokers. Furthermore, in the young and older volunteers, the total numbers of BALF cells and the percentage of BALF macrophages were significantly increased in current smokers compared with lifelong nonsmokers.

Among young subjects, Nrf2 mRNA expression in BALF alveolar macrophages did not differ between lifelong nonsmokers and current smokers (Figure 3A). Among older subjects, Nrf2 mRNA expression in BALF alveolar macrophages was significantly down-regulated in current smokers compared with lifelong nonsmokers ($P < 0.001$) (Figure 3A). Although there was no difference in the BALF macrophages' Nrf2 mRNA levels between young and older lifelong nonsmokers, Nrf2 mRNA expression was significantly down-regulated in older current smokers compared with young current smokers ($P = 0.02$) (Figure 3A). The issue of whether the basal transcriptional level of Nrf2 affects the levels of Nrf2 target genes in alveolar macrophages was then considered, and it was demonstrated that Nrf2 translocation into the nucleus is the key event in the induction of Nrf2 target genes in response to CS (*see* Figure 1). To address this issue, the expression of Nrf2 target genes in BALF alveolar macrophages was examined. In addition to Nrf2 mRNA, HO-1 mRNA in BALF alveolar macrophages was significantly down-regulated in older current smokers compared with older lifelong nonsmokers ($P = 0.02$) and young current smokers ($P = 0.009$) (Figure 3B). NQO1, GCLM, and GSR mRNAs were significantly up-regulated in young current smokers compared with young lifelong nonsmokers, whereas in the older subjects, NQO1, GCLM, and GSR mRNAs did not differ by smoking status (Figures 3C–3E).

Expression of Nrf2 in Alveolar Macrophages and Oxidative Stress Markers in BALF

We previously reported that oxidized glutathione (GSSG) and protein carbonyl levels were elevated in BALF obtained from older smokers with long smoking histories (21). This suggests that endogenous antioxidant defenses are overwhelmed in older

TABLE 1. CHARACTERISTICS OF THE BAL STUDY SUBJECTS

	Young		Older	
	Lifelong Nonsmokers	Current Smokers	Lifelong Nonsmokers	Current Smokers
n	7	7	9	14
Sex, M/F	7/0	7/0	9/0	12/2
Age, yr	23 ± 2	23 ± 1	68 ± 3	51 ± 2*
Pack-years	0	5 ± 1	0	44 ± 5
FEV_1/FVC, %	96 ± 2	86 ± 3†	81 ± 3	81 ± 2
FEV_1, % predicted	84 ± 5	89 ± 5	121 ± 7	103 ± 4*
BAL findings				
Recovery rate, %	80 ± 3	66 ± 4†	67 ± 5	50 ± 4*
Total cells, $\times 10^4$/ml	9 ± 1	14 ± 2†	12 ± 2	39 ± 9*
Macrophages, %	87 ± 2	94 ± 2†	84 ± 5	96 ± 1*

* $P < 0.05$ versus older lifelong nonsmokers.
† $P < 0.05$ versus young lifelong nonsmokers (mean ± SE).

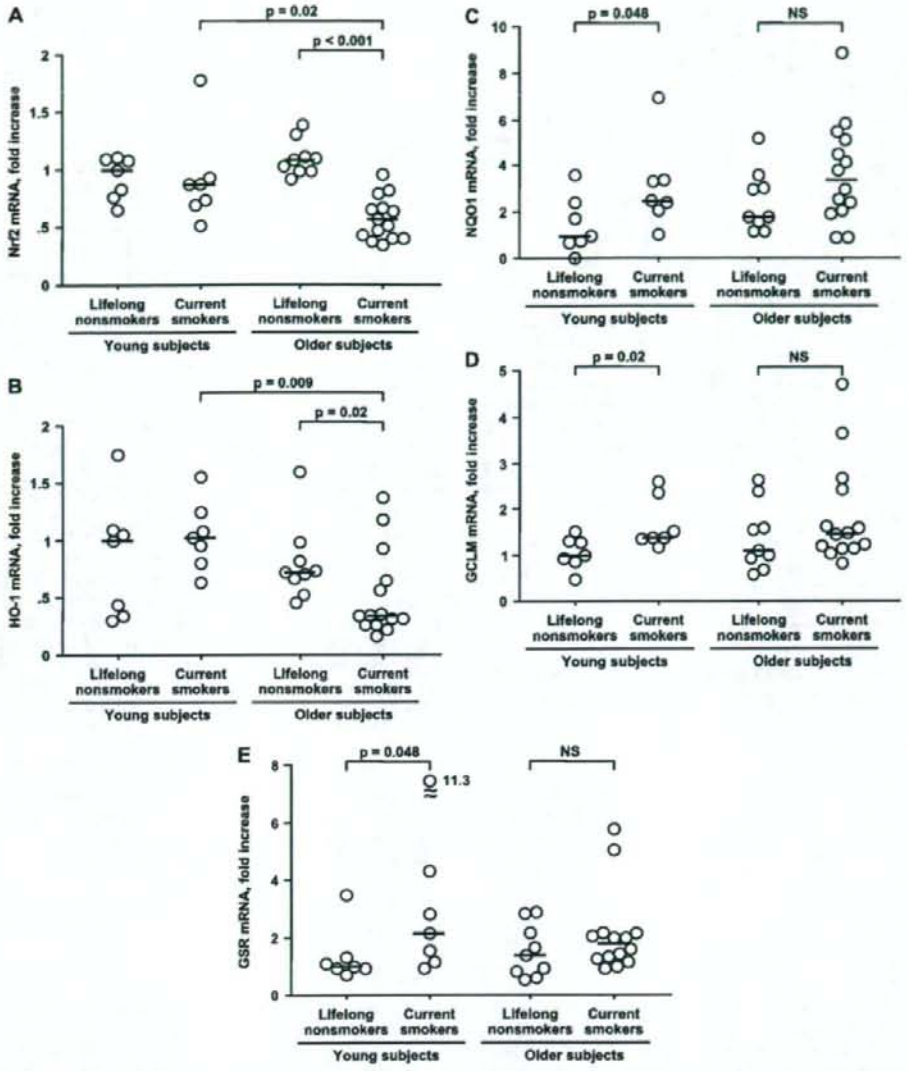

Figure 3. mRNA expression of Nrf2 and Nrf2 target genes in human alveolar macrophages harvested by BAL. (*A*) Nrf2 mRNA. (*B*) HO-1 mRNA. (*C*) NQO1 mRNA. (*D*) GCLM mRNA. (*E*) GSR mRNA. Medians are indicated by *horizontal lines*. The values are corrected for GAPDH and are expressed as fold increases against the median value of lifelong nonsmokers.

current smokers. To investigate whether the down-regulation of Nrf2 mRNA in alveolar macrophages is related to the presence of excessive oxidative stress in the lungs, the relationships between macrophage Nrf2 mRNA levels and BALF GSSG and carbonylated albumin levels were examined. Among the older subjects, it was found that, in the BALF, macrophage Nrf2 mRNA levels were significantly correlated with the GSSG level ($r = -0.57$; $P = 0.007$) (Figure 4A) and the ratio of GSSG per total glutathione ($r = -0.44$; $P = 0.03$). Data on BALF GSSG levels and the ratio of GSSG per total glutathione of seven older lifelong nonsmokers and 14 older current smokers were obtained from a previous paper (21). BALF macrophage Nrf2 mRNA levels were significantly correlated with carbonylated albumin levels ($r = -0.57$; $P = 0.008$) (Figure 4B). Data on BALF carbonylated albumin levels of seven older lifelong nonsmokers and nine older current smokers were obtained from a previously published paper (21). These findings suggest that down-regulation of Nrf2 in alveolar macrophages obtained from older current smokers is associated with diminished expression of Nrf2 target genes, leading to the appearance of excessive oxidative stress markers in the epithelial lining fluid.

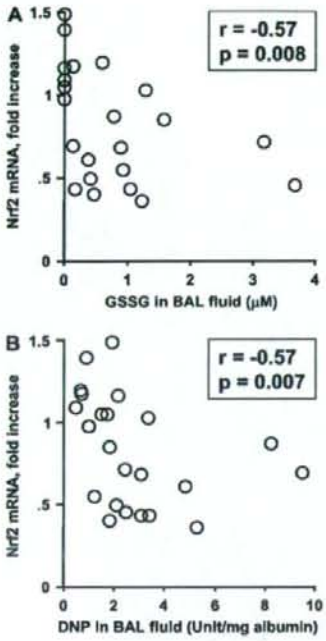

Figure 4. Correlations between Nrf2 mRNA levels in alveolar macrophages and oxidative stress markers in BAL fluid (BALF). (*A*) Correlation between BALF macrophage Nrf2 mRNA levels and BALF oxidized glutathione (GSSG) levels. (*B*) Correlation between BALF macrophage Nrf2 mRNA levels and BALF carbonylated albumin levels. These figures incorporate the data of 21 and 16 subjects from reference (17), respectively.

Expression of Nrf2 and Nrf2 Target Genes in Response to CSE Exposure in Mouse Alveolar Macrophages of Different Age Groups

The human BAL study showed that expression of Nrf2 and Nrf2 target genes in human alveolar macrophages differed between young and older current smokers. Thus, it was hypothesized that aging affects the regulation of alveolar macrophages' expression of Nrf2 and Nrf2 target genes in response to CS. To investigate this hypothesis, *in vitro* experiments were done using mouse alveolar macrophages. Alveolar macrophages were obtained from two age groups (young/adult and older groups) of C57BL/6 and ICR mice, and the alveolar macrophages were exposed to 2.5% CSE for 6 or 24 hours. Nrf2 was temporarily down-regulated in alveolar macrophages exposed to CSE for 6 hours in both age groups; the Nrf2 mRNA level was lower in older mice than in young mice (Figure 5A). CS-induced Nrf2 up-regulation was observed in alveolar macrophages at 24 hours only in young/adult mice but not in older mice independent of the strain (C57BL/6 or ICR) (Figures 5A and 5B). On Western blotting, the intensity of band corresponding to Nrf2 was weaker in the CSE-exposed alveolar macrophages of the older mice than of the young mice. Therefore, changes in the mRNA levels seem to mirror the Nrf2 protein levels (Figure 5C).

The effects of aging on the expression of Nrf2 target genes in mouse alveolar macrophages exposed to CSE for 6 hours were investigated. HO-1, GCLM, and GSR mRNAs were significantly decreased in CSE-exposed alveolar macrophages of older mice compared with those of young mice (Figure 6), whereas NQO1 mRNA did not change between the two age groups (data not shown). These results suggest that aging impairs induction of Nrf2 and its target genes in alveolar macrophages in response to CS in mice.

Site-Specific Nrf2 mRNA Expression in the Human Lung

To investigate Nrf2 mRNA levels in macrophages and in other lung sites in association with COPD, a site-specific gene expression analysis using dissection techniques was done. For this surgical tissue study, 20 patients who had a lung resection for small peripheral tumors were recruited; 10 of these had normal respiratory functions (five lifelong nonsmokers and five former smokers) and served as control subjects, and 10 were former smokers who had COPD (Table 2). All former smokers in both groups had quit smoking at least 1 month before surgery. There was no difference in age between the two groups. The FEV_1/FVC and FEV_1% predicted values were significantly lower in subjects with COPD than in control subjects. None of the subjects in the surgical tissue study had a history of asthma or had a respiratory infection during the month before enrollment. The GOLD guideline was used to make the diagnosis and to grade COPD severity (23).

Based on the whole lung analysis, subjects with COPD showed a significant decrease in Nrf2 mRNA expression compared with control subjects ($P = 0.01$) (Figure 7A). In particular, the Nrf2 mRNA of LCM-retrieved pulmonary macrophages was markedly down-regulated in subjects with COPD compared with control subjects ($P = 0.001$) (Figure 7B); it was significantly down-regulated in subjects with COPD compared with lifelong nonsmokers ($P = 0.02$) and in subjects with COPD compared with former smokers ($P = 0.003$). The Nrf2 mRNA level of LCM-retrieved bronchiolar epithelial cells also tended to be down-regulated in subjects with COPD compared with control subjects ($P = 0.096$) (Figure 7C); however, in the alveolar septa, there was no difference in the Nrf2 expression between the two groups (Figure 7D). When all of the subjects' data were analyzed together, there were significant correlations between FEV_1/FVC and Nrf2 mRNA levels in the whole lung ($r = 0.60$; $P = 0.009$), pulmonary macrophages ($r = 0.76$; $P < 0.001$), and bronchiolar epithelial cells ($r = 0.47$; $P = 0.04$), whereas FEV_1% predicted was significantly correlated only with Nrf2 mRNA levels in pulmonary macrophages ($r = 0.60$; $P = 0.009$).

Immunohistochemistry of Nrf2 in the Human Lung

On immunohistochemistry, Nrf2 protein was predominantly located in the cytoplasm of alveolar macrophages and bronchiolar epithelial cells, whereas Nrf2-positive cells were sparsely seen within the alveolar septa in lung tissue obtained from lifelong nonsmokers (Figure 8A). In contrast, Nrf2 staining intensity was weak in alveolar macrophages and bronchiolar epithelial cells in the lung tissue of subjects with COPD (Figures 8B and 8C).

DISCUSSION

In the present study, CSE exposure to macrophages rapidly induced nuclear accumulation of Nrf2 and activated the transcription of Nrf2 target genes. This suggests that the Nrf2 present in macrophages plays a role in the human defense system against CS exposure. The basal Nrf2 mRNA levels and Nrf2 target gene expressions were significantly lower in alveolar macrophages obtained from older current smokers than from lifelong nonsmokers, and basal Nrf2 mRNA levels in pulmonary macrophages were significantly lower in patients with COPD than in nonsmokers and former smokers without COPD.

Under normal conditions, Nrf2 resides in the cytoplasm and is bound to its negative regulator, the Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), which leads to the degradation of Nrf2. When cells are exposed to oxidative or xenobiotic stress, Nrf2 is released from Keap1 and rapidly translocates into the nucleus, where it activates its target genes (24–27). In macrophages, Nrf2 activation occurs in the presence of oxidative stress, such as 4-hydroxynonenal and diesel exhaust chemicals (15, 16). The present results show that macrophages also activate Nrf2-regulated genes in

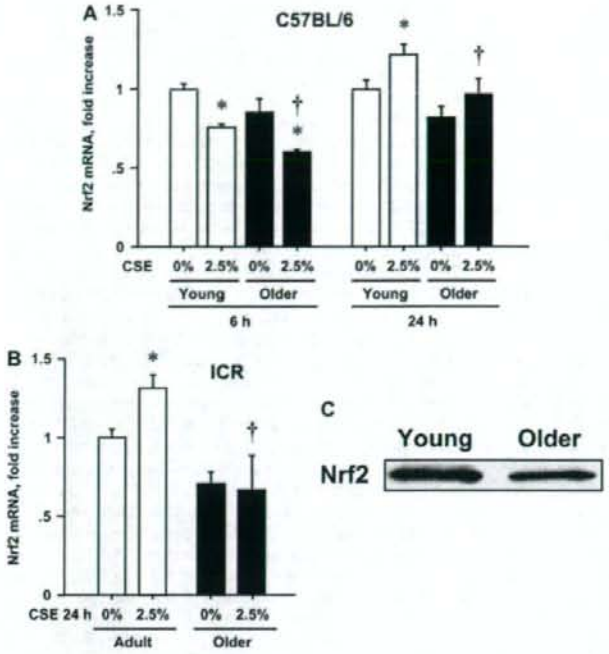

Figure 5. Nrf2 expression in response to CSE in mouse alveolar macrophages of different age groups. (*A, B*) Nrf2 mRNA expression in response to CSE in mouse alveolar macrophages. (*A*) C57BL/6 mice. (*B*) Imprinting Control Region (ICR) mice. Mouse alveolar macrophages stimulated with 2.5% CSE or medium only for 6 or 24 hours. The data are shown as means ±SE of three experiments ($n = 3$; each sample was pooled from three mice). The values are corrected for β2-microglobulin and expressed as fold increases against the median value of the nontreatment young/adult control mice. $^*P < 0.05$ compared with the nontreatment control mice. $^\dagger P < 0.05$ compared with CSE-exposed alveolar macrophages of young/adult mice. (*C*) Immunoblot analysis of whole-cell lysates isolated from pooled alveolar macrophages of eight C57BL/6 mice exposed to CSE for 24 hours with anti-Nrf2 antibody. Nrf2 protein (~110 kD) in CSE-exposed alveolar macrophages of older mice was less than in those of young mice.

response to CS. Nrf2 activation seems to occur mainly by protein stabilization and translocation into the nucleus rather than by transcriptional up-regulation. However, Nrf2 mRNA was slightly up-regulated in human alveolar macrophages exposed to CSE for 2 hours (Figure 1C) and in alveolar macrophages obtained from young/adult mice after 24 hours of CSE exposure (Figures 5A and 5B). Because an ARE-like element is found in the Nrf2 gene promoter region (28, 29), Nrf2 transcription seems to be, at least in part, self-regulated.

No previous studies have analyzed basal Nrf2 transcriptional levels in human lungs. Therefore, to evaluate the effects of current smoking on *in vivo* Nrf2 expression in alveolar macrophages, a BAL study of healthy volunteers was done. The amount of time between the last exposure to cigarette smoke and the harvesting of macrophages in current smokers was strictly controlled. Nrf2 mRNA and Nrf2 target gene expressions were significantly lower in alveolar macrophages obtained from healthy older current smokers than in alveolar macrophages obtained from older lifelong nonsmokers or young current smokers (Figure 3). Taken together with the findings in the mouse experiments, it appears that, in alveolar macrophages, aging suppresses Nrf2 up-regulation and/or activation in response to CS exposure.

We previously reported that the potential antioxidant activity of alveolar macrophages was impaired and that oxidized glutathione and protein carbonyl levels were elevated in the BALF obtained from older smokers with long-term smoking histories

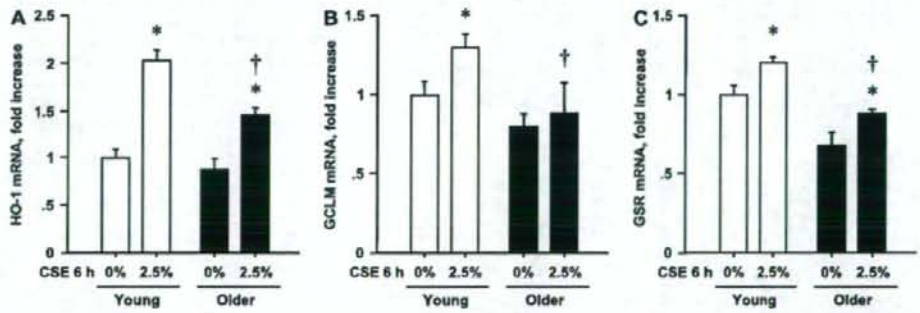

Figure 6. mRNA expression of Nrf2 target genes in response to CSE in mouse alveolar macrophages of different age groups (*A*) HO-1 mRNA. (*B*) GCLM mRNA. (*C*) GSR mRNA. Alveolar macrophages of C57BL/6 mice stimulated with 2.5% CSE or medium only for 6 hours. The data are shown as means ± SE of three experiments ($n = 3$; each sample was pooled from three mice). The values are corrected for β2-microglobulin and expressed as fold increases against the median value of the nontreatment young control mice. $^*P < 0.05$ compared with the nontreatment control mice. $^\dagger P < 0.05$ compared with CSE-exposed alveolar macrophages of young mice.

TABLE 2. CHARACTERISTICS OF THE SURGICAL TISSUE STUDY SUBJECTS

	Control Subjects	Subjects with COPD
n	10	10
Sex, M/F	4/6	10/0
Former smokers	5	10
Age, yr	61 ± 5	68 ± 3
FEV_1/FVC, %	82 ± 2	58 ± 3*
FEV_1, % predicted	119 ± 5	87 ± 10*
GOLD stage, I/II/III/IV	—	8/1/0/1

Definition of abbreviation: COPD, chronic obstructive pulmonary disease.
* $P < 0.05$ versus control subjects (mean ± SE).

(21, 30). Indeed, among older subjects, BALF macrophage Nrf2 mRNA levels were inversely correlated with oxidized glutathione and carbonylated albumin levels (Figures 5B and 5C). This implies that, with age, endogenous antioxidant defenses are overwhelmed in the lungs of subjects with long-term smoking histories. Based on our recent finding that lowering basal Nrf2 mRNA levels using Nrf2 siRNA abrogated CS-induced Nrf2 target gene expressions in C22 cells (the mouse Clara cell line) (22) and on similar findings found by others in various cells (31, 32), we wondered whether the expression of Nrf2 target genes might be lowered in alveolar macrophages whose Nrf2 mRNA level was down-regulated. As was the case for Nrf2 mRNA, HO-1 mRNA was significantly lower in macrophages from current smokers compared with never smokers only in older subjects. On the other hand, increased NQO1, GCLM, and GSR mRNA levels were observed only in young current smokers (*see* Figure 4). Therefore, the down-regulation of basal Nrf2 mRNA levels in alveolar macrophages might be related to impaired antioxidant capacity in older current smokers' lungs via impaired induction of Nrf2 target genes in those cells.

Next, in the surgical tissue study, site-specific Nrf2 mRNA levels in lung tissue obtained from patients with COPD and control subjects were determined. Among the cell types/sites that were examined, Nrf2 mRNA levels in pulmonary macrophages were markedly down-regulated in subjects with COPD compared with nonsmokers and former smokers without COPD (Figure 7B); in fact, they were significantly correlated with FEV_1/FVC and FEV_1% predicted values. These are the first human data supporting previous animal studies to suggest that Nrf2 deficiency is related to susceptibility for pulmonary emphysema (7–9) and that macrophages play a crucial role via the Nrf2-dependent system in protecting against the development of emphysema (9). This finding is also in line with previous reports showing that the expression of HO-1, one of the Nrf2 target genes, was diminished in pulmonary macrophages obtained from patients with COPD (33, 34). There is increasing evidence that there is an elevated oxidative stress level in the lungs of patients with COPD (35); the present data imply that decreased Nrf2 expression levels in pulmonary macrophages may contribute to the oxidant/antioxidant imbalance observed in COPD. It was found in the present study that, in subjects with COPD, down-regulation of Nrf2 mRNA in pulmonary macrophages is sus-

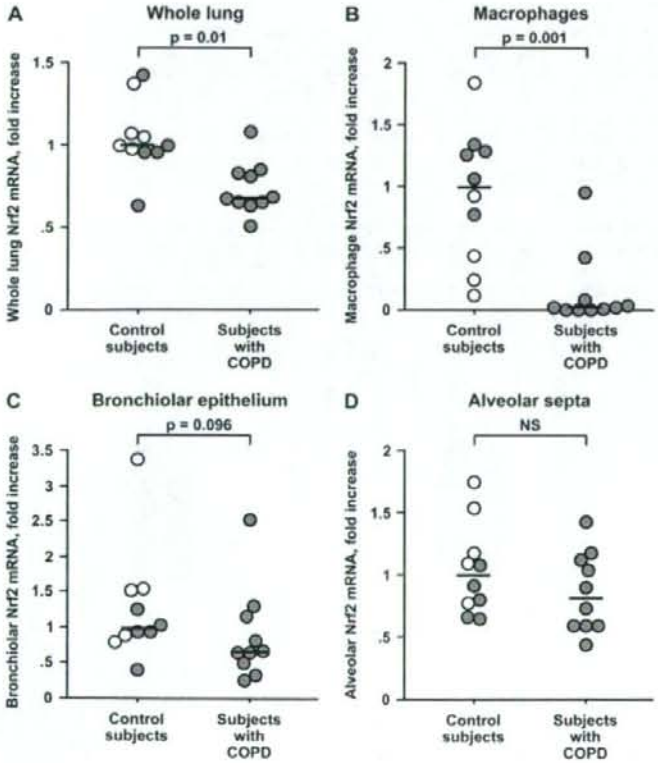

Figure 7. Site-specific Nrf2 mRNA expression in the human lung. (*A*) Whole lung homogenates. (*B*) Pulmonary macrophages collected by immuno–laser capture microdissection (LCM). (*C*) Bronchiolar epithelial cells collected by LCM. (*D*) Alveolar septa collected by manual dissection. *White circles,* lifelong nonsmokers; *gray circles,* former smokers. Medians are indicated by *horizontal lines*. The values are corrected for GAPDH and expressed as fold increases against the median value of the control subjects.

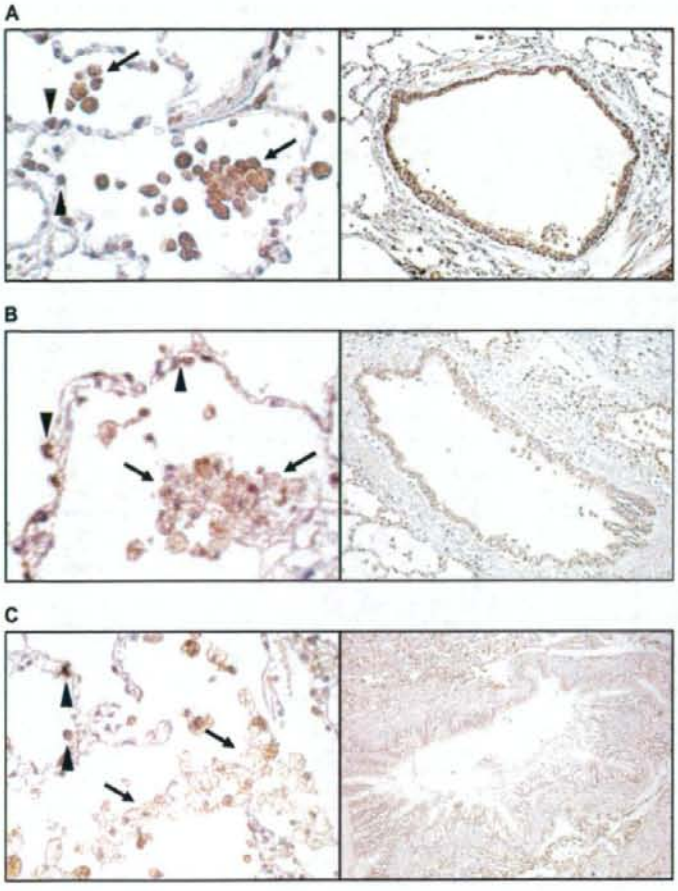

Figure 8. Nrf2 immunohistochemistry in the human lung. In lung tissue obtained from lifelong nonsmokers, Nrf2 has a cytoplasmic localization in alveolar macrophages (*left panel, arrows*), dome-shaped cells within alveolar septa (*left panel, arrowheads*), and bronchiolar epithelial cells (*right panel*) (*A*). Nrf2 staining intensity is weak in alveolar macrophages (*left panel, arrows*) and bronchiolar epithelial cells (*right panel*) in the lung tissue obtained from patients with GOLD stage I COPD (*B*) and stage IV COPD (C). Nrf2-positive cells within the alveolar septa are present in the lung tissue obtained from patients with COPD (*B, C, left panels, arrowheads*). Positive immunohistochemical staining appears brown. Original magnification: ×400 (*left panels*), ×100 (*right panels*).

tained despite smoking cessation. Recently, the Nrf2-ARE system was shown to play an important role in the innate immune response (36); this indicates that reduced Nrf2 levels might affect inflammatory responses in older current smokers and/or patients with COPD.

Some polymorphisms in the Nrf2 gene promoter region have been reported; a −617 C/A polymorphism in the ARE-like sequence is functionally related to a decrease in Nrf2 at the transcriptional level, and patients with this polymorphism had a significantly higher risk of developing acute lung injury after major trauma (29). The same polymorphism was found in a Japanese population, although it was not significantly associated with COPD risk (37). Although the mechanisms of decreased Nrf2 expression in alveolar macrophages associated with chronic smoking, aging, and/or COPD remain to be determined, some polymorphisms of the Nrf2 gene might affect basal Nrf2 mRNA levels.

There are some limitations to the present study. First, in the BAL study, among the older subjects, the current smokers were statistically significantly younger than lifelong nonsmokers. However, this is not likely to have affected the results because there was no evidence of an aging effect with respect to basal Nrf2 expression levels in alveolar macrophages when young and older lifelong nonsmokers were compared. Second, in the surgical tissue study, most of the subjects with COPD had only mild COPD (GOLD stage I). Nrf2 mRNA levels in the pulmonary macrophages were significantly correlated with FEV_1/FVC and $FEV_1\%$ predicted values. A larger sample of patients with more advanced disease should be assessed to further our understanding of the involvement of Nrf2 regulation in the mechanism of disease progression, given that the present study's findings indicate that decreased basal Nrf2 mRNA levels occur even with mild COPD. This supports the notion that decreased Nrf2 plays a role in the development of early COPD. Last, the results of the mouse experiments should be interpreted with caution because the alveolar macrophages' Nrf2 gene expression responses against CSE exposure differed between mice and humans. However, aging seems to be a common regulatory factor for the expression of Nrf2 and Nrf2 target genes in response to CS.

In summary, it was found that acute CSE exposure leads to Nrf2 activation in human alveolar macrophages. Nrf2 expression was attenuated in pulmonary macrophages obtained from older current smokers and patients with COPD. Although the mechanism of the down-regulation of Nrf2 mRNA remains to be elucidated, the pharmacologic activation of the Nrf2-ARE system might be a useful strategy for preventing and treating COPD.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Functional single nucleotide polymorphisms of the *CCL5* gene and nonemphysematous phenotype in COPD patients

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ABSTRACT: **It was previously reported that the gain-of-function -28 guanine allele of the promoter single nucleotide polymorphism (SNP; cytosine to guanine substitution of nucleotide -28 (-28C>G)) in the CC chemokine ligand 5 gene (*CCL5*) was associated with susceptibility to late-onset asthma in patients who developed asthma at age ⩾40 yrs. The clinical diagnosis of chronic obstructive pulmonary disease (COPD) includes emphysema and small airway disease, and upregulation of CCL5 has been described in the airways of patients with COPD. It was hypothesised that *CCL5* has a genetic impact upon the variable expression of emphysema in patients with COPD.**

Patients with COPD were studied (n=267). All of the patients underwent pulmonary high-resolution computed tomography (CT), and visual scoring (CT score) was performed to determine emphysema severity. Three SNPs of *CCL5* were genotyped, including -403G>A, -28C>G and 375T>C.

A significant difference was found in CT score according to *CCL5* genotype; the -28G allele was inversely associated with CT score. When the analysis was confined to 180 patients with bronchial reversibility of <15%, even stronger evidence for this association was noted.

Functional single nucleotide polymorphisms in the CC chemokine ligand 5 gene were associated with milder emphysema. Together with previous findings, the present study may identify the CC chemokine ligand 5 gene as part of a common pathway in the pathogenesis of late-onset asthma and chronic obstructive pulmonary disease with milder emphysema.

KEYWORDS: **Chronic obstructive pulmonary disease, emphysema, genetics, late-onset asthma, small airway disease**

It was previously found that the cytosine (C) to guanine (G) substitution of nucleotide -28 (-28C>G) promoter polymorphism of the CC chemokine ligand 5 gene (*CCL5*), which has been associated with increased levels of mRNA and protein expression *in vitro* [1], was associated with susceptibility to late-onset asthma among patients who developed the disease after the age of 40 yrs [2]. In general, late-onset asthma is not strongly associated with specific allergen sensitisation. Rather, infections, including respiratory viruses, may be more likely to be involved in the pathophysiology of late-onset asthma through host response mechanisms [3]. Viral infections are associated with most exacerbations of asthma and chronic obstructive pulmonary disease (COPD) [4–8], and the most prominent aspect of the epithelial immune response towards viral respiratory infections consists of the production and release of CCL5 [9–12]. Indeed, exacerbation of mild COPD is associated with the upregulation of CCL5 in both the inflammatory and epithelial cells of the bronchial mucosa [11, 13].

The chronic airflow limitation associated with COPD is caused by a mixture of small airway disease and emphysema, the relative contributions of which vary from person to person [14]. These phenotypic variations of COPD may be influenced by several innate susceptibility factors to environmental stimuli, including tobacco smoking and viral respiratory infections. However, the

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relative importance of genetic factors in the pathogenesis of airway disease and emphysematous components of COPD is unknown. Given that accumulation of inflammatory immune cells and airway wall remodelling processes are common characteristics in the small airways of patients with asthma and COPD [15], a common genetic susceptibility may be present, with latent viral infections predisposing some patients to experience increased airway inflammation. *CCL5* may be involved in the pathogenesis of epithelial remodelling and chronic hyperreactivity in response to viral infections.

In the present study, using a well-characterised COPD cohort of Japanese subjects [16], the specific hypothesis that functional single nucleotide polymorphisms (SNPs) in the regulatory region of *CCL5* and their haplotypes have a genetic impact upon the variable expression of the emphysematous phenotype in patients with COPD was examined.

METHODS

Further details of all methods and procedures can be found in the supplementary material.

Study subjects

Among 274 patients with COPD recruited for the Hokkaido COPD cohort study [16], a total of 267 patients, for whom genetic samples were available, were examined in the present study. Study approval was obtained from governing ethics committees for each study centre, and all subjects provided written informed consent.

Lung computed tomography scans

Information regarding the computed tomography (CT) scanners and parameters assessed has been described previously [16]. The severity of emphysema was assessed visually by three independent pulmonologists according to the modified [16] scoring system of GODDARD *et al.* [17]; the pulmonologists were blinded to any clinical information regarding the patient. Six images in three slices were analysed in the lungs, including the aortic arch, the carina and 1–2 cm above the highest hemidiaphragm. Each image was scored on a scale of 0–4 (0: normal; 0.5: ⩽5% affected; 1: ⩽25% affected; 2: ⩽50% affected; 3: ⩽75% affected; 4: >75% affected); the mean score from six images was considered representative of the severity of emphysema in the lungs. When the three independent pulmonologists were split in their evaluation, the score assessed by the majority was used.

In order to confirm the accuracy and reliability of the present visual assessment, the severity of emphysema assessed visually was compared with that assessed by three-dimensional computerised analyses. The method of computerised assessment of emphysema for the whole lung has been described previously and is given in detail in the supplementary material. A strong correlation between the two methods of assessment was found (n=137; r=0.835; p<0.0001) [16].

Pulmonary function test

Spirometry was performed before and 30 min after bronchodilator (400 μg salbutamol) administration. Bronchial reversibility was expressed as the percentage change in forced expiratory volume in one second (FEV1) following salbutamol administration. The carbon monoxide diffusing capacity of the lung ($D_{L,CO}$) test was also performed, and $D_{L,CO}$/alveolar volume (V_A), adjusted for haemoglobin levels, was calculated.

Allele-specific PCR and detection of fluorescence-labelled PCR fragments

Three SNPs (-403G>A (National Center for Biotechnology Information SNP ID rs2107538), -28C>G (rs2280788) and 375T>C (rs2280789)) in the regulatory region were genotyped using an assay that combined kinetic (real-time quantitative) PCR with allele-specific amplification, as described previously [2].

Statistical analysis

The linkage disequilibrium between the three SNPs was analysed, and all of the SNPs were tested for conformation with Hardy–Weinberg expectations in patients with COPD using Haploview software, version 3.2 (Broad Institute, Cambridge, MA, USA) [18]. The genetic impact of the three regulatory SNPs on CT score was examined using a multivariate stepwise linear regression model. The model included sex, age, smoking status (current or ex-), cumulative smoking history in pack-years, body mass index, $D_{L,CO}/V_A$, FEV1 (percentage of the predicted value), levels of total serum immunoglobulin (Ig) E, and peripheral blood eosinophil counts. The genetic effects of the regulatory SNPs on CT score were also examined, using only the 180 patients whose bronchial reversibility was <15% (model 2), since the possibility might remain that the presence of bronchodilator reversibility indicates the presence of coexistent asthma, although patients with physician-diagnosed asthma were carefully excluded from the present study [16].

The association between the extent of emphysema as judged by a low attenuation volume (LAV), automatically assessed by three-dimensional CT, and the three SNPs was also examined (model 3; n=105).

The association between common haplotypes with a frequency of >1% and CT score was tested using global and haplotype-specific statistics using the Haplo.Score program (Mayo Clinic, Rochester, MN, USA) [19].

RESULTS

The clinical characteristics of the subjects are summarised in tables 1 and 2. The genotypic distributions of all of the three SNPs were in Hardy–Weinberg equilibrium. Among COPD-related phenotypes, including body mass index, FEV1 (% pred), bronchial reversibility and CT score, a significant difference was found in CT score for the -28C>G ($p<0.05$) and the -403G>A SNPs ($p<0.05$; table 2). On multiple linear regression analysis using 267 patients (model 1), the -28C>G *CCL5* SNP, but neither the -403G>A nor the 375T>C SNP, was significantly associated with CT score. The presence of the -28G allele was significantly associated with a lower CT score; mean±SD CT scores were 1.49±0.93, 1.15±0.87 and 0.93±0.69 for -28CC homozygotes, -28CG heterozygotes and -28GG homozygotes, respectively ($p=0.00038$; table 3; fig. 1E of the online supplementary material). In the subgroup analysis (model 2), the association between the -28G allele and a lower CT score was the most significant in the 180 patients who exhibited bronchial reversibility of <15% ($p=0.00002$; table 3). A linear dose–response relationship between genotype and phenotype was consistently found between CT score and

TABLE 1 Baseline characteristics of chronic obstructive pulmonary disease (COPD) patients

	All subjects	Bronchial reversibility <15%
Subjects n	267	180
Age yrs	69.6±8.1	68.9±8.3
Sex M/F n	251/16	168/12
BMI kg·m^{-2}	22.3±3.2	22.1±3.1
Current smoker %	27.3	30.6
Smoking history pack-yrs	56.0 (12–220)	54.5 (12–220)
FEV1/FVC %	50.2±12.1	53.3±11.5
FEV1 % pred	63.4±21.7	68.7±22.4
Bronchial reversibility# %	13.3±13.3	5.87±4.85
CT score	1.40±0.92	1.41±0.93
***D*L,CO/*V*A**	63.2±24.2	62.3±24.0
Atopy %	24.1	24.9
Log [total serum IgE] IU·mL^{-1}	1.78±0.69	1.81±0.71
Log [eosinophils] cells·mm^{-3}	2.20±0.33	2.18±0.32
-403G>A n		
GG	106	69
GA	122	82
AA	36	28
-28C>G n		
CC	196	133
CG	61	41
GG	6	3
375T>C n		
TT	117	75
TC	116	79
CC	34	26

Data are presented as mean±SD or median (range) unless otherwise indicated. It was not possible to determine cytosine (C) to guanine (G) substitution of nucleotide -28 (-28C>G) single nucleotide polymorphism (SNP) genotype in four COPD patients and -403G>A SNP genotype in three. M: male; F: female; BMI: body mass index; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; % pred: % predicted; CT: computed tomography; *D*L,CO: diffusing capacity of the lung for carbon monoxide; *V*A: alveolar volume; Ig: immunoglobulin; A: adenine; T: thymidine; #: (post-bronchodilator FEV1 – prebronchodilator FEV1)/prebronchodilator FEV1.

-28C>G genotype. Three independent variables, including smoking status (current or ex-), baseline FEV1 (% pred) and *D*L,CO/*V*A, were consistently associated with CT score in both models, and other variables were excluded from the models. The adjusted R^2 associated with these fitting models (models 1 and 2) was 40 and 47%, respectively. This indicated that the models including smoking, baseline FEV1 (% pred), *D*L,CO/*V*A and the -28C>G genotype explain only 40–47% of the variance in CT score, and that unidentified factors other than those included in the present study also significantly influence the extent of emphysema in patients with COPD.

Using LAV, assessed by computerised analysis as an index of the severity of emphysema, the inverse association with the -28G allele remained significant (p=0.0015; fig. 1E of the online supplementary material) even though the number of patients in this analysis was limited (n=105). The data were also analysed by combining this group with that of the heterozygote because of the low frequency of the homozygous mutant genotype. When the homozygous wild-type was compared to the combined genotype (heterozygous plus homozygous mutant), a similar inverse association was found between emphysema score and the presence of the -28G allele in models 1 (p=0.00059), 2 (p=0.00005) and 3 (p=0.0049).

The three regulatory SNPs (-403G>A, -28C>G and 375T>C) were in significant linkage disequilibrium and were shown to be part of a single haplotype, with values of the normalised disequilibrium constant *D'* ranging 0.94–0.98. The four most common haplotypes constituted 95.9% of haplotypes in the 267 patients with COPD (table 4). The haplotype comprising three SNPs was significantly associated with CT score (global p-value=0.0023). The haplotype -403A/-28G/375C was most strongly associated with lower CT scores (p=0.0010), as judged by haplotype-specific scores on the basis of 10,000 simulations, whereas the -403G/-28C/375T haplotype was associated with higher CT scores (p=0.014; table 4). Confining the analysis to the 180 patients without bronchial reversibility strengthened the association between the haplotype and CT score (global p-value=0.00075); the -403A/-28G/375C haplotype was inversely associated with CT score (p=0.00015), and the -403G/-28C/375T haplotype was associated with CT score (p=0.0011). However, the association observed in the haplotype analysis was not stronger than that observed in the single-locus analysis using the -28C>G SNP.

DISCUSSION

COPD is a heterogeneous condition including emphysema and small airway disease. In the present study, the genetic effects of functional alleles in the *CCL5* regulatory region were investigated in a well-characterised cohort of 267 patients, and it was found that the gain-of-function allele was inversely associated with severity of emphysema in patients with COPD. Given that upregulation of CCL5 in the airways has been associated with exacerbation of COPD, and a significant association between the CCL5 -28G allele and late-onset asthma having been identified [2], it was possible to test whether or not the allele has a genetic effect on variable COPD phenotypes in a hypothesis-driven association study. This type of study is statistically more powerful than the typical association study that tests multiple genes with no *a priori* hypothesis. By investigating patients with asthma and COPD, a series of these studies may identify *CCL5* as a shared genetic risk factor for these chronic inflammatory airway diseases.

Three common SNPs of functional relevance have been identified in the regulatory region of *CCL5* (-403G>A, -28C>G and 375T>C); these three SNPs influence transcriptional activity *in vitro* and subsequent CCL5 expression in human cell lines [1, 20, 21]. These SNPs were associated with increased levels of CCL5 [2, 22], as well as increased blood eosinophil counts [23]. These SNPs have also been associated with several inflammatory immune diseases, including asthma, allergic rhinitis and atopic dermatitis [2, 20, 22, 24]. In the context of haplotypes involving these SNPs, the -28G/-403G haplotype has been associated with near-fatal asthma in Chinese children [23]. In addition, the -403A/-28G haplotype has been shown to be

TABLE 2 Patient characteristics by CC chemokine ligand 5 polymorphism

	-403G>A#			-28C>G¶			375T>C+		
	GG	GA	AA	CC	CG	GG	TT	TC	CC
Age yrs	69.5±8.09	69.9±8.08	68.2±8.40	69.4±7.96	70.7±8.60	66.0±7.52	69.8±7.95	69.7±8.23	68.6±8.36
Sex M/F n	104/2	113/9	31/5*	184/12	58/3	5/1	115/2	106/10	30/4*
BMI kg·m^{-2}	22.4±3.35	22.4±3.24	21.7±2.71	22.5±3.24	21.8±3.18	21.5±3.31	22.3±3.30	22.5±3.27	21.6±2.72
Current smoker %	26.4	26.2	33.3	27.0	26.2	66.7	27.4	25.9	32.4
Smoking history pack-yrs	57.0 (12.5–160.0)	55.0 (12.0–220.0)	56.0 (19.0–132.0)	57.0 (12.5–220.0)	50.0 (12.0–174.0)	76.5 (38.3–105.0)	57.0 (12.5–57.0)	55.0 (12.0–220.0)	59.9 (19.0–132.0)
FEV$_1$ % pred	54.6±19.6	58.4±25.5	63.3±19.1	57.5±23.3	58.0±21.0	61.0±76.9	55.1±19.0	58.5±26.2	62.1±18.9
Bronchial reversibility %	10.53 (-10.1–69.2)	10.22 (-2.99–60.0)	6.09 (-5.19–52.7)	9.52 (-10.1–69.2)	9.27 (-5.19–60.0)	11.21 (-0.64–25.9)	10.78 (-10.1–69.2)	9.71 (-2.99–60.0)	6.59 (-5.19–52.7)
***D*$_L$CO/*V*$_A$**	61.9±24.2	64.2±25.1	64.1±22.4	65.0±24.3	58.8±23.2	61.0±24.6	61.2±24.1	65.3±24.7	63.1±22.5
CT score	1.53±0.94	1.38±0.89	1.17±0.90*	1.46±0.93	1.26±0.87	0.88±0.69*	1.51±0.92	1.36±0.92	1.20±0.90
Atopy %	25.0	22.1	30.6	26.0	18.0	33.3	25.2	21.6	29.4
Log [total serum IgE] IU·mL^{-1}	1.82±0.68	1.75±0.70	1.80±0.72	1.78±0.70	1.81±0.65	1.67±0.87	1.83±0.68	1.75±0.71	1.74±0.68
Log [eosinophils] cells·mm^{-3}	2.20±0.32	2.23±0.32	2.13±0.36	2.19±0.34	2.24±0.29	2.19±0.20	2.20±0.32	2.24±0.32	2.12±0.36
COPD stage n									
I	18	33	10	47	14	0	20	32	9
II	53	49	19	86	30	6	62	44	18
III	31	33	6	52	16	0	31	33	6
IV	4	7	1	11	1	0	4	7	1

Data are presented as mean±SD or median (range) unless otherwise indicated. -403G>A: guanine (G) to adenine (A) substitution of nucleotide -403; C: cytosine; T: thymidine; M: male; F: female; BMI: body mass index; FEV$_1$: forced expiratory volume in one second; % pred: % predicted; *D*$_L$CO: diffusing capacity of the lung for carbon monoxide; *V*$_A$: alveolar volume; CT: computed tomography; Ig: immunoglobulin; COPD: chronic obstructive pulmonary disease. #: 264 subjects; ¶: 263 subjects; +: 267 subjects. *: $p<0.05$ (one-way ANOVA or the Chi-squared test was used as appropriate).

associated with a slower rate of CD4+ T-cell depletion in HIV-1-infected Japanese subjects [1], and haplotypes that included 375C displayed a strong dominant association with rapid progression to AIDS among HIV-1-infected individuals in African-American, European-American and combined cohorts [21]. Therefore, the present authors believe that the genetic association observed in the present study is due to the functional consequences that these functional SNPs have on *CCL5* transcriptional activity, although the possibility that they act as markers of another important genetic abnormality without themselves being functionally relevant cannot be excluded. It is also interesting to note that the frequency of the -28G allele differs according to ethnicity; its frequency is ~15–20% in Asians, including Japanese, Chinese and Koreans, whereas it is very low (⩽2%) in Caucasians and African-Americans. Therefore, the genetic impact of the -28G allele observed in the present studies seems to be clinically important, especially in Asian populations.

In patients with COPD, the relative contributions of small airway disease and emphysema to the degree of airflow limitation vary [14]. Indeed, it has been shown that severity of emphysema varies widely, even in the same stage of COPD [16], and thus COPD patients with milder emphysema despite severe airflow limitation could be considered as having predominantly small airway disease. Within the context of the previous finding that the -28G allele was associated with late-onset asthma, the current observation of an inverse association between this allele and CT score in patients with COPD leads to a specific hypothesis that increased severity of small airway disease caused by a gain effect of the -28G allele may underlie the chronic inflammation and remodelling of the small airways of late-onset asthma and COPD with milder emphysema. Alternatively, a low attenuation area may reflect hyperlucency due to air trapping rather than emphysema itself, thus confounding the assumption that the CT score purely indicates the extent of emphysema [25]. However, high-resolution CT scans were performed at full inspiration; thus this latter possibility would be less likely than is the case when conventional CT scans are performed at expiration. In addition, if low attenuation areas reflected not only emphysema but also hyperlucency due to air trapping, a good correlation between CT score and airflow limitation would be expected; however, this was not the case in the present population [16].

An increased prevalence of viral infections, as well as the persistence of cells expressing viral proteins in patients with asthma [4, 5] or COPD [6–8], has been reported, suggesting that viral infections, such as human rhinovirus and respiratory syncytial virus (RSV), may play a critical role in the pathogenesis of airway inflammation and the subsequent deterioration in lung function in patients with asthma and COPD. Studies of respiratory secretions from individuals with

TABLE 3 Linear regression analyses of the functional CC chemokine ligand 5 polymorphisms with computed tomography (CT) score

	Subjects n	CT score	p-value
Model 1#			
-403GG	105	1.48±0.94	0.063
-403GA	121	1.39±0.89	
-403AA	36	1.24±0.90	
-28CC	196	1.49±0.93	0.00038
-28CG	61	1.15±0.87	
-28GG	6	0.93±0.69	
375TT	116	1.44±0.92	0.12
375TC	115	1.40±0.90	
375CC	34	1.24±0.92	
Model 2¶			
-403GG	69	1.57±0.94	0.006
-403GA	82	1.35±0.89	
-403AA	28	1.15±0.90	
-28CC	133	1.53±0.93	0.00002
-28CG	41	1.05±0.87	
-28GG	3	0.70±0.69	
375TT	75	1.54±0.92	0.009
375TC	79	1.36±0.90	
375CC	26	1.14±0.92	

Data are presented as mean±SD. Linear regression models were applied to test the association between the three polymorphisms and the CT score. Smoking status, diffusing capacity of the lung for carbon monoxide and percentage predicted prebronchodilator forced expiratory volume in one second were consistently associated with CT score in models 1 and 2. G: guanine; A: adenine; C: cytosine; T: thymidine. #: all subjects; ¶: bronchial reversibility <15%.

RSV bronchiolitis showed that CCL5 was highly expressed [26], suggesting a special role for this chemokine in antiviral defence. Interestingly, a genetic variant of the key receptor for CCL5, CC chemokine receptor 5, has been associated with the severity of bronchiolitis caused by RSV [27]. Viral infections are the most likely cause of CCL5 upregulation, and the epithelium of the small airways is a considerable source of CCL5. The presence of the gain-of-function allele as a common susceptibility factor to asthma and COPD with milder emphysema predisposes patients to greater expression of CCL5 in response to prolonged and repeated exogenous stimuli, including viral antigens, leading to amplified inflammation of the small airways.

In a mouse model with targeted disruption of *CCL5*, immunoregulatory and antiapoptotic effects of CCL5 have been suggested [28], which are distinct from those that have previously been identified in the setting of infection, such as the initiation of antiviral responses and airway inflammation *via* enhancement of inflammatory cell recruitment. The functional properties of CCL5, if any, in humans may provide an alternative explanation for the present findings, such as that the presence of the -28G allele predisposes an individual to some type of protection against the development of emphysema. However, the present authors believe that this possibility is less likely given that the -28G allele has also been associated with late-onset asthma and that increased small airway pathology is a common cardinal feature of asthma and COPD.

Although it is difficult to discriminate asthma from COPD in some older patients, the present authors believe that the findings of the present study were not the result of untoward inclusion of patients with late-onset asthma carrying the -28G allele. Among 267 patients with COPD, no correlation was found between CT score and levels of bronchial reversibility, levels of total serum IgE, peripheral blood eosinophil counts or

TABLE 4 Haplotypes comprising three CC chemokine ligand (CCL) 5 single nucleotide polymorphisms and computed tomography (CT) score

Patients	Nucleotide			Frequency	Haplotype-specific score	Empirical p-value
	-403	-28	375			
All subjects#	A	G	C	0.132	-3.218	0.00101
	A	C	T	0.017	-0.344	0.72
	A	C	C	0.210	1.021	0.31
	G	C	T	0.609	2.450	0.0143
Bronchial reversibility <15%¶	A	G	C	0.125	-3.746	0.00015
	A	C	T	0.017	-0.017	0.99
	A	C	C	0.235	-0.084	0.93
	G	C	T	0.594	3.234	0.0011

The frequencies of the haplotypes comprising the three *CCL5* polymorphisms were significantly associated with CT score based on p-values from 10,000 simulations of global score tests, as implemented in Haplo.Score [19]. The analyses were adjusted for smoking status, diffusing capacity of the lung for carbon monoxide and percentage predicted prebronchodilator forced expiratory volume in one second. The haplotype-specific score permits the evaluation of which haplotypes have the strongest association with a trait. It should be noted that the global score test does not give effect estimates, whereas negative haplotype-specific scores are associated with a protective effect and positive haplotype-specific scores are associated with an increased risk. #: global p=0.0023 (n=267); ¶: global p=0.00075 (n=180).

frequency of atopy, which makes it unlikely that inclusion of asthmatic patients occurred more often in a group with milder emphysema. In addition, the genetic association between the -28G allele and CT score became even stronger when the analysis was limited to patients who exhibited bronchial reversibility of <15% in order to reduce the risk of unknowingly including patients with asthma.

Although the $D_{L,CO}$ appears to be the best single physiological measure of emphysema severity, it was found that the -28C>G SNP was significantly associated only with visually assessed CT score and not with $D_{L,CO}/V_A$. Wide variations in CT score, even among patients who had the same $D_{L,CO}/V_A$, were noted (fig. 2E of the online supplementary material) despite the finding that $D_{L,CO}/V_A$ was significantly associated with CT score ($p<0.0001$). $D_{L,CO}$ are usually influenced by parenchymal destruction involving respiratory bronchioles, alveoli and the pulmonary capillary system, and are reduced in patients with emphysema because of the loss of alveolocapillary surface. However, $D_{L,CO}$ may be relatively insensitive to the loss of surface area for gas exchange when ventilation and perfusion remain well matched in the lung. $D_{L,CO}$ may also be influenced by several other factors, including the pathology of alveolar septa, inequality of blood/gas distribution in the lung and lung volume at the time of measurement, even if correction is made for V_A. Therefore, the present authors believe that the CT score indicate emphysema severity more specifically than $D_{L,CO}/V_A$ in the present study.

A visual scoring system was used to assess the extent of emphysema according to the modified [16] scoring system of GODDARD *et al.* [17]. Given that automatically calculated parameters, such as LAV, may be a more sensitive technique for the detection and quantification of pulmonary emphysema *in vivo*, visual assessment of emphysema is a limitation of the present study that could bias the results. However, when the severity of emphysema assessed by visual score was compared with that assessed by computerised analysis, a strong correlation was found between these two approaches (fig. 3E of the online supplementary material) [16]. In addition, subanalysis using automatically calculated emphysema scores confirmed the inverse association between the functional -28G allele and the severity of emphysema. In addition, computerised analysis may not be easy to obtain in many centres, and, therefore, may not be suitable for genetic studies of complex diseases such as COPD, especially since these studies usually require a large number of subjects in order to identify rather small effects of a gene or genes and because they also require replication studies in independent centres.

In conclusion, together with the previous finding of an association between the -28G allele and the development of late-onset asthma, the present study indicates that the CC chemokine ligand 5 gene may be involved in the common pathogenesis underlying late-onset asthma and chronic obstructive pulmonary disease with milder emphysema. The present findings led to speculation that specific components of the innate immune system may manifest an aberrant antiviral response as a basis for chronic inflammatory lung diseases, such as asthma and chronic obstructive pulmonary disease. Further studies of the disease phenotype presented in the current study are needed in order to improve understanding of the underlying pathophysiology and elucidate potential treatment modalities for the complex disease labelled chronic obstructive pulmonary disease.

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