

Matrix Metalloproteinase-12 and Cathepsin D Expression in Pulmonary Macrophages and Dendritic Cells of Cigarette Smoke-Exposed Mice

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Key Words

COPD · Emphysema · Cigarette smoke · MMP-12 · Cathepsin D · Dendritic cells · Macrophages

Abstract

An imbalance between proteinases and their inhibitors is believed to play an essential role in the development of chronic obstructive pulmonary disease (COPD) and pulmonary emphysema. COPD is mainly caused by cigarette smoking, and is characterized by an increase in inflammatory cells in small airways and lung parenchyma. We examined the mRNA expression of several proteinases in lungs of mice exposed to cigarette smoke or control air. After 1, 3 and 6 months' smoke exposure there was a significant increase of matrix metalloproteinase (MMP)-12 and Cathepsin D mRNA, compared to air-exposed mice. To determine the cellular origin of MMP-12 and Cathepsin D, we isolated dendritic cells (DCs) and macrophages from the lungs of mice. There was an increase in MMP-12 mRNA after smoke exposure in both macrophage and DC populations, whereas Cathepsin D was predominantly expressed in macrophages. Immunohistochemistry clearly revealed the expression of Cathepsin D protein in alveolar macrophages of cigarette

smoke-exposed mice, in contrast to air-exposed littermates. Western blots on lung tissue demonstrated an increase of MMP-12 protein in cigarette smoke-exposed animals. These results indicate that cigarette smoke increases the expression of MMP-12 and Cathepsin D in the lungs of mice, and that not only macrophages but also DCs produce MMP-12.

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Introduction

Cigarette smoking is the major risk factor for the development of chronic obstructive pulmonary disease (COPD). COPD is one of the leading causes of mortality and morbidity and its prevalence is increasing worldwide [1, 2]. The disease is characterized by slowly progressive development of airflow limitation that is poorly reversible. The airflow limitation is due to chronic obstructive bronchiolitis and loss of elastic recoil caused by destruction of lung parenchyma (emphysema). The molecular and cellular mechanisms that are responsible for the development of COPD are not well understood. It has been shown that chronic exposure to cigarette smoke leads to lung inflammation with recruitment of inflammatory

cells such as macrophages [3, 4], neutrophils [5, 6] and dendritic cells (DCs) [7, 8] to the airways and pulmonary tissue. These cells are all capable of releasing proteinases in excess of their inhibitors, causing damage to the extracellular matrix (ECM) of the lung. Ineffective repair of elastin and other ECM components results in air space enlargement that defines pulmonary emphysema. This 'proteinase/antiproteinase imbalance' hypothesis was first proposed 40 years ago, based on the observations that smokers with a deficiency of α_1 -antitrypsin were at increased risk for pulmonary emphysema [9], and that intratracheal administration of papain (a plant proteinase) resulted in emphysema in experimental animals [10]. Later, it was described that instillation of neutrophil elastase causes pulmonary emphysema in animal experiments [11, 12]. Since then, many other proteinases, including serine proteinases, cysteine proteinases and matrix metalloproteinases (MMPs) have been linked in one way or another to COPD or emphysema.

We have previously demonstrated that MMP-9 and its active form are present in increased amounts in COPD sputum [13]. Other authors showed higher levels of MMP-1, -2, -9 and -14 in lungs of COPD patients [14–16]. Animal models also afford strong arguments for a significant role of proteinases such as MMPs in emphysema. Tissue inhibitor of matrix metalloproteinases (TIMP)-3-deficient mice demonstrate a slowly progressive spontaneous enlargement of alveolar air spaces with increasing age [17] and MMP-12-deficient mice do not develop air space enlargement in response to long-term exposure to cigarette smoke, in contrast to their wild-type littermates [18]. Inducible targeting of IL-13 or IFN- γ to the lungs of mice causes emphysema and is associated with an increased expression of MMPs and cathepsins [19, 20].

To further elucidate which are the key proteinases in the development of pulmonary emphysema, we examined the mRNA expression of several proteinases and antiproteinases *in vivo* in the lungs of mice either exposed to cigarette smoke or to control air for 1, 3 and 6 months. Secondly, to determine the cellular sources of these proteinases, we examined the mRNA expression in DCs and macrophages isolated from lung tissue of mice exposed to cigarette smoke or control air. Thirdly, we studied the influence of cigarette smoke extract (CSE) on bone marrow-derived DCs *in vitro*, to elucidate whether the observed effects of cigarette smoke exposure on proteinase expression in DCs were due to a direct effect (by cigarette smoke itself) or due to an indirect mechanism (e.g. via lung inflammation).

Materials and Methods

Animals

Male C57BL/6 mice, 6–8 weeks old, were purchased from Harlan (Zeist, The Netherlands). The local Ethics Committee approved all *in vivo* manipulations.

Cigarette Smoke Exposure

Mice were exposed to the whole smoke of 5 cigarettes (Standard Cigarette 1R3, Kentucky University) using a standard smoking apparatus [21] with the chamber adapted for a group of mice (chamber dimensions: $24 \times 14 \times 14 \text{ cm} = 4,700 \text{ cm}^3$). An optimal smoke: air ratio of 1:12 was obtained. Smoke exposure is repeated 4 times/day, with a smoke-free interval of 30 min, 5 days/week for 1, 3 or 6 months. Control mice were air-exposed.

Preparation of CSE

To prepare CSE, the smoke of 10 cigarettes (Standard Cigarette 2R4F without filter, University of Kentucky) was bubbled through 30 ml of culture medium. This treatment did not alter the pH of the medium. The resulting suspension was filter-sterilized. This solution, considered to be 100% CSE, was diluted to a 5% concentration and applied to the DC cultures within 30 min of preparation.

Tissue Processing and Cellular Recovery

Twenty-four hours after the last smoke exposure, mice were anesthetized using an intraperitoneal pentobarbital injection. To obtain bronchoalveolar lavage (BAL) fluid, the trachea are cannulated and 1 ml of HBSS without Ca^{2+} or Mg^{2+} , supplemented with 0.5 mM EDTA, was instilled 4 times via the tracheal cannula and recovered by gentle manual aspiration. This BAL fluid was centrifuged, and the cell pellet was subjected to red blood cell lysis (ammonium chloride) and kept on ice until RNA extraction. To obtain lung tissue, right heart catheterization and perfusion with saline-EDTA was performed to remove the pulmonary intravascular pool of cells. Lungs were removed and collected in ice-cold tissue culture medium for preparation of single-cell suspensions, or in RNA later stabilization fluid (Qiagen) for RNA extraction. For protein analysis, lungs were frozen in liquid nitrogen until further processing.

Morphometric Quantification of Emphysema

Lungs were fixated by gentle infusion of fixative (4% paraformaldehyde) through the tracheal cannula by a continuous-release pump under constant pressure for 10 min. The lungs were resected and fixed for an additional 4 h. After routine paraffin embedding, 3- μm sections were stained with hematoxylin and eosin (Klinipath, Geel, Belgium).

Quantification of emphysema was determined by mean linear intercept (L_m). For each animal, 10 fields at a magnification of $200\times$ were captured in a blinded fashion and studied via a computerized image analysis system (KS400, Zeiss, Oberkochen, Germany) with a custom-made macro-command. A $100 \times 100 \mu\text{m}$ grid was drawn on the image and the L_m was measured [22, 23].

Buffers and Media for Preparation of Single-Cell Suspensions and Flow Cytometry

Tissue culture medium was prepared using RPMI-1640 supplemented with 5% FCS, penicillin/streptomycin, *L*-glutamine and 2-mercaptoethanol (all from GibcoBRL). Digestion medium consisted of tissue culture medium supplemented with 1 mg/ml colla-

genase type 2 (Worthington Biochemical Corp., Lakewood N.J., USA) and 0.02 mg/ml DNase I (grade II from bovine pancreas, Boehringer Mannheim, Brussels, Belgium). FACS-EDTA buffer contained PBS without Ca^{2+} or Mg^{2+} , 0.1% azide, 1% bovine serum albumin (Sigma) and 5 mM EDTA.

Preparation of Lung Single-Cell Suspensions

Lungs were minced using fine scissors and incubated in digestion medium in a humidified incubator at 37°C and 5% CO_2 . After 30 min incubation, samples were vigorously agitated using glass pipettes, fresh digestion medium was added and incubation prolonged for another 15 min. Subsequently, the samples were agitated, centrifuged and incubated in calcium and magnesium-free PBS containing 10 mM EDTA. Finally, the cells were subjected to red blood cell lysis, washed in FACS-EDTA, passed through a 50- μm cell strainer and kept on ice until fluorescent labeling.

Labeling of Single-Cell Suspensions for Flow Cytometry

Cells were preincubated with Fc-receptor blocking antibody (anti-CD16/CD32, clone 2.4G2) to reduce non-specific binding. Monoclonal antibodies used to identify mouse DC and macrophage populations were: biotinylated anti-CD11c (N418), followed by streptavidin-APC, and PE-conjugated anti-MHCII/I-A^b (AF6-120.1). All reagents were obtained from BD-Pharmingen (Erembodegem, Belgium) except clone N418 (kindly provided by M. Moser, ULB, Brussels, Belgium). Flow cytometry data acquisition was performed on a FACS Vantage SE equipped with 488- and 633-nm lasers and running Cellquest 3.3 software (Becton Dickinson, Franklin Lakes, Calif., USA). Lung DCs were defined as CD11c⁺/low-autofluorescent/MHCII⁺ cells. Lung macrophages were defined as CD11c⁺/high-autofluorescent cells [24].

Purification and Separation of Pulmonary CD11c⁺ Cell

Subsets

CD11c⁺ cell subsets were isolated according to Vermaelen and Pauwels [24]. To obtain sufficient amounts of DCs and macrophages for RNA preparation, the single-cell suspensions of 8–12 mice were pooled. Pulmonary single-cell suspensions were first incubated with Fc-receptor block, followed by anti-CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD11c⁺ lung cells were enriched after one passage through a VarioMACS magnetic cell separator according to manufacturer's instructions. Subsequently, the CD11c⁺ fraction was kept on ice until RNA extraction. The CD11c⁺ fraction was labelled with biotinylated anti-CD11c, followed by streptavidin-APC, and PE-conjugated anti-MHCII. On the FACS Vantage SE, the CD11c⁺/low-autofluorescent/MHCII⁺ and CD11c⁺/high-autofluorescent cells, representing DC and macrophage populations respectively, were separated by fluorescence-activated cell sorting with a 70-nm nozzle.

Generation of Bone Marrow-Derived DCs

The procedure used for the generation of DCs was that described by Lutz et al. [25]. Briefly, single-cell suspensions were obtained from bone marrow by flushing the femurs of mice. At day 0 the cells were seeded at 2×10^6 per 100 mm² Petri dish in 10 ml medium (RPMI-1640, 10% FCS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM *L*-glutamine and 50 μM 2-mercaptoethanol (all from GibcoBRL)), supplemented by 20 ng/ml GM-CSF. At day 3, an equal volume of fresh medium and GM-CSF was added to the plate. At day 6, half of the culture supernatant was collected,

centrifuged, and the cell pellet was resuspended in an equal volume of fresh medium containing GM-CSF. At day 8, the DCs were harvested and depleted of GR1⁺ cells to remove contamination of granulocytes (using purified anti-GR1 and Dynabeads m450). Part of the DCs were kept on ice until RNA extraction, and part of the cells were stimulated with LPS (1 $\mu\text{g}/\text{ml}$ culture medium) or 5% CSE and incubated overnight in a humidified incubator at 37°C and 5% CO_2 . On day 9 these LPS- or CSE-stimulated cells were harvested and kept on ice until RNA extraction.

RNA Extraction

RNA from lung tissue was extracted with the Qiagen RNeasy Midi Kit. RNA from cultured cells, BAL cells or sorted cells was extracted with the Qiagen RNeasy Mini Kit. All RNA extractions included an additional DNase step.

RT-PCR

RT-PCR for different proteinases and antiproteinases was performed by means of in-house designed primer combinations or the Assays-on-Demand™ Gene Expression Products (Applied Biosystems, Bedford, Mass., USA). All reactions were performed starting from 10 ng of total RNA.

For MMP-2, -9 and TIMP-1 and -2, a known copy number of external control RNA template (synthetic RNA) was introduced in each sample to monitor the RT-PCR reaction and to allow the quantitation of each endogenous mRNA [26]. RT-PCR was performed using the GeneAmp ThermoStable rTth reverse transcriptase RNA PCR kit (PerkinElmer, Branchburg, N.J., USA) and two pairs of primers (GibcoBRL-Life Technologies) (table 1). Reverse transcription was performed at 70°C for 15 min followed by 2 min incubation at 95°C for denaturation of RNA-DNA heteroduplexes. Amplification started at 94°C for 15 s, 68°C for 30 s and 72°C for 10 s and RT-PCR terminated by 2 min at 72°C. RT-PCR products were resolved on 10% acrylamide gels and analyzed using a Fluor-S MultiImager (Bio-Rad, Hercules, Calif., USA) after staining with Gelstar dye (FMC Bioproducts, Rockland, Me., USA). Quantitative RT-PCR results are expressed as a ratio between the intensities of the bands corresponding to the endogenous RNA and to a synthetic RNA added in the RT-PCR reaction in known amounts [26, 27]. For normalization of the samples, this ratio calculated for a given MMP is further divided by the same ratio calculated for 28S rRNA in the same sample. The results shown are a mean of duplicates.

Expression of MMP-8 and -12, Cathepsins B, D and L and Cystatin C mRNA, relative to hypoxanthine guanine phosphoribosyltransferase (hprt) mRNA or transferrin receptor (tfrc) mRNA, was analyzed with the Assays-on-Demand™ Gene Expression Products (Applied Biosystems). RT-PCR was performed on an ABI PRISM 7700 Sequence Detection System with MuLV RTase (Applied Biosystems). Reverse transcription was performed at 48°C for 30 min followed by 12 min incubation at 95°C for denaturation of RNA-DNA heteroduplexes, and 50 cycles of 95°C for 15 s and 60°C for 60 s. Monitoring of the RT-PCR occurred in real-time using a FAM/TAMRA probe.

Western Blotting for MMP-12

Proteins were isolated from lung tissue crushed in liquid nitrogen using a urea solution [28]. Samples were electrophoretically separated in SDS-12% polyacrylamide gels in the presence of 5% β -mercaptoethanol. Proteins were transferred to a PVDF mem-

Table 1. Primers and cycling conditions for RT-PCR

	Oligonucleotide sense	Oligonucleotide antisense	Number of PCR cycles	Expected RNA size, bp	
				control	endogenous
MMP-2	5'-AGATCTTCTTCTTCAAGGACCGGT-3'	5'-GGCTGGTCAGTGGCTTGGGGTA-3'	36	271	225
MMP-9	5'-CCCACATTGACGTCCAGAGAAGAA-3'	5'-GTTTTTGATGCTATTGCTGAGATCCA-3'	36	266	208
TIMP-1	5'-GGCATCCTCTGTGCTATCACTG-3'	5'-GTCATCTTGATCTCATAACGCTGG-3'	35	295	168
TIMP-2	5'-CTCGCTGGACGTTGGAGGAAAGAA-3'	5'-AGCCCATCTGGTACCTGTGGTTCA-3'	30	269	155
28S	5'-GTTACCCACTAATAGGGAACGTGA-3'	5'-GATTCTGACTTAGAGGCGTTCAGT-3'	19	269	212

RT-PCR for MMP-8 and -12, Cathepsins B, D and C, Cystatin C, hprt and tfr was performed with the Applied Biosystems Assays-on-Demand Gene Expression Products™.

brane (NEN Life Science Products, Downers Grove, Ill., USA) which was subsequently incubated for 1 h at room temperature in Tris-buffered saline (TBS) containing 5% skimmed milk. The blots were then incubated for 1 h at room temperature with a rabbit polyclonal anti-MMP-12 antibody (Biomol, Plymouth Meeting, Pa., USA) diluted 1:100 in TBS, washed 3 times in TBS-Tween (0.1%) and finally incubated for 1 h with peroxidase-conjugated swine-anti-rabbit IgG (DAKO, Glostrup, Denmark) diluted 1:1,000. According to the manufacturer's data, this antibody showed no cross-reactivity with other MMPs. The peroxidase activity was assessed using an enhanced chemiluminescence kit (NEN Life Science Products).

Immunohistochemistry for Cathepsin D

Sections obtained from formalin-fixed, paraffin-embedded lung lobes were subjected to the following immunohistological staining sequence: blocking reagent (Boehringer Mannheim, Germany) plus Triton X-100, goat-anti-mouse Cathepsin D (R&D Systems Europe, Abingdon, UK) or goat IgG isotype control, rabbit-anti-goat biotin (DAKO), streptavidin HRP (DAKO) and DAB substrate (DAKO). Sections were counterstained with hematoxylin.

Statistical Analysis

Reported values are expressed as means $\pm$ SD. The different experimental groups were compared by Kruskal-Wallis test for multiple comparisons. Pairwise comparisons were made by means of a Mann-Whitney U test with the Bonferroni correction. A *p* value <0.05 was considered significant.

Results

Morphometric Quantification of Emphysema

Microscopic analysis of lung tissue sections revealed a significant degree of emphysema after 6 months of smoke exposure. The L_m value was significantly higher in smoke-exposed mice as compared to air-exposed animals (L_m at 6 months' smoke $42.44 \pm 0.69 \mu\text{m}$ vs. air $38.21 \pm 0.87 \mu\text{m}$; *p* < 0.01) (fig. 1).

Proteinase mRNA Expression in Lung Tissue

Mice were exposed to cigarette smoke or control air for 1, 3 and 6 months. Expression of proteinase mRNA in total non-lavaged lung tissue was examined by quantitative RT-PCR at the three different time intervals. For MMP-12, there was a 6- to 7-fold increase in mRNA expression after 1, 3 and 6 months' smoke exposure, compared to air-exposed mice (fig. 2A). The Cathepsin D mRNA expression increased 2-fold after 1, 3 and 6 months' smoke exposure, compared to air-exposed mice (fig. 2B). After 6 months of smoke exposure there was also a slight increase in MMP-2 expression (data not shown).

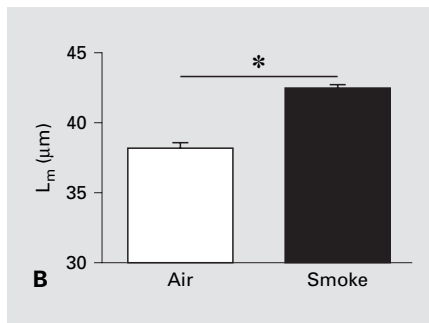
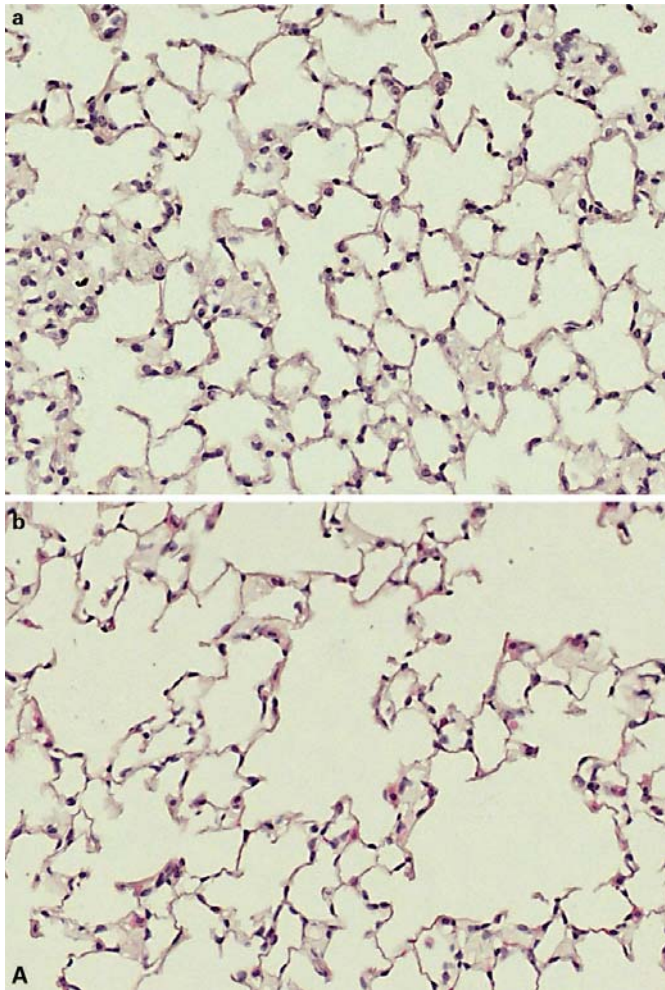
No differences in the expression of MMP-8 and -9 and Cathepsins B and L could be detected (data not shown). Importantly, there were no differences in the mRNA expression of the proteinase inhibitors TIMP-1, TIMP-2 and Cystatin C after smoke exposure (data not shown).

Proteinase mRNA Expression in BAL Cells

Mice were exposed to cigarette smoke or control air for 1 month. Expression of proteinase mRNA in BAL cells was examined by quantitative RT-PCR. There was a 30-fold increase in MMP-12 mRNA after smoke exposure, compared to air-exposed mice (fig. 3A). Smoke exposure induced a 2-fold increase in Cathepsin D mRNA levels (fig. 3B).

Western Blotting for MMP-12 Protein in Lung Tissue

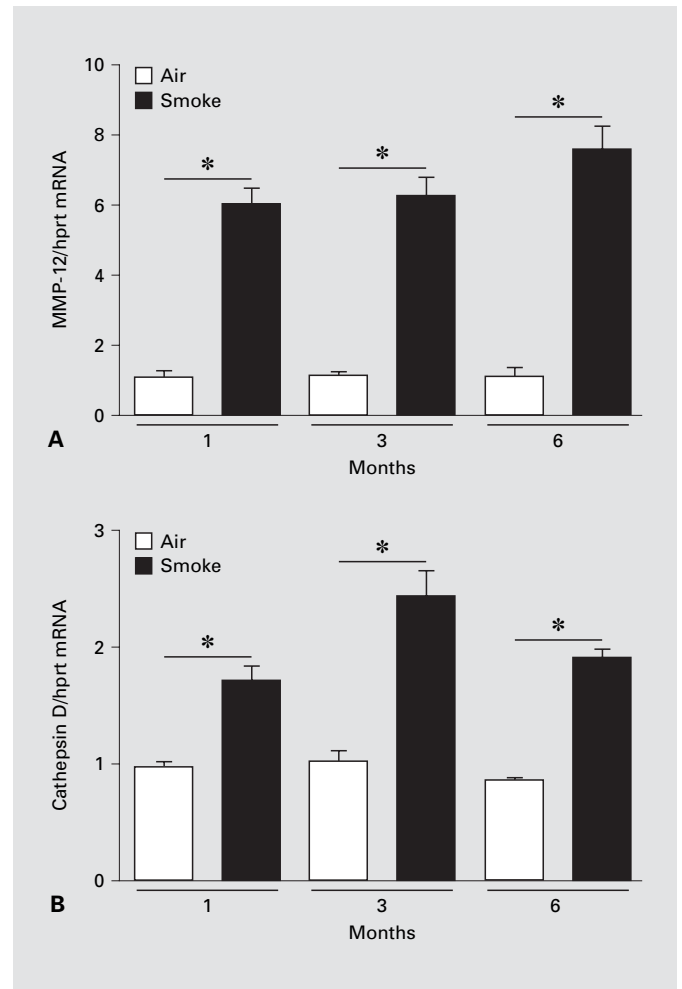
The presence of MMP-12 protein was examined by Western blotting on total, non-lavaged lung tissue. The used antibody recognized mainly the 54-kDa latent form of MMP-12. There was a significant increase in MMP-12 protein in the lungs of mice exposed to cigarette smoke for 1 month, compared to air-exposed mice (fig. 4).



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Immunohistochemistry for Cathepsin D Protein on Lung Tissue

The presence of Cathepsin D protein was examined by immunohistochemistry on paraffin-embedded lung sections of mice exposed to cigarette smoke or control air for 1 month. While there was no Cathepsin D protein detectable in the lungs of air-exposed mice (fig. 5B), there was



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Fig. 1. Morphometric quantification of emphysema. **A** Representative photomicrographs of hematoxylin and eosin stained lung tissue of air-exposed (**a**) and smoke-exposed (**b**) mice. **B** Mean linear intercept (L_m) values of mice exposed to cigarette smoke for 6 months, compared to air-exposed mice. $n = 6$ animals per group. * $p < 0.05$.

Fig. 2. RT-PCR for the expression of MMP-12 mRNA (**A**) and Cathepsin D mRNA (**B**) in lung tissue of mice exposed to cigarette smoke for 1, 3 and 6 months, compared to air-exposed mice. Results are expressed as a ratio with hprt mRNA (mean $\pm$ SD). $n = 5$ animals per group. * $p < 0.05$. hprt = Hypoxanthine guanine phosphoribosyltransferase.

a strong staining for Cathepsin D protein in the alveolar macrophages of the smoke-exposed animals (fig. 5C, D).

MMP-12 and Cathepsin D mRNA Expression in DCs and Macrophages Isolated from Lung Tissue

Mice were exposed to cigarette smoke or control air for 1, 3 and 6 months. Smoke exposure induced a sig-

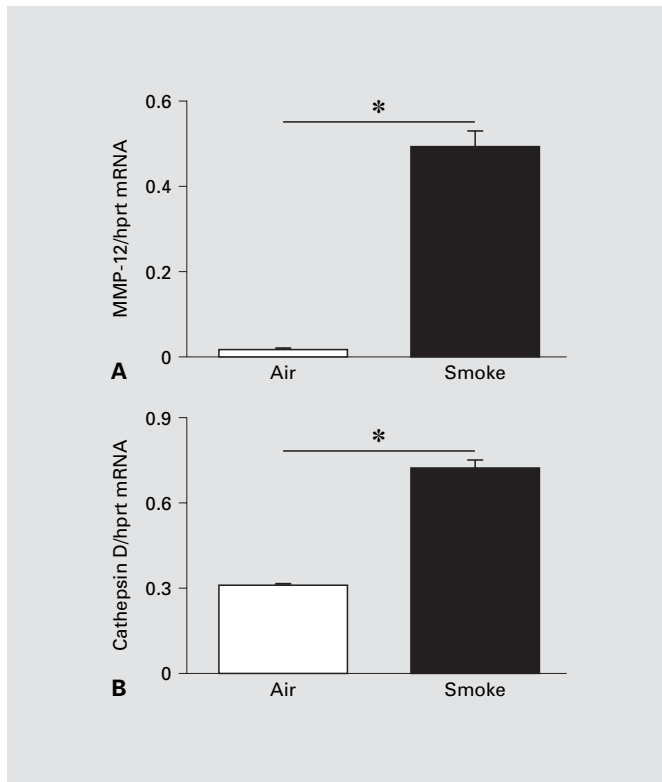


Fig. 3. RT-PCR for the expression of MMP-12 mRNA (**A**) and Cathepsin D mRNA (**B**) in bronchoalveolar (BAL) cells of mice exposed to cigarette smoke for 1 month, compared to air-exposed mice. Results are expressed as a ratio with hrpt mRNA (mean $\pm$ SD). n = 8 animals per group. * p < 0.05. hrpt = Hypoxanthine guanine phosphoribosyltransferase.

nificant increase in DCs and macrophages in BAL fluid (total number of DCs at 6 months: smoke $50.6 \pm 2.6 \times 10^3$ vs. air $10.2 \pm 1.5 \times 10^3$, p < 0.001; total number of macrophages at 6 months: smoke $11.5 \pm 1.1 \times 10^5$ vs. air $4.1 \pm 0.4 \times 10^5$, p < 0.001). Also in lung parenchyma, the total number of DCs and macrophages increased significantly upon smoke exposure (DCs at 6 months: smoke $2.9 \pm 0.3 \times 10^5$ vs. air $1.9 \pm 0.2 \times 10^5$, p < 0.05; macrophages at 6 months: smoke $8.7 \pm 0.8 \times 10^5$ vs. air $4.8 \pm 0.8 \times 10^5$, p < 0.01).

DCs and macrophages were isolated from total non-lavaged lung tissue by FACS cell sorting. The MMP-12 and Cathepsin D mRNA expression was examined by quantitative RT-PCR in DCs (CD11c⁺/low-autofluorescent/MHCII⁺ cells), in macrophages (CD11c⁺/high-autofluorescent cells) and in the CD11c⁻ population [24].

We found MMP-12 mRNA expression not only in macrophages, but also in DCs (fig. 6A). In both cell populations

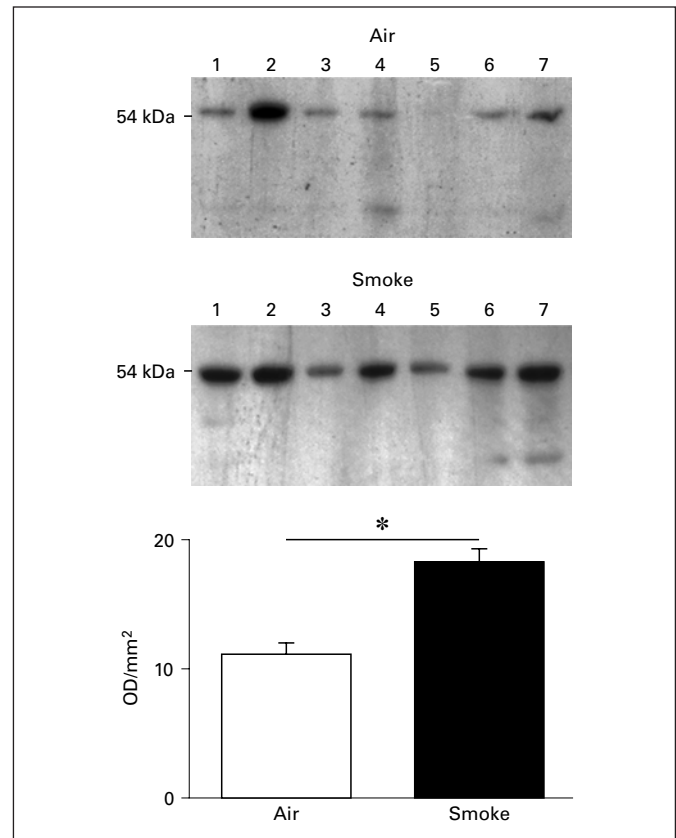


Fig. 4. Western blot for the presence of MMP-12 protein in the lungs of mice exposed to cigarette smoke for 1 month, compared to air-exposed mice. Results are expressed as OD/mm² (mean $\pm$ SD). n = 7 animals per group. * p < 0.05.

there was a strong increase in MMP-12 expression after 1, 3 and 6 months' smoke exposure. Only a faint expression of MMP-12 was detected in the CD11c⁻ population that was depleted from DCs and macrophages (fig. 6A).

Cathepsin D mRNA was expressed predominantly in macrophages, with only very low levels of mRNA expression in the DC and CD11c⁻ populations (fig. 6B). After 1, 3 and 6 months' smoke exposure there was a strong increase in Cathepsin D mRNA in the macrophage population (fig. 6B).

Effects of CSE on Bone Marrow-Derived DCs

Expression of MMP-12 mRNA in in vitro cultured, bone marrow-derived DCs was examined by quantitative RT-PCR. Expression was compared between immature DCs (harvested on days 8 and 9) and LPS- or CSE-stimulated DCs (harvested on day 9).

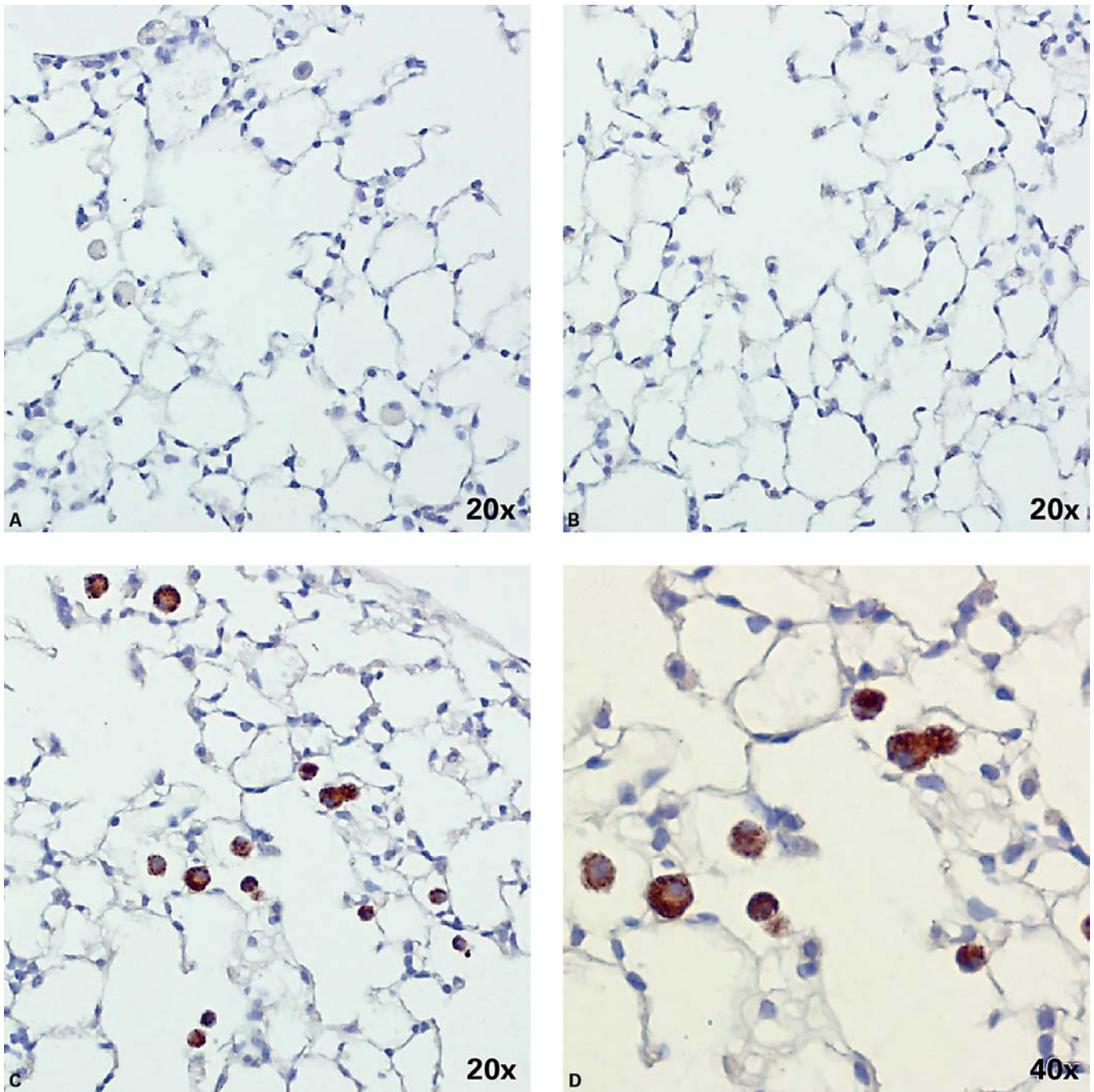


Fig. 5. Immunohistochemistry for Cathepsin D protein on lung tissue of mice exposed to cigarette smoke or control air for 1 month: **A** isotype control, **B** air-exposed mice, **C** smoke-exposed mice on a 20 × magnification and **D** smoke-exposed mice on a 40 × magnification.

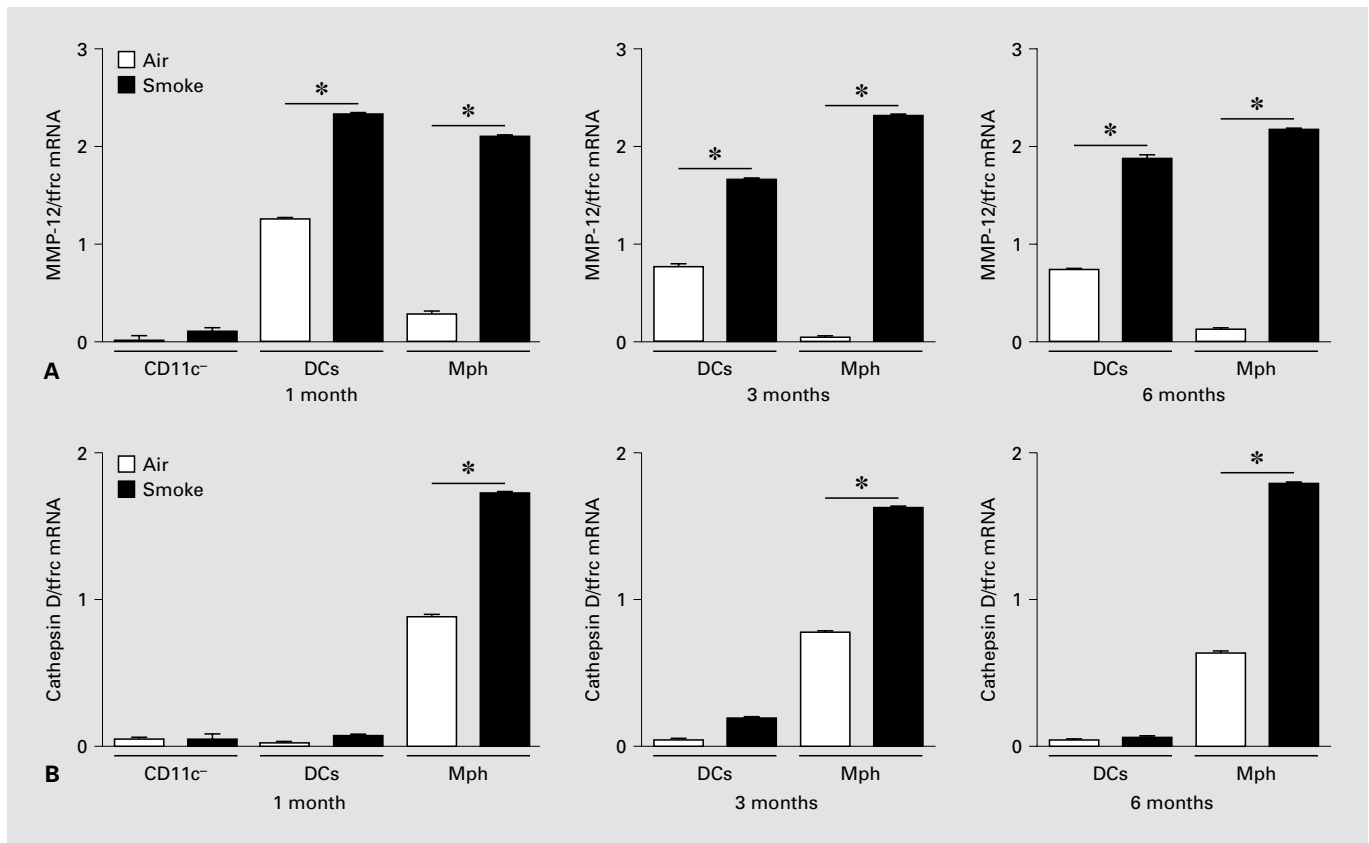
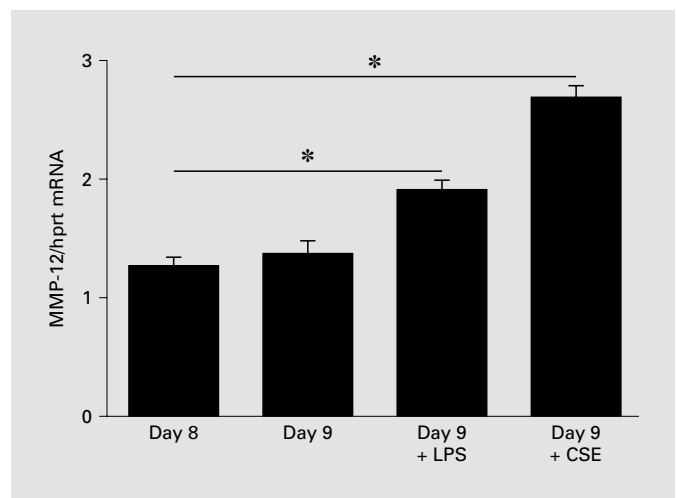


Fig. 6. MMP-12 (A) and Cathepsin D mRNA (B) expression in dendritic cells (DCs) and macrophages (Mph) obtained by cell sorting of non-lavaged lung tissue from mice-exposed to cigarette smoke for 1, 3 and 6 months, compared to air-exposed mice, and the MMP-12 and Cathepsin D mRNA expression in the CD11c⁻ population that is depleted from DCs and macrophages. Results are expressed as a ratio with tfr mRNA. Data are representative of three independent experiments. * $p < 0.05$. tfr = Transferrin receptor.

MMP-12 expression was detected in all DC populations, with a significantly higher expression in the LPS- and CSE-stimulated populations (fig. 7).

For Cathepsin D, there was no significant difference in expression between the DC populations (data not shown).

Fig. 7. MMP-12 mRNA expression in bone marrow-derived DCs, harvested at days 8 and 9 after LPS or CSE stimulation. Results are expressed as a ratio with hprt mRNA (mean $\pm$ SD). $n = 5$ cultures of bone marrow-derived DCs. Data are representative of three independent experiments. * $p < 0.05$. LPS = Lipopolysaccharide; CSE = cigarette smoke extract; hprt = hypoxanthine guanine phosphoribosyltransferase.



Discussion

Airflow limitation in COPD is largely due to airway inflammation and loss of elastic recoil due to destruction of lung parenchyma (emphysema). An imbalance between proteinases and their inhibitors is believed to play an essential role in the development of pulmonary emphysema. To elucidate which proteinases play a key role in this process, we examined the mRNA expression of several proteinases and antiproteinases in the lungs of mice either exposed to cigarette smoke or to control air. Histological analysis of the lungs of these mice showed clear signs of pulmonary emphysema after 6 months of smoke exposure, in contrast to air-exposed mice.

In both lung tissue and BAL cells there was a strong increase in MMP-12 mRNA expression after smoke exposure, while there was no difference in the expression levels of the inhibitors TIMP-1 and -2. Therefore, the MMP-12/TIMP ratio will be increased, contributing to the proteinase/antiproteinase imbalance. Western blot analysis revealed a significant increase in MMP-12 protein in lung extracts of smoke-exposed mice. Taken together with the fact that MMP-12-deficient mice do not develop emphysema in response to long-term cigarette smoke [18], these findings further establish the involvement of MMP-12 in the development of pulmonary emphysema. Moreover, it has been described that the development of age-related emphysema in *Itg β 6*-deficient mice is accompanied by a marked induction of MMP-12 [29]. It should however be noted that, to date, the involvement of MMP-12 in pulmonary emphysema has only been demonstrated in mice, and not yet in humans. This could point to species-specific effects, although it has recently been described that human bronchial epithelial cells may be an important source of MMP-12 in COPD and tissue remodelling [30].

Lung inflammation in COPD is characterized by the recruitment of several inflammatory cells such as macrophages, neutrophils and DCs. In our mouse model of COPD, these cell types are increased in mice exposed to cigarette smoke, compared to air-exposed mice [21]. To determine the cellular sources of MMP-12, we examined the mRNA expression in DCs and macrophages isolated from the lungs of mice either exposed to cigarette smoke or to control air. We found MMP-12 expression in similar amounts in both DC and macrophage populations. Since we found almost no MMP-12 expression in the population that was depleted from DCs and macrophages, it is likely that DCs and macrophages are among the main sources for MMP-12 in the lungs of mice. In both DC and

macrophage populations, there was a strong increase in MMP-12 expression after 1, 3 and 6 months' smoke exposure, compared to air exposure. The higher MMP-12 levels in the lungs of smoking mice can thus be attributed to the combination of increased numbers of macrophages and DCs, and a higher expression of MMP-12 in these cells.

We confirmed expression of MMP-12 in DCs derived from bone marrow. According to Lutz et al. [25], cells harvested at day 8 were mostly immature DCs. After overnight stimulation of bone marrow-derived DCs with LPS or CSE, there was a significant increase in MMP-12 mRNA expression. This indicates that MMP-12 expression increases with LPS- or CSE-induced maturation of DCs, and correlates with the increased MMP-12 expression in the *in vivo* DCs isolated from lungs of mice after smoke exposure. Moreover, this increased expression of MMP-12 in bone marrow-derived DCs clearly shows a direct influence of CSE on DCs. The expression of MMP-12 by DCs has also been described in human blood monocyte-derived DCs [31].

Although the relative numbers of macrophages are higher than those of DCs, the observation that MMP-12 (also called macrophage metalloelastase) is not only produced by macrophages but also by DCs, could be of particular importance, since DCs are migratory cells. In steady-state conditions, respiratory DCs are continuously replenished by a fast recruitment of DC precursors from the circulation [32]. In response to environmental stimuli (e.g. antigen) or stress signals, respiratory DCs emigrate from the airways towards thoracic lymph nodes [33]. This airway trafficking is further accelerated in mouse models of ongoing allergic airway inflammation [34]. During their recruitment from the circulation into the lungs, or during their emigration from the respiratory tract, DCs could thus harm the surrounding tissue and degrade cross-linked elastin networks by releasing MMP-12. Moreover, MMPs not only degrade components of the ECM, but also have several cytokines and chemokines among their substrates [35]. MMP-12 in particular, acts as a TNF- α -converting enzyme, releasing active TNF- α [36]. It has been shown that TNF- α plays an important role in the pathogenesis of cigarette smoke-induced emphysema [37].

Besides the important role of MMP-12, there are most certainly other proteinases involved in the development of pulmonary emphysema. We showed an increase in Cathepsin D mRNA expression after 1, 3 and 6 months' smoke exposure, while the Cathepsin inhibitor Cystatin C was not influenced by the smoke exposure. The in-

creased ratio of Cathepsin D to Cystatin C adds further to the imbalance between proteinases and antiproteinases. We found Cathepsin D mRNA and protein to be predominantly expressed by macrophages, as evidenced by RT-PCR and immunohistochemistry respectively. Again, the increase in Cathepsin D mRNA in the lungs of smoking mice can be attributed to both the higher number of macrophages and their higher expression of Cathepsin D per cell. It has been described earlier that induction of emphysema by IFN- γ is accompanied by an increased expression of Cathepsin D [20].

We also found a slight increase in MMP-2 expression after 6 months of smoke exposure, but no difference in the mRNA expression of MMP-8 and -9 and Cathepsins B and L could be detected. Next to the MMPs and cathepsins, there is evidence that neutrophil elastase is important, as neutrophil elastase-deficient mice are significantly protected from the development of pulmonary emphysema in response to cigarette smoke [38].

In conclusion, we have demonstrated an increase in MMP-12 and Cathepsin D – both at the mRNA and protein level – in cigarette smoke-exposed mice. Moreover, we have shown that MMP-12 is not only produced by macrophages, but also by pulmonary DCs. Our results further support the hypothesis that MMP-12 plays a central role in the cascade of events leading to perpetuated inflammation and pulmonary emphysema related to cigarette smoke exposure.

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