The Journal of Immunology

YKL-40 Is Elevated in Patients with Chronic Obstructive Pulmonary Disease and Activates Alveolar Macrophages

Séverine Létuvé, Alexander Kozhich, Nassim Arouche, Martine Grandsaigne, Jennifer Reed, Marie-Christine Dombret, Peter A. Kiener, Michel Aubier, Anthony J. Coyle and Marina Pretolani

J Immunol 2008; 181:5167-5173; ;
doi: 10.4049/jimmunol.181.7.5167
http://www.jimmunol.org/content/181/7/5167

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

YKL-40 Is Elevated in Patients with Chronic Obstructive Pulmonary Disease and Activates Alveolar Macrophages[1]

Séverine Létuvé,[2]*† Alexander Kozhich,[2]‡ Nassim Arouche,*† Martine Grandsaigne,*† Jennifer Reed,‡ Marie-Christine Dombret,§¶ Peter A. Kiener,‡ Michel Aubier,*†§¶ Anthony J. Coyle,‡ and Marina Pretolani[3]*†¶

YKL-40 is a chitin-binding protein that is elevated in patients with various inflammatory conditions associated with ongoing remodeling. We investigated whether the levels of YKL-40 were up-regulated in the circulation and the airways of patients with chronic obstructive pulmonary disease (COPD), and whether it promoted the production of inflammatory mediators from macrophages. Serum, bronchoalveolar lavage (BAL), bronchial biopsies, lung tissue specimens, and alveolar macrophages from never-smokers ($n = 15$), smokers without COPD ($n = 20$), and smokers with COPD ($n = 30$) were assessed for YKL-40 levels and immunolocalization. In addition, YKL-40-induced mediator release from alveolar macrophages was examined. We found that smokers with COPD had elevated levels of YKL-40 in serum ($p \leq 0.027$) and BAL ($p \leq 0.007$), more YKL-40-positive cells in bronchial biopsies ($p \leq 0.03$), and a greater proportion of alveolar macrophages expressing YKL-40 than smokers without COPD or never-smokers. YKL-40 levels in serum and BAL were associated with airflow obstruction (pre-β_2 agonist forced expiratory volume in 1 s, $r_s = -0.3892$, $p = 0.0072$ and $r_s = -0.5491$, $p < 0.0001$, respectively) and impaired diffusion lung capacity (transfer factor of the lung for carbon monoxide, $r_s = -0.4667$, $p = 0.002$ and $r_s = -0.3854$, $p = 0.0045$, respectively). TNF-α stimulated YKL-40 synthesis in alveolar macrophages from smokers with COPD, and exposure of these cells to YKL-40 promoted the release of IL-8, MCP-1, MIP-1α, and metalloproteinase-9. We conclude that YKL-40 is up-regulated in COPD, in which it may contribute to tissue inflammation and remodeling by sustaining the synthesis of proinflammatory and fibrogenic chemokines and of metalloproteinases by alveolar macrophages. *The Journal of Immunology,* **2008, 181: 5167–5173.**

Chronic obstructive pulmonary disease (COPD)[4] is a chronic respiratory disorder characterized by the development of progressive, largely irreversible, airflow limitation and emphysematous degeneration. Destruction of lung parenchyma leading to loss of elastic recoil and peribronchiolar fibrosis, which both contribute to fixed airway obstruction, are secondary to a chronic inflammatory response to noxious particles or gases, most often related to cigarette smoking (1, 2). Several mediators, including cytokines, chemokines, growth factors, and reactive oxygen species are increased in patients with COPD, either in the circulation, or in the airways, or in both compartments (3). However, most of these mediators are already elevated in asymptomatic smokers, as a result of their long-term exposure to cigarette smoking (3). Therefore, there is a need to identify novel molecular targets associated with pathophysiologic changes found in patients with COPD.

YKL-40 is a highly phylogenetically conserved chitin-binding glycoprotein that belongs to the family of chitinase-like proteins, defined by their structural similarity to bacterial chitinases, although lacking their characteristic enzymatic activity (4). YKL-40 controls fundamental functions in mammalian cells, such as mitogenesis, differentiation, and extracellular matrix homeostasis (5–7). Prominent expression of YKL-40 has been associated with pathologic conditions characterized by tissue inflammation and remodeling and by aberrant cell growth, such as atherosclerosis, asthma, liver fibrosis, and several malignancies (8–14). For some of these diseases, the assessment of serum YKL-40 has proven to be of both diagnostic and prognostic value (9, 12).

In the current study, we hypothesized that YKL-40 is up-regulated in COPD patients and contributes to tissue inflammation and remodeling by activating alveolar macrophages, the main orchestrators of these responses (3, 15).

Therefore, we compared levels of YKL-40 in serum and in proximal and peripheral airways of healthy never-smokers and of heavy smokers with and without COPD, and tested whether these values correlate with clinical outcomes of disease severity and with smoking history. In addition, we examined the capacity of healthy and diseased alveolar macrophages to synthesize YKL-40 and to respond to this glycoprotein by releasing factors involved in chronic airway inflammation and remodeling, such as proinflammatory and fibrogenic chemokines and the matrix-degrading metalloproteinase (MMP), MMP-9.

*Institut National de la Santé et de la Recherche Médicale Unité 700, Physiopathology and Epidemiology of Respiratory Insufficiency, Paris, France; †Université Paris 7, Faculté de Médecine Denis Diderot, Site Bichat, Paris, France; ‡Department of Autoimmunity, Inflammation and Respiratory Disease, MedImmune, Gaithersburg, MD 20878; §Département de Pneumologie A, Centre Hospitalier Universitaire Bichat-Claude Bernard, Paris, France; and ¶Assistance Publique Hôpitaux de Paris, Paris, France

Received for publication January 10, 2008. Accepted for publication August 4, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Fondation pour la Recherche Médicale (fellowship to S.L.) and by grants from the Agence Nationale de la Recherche (Santé et Environnement-Santé Travail) and from MedImmune.

[2] S.L. and A.K. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Marina Pretolani, Inserm Unité 700, Faculté de Médecine Denis Diderot, Site Bichat - 16, rue Henri Huchard, 75018 Paris, France. E-mail address: marina.pretolani@inserm.fr

[4] Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; BAL, bronchoalveolar lavage; DL_{CO}, transfer factor of the lung for carbon monoxide; GOLD, Global Initiative for Obstructive Lung Disease; MMP, matrix metalloproteinase.

Table I. *Characteristics of never-smokers and of smokers with and without COPD*

	Never-Smokers	Smokers without COPD	Smokers with COPD	*p* Value[a]
Subjects examined	15	20	30	
Age (years)	58.0 (50.0–54.5)[b]	56.5 (48.5–68.0)	63.5 (57.3–68.5)	0.4903
Gender (male/female)	8/7	12/8	23/7	
Smoking history (packs/year)	0	47.5 (35.0–52.5)	60.0 (40.0–67.5)	0.2785
Smoking status (current/former)	0	20/0	14/16	
Diagnosis: lung cancer	2	4	5	
Pre-β_2 agonist FEV_1[c] (%)	101.0 (100.0–117.0)	95.0 (86.3–100.0)	61.5 (50.8–64.8)*†	<0.0001
Post-β_2 agonist FEV_1 (%)	108.0 (104.3–123.8)	99.5 (89.3–101.8)	66.0 (56.3–71.8)*†	<0.0001
Pre-β_2 agonist FEV_1/FVC (%)	80.9 (79.6–84.6)	76.3 (72.9–80.8)	53.6 (46.5–60.1)*†	<0.0001
Post-β_2 agonist FEV_1/FVC (%)	83.7 (80.8–86.1)	77.9 (73.8–82.5)	56.2 (40.7–59.3)*†	<0.0001
PaO_2 (mmHg)	N.D.	98.0 (97.0–99.0)	82.0 (69.3–87.0)	<0.0001
$PaCO_2$ (mmHg)	N.D.	39.0 (38.8–40.0)	40.0 (38.1–41.3)	0.2254
pH	N.D.	7.40 (7.40–7.41)	7.41 (7.40–7.43)	0.1968
FRC (% predicted)	N.D.	102.0 (93.0–119.5)	121.0 (105.8–146.5)	0.0070
RV (% predicted)	N.D.	94.0 (82.5–108.5)	119.0 (100.3–134.3)	0.0012
TLC (% predicted)	N.D.	99.0 (88.0–105.0)	105.0 (94.3–120.0)	0.1007
DL_{CO} (% predicted)	N.D.	89.0 (86.0–98.5)	72.0 (59.0–80.5)	<0.0001
On inhaled steroids	0	0	2	
On anticholinergics	0	0	30	

[a] Significance was assessed with the use of Kruskal-Wallis test, except for smoking history; pre- and post-β_2 agonists FEV_1, pre- and post-β_2 agonists FEV_1/FVC, PaO_2, $PaCO_2$, pH, FRC, RV, TLC, and DL_{CO}, for which the Mann-Whitney *U* test was applied.

[b] Results are expressed as median (interquartile range) of the indicated number of values. *, $p < 0.05$, as compared with never-smokers; †, $p < 0.05$, as compared with smokers without COPD.

[c] FEV_1, Forced expiratory volume in 1 s; FVC, forced vital capacity; Pa, partial pressure; FRC, functional residual capacity; RV, residual volume; TLC, total lung capacity; N.D., not done.

Materials and Methods

Subjects

Fifteen healthy never-smoker volunteers, 20 current heavy smokers without COPD, and 30 heavy smokers (14 current and 16 former smokers) with COPD were recruited (Table I). COPD severity was graded into stages II ($n = 23$), III ($n = 4$), and IV ($n = 3$) following the Guidelines of the Global Initiative for Obstructive Lung Disease (GOLD) (16). Heavy smokers without COPD reported chronic cough and sputum, but had normal measurements on spirometry. None of the patients were taking oral steroids or statins, and none of them had other inflammatory diseases. In parallel, 40 nonsmoker asthmatic subjects (15 mild, 10 moderate, and 15 severe), mainly atopics and fulfilling the criteria of the Guidelines for the Diagnosis and Management of Asthma of the National Heart, Lung, and Blood Institute/World Health Organization (17), were recruited. These asthmatic subjects have already been included in a recent study (14). Bronchoalveolar lavage (BAL) was collected by bronchoscopy by instilling and recovering 150 ml of 0.9% NaCl (18, 19), then centrifuged (400 × *g*, 5 min, 4°C) and stored at −80°C until use. Cytospin preparations from BAL cells were fixed in acetone for 10 min at room temperature and stored at −20°C until use. Differential cell counting was performed by microscopic examination using Diff-Quick staining (Baxter Dade).

Four biopsy specimens were taken from the subcarinae in 10 never-smokers, in 12 smokers without COPD, and in 12 smokers with COPD, and they were immediately frozen in liquid nitrogen in Tissue-Tek compound (Sakura Finetek). These subjects were matched in terms of sex and age, and smokers with and without COPD had comparable smoking history (Table I). Lung tissue specimens were obtained from 2 never-smokers and from 4 and 5 smokers with and without COPD included in this study and undergoing lung lobectomy for localized lung carcinoma. Specimens were dissected at distance of the tumor (from 7th to 10th bronchi), immediately fixed in 10% formol, and processed to paraffin wax.

This protocol was approved by the Human Ethics Committee of the Bichat Hospital, and all subjects gave their written informed consent.

Ab and protein

The 293-F cell line (Invitrogen) was transfected with a plasmid-encoding human YKL-40, and His Tag was expressed at C terminus of this protein. rYKL-40 was purified from supernatant using Ni columns and was characterized by SDS PAGE, Western blot, protein assay, absorption at 280 nm, and mass spectrometry. Preparations were sterilized by passage through 0.2-μm filters. Endotoxin content was below 3 EU/mg protein, as tested by Lymulus Amoebocyte Lysate assay (Associates of Cape Cod).

Rabbits were immunized with rYKL-40 in CFA, and serum IgG was purified on protein G columns. Specificity and titers of the immune sera and the purified IgG were determined by Western blot and ELISA, using rYKL-40 and the unrelated His-tagged protein (14).

Immunohistochemistry and immunofluorescence

Serial 5-μm acetone-fixed sections from frozen bronchial tissue and cytospin preparations from BAL were sequentially incubated with 2 μg/ml rabbit polyclonal Ab directed against human YKL-40, biotin-conjugated goat anti-rabbit Ab, and streptavidin-alkaline phosphatase complex from the Vectastain kit (Vector Laboratories). Reaction was developed using Fast Red as a substrate (DakoCytomation), and tissues were counterstained using light nuclear Mayer's hematoxylin (Sigma-Aldrich). Staining of formalin-fixed paraffin-embedded sections was performed using 4 μg/ml YKL-40 Ab. Immunostaining specificity was determined using the corresponding control isotype, i.e., rabbit IgG (DakoCytomation), instead of the primary Ab. Biopsy areas were determined by morphometry and computer-assisted image analysis (Microvision Instruments, Histolab), and results were expressed as numbers of YKL-40-positive cells/mm^2 bronchial tissue (14, 19). Biopsy area was comparable in the three patient groups (median values ranging from 0.446 to 0.496 mm^2; overall p value = 0.168). For some subjects, the tissues recovered during the fibroscopy were not of good enough quality to perform or to quantify immunostaining.

The proportion of YKL-40-positive BAL macrophages in cytospin preparations was determined after counting at least 200 cells in randomly selected fields, by moving the objective of the light microscope onto the cytospin spot in a cross fashion, from the top to the bottom and from the left to the right.

Immunofluorescence double staining was performed on paraffin-embedded lung tissue sections using a mouse anti-human CD68 mAb (clone PG-M1; DakoCytomation), or its control isotype, mouse IgG3κ, in combination with the anti-YKL-40 Ab, followed by goat anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 568 Abs (Invitrogen). Sections were examined using a Plan Apochromat 63 × 1.4 oil differential interference contrast objective on the LSM 510 confocal microscope, and fluorescence was analyzed with the LSM META Acquisition and Image Browser softwares (all from Carl Zeiss).

Alveolar macrophage isolation and stimulation

Alveolar macrophages were isolated by adhesion on plastic from BAL obtained by bronchoscopy (20). Because the number of alveolar macrophages in most of the BAL samples was not sufficient to perform the whole series of in vitro stimulations, final study groups were composed of cells obtained from 8 healthy never-smokers, 12 smokers without COPD (stage 0), and 12 smokers with COPD (GOLD stage II, $n = 9$, and GOLD stage III, $n = 3$). Smokers with COPD were former ($n = 5$) or current smokers ($n = 7$). BAL cells were resuspended in RPMI 1640 medium containing 25

mM HEPES, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (all from Invitrogen), and were seeded at 500,000 macrophages/1 ml in 12-well plates (Corning-Costar). After 24-h incubation at 37°C in a humidified 5% CO_2 incubator, medium was removed and cells were either collected for RNA isolation or further stimulated for 24 h with 0.1, 1, and 10 μg/ml purified YKL-40, or with 10 ng/ml human rTNF-α (R&D Systems), or with the medium alone. Cell-free supernatants were collected and stored at −80°C until use.

Quantitative real-time PCR

Total RNA was extracted from alveolar macrophages using the Nucleospin RNA II kit (Macherey-Nagel), according to the manufacturer's instructions, and reverse transcribed using Moloney murine leukemia virus enzyme (Invitrogen), as described (21). The levels of the transcript encoding YKL-40 were assessed by quantitative real-time PCR, using an Mx 3000P apparatus (Stratagene Europe). The expression of the transcript was normalized to that of ubiquitin C, used as the most stable housekeeping gene. Calculation and normalization were performed using GeNorm software (21). Primers were designed using Primer Express 2 Software (Applied Biosystems) and were synthesized by Sigma-Genosys. Primer sequences were as follows: YKL-40 (GenBank Identifier M80927), sense 5′-TGC CCTTGACCGCTCCTCTGTACC-3′, antisense 5′-GAGCGTCACAT CATTCCACTC-3′; ubiquitin C (GenBank Identifier NM_004168), sense 5′-CACTTGGTCCTGCGCTTGA-3′, antisense 5′-TTTTGGGAATG CAACAACTTT-3′. Their sequences were blasted against Basic Local Alignment Search Tool database (http://www.ncbi.nlm.nih.gov/BLAST).

Mediator assessment

Concentrations of YKL-40 in serum, BAL, and macrophage supernatants were measured by sandwich-type ELISA developed in-house. Anti-YKL-40 mAb (MedImmune) was used for capture, and biotinylated anti-YKL-40 polyclonal Ab, followed by HRP-labeled streptavidin (Amersham, GE Healthcare), was used for detection. This ELISA allowed to decrease the detection limit of human YKL-40 from 250 pg/ml, in the commercially available kit (14, http://www.quidel.com) down to 100 pg/ml. IL-8, MCP-1, and MIP-1α were assessed by commercially available ELISA (R&D Systems), and MMP-9 was measured by Biotrak activity assay (GE Healthcare). Sensitivities were of 250 pg/ml (for MMP-9), 6.5 pg/ml (for IL-8), and 12.5 pg/ml (for MCP-1 and MIP-1α).

Statistical analysis

Data were analyzed statistically using the GraphPad Prism, version 4.0 for MacIntosh (GraphPad, eBizcuss). Results are expressed as median (interquartile range), except those obtained with purified alveolar macrophages, which represent means ± SEM. ANOVA was assessed with use of the nonparametric Kruskal-Wallis test. When an overall significant difference was detected ($p \leq 0.05$), pairwise group tests were performed using the Mann-Whitney *U* test.

Univariate regression analyses were performed by the Spearman's rank-order method, and correlation coefficients (r_s) were calculated. Only smokers with and without COPD were included in the analysis. The ANOVA was assessed with the use of the nonparametric Kruskal-Wallis test. To account for multiple tests because of the eight comparisons across the three study groups, we used a Benjamini and Hochberg correction with a family-wise error rate of 0.05 (22). The *p* values were sorted in ascending order, and the smallest *p* value, *P*1, was compared with $(0.05 \times 1)/6 = 0.0063$, and if *P*1 was less than 0.0063, *P*2 was compared with $(0.05 \times 2)/8 = 0.0125$, etc.

Results

Circulating and BAL levels of YKL-40

YKL-40 was detectable in serum and BAL samples from healthy never-smoker individuals (median (interquartile range), 13.5 ng/ml (10.1–40.3) and 2.8 ng/ml (1.4–5.1), respectively; Fig. 1). BAL, but not serum, levels of YKL-40 were slightly, but significantly increased in smokers without COPD (7.6 ng/ml (2.4–12.0), $p = 0.0007$; Fig. 1). Smokers with COPD had highly augmented levels of YKL-40 in serum (85.6 ng/ml (17.3–140.1)) and in BAL (19.0 ng/ml (10.0–34.1); Fig. 1).

Mild, moderate, and severe asthmatics had low levels of YKL-40 in their BAL fluid (median (interquartile range), 2.5 ng/ml (1.9–6.3), 3.0 ng/ml (1.1–7.4), and 3.0 ng/ml (2.7–6.8), respectively), and no significant changes were noted when these values were compared with those measured in never-smokers ($p = 0.453$).

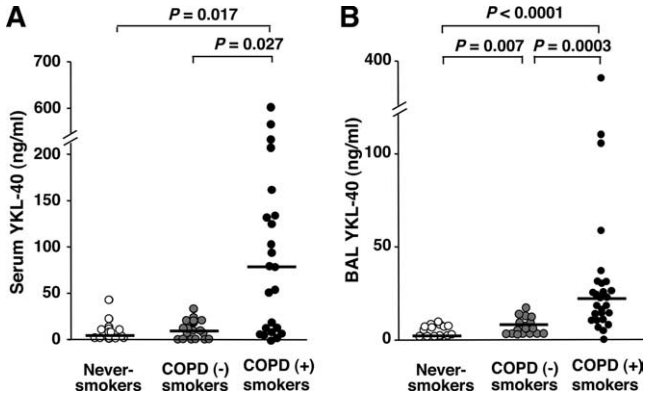

FIGURE 1. YKL-40 is elevated in serum and BAL of smokers with COPD. Levels of YKL-40 levels in serum (*A*) and in BAL fluid (*B*) collected in never-smoker healthy subjects, smokers without COPD, and smokers with COPD (defined as COPD (−) and COPD (+), respectively). The horizontal bars represent median values.

Univariate regression analyses in smokers with and without COPD ($n = 50$ subjects included) demonstrated that the levels of YKL-40 in serum and BAL were strongly associated with airflow obstruction, as determined by the assessment of percentage of predicted prebronchodilator, forced expiratory volume in 1 s (Table II). YKL-40 levels in serum and BAL also correlated significantly with transfer factor of the lung for carbon monoxide (DL_{CO}) (Table II). Neither YKL-40 in serum, nor in BAL, correlated with total lung capacity or smoking history (Table II). In a further subanalysis performed to account for smoking exposure, we found similar serum and BAL levels of YKL-40 in patients with COPD, either former or current smokers. Indeed, levels of YKL-40 in the serum of former ($n = 14$) and current ($n = 16$) smokers were 86.7 (19.5–154.0) and 61.0 (19.9–132.0) ng/ml, respectively ($p = 0.403$), and those found in BAL were 30.0 (17.5–96.8) and 23.8 (9.2–41.3) ng/ml, respectively ($p = 0.140$).

Finally, serum and BAL levels of YKL-40 significantly correlated between each other ($r_s = 0.376$, $p = 0.011$).

YKL-40 expression in bronchial biopsy and in lung tissue sections

Immunohistochemical detection of YKL-40 in bronchial biopsies from never-smokers showed only scarce, if any, positive cells (median (interquartile range), 3.1 cells/mm^2 (2.1–5.9); Fig. 2, *A* and *B*). In smokers without COPD, the number of cells expressing YKL-40 tended to increase (4.1 cells/mm^2 (0.0–6.3), $p = 0.191$;

Table II. *Correlation analyses in smokers with and without COPD*[a]

Parameter	YKL-40 in Serum		YKL-40 in BAL	
	r_s	*p* Value	r_s	*p* Value
Pre-β_2 agonist FEV_1[b] (% predicted)	−0.3892	**0.0072**[c]	−0.5491	**<0.0001**
TLC (% predicted)	−0.2955	0.0339	−0.1771	0.1168
DL_{CO} (% predicted)	−0.4667	**0.0020**	−0.3854	**0.0045**
Smoking history (packs/year)	0.2239	0.0853	0.1341	0.0846

[a] Spearman's rank order method ($n = 50$ subjects included).
[b] FEV_1, Forced expiratory volume in 1 s; TLC, total lung capacity.
[c] Bold denotes significant correlation, after Benjamini and Hochberg correction.

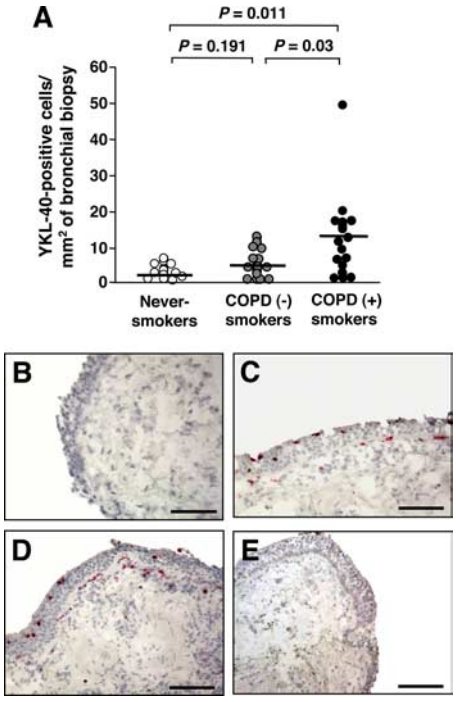

FIGURE 2. The number of YKL-40-expressing cells is increased in bronchial biopsies from smokers with COPD. Frozen bronchial tissue sections were reacted with a rabbit anti-human polyclonal Ab directed against YKL-40, using streptavidin-alkaline phosphatase complex and Fast Red staining and *A*, numbers of YKL-40-positive cells per mm^2 were determined in never-smokers, in smokers COPD, and in COPD subjects. Horizontal bars represent median values. *B*, *D*, and *E*, Representative immunostaining of YKL-40 in bronchial biopsies from a never-smoker (*B*), a smoker without COPD (*C*), and a smoker with COPD (*D*). *E*, Isotype (rabbit IgG)-reacted tissue section from a smoker with COPD showing the absence of immunostaining. Scale bars = 200 μm.

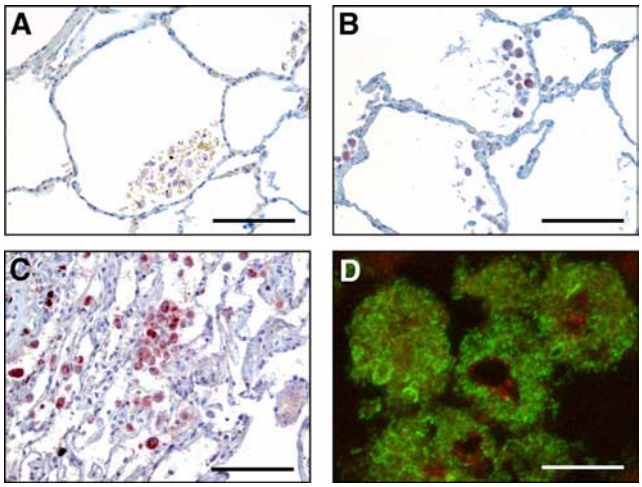

FIGURE 3. YKL-40 is expressed in lung tissue sections and localizes within macrophages. Lung tissue sections were reacted for YKL-40 and CD68 (which labels macrophages). Representative immunostaining shows the following: *A*, low numbers of inflammatory cells consistently negative for YKL-40 in the alveolar septa of a never-smoker subject; *B*, an accumulation of cells expressing YKL-40 (red deposit) in the alveoli of a smoker without COPD; and *C*, high numbers of YKL-40-positive cells in the lung parenchyma of a smoker with COPD. *D*, Confocal microscopy showing localization of YKL-40 (red fluorescence) within CD68-positive macrophages (green fluorescence) in lung tissue sections from a smoker with COPD. Scale bars: 400 (*A–C*) and 10 (*D*) μm.

Fig. 2, *A* and *C*), and it was highly augmented in smokers with COPD as compared with never-smokers and to smokers without COPD (13.6 cells/mm^2 (9.3–14.2), $p = 0.011$ and $p = 0.03$, respectively; Fig. 2, *A* and *D*). YKL-40 mainly localized to cells infiltrating the bronchial epithelium and the submucosa (Fig. 2, *C* and *D*), but was neither expressed by the bronchial epithelium (Fig. 2, *C* and *D*), nor by vascular endothelium, or airway smooth muscle (data not shown). No immunostaining was observed in control isotype rabbit IgG-treated tissue sections (Fig. 2*E*).

Examination of lung tissue sections from patients undergoing lung lobectomy for peripheral carcinoma showed an accumulation of YKL-40-containing cells in smokers with COPD, as compared with smokers without COPD and, to a higher extent, to never-smokers (Fig. 3, *A* and *B*, and Fig. 4*C*). Double immunofluorescence coupled to confocal microscopy demonstrated that macrophages in the alveolar septa and the interstitium were the main cell sources of YKL-40 in the airways of smokers with and without COPD (Fig. 3*D*). Rare neutrophils were found in these specimens, but when present, they were also strongly stained for YKL-40 (data not shown). YKL-40 was undetectable in bronchiolar and alveolar epithelium, or vascular endothelium of any of the three patient groups (data not shown).

Expression and production of YKL-40 by alveolar macrophages

Because alveolar macrophages were the major cell source of YKL-40, we next examined whether its expression was differentially regulated in relation with the disease status of the donors. The proportion of alveolar macrophages in BAL cells was higher in smokers with and without COPD (91.3 and 95.1%, respectively), as compared with never-smokers (81.3%). Immunostaining of cytospin preparations of BAL cells revealed weak or no expression of YKL-40 in the cytosol of alveolar macrophages of never-smokers (Fig. 4*A*), a consistent increment in smokers without COPD (Fig. 4*B*) and a strong immunostaining in smokers with COPD (Fig. 4*C*). Significantly higher proportion of YKL-40-positive alveolar macrophages was found in smokers without COPD (mean ± SEM, 26.7 ± 6.0%) in relation to never-smokers (3.3 ± 1.2%). This proportion was further increased in smokers with COPD (69.3 ± 5.8) (Fig. 4*D*).

To determine whether the increased expression of YKL-40 resulted from its elevated intrinsic baseline and stimulated synthesis, we examined the levels of YKL-40 transcript and protein in unstimulated and in TNF-α-exposed healthy and diseased alveolar macrophages. By quantitative real-time PCR, we found higher basal levels of YKL-40 mRNA in alveolar macrophages from smokers with and without COPD, in relation to never-smokers (Fig. 4*E*). This was accompanied with elevated amounts of the YKL-40 protein in the supernatants, as assessed by ELISA (Fig. 4*F*). The expression of both YKL-40 mRNA and protein did not differ significantly between never-smokers and smokers without COPD, whereas it was increased significantly in alveolar macrophages from smokers with COPD (Fig. 4, *E* and *F*). Stimulation of alveolar macrophages during 24 h with 10 ng/ml TNF-α up-regulated the levels of YKL-40 mRNA and secreted protein in never-smokers and in smokers with and without COPD (Fig. 4, *E* and *F*). However, these levels were substantially increased in smokers with COPD in relation to the other patient groups (Fig. 4, *E* and *F*).

Effect of YKL-40 on alveolar macrophages

Finally, we examined the ability of YKL-40 to promote the release of proinflammatory and fibrogenic chemokines and of MMP-9 by alveolar macrophages. BAL alveolar macrophages were incubated during 24 h with 0.1, 1, and 10 μg/ml purified human YKL-40, or

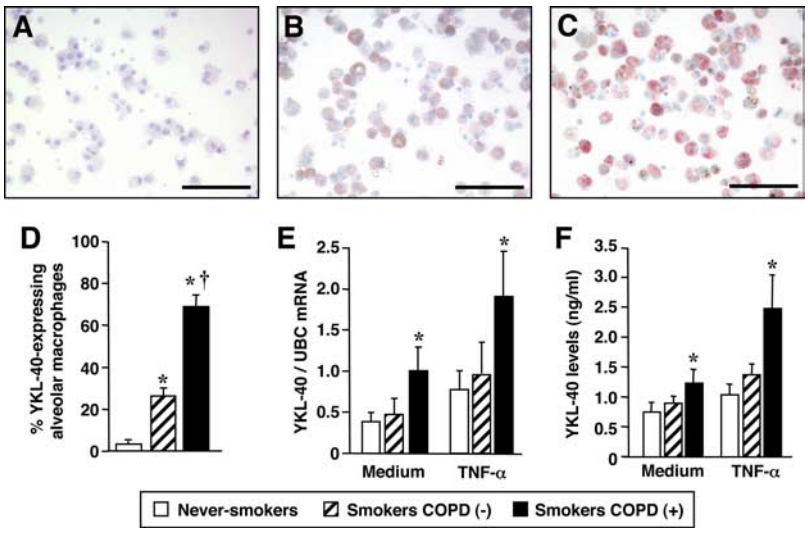

FIGURE 4. YKL-40 is differentially expressed in healthy and diseased alveolar macrophages. *A–C*, Photomicrographs illustrating YKL-40 immunostaining (red deposit) in BAL macrophages from a never-smoker (*A*), a smoker without COPD (*B*), and a smoker with COPD (*C*). Scale bar = 200 μm. *D*, Percentage of alveolar macrophages expressing YKL-40 in never-smokers and in smokers with and without COPD. *E*, Levels of YKL-40 mRNA, normalized according to the amount of ubiquitin C (UBC) mRNA, in medium- and in TNF-α (10 ng/ml)-stimulated alveolar macrophages from never-smokers and from smokers with and without COPD. *F*, Levels of YKL-40 in the supernatant of medium- and TNF-α-activated alveolar macrophages after 24 h of culture. Results are means ± SEM of six to nine independent experiments. *, $p < 0.05$, as compared with never-smokers; †, $p < 0.05$, as compared with smokers without COPD.

with 10 ng/ml TNF-α, used as positive internal control. Higher levels of IL-8, MCP-1, and MIP-1α were found in the supernatant of unstimulated alveolar macrophages from smokers with and without COPD than in that from never-smokers (Fig. 5). YKL-40 dose-dependently promoted mediator release, with a maximal increase at the concentration of 10 μg/ml (Fig. 5).

The effect of YKL-40 was more pronounced in alveolar macrophages obtained from smokers with and without COPD, as compared with never-smokers. Indeed, the net levels of secreted IL-8 induced by YKL-40 (defined as the levels of IL-8 in the supernatant of alveolar macrophages stimulated with 10 μg/ml YKL-40 minus the levels detected in medium-stimulated cells) were higher in smokers with and without COPD (1347.0 and 1983.0 pg/ml, respectively), than in never-smokers (896.0 ng/ml) ($p \leq 0.02$). Similarly, YKL-40 promoted the release of higher quantities of MCP-1 in smokers with and without COPD (mean net levels of 39.9 and 56.0 pg/ml, respectively), as compared with never-smokers (15.6 pg/ml, $p \leq 0.04$), as well as of MIP-1α (mean net levels of 100.2, 368.0, and 466.3 pg/ml in never-smokers, smokers with COPD, and smokers without COPD, respectively, $p \leq 0.03$).

The ability of YKL-40 (10 μg/ml) to promote chemokine and MMP-9 release from alveolar macrophages was comparable to that observed with TNF-α (Fig. 5).

Discussion

The diagnosis and course of COPD are currently evaluated by conventional clinical information and changes in pulmonary function, arterial blood gases, and chest radiographs. However, none of these criteria provide direct information concerning lung inflammation and remodeling, which are believed to play a central role in the pathogenesis and progresssion of COPD. In the current study, we showed significantly elevated levels of the chitinase-like protein, YKL-40, in the serum and BAL fluid and greater numbers of YKL-40-expressing cells in bronchial biopsies of smokers with COPD, in relation to smokers without COPD and to never-smoker healthy individuals. It is noteworthy that the expression of YKL-40 was low in smokers without COPD, suggesting that the induction of this glycoprotein plays only a limited part in the ongoing inflammatory response elicited by cigarette smoking. In support with this observation, serum and BAL levels of YKL-40 were similar in

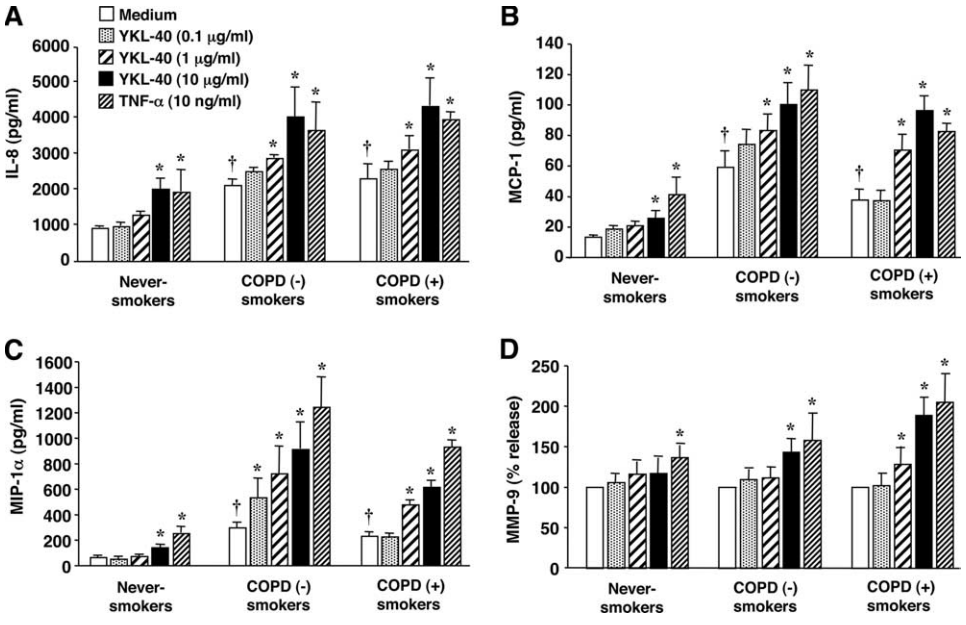

FIGURE 5. YKL-40 promotes the release of inflammatory and fibrogenic mediators by alveolar macrophages. Levels of IL-8 (*A*), MCP-1 (*B*), MIP-1α (*C*), and MMP-9 (*D*) in the supernatant of alveolar macrophages obtained from never-smokers and from smokers with and without COPD that had been stimulated over 24 h with the medium alone, or supplemented with 0.1, 1, and 10 μg/ml YKL-40, or with 10 ng/ml TNF-α. Results are means ± SEM of six to eight independent experiments. *, $p < 0.05$, as compared with medium-stimulated alveolar macrophages within each group of subjects; †, $p < 0.05$ for medium-stimulated alveolar macrophages from smokers and COPD subjects, as compared with never-smokers.

former and in current smokers with COPD and, irrespective of the compartment analyzed, i.e., the circulation or BAL fluid, YKL-40 failed to correlate with smoking history. In contrast, univariate regression analyses in smokers with and without COPD demonstrated a strong correlation between the levels of YKL-40 in serum and BAL and airflow obstruction, which reflects, by a large part, disease severity (1, 2, 16). Levels of YKL-40 in these compartments were also significantly related to DL_{CO}, an index that statistically correlates with the degree of emphysema in COPD subjects. However, because DL_{CO} cannot be used as a quantitative measure of emphysema in a given patient, additional studies with high resolution computerized tomography scan are required to further address the relationship between YKL-40 levels and the extent of emphysema.

Recently, we have demonstrated that serum levels and number of cells expressing YKL-40 in bronchial biopsies were elevated in subjects with severe asthma (14), another debilitating respiratory disease characterized by airway inflammation and remodeling (23). Although in these subjects serum levels of YKL-40 were in the same range as those presently reported in subjects with COPD, this glycoprotein was almost undetectable in BAL fluid. This difference may simply reflect the airway infiltration by different cell types (e.g., neutrophils vs eosinophils) between these two diseases.

To determine YKL-40 localization in the airways, immunohistochemical detection was next performed on bronchial biopsy and lung tissue sections. In both types of specimens, very few YKL-40-positive cells were found in never-smokers, whereas these numbers were higher in smokers without COPD and further augmented in smokers with COPD. Study of the staining pattern of YKL-40 by double immunofluorescence coupled to confocal microscopy led to the identification of alveolar macrophages as the main cell source of YKL-40 in the lung of smokers with and without COPD. However, when present, neutrophils also stained positively for YKL-40. Together, these findings extend previous observations showing that phagocytes synthesize abundantly YKL-40, both in the systemic circulation and in normal and diseased tissues (8, 24, 25).

The number of macrophages in the lung tissue and the alveolar compartment is higher in COPD (26), and this may account, at least in part, for the increase in BAL levels of YKL-40 observed in smokers with COPD. However, we also found that the proportion of alveolar macrophages containing YKL-40 was substantially increased in smokers with COPD, when compared with smokers without COPD or to never-smokers, with ~70% of cells expressing this protein. In addition, alveolar macrophages from smokers with and without COPD showed increased baseline mRNA and higher spontaneous as well as TNF-α-induced secretion of YKL-40, as compared with never-smokers. These observations extend previous results showing strong up-regulation of YKL-40 expression in macrophages trapped in vessel atherosclerotic plaques (8) and suggest that elevated numbers of alveolar macrophages, associated with an intrinsically greater potential of synthesizing YKL-40, participate in the elevation of this glycoprotein in BAL from smokers with COPD.

Intriguingly, visual inspection also revealed that certain subsets of BAL alveolar macrophages failed to stain for YKL-40, suggesting that the expression of this glycoprotein reflects differences in phenotype within the whole macrophage population, or in the activation status, or in the degree of maturation, as previously proposed (27).

Recent studies conducted on articular chondrocytes demonstrated that IL-1β and TNF-α efficiently promote the synthesis of YKL-40 (28). These proinflammatory cytokines are elevated in the lung of patients with COPD (11), suggesting that chronic exposure of alveolar macrophages to the inflammatory airway environment augments YKL-40 levels. Whether other factors that play a role in the pathogenesis of COPD regulate YKL-40 expression is an area for further investigation.

Once generated within the airways, YKL-40 may interact in an autocrine and/or paracrine manner to promote the generation of factors involved in COPD pathogenesis. Therefore, we tested the possibility that YKL-40 induced the release, by alveolar macrophages, of IL-8, MCP-1, and MIP-1α, three chemokines that sustain tissue inflammation and fibrosis, and of MMP-9, which is primarily involved in matrix degradation and in the accompanying alveolar destruction seen in emphysema (11). Extending previous observations (19, 29), alveolar macrophages from smokers with and without COPD constitutively produced higher amounts of IL-8, MCP-1, and MIP-1α than those from never-smokers. Exposure of alveolar macrophages to YKL-40 promoted chemokine and MMP-9 release, and this stimulatory effect was comparable to that obtained upon cell activation with TNF-α. It is noteworthy that YKL-40 was biologically active at 1 and, more so, at 10 μg/ml. These apparent high concentrations are, however, in the lower range of those routinely found in the serum of patients with chronic inflammatory diseases characterized by aberrant macrophage activation and tissue fibrosis (9–11).

Peribronchiolar fibrosis is another important histopathologic feature of tissue remodeling in COPD that is strongly associated with airflow obstruction and emphysema (2, 30). Recent studies have shown that YKL-40 is preferentially expressed in areas with active fibrogenesis in patients with hepatic fibrosis (10), where it may act synergistically with insulin-like growth factor I to stimulate the growth of fibroblasts (6). YKL-40 may also contribute to fibrosis by modulating the rate of type I collagen fibril formation (31). Whether YKL-40 participates in the onset of fibrosis of the small airways in patients with COPD remains to be determined. However, our present findings suggest that YKL-40 may influence extracellular matrix deposit and turnover by inducing MMP-9 production by alveolar macrophages (32, 33).

In conclusion, the present findings demonstrate that circulating and alveolar levels of YKL-40 are elevated in patients with COPD in association with disease severity. In addition, the in vitro data showing that alveolar macrophages are both source and target for YKL-40 make this glycoprotein a plausible candidate for playing a role in COPD pathogenesis. Overall, the current report brings a novel view on the pathophysiology of COPD and may lead to develop new therapeutic strategies for better management and benefit of the patients.

Acknowledgments

We thank the patients who participated in the study, and Pr. France Mentré (Institut National de la Santé et de la Recherche Médicale Unité 738, Centre Hospitalier Universitaire Bichat-Claude Bernard) for expert statistical advice.

Disclosures

The authors have no financial conflict of interest.

References

1. Barnes, P. J. 2000. Chronic obstructive pulmonary disease. *N. Engl. J. Med.* 343: 269–280.
2. Hogg, J. C. 2004. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet* 364: 709–721.
3. Barnes, P. J. 2004. Mediators of chronic obstructive pulmonary disease. *Pharmacol. Rev.* 56: 515–548.
4. Bleau, G., F. Massicotte, Y. Merlen, and C. Boisvert. 1999. Mammalian chitinase-like proteins. *EXS* 87: 211–221.
5. Badariotti, F., M. Kypriotou, C. Lelong, M. P. Dubos, E. Renard, P. Galera, and P. Favrel. 2006. The phylogenetically conserved molluscan chitinase-like protein

1 (Cg-Clp1), homologue of human HC-gp39, stimulates proliferation and regulates synthesis of extracellular matrix components of mammalian chondrocytes. *J. Biol. Chem.* 281: 29583–29596.
6. Recklies, A. D., C. White, and H. Ling. 2002. The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signaling pathways. *Biochem. J.* 365: 119–126.
7. Jacques, C., A. D. Recklies, A Levy, and F. Berenbaum. 2007. HC-gp39 contributes to chondrocyte differentiation by inducing SOX9 and type II collagen expressions. *Osteoarthritis Cartilage* 15: 138–146.
8. Boot, G. R., A. E. van Achterberg, B. E. van Aken, G. H. Renkema, M. J. H. M. Jacobs, J. M. Aerts, and C. J. de Vries. 1999. Strong induction of members of the chitinase family of proteins in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 19: 687–694.
9. Johansen, J. S., B. V. Jensen, A. Roslind, D. Nielsen, and P. A. Price. 2006. Serum YKL-40, a new prognostic biomarker in cancer patients? *Cancer Epidem. Biomarkers Prev.* 15: 194–202.
10. Johansen, J. S., P. Chrisoffersen, S. Moller, P. A. Price, J. H. Henriksen, C. Garbarsch, and F. Bendtsen. 2006. Serum YKL-40 is increased in patients with hepatic fibrosis. *J. Hepatol.* 32: 911–920.
11. Kucur, M., F. K. Isman, B. Karadag, V. A. Vural, and S. Tavsanoglu. 2007. Serum YKL-40 levels in patients with coronary artery disease. *Coron. Artery Dis.* 18: 391–396.
12. Kim, S. H., K. Das, S. Noreen, F. Coffman, and M. Hameed. 2007. Prognostic implications of immunohistochemically detected YKL-40 expression in breast cancer. *World J. Surg. Oncol.* 5: 17.
13. Rathcke, C. N., J. S. Johansen, and H. Vestergaard. 2006. YKL-40, a biomarker of inflammation, is elevated in patients with type 2 diabetes and is related to insulin resistance. *Inflamm. Res.* 55: 53–59.
14. Chupp, G. L., C. G. Lee, N. Jarjour, Y. M. Shim, C. T. Holm, S. He, J. D. Dziura, J. Reed, A. J. Coyle, P. Kiener, et al. 2007. A chitinase-like protein in the lung and circulation of patients with severe asthma. *N. Engl. J. Med.* 357: 2016–2027.
15. Barnes, P. J. 2004. Alveolar macrophages as orchestrators of COPD. *COPD* 1: 59–70.
16. Rabe, K. F., S. Hurd, A. Anzueto, P. J. Barnes, S. A. Buist, P. Calverley, Y. Fukuchi, C. Jenkins, R. Rodriguez-Roisin, C. van Weel, et al. 2007. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. GOLD executive summary. *Am. J. Respir. Crit. Care Med.* 176: 532–555.
17. National Asthma Education and Prevention Program. 1997. Guidelines for the Diagnosis and Management of Asthma. Expert Panareport 2. Atlanta, GA: National Institutes of Health; National Heart, Lung and Blood Institute Publication No. 97-4051A.
18. 1985. Summary and recommendations of a workshop on the investigative use of fiberoptic bronchoscopy and bronchoalveolar lavage in asthmatics. *Am. Rev. Respir. Dis.* 132: 180–182.
19. Benayoun, L., A. Druilhe, M. C. Dombret, M. Aubier, and M. Pretolani. 2003. Airway structural alterations selectively associated with severe asthma. *Am. J. Respir. Crit. Care Med.* 167: 1360–1368.
20. Culpitt, S. V., D. F. Rogers, P. Shah, C. De Matos, R. E. Russell, L. E. Donnelly, and P. J. Barnes. 2003. Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 167: 24–31.
21. Pégorier, S., N. Arouche, M. C. Dombret, M. Aubier, and M. Pretolani. 2007. Augmented epithelial endothelin-1 expression in refractory asthma. *J. Allergy Clin. Immunol.* 120: 1301–1307.
22. Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B. Methodol.* 57: 289–300.
23. Bousquet, J., P. Jeffery, W. W. Busse, M. Johnson, and A. M. Vignola. 2000. Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am. J. Respir. Crit. Care Med.* 161: 1720–1745.
24. Renkema, G. H., R. G. Boot, F. L. Au, W. E. Donker-Koopman, A. Strijland, A. O. Muijsers, M. Hrebicek, and J. M. Aerts. 1998. Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur. J. Biochem.* 251: 504–509.
25. Volck, B., P. A. Price, J. S. Johansen, O. Sorensen, T. L. Benfield, H. J. Nielsen, J. Calafat, and N. Borregaard. 1998. YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. *Proc. Assoc. Am. Physicians* 110: 351–360.
26. Retamales, I., W. M. Elliott, B. Meshi, H. O. Coxson, P. D. Pare, F. C. Sciurba, R. M. Rogers, S. Hayashi, and J. C. Hogg. 2001. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am. J. Respir. Crit. Care Med.* 164: 469–473.
27. Rehli, M., S. W. Krause, and R. Andreesen. 1997. Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation. *Genomics* 43: 221–215.
28. Recklies, A. D., H. Ling, C. White, and S. M. Bernier. 2005. Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes. *J. Biol. Chem.* 280: 41213–41221.
29. Lim, S., N. Roche, B. G. Oliver, W. Mattos, P. J. Barnes, and K. F. Chung. 2000. Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers: regulation by interleukin-10. *Am. J. Respir. Crit. Care Med.* 162: 1355–1360.
30. Martin-Mosquero, C., G. Peces-Barba, M. L. Rubio, M. Ortega, M. J. Rodriguez-Nieto, L. Martinez Galan, and N. Gonzalez-Mangado. 2006. Increased collagen deposition correlated with lung destruction in human emphysema. *Histol. Histopathol.* 21: 823–828.
31. Bigg, H. F., R. Wait, A. D. Rowan, and T. E. Cawston. 2006. The mammalian chitinase-like lectin, YKL-40, binds specifically to type I collagen and modulates the rate of type I collagen fibril formation. *J. Biol. Chem.* 281: 21082–21095.
32. Atkinson, J. J., and R. M. Senior. 2003. Matrix metalloproteinase-9 in lung remodeling. *Am. J. Respir. Cell Mol. Biol.* 28: 12–24.
33. Chapman, H. A. 2004. Disorders of lung matrix remodeling. *J. Clin. Invest.* 113: 148–157.