Chemokine Expression by Small Sputum Macrophages in COPD

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Small sputum macrophages represent highly active cells that increase in the airways of patients with inflammatory diseases such as chronic obstructive pulmonary disease (COPD). It has been reported often that levels of cytokines, chemokines and proteases are increased in sputum supernatants of these patients. In COPD, the small sputum macrophages may contribute to these supernatant proteins and recruit additional cells via specific chemokine expression patterns. We therefore investigated the expression profile of chemokines in sputum macrophages obtained from COPD patients in comparison to cells from healthy donors and cells isolated after inhalation of lipopolysaccharide (LPS). We used the minimally invasive procedure of sputum induction and have purified macrophages with the RosetteSep technology. Using macrophage purification and flow cytometry we show that in COPD small sputum macrophages account for 85.9% ± 8.3% compared with 12.9% ± 7.1% of total macrophages in control donors. When looking at chemokine expression we found, for the small macrophages in COPD, increased transcript and protein levels for CCL2, CCL7, CCL13 and CCL22 with a more than 100-fold increase for CCL13 mRNA ($P < 0.001$). Looking at active smokers without COPD, there is a substantial increase of small macrophages to 60% ± 15% and, here, chemokine expression is increased as well. In a model of airway inflammation healthy volunteers inhaled 20 μg of lipopolysaccharide (LPS), which resulted in an increase of small sputum macrophages from 18% ± 19% to 64% ± 25%. The pattern of chemokine expression was, however, different with an upregulation for CCL2 and CCL7, while CCL13 was downregulated three-fold in the LPS-induced small macrophages. These data demonstrate that sputum macrophages in COPD show induction of a specific set of CCL chemokines, which is distinct from what can be induced by LPS.

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INTRODUCTION

Analysis of sputum is a noninvasive approach to understanding disease processes in inflammatory airway disease (1). Many studies have looked at cell-free sputum supernatants and have reported increased levels of cytokines, chemokines and proteases (2–4).

When looking at the cellular composition of sputum, granulocytes and macrophages predominate in samples from healthy individuals (5). With airway inflammation, for instance in chronic obstructive pulmonary disease (COPD), there often is a dramatic increase of neutrophils (5). Also, determination of eosinophils can be informative in diagnosis and for monitoring of therapy (6,7).

When looking at macrophages in patients with airway disease, a shift from large macrophages to small macrophages has been noted in COPD and in cystic fibrosis (8,9). While these cells express higher levels of CD14 and HLA-DR, they show reduced scavenger receptor expression and reduced phagocytosis (8,9). The small macrophages may represent cells newly attracted by chemokines from the blood monocyte pool. Such newly recruited cells may themselves recruit additional leukocytes to the site of infection and inflammation in that they express various types of chemokines (10).

Chemokines are peptide mediators released by many tissue cells, including leukocytes, in response to tissue damage and infection (11). They act by binding to 7-transmembrane G-protein coupled receptors on leukocytes. Signaling via these receptors leads to migration of the white cells into the area of damage and infection

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to fight the microbes and clear the damaged tissue. Chemokines can be assigned to two major groups, that is, the CXC chemokines and the CC chemokines, depending on whether there is an additional amino acid between the two N-terminal cysteines or not. The respective receptors are classified as CC receptors or CXC receptors. A given chemokine can bind to several receptors and a given receptor can bind several chemokines. Still, there is functional specificity and the expression of a specific chemokine will lead, via binding to its respective receptors, to immigration of a specific set of leukocytes. We now have analyzed the expression of chemokines by sputum macrophages and focused on those targeting CCR1-4, since these receptors show a broad expression on lymphocytes, monocytes and granulocytes (12). When testing the set of 15 chemokines, we found an increased expression for CCL2, 7, 13 and 22 in COPD while there was no significant change in expression of the remaining 11 chemokines. CCL2 (MCP-1; monocyte chemoattractant protein-1) is produced by many cell types, including fibroblasts, endothelial, epithelial, smooth muscle, mesangial, astrocytic, monocytic and microglial cells. It has been shown to be elevated in tissue sections in ex-smokers with, as compared to ex-smokers without, COPD (13). CCL7, previously called MCP-3 (monocyte chemoattractant protein-3), has an expression pattern that is more restricted compared with CCL2. mRNA expression of CCL7 is constitutive in platelets and has been induced by various inflammatory cytokines in several cell types including monocytes (14). To date nothing is known regarding expression of CCL7 in COPD. CCL13, also called MCP-4 (monocyte chemoattractant protein-4), is a CC chemokine with potent eosinophil chemoattractant activity. Taha *et al*. demonstrated an increased expression of CCL13 in induced sputum samples of asthmatic patients compared with control subjects (15). CCL22, originally called MDC (macrophage-derived chemokine), is a chemokine produced mainly by macrophages and dendritic cells (16). An increase of CCL22 mRNA in tissue and of protein in lavage fluid was reported for COPD (17). These limited data show that a systematic analysis of expression of these chemokines in COPD is warranted.

Table 1. Demographic data of control donors.

Control sample #	Male/female	Age	Smoker status	% Small MΦ in sputum	% Small MΦ after LPS inhalation
0578	female	56	never	4.9	
0673	female	31	never	7	
0744	female	27	never	9.5	
0891	female	30	never	23.9	
0939	male	30	never	19.5	
0945	male	28	never	7	
1028	female	30	never	12	
1173	male	30	never	19.8	
1594/1596[a]	female	31	never	13.4	66.5
1595/1597[a]	male	76	never	46.8	93.1
1761/1764[a]	female	46	never	4.7	64.6
1808/1811[a]	male	33	never	8.4	30.4
0526	male	42	active	81.7	
0534	female	67	active	57.8	
0562	male	58	active	51.1	
0574	male	58	active	48.7	

[a]After LPS inhalation.

Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria. By binding to toll-like-receptor-4 (TLR-4), LPS can activate the NF-κB signaling pathway, and this leads to induction of many genes involved in inflammation, including the expression of various chemokines. LPS inhalation has been used as a model for inflammation of the lung (18). LPS-induced inflammatory responses include systemic effects such as fever and granulocytosis in blood and local effects such as an increase of cytokines in sputum and influx of leukocytes (18) and, in the mouse model, an LPS-induced influx of small macrophages has been documented (19). We can demonstrate herein that LPS targeted to the airways of the lung (Moeller *et al*., submitted) will lead to an influx of small macrophages. When compared with expression of chemokines in sputum macrophages from COPD patients, LPS inhalation leads to a clearly distinct pattern of chemokine expression. This indicates that small sputum macrophages can have different properties dependent on the circumstance of their induction.

MATERIALS AND METHODS

Study Population

The study population consisted of healthy control donors (Table 1) (n = 8, 3 males, 5 females, aged 32 ± 9.4 years) recruited from the hospital staff or colleagues, healthy smokers (n = 4, 3 males, 1 female, ages 56.2 ± 10.4 years) and COPD patients (details given in Table 2) (n = 11, 10 males, 1 female, ages 66.6 ± 11.1 years, mean FEV_1 in % predicted of 41% ± 8.3%, 10 were ex-smokers for more than 3 years, one was a current smoker) recruited from the inpatient department of the Asklepios Hospital Munich-Gauting. The latter were examined immediately before discharge and at least 10 d after the last exacerbation.

Written informed consent was obtained from all donors. The study was approved by the Ethics Committee of the Ludwig-Maximilians University Munich.

LPS Inhalation

Four healthy nonatopic never-smokers (2 male, 2 female, ages 47.2 ± 20.9 years) were recruited for the inhalation of 20 μg of LPS. Respiratory symptoms were obtained by using a questionnaire (20) and pulmonary function parameters were measured by spirometry and body

Table 2. Demographic data, lung function and therapy of patients.

Patient #	Male/ female	Age	FEV_1 (L)	FVC (L)	FEV_1/ FVC	GOLD stage I-IV	Smoker status	% Small MΦ in sputum	Treatment	
									Inhaled	Oral
0380	male	69	0.74	1.58	0.47	IV	ex	97.6	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	25 mg glucocorticoid/day
0436	male	39	1.57	3.72	0.42	II	ex	85.7	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	35 mg glucocorticoid/day, theophyllin
0438	male	59	0.8	3.12	0.26	IV	ex	95.3	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	25 mg glucocorticoid/day, theophyllin
0449	male	67	0.98	2.51	0.39	III	ex	95.6	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	40 mg glucocorticoid/day, theophyllin
0462	female	62	1.03	2.26	0.46	II	ex	84	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	5 mg glucocorticoid/day, theophyllin
0463	male	64	1.48	3.07	0.48	III	ex	88.1	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	15 mg glucocorticoid/day, theophyllin
0487	male	73	0.96	2.33	0.41	III	ex	81.2	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	15 mg glucocorticoid/day
0565	male	81	1.15	2.92	0.39	II	ex	84.9	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	30 mg glucocorticoid/day
0834	male	72	0.96	2.33	0.41	III	ex	68	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist	
1315	male	76	1.1	3.92	0.28	III	ex	84	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist	30 mg glucocorticoid/day
1336	male	71	0.86	3.15	0.27	III	ex	80.6	short acting β_2 agonist, anti-cholinergic, long acting β_2 agonist, glucocorticoids	10 mg glucocorticoid/day

plethysmography (Jäger Masterlab, Erich Jaeger GmbH, Höchberg, Germany) (21). Bronchial hyperresponsiveness (BHR) was assessed by a methacholine challenge test according to the guidelines for bronchial challenges of the European Respiratory Society (22). Subjects included in the study showed no or only weak responses (less than two-fold increase of airway resistance) at the highest dose of inhaled methacholine. Sputum induction was performed 24 h prior to and 24 h after LPS inhalation.

Targeted delivery of aerosolized LPS to the airways was done between 0900 and 1100 by shallow aerosol bolus inhalation using the AKITA device (Activaero GmbH, Gemünden, Germany). Previous studies have shown that the AKITA device shows little inter- and intrasubject variation of aerosol deposition in the lung (23). LPS from von *Salmonella abortus equi*, S-form (TLRgrade, ALX-581-009, Alexis Biochemicals via Enzo Life Sciences, Lörrach, Germany) was used in all subjects. A 100 mL bolus was inhaled to 180 mL bolus penetration front depth. The pressure of the jet nebulizer was 1.0 bar producing 4.5 μm mass median aerodynamic diameter (MMAD) droplets. The inhalation flow rate was 200 mL/sec and an 8-sec breath-holding was performed at the end of inhalation. Previous deposition measurements using radiolabeled DTPA (diethylenetriaminepentaacetic acid) particles revealed 80% deposition efficiency for this inhalation maneuver. By use of the nebulizer output, the deposition efficiency and the LPS concentration in the nebulizer, the number of breaths was calculated for a deposited dose of 20 μg LPS in the airways. The protocol was approved by the Ethics Committee of the Ludwig-Maximilians University Munich, and informed consent was obtained from each subject.

Sputum Induction and Processing

Sputum induction and processing were carried out as described previously (8,9) with some modifications. Briefly, the subjects were asked to inhale sterile saline solutions from 0.9% up to 3% in COPD and to 5% in healthy controls. The aerosol was generated by using a multisonic nebulizer at maximum output for 5 min. Donors then were asked to blow their noses and rinse their mouths with water. The participants were encouraged to cough vigorously into a Petri dish. This was repeated for a further two inhalations. In total, saline inhalation for COPD and controls was performed for no longer than 15 min, and sputum was processed immediately on ice. Sputum from COPD patients and control donors was processed as a whole to have equal conditions. Donated sputum (1 volume) was then admixed with four times the volume of Sputolysin, reconstituted according to the manufacturer's instructions (Calbiochem, San Diego, CA, USA). The sputum/Sputolysin mixture was placed in a waterbath at 37°C for up to 20 min. An equal volume of PBS (pH 7.4)

was added subsequently. The resultant cell suspension was then filtered through 100 μm and 40 μm Falcon cells sieves (#352360 and #352340, BD Sciences, Mannheim, Germany) to remove aggregates. Cells were centrifuged at 800*g* for 10 min at 4°C. The supernatant was collected for detection of proteins and the cell pellet was assessed for cell viability by using the trypan blue exclusion method. Cells were pelleted and resuspended in PBS (2% FCS) for flow cytometry staining before the RosetteSep procedure.

RosetteSep Isolation of Sputum Macrophages

To obtain highly purified sputum macrophages, a further isolation step was added by using the RosetteSep method. For this 3-mL cell suspension, mononuclear cell depleted erythrocytes (30 μL; obtained following centrifugation over LymphoPrep gradient) and 50 μL monocyte enrichment cocktail (RosetteSep monocytes enrichment reagent, #15068, Stem Cell Technologies via Cell Systems, St. Katharinen, Germany) were added and incubated at room temperature for 20 min. Cells were then diluted 1:1 with tissue-culture grade PBS, layered over an equal volume of LymphoPrep density gradient medium (Progen, Heidelberg, Germany) and centrifuged at 800*g* for 30 min to generate a mononuclear layer, which was aspirated, washed and resuspended in LPS-free PBS/2% FCS solution. Cells were counted, and viability was determined by using trypan blue as stated previously. Isolated sputum macrophages were reanalyzed by flow cytometry to determine the purity of the population and then further were used for reverse transcription polymerase chain reaction (RT-PCR).

Flow Cytometry

For determination of small and large sputum macrophages before and after RosetteSep isolation we used the following monoclonal antibodies according to the manufacturer's instructions: anti-CD66b-FITC (#0531, Immunotech, Coulter, Krefeld, Germany), anti-CD16b-PE (#550868, Pharmingen, BD Sciences, Heidelberg, Germany), and anti-CD14-PC5 (#A07765, Coulter, Krefeld, Germany). Small and large macrophages were determined in percent of all macrophages by first excluding the CD66b/CD16b-positive granulocytes and further gating on the CD14-positive macrophages. Within this population, small and large macrophages were regated in a forward versus side scatter plot to define their percentage and purity. The sputum macrophages used for further molecular analysis had a purity of at least 90% as determined by the three color immunofluorescence described above.

RT-PCR

Semiquantitative RT-PCR for selected genes was performed by using the LightCycler system as described previously (9). The MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (24) were followed as far as applicable. In brief, 2×10^4 cells each were lysed in 200 μL TRI-Reagent (#T-9424 Sigma, Taufkirchen, Germany) and spiked with 15 μg tRNA from brewer's yeast (#109527 Roche Diagnostics, Mannheim, Germany) for precipitation of the RNA. Equal amounts of each sample were reverse transcribed with oligo (dT) as primer. Semiquantitative PCR was performed by using the LightCycler system (Roche Diagnostics) with 3 μL cDNA/capillary in the SYBR Green format using the LightCycler-FastStart DNA Master SYBR Green I kit from Roche Diagnostics (Catalogue #2 239 264) in a total volume of 20 μL. As an external control, the reference gene α-enolase was amplified under the same conditions (annealing at 60°C). Amplification was done with the following primers that have been chosen with the online program http://frodo.wi.mit.edu/primer3/. We recommend that use of this software be cited in publications as Rozen *et al*., 2000 (25):

CCL2: forward, 5′-AGC AGC AAG TGT CCC AAA GAA G; reverse, 5′-CAA AAC ATC CCA GGG GTA GAA C (product size 386 bp). CCL7: forward, 5′-AGC ACC TGG ACA AGA AAA CCC, reverse, 5′-CCC CCA TGA GGT AGA GAA GG (product size 327 bp). CCL13: forward, 5′-GGA AAG CTC ACA CCC TGA AGA; reverse, 5′-CCA AAC CAG CAA CAA GTC AAA TA (product size 214 bp). CCL22: forward, 5′-TGT GCC AAC TCT CTG CAT TCC; reverse, 5′-AGA CCC AGA AGT GGG ATG TGT (product size 335 bp). α-Enolase: forward, 5′-GTT AGC AAG AAA CTG AAC GTC ACA; reverse, 5′-TGA AGG ACT TGT ACA GGT CAG (product size 619 bp). All amplicons were monitored on a 2% agarose gelelectrophoresis in 0.5 × TBE buffer to reveal the estimated product size in a single band (see Supplemental Figure 3).

ELISA

Supernatants of fresh sputum samples were prepared as described above in "sputum induction and processing." Enzyme-liked immunosorbent assays (ELISAs) for CCL2, CCL7, CC13 and CCL22 (all from R&D Systems, Wiesbaden, Germany) were performed according to the manufacturer's instructions while applying samples in duplicates and several titration steps.

Statistics

All data are expressed as mean ± standard deviation. For statistical analysis of the data, we used the Student *t* test. Results were considered significant when $P < 0.05$.

All supplementary materials are available online at www.molmed.org

RESULTS

Increased Small Macrophages in COPD Sputum Samples

As has been reported earlier (8,9) patients with COPD exhibit a strong increase of small macrophages in their airways as detected in induced sputum samples. We now have employed a novel technique for the study of these

cells which involves depletion of all nonmacrophages from sputum samples by using the RosetteSep purification. With this technique, granulocytes are removed and less artifactual events are seen in the flow cytometry analysis, and cell populations are much more clearly defined (see Supplemental Figures 1 and 2).

By use of the RosetteSep technique in a cohort of 10 patients, the percentages of small macrophages in samples before and after RosetteSep purification were found to be 71.9% ± 0.18% and 60.4% ± 0.23%, respectively. This finding is explained by the fact that granulocytes in the native sputum samples are not removed completely by FACS gating techniques and contaminate the small macrophage window. A precise determination of small versus large macrophages can only be achieved after physical removal of granulocytes by RosetteSep.

We therefore have used the RosetteSep purification as a standard approach to sputum samples for COPD and healthy donors.

As revealed in Figure 1A, the macrophage population in a healthy donor shows predominantly large macrophages with high granularity, while, in COPD, there are mainly cells of small size and lower granularity. In this example, small macrophages in COPD account for 80.6%, while in the control there are 4.9% small macrophages (Figure 1A).

Chemokine Gene Expression in COPD Sputum Macrophages

RosetteSep not only provides an improved approach for definition of macrophage populations by flow cytometry, but this purification technique also makes these cells amenable to the study of gene expression. Since one of the main functions of macrophages in inflammation is to recruit additional leukocytes, we have studied expression of chemokines, and for this we screened for CCL chemokines that target CCR1-4 in a pilot study on up to five COPD patients (data not shown). Among these CCL 3, 4, 5, 8, 11, 14, 15, 17, 23, 24 and 26 showed no expression, or the difference in expression level between COPD samples with high percentage of small macrophages and control samples with low percentages of small macrophages was less than three-fold (data not shown).

By contrast, we found a strongly increased level of mRNA expression for CCL2, 7, 13 and 22 in COPD patients as compared with control donors. The cohort of COPD patients used for these analyses contained an average of 85.9% ± 8.3% small macrophages (n = 11) compared with 12.9% ± 7.2% small macrophages in controls (n = 8) ($P < 0.01$). For CCL2 we found an average 16-fold increase, for CCL7 it was 13-fold and for CCL22 it was 12-fold. The strongest increase of CCL expression in COPD sputum macrophages versus healthy control donor macrophages was seen for CCL13, which was more than 100-fold (Figure 1B).

Chemokine Gene Expression in Sputum Macrophages from Smokers

Smoking is the main cause of COPD and it is known to induce inflammation of the airways as evidenced by cough and increased sputum production. We therefore have studied the impact of smoking on sputum macrophages and their gene expression in asymptomatic smokers. As shown in Figure 2A, smoking does induce a pronounced increase of small sputum macrophages. On average, the percentage was 59.8% ± 15 % ($P < 0.05$ compared with healthy controls). When looking at chemokine gene expression we found increased expression for CCL2, 7, 13 and 22—similar to what is seen in COPD patients. The increase was, however, less pronounced for CCL2 (six-fold), CCL7 (2.5-fold) and CCL13 (31-fold). By contrast, induction of CCL22 was clearly higher in smokers with a 23-fold higher expression (Figure 2B) in smokers compared with the 12-fold induction seen in COPD. Still, the pattern of chemokine gene expression in sputum macrophages in smokers was very similar to COPD patients.

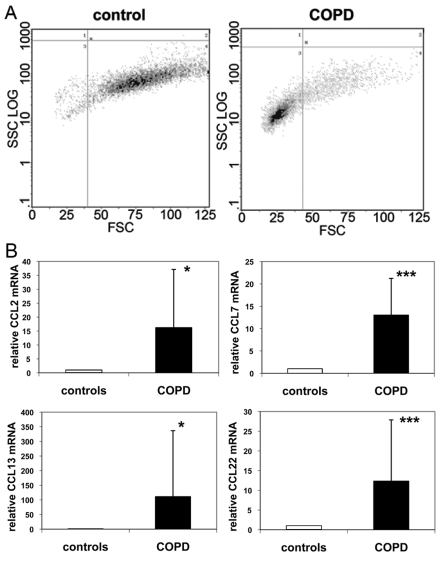

Figure 1. (A) Enrichment of sputum macrophages by RosetteSep technique. Induced sputum samples from a healthy individual (left panel) and a COPD patient (right panel) were used to isolate the macrophage populations by using the RosetteSep technique. Purified cells were stained with CD66b/CD16b/CD14 and analyzed by flow cytometry. Resulting macrophage populations were regated on a forward versus side scatter plot to show the differential properties in size and granularity. In the given example, the proportion of small macrophages in the control donor is 4.9% (left) whereas the COPD patient shows an increase to 80.6% (right) of all macrophages. Shown is one example out of 11 for COPD and of 8 for controls donors. (B) Chemokine mRNA expression in controls and COPD. Purified sputum macrophages (>90%) from controls (left bar in each plot) and COPD patients (right bar) were analyzed by RT-PCR for mRNA expression for CCL2, CCL7, CCL13 and CCL22. Shown is the relative fold difference in expression of COPD patients as compared with controls that have been set as one. Values have been normalized to the expression of the house keeping gene α-enolase. Results were in fold difference 16.2 ± 20.9 for CCL2, 13.01 ± 8.2 for CCL7, 110 ± 225 for CCL13 and 12.3 ± 15.5 for CCL22. Shown are the mean values of 8 controls and 9–11 COPD patients. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The average percentage of small sputum macrophages in the two cohorts used for RT-PCR analysis was 12.9% ± 7.2% in controls and 85.9% ± 8.3% in COPD ($P < 0.01$).

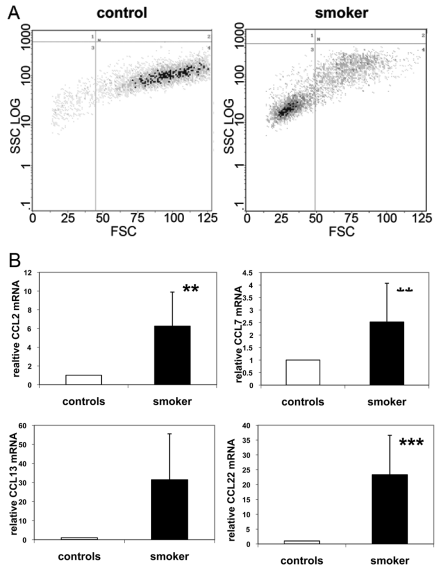

Figure 2. (A) Proportion of sputum macrophages in controls and smokers. Shown are purified macrophages from induced sputum samples of a control donor (left plot) and a healthy smoker (right plot) in a forward versus side scatter analysis after staining with CD66b/CD16b/CD14. The percentage of small sputum macrophages in the control is 7% and 57.8% in the smoker. Shown is one example of four. The average percentage of small sputum macrophages in the two cohorts was 12.9% ± 7.2% in controls and 59.8% ± 15% in smokers ($P < 0.02$). (B) Chemokine mRNA expression in controls and healthy smokers. Purified sputum macrophages (>90%) from controls (left bar in each plot) and smokers (right bar) were analyzed by RT-PCR for mRNA expression for CCL2, CCL7, CCL13 and CCL22. Shown is the relative fold difference in expression of COPD patients as compared with controls that have been set as one. Values have been normalized to the expression of the house keeping gene α-enolase. Results are in fold difference 6.25 ± 3.6 for CCL2, 2.5 ± 1.5 for CCL7, 31.4 ± 24 for CCL13 and 23.3 ± 13.3 for CCL22. Shown are the mean values of 8 controls and 4 smokers. $^{**}P < 0.01$, $^{***}P < 0.001$.

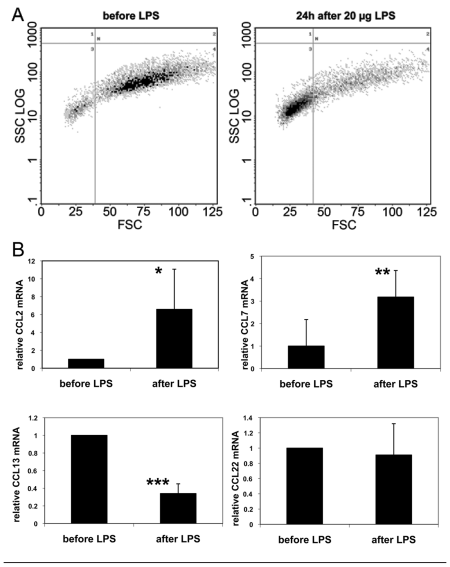

Figure 3. (A) Proportion of small sputum macrophages after LPS inhalation. Sputum induction was performed in healthy control donors 24 h before (left plot) and 24 h after (right plot) inhalation of 20 µg LPS. Sputum macrophages were purified by RosetteSep and regated in a forward versus side scatter plot after staining with CD66b/CD16b/CD14. Percentage of small sputum macrophages before LPS inhalation is 13.4% and 66.5% 24 h after inhalation. Shown is 1 out of 4 donors. The average percentage of small sputum macrophages was 18.3% ± 19.3% before and 63.7% ± 25.7 % after LPS inhalation ($P < 0.05$). (B) Chemokine expression before and after LPS inhalation. Macrophages from induced sputum were purified 24 h before and 24 h after LPS inhalation. RT-PCR was performed for CCL2, CCL7, CCL13 and CCL22. mRNA expression before LPS inhalation (left bars) was set as one and shown is the fold difference after LPS challenge (right bars). This was 6.5 ± 4.5 for CCL2, 3.2 ± 1.2 for CCL7, 0.3 ± 0.1 for CCL13 and 0.9 ± 0.4 for CCL22. Shown are the mean values from four donors. $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$.

Chemokine Gene Expression in Sputum Macrophages Induced by LPS Inhalation

LPS is a constituent of gram-negative bacteria and a potent activator of innate immunity. Since its application can mimic infection and inflammation we used inhalation of LPS to study the impact on macrophage composition and gene expression. For this, healthy volunteers inhaled 20 µg of *S. abortus equii* LPS, and this was deposited to the airways by using the AKITA inhalation device as described (Möller *et al.*, submitted). Induced sputum was obtained 24 h before and 24 h after inhalation. As shown in Figure 3A, there was a dramatic increase in the small sputum macrophages after LPS inhalation, very similar to what was seen in COPD patients and in smokers. On average, of four donors, the percentage of small sputum macrophages referred to all macrophages increased from 18.3% ± 19.3% at 24 h before LPS inhalation to 63.7% ± 25.7% at 24 h after LPS inhalation ($P < 0.05$).

We then asked whether these small macrophages would show the same pattern of gene expression as seen in COPD patients and healthy smokers. While there was an increase of both CCL2 and CCL7 mRNA in the LPS-induced cells, CCL22 remained unchanged. Surprisingly, CCL13 showed a three-fold decrease (Figure 3B). This is in strong contrast to the more than 100-fold induction seen in COPD. Taken together, these data indicate that the LPS-induced small macrophages show a unique pattern of gene expression, which is clearly distinct to what is seen in small sputum macrophages from COPD.

Increased Chemokine Protein in COPD Sputum Samples

Finally, we asked whether the increased mRNA expression in small sputum macrophages in COPD will result in increased production of the respective proteins. For this, supernatants of induced sputum samples were recovered and tested by ELISA. As shown in Figure 4, there were significantly higher levels of protein for all of the chemokines tested in samples obtained from COPD patients compared with healthy control donors.

DISCUSSION

Cells of the monocyte/macrophage system derive from myelomonocytic stem cells in bone marrow. These progenitor cells develop into monoblasts, which then give rise to monocytes. Monocytes migrate into tissues and give rise to macrophages that show different phenotype and function dependent on the type of tissue, such as brain (microglia cells), bone (osteoclasts) and alve-

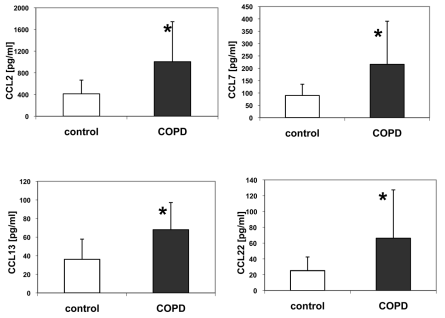

Figure 4. Chemokine protein in sputum in COPD and control donors. Supernatants from induced sputum samples were assessed by ELISA for CCL2, CCL7, CCL13 and CCL22 protein. Shown are the mean values in pg protein/mL in n = 8 controls (left bars) and in n = 5 COPD patients (right bars). *$P < 0.05$ for all proteins.

olar space of the lung. The alveolar macrophage is characterized by rounded shape and shows larger size and higher granularity compared with blood monocytes. The same is true for macrophages, which reside on the surface to the airway epithelium.

Airway leukocytes can be recovered easily via sputum induction, but to date little is known about the phenotype and function of macrophages in sputum. Studies in inflammatory disease have revealed an increase in the percentage of small macrophages in induced sputum samples of patients with inflammation as compared with apparently healthy controls (8,9,26).

The analysis of the sputum macrophages in health and disease is hampered by the contamination of samples with other leukocytes, notably with abundant granulocytes. We therefore have used the novel RosetteSep technology to remove all granulocytes and lymphocytes by using an antibody-mediated rosetting with autologous erythrocytes followed by ficoll hypaque separation. This procedure generates highly purified cells for phenotypic and functional analysis.

When looking at the light scatter profiles of such purified cells, we noted that small macrophages were the predominant cells in COPD, in active smokers and also in a model of airway inflammation induced by inhalation of LPS.

We then studied gene expression in purified sputum macrophages and focused on chemokines, which can attract additional leukocytes such as neutrophils (CXC-chemokines), lymphocytes and monocytes (CC-chemokines).

In initial pilot studies on CXC-chemokines in small sputum macrophages of COPD patients versus the large sputum macrophages of healthy controls in three donors each, we found no difference for CXCL1 and only a two-fold increase for CXCL2 (data not shown).

Looking at CC-chemokines we focused on chemokines that target CCR1-4 (that is, CCL 2, 3, 4, 5, 7, 8, 11, 13, 14, 15, 17, 22, 23, 24 and 26) we found a greater than threefold induction in COPD versus healthy controls only for CCL2, 7, 13 and 22.

CCL2, also called MCP-1 (monocyte chemoattractant protein-1), is produced by many cell types, including endothelial, fibroblast, epithelial, smooth muscle, mesangial, astrocytic, monocytic and microglial cells. However, monocytes/macrophages are the major source of CCL2 (27). In line with this cellular pattern of CCL2 expression, De Boer *et al*. reported a strong signal for CCL2 in macrophages and a weak signal in alveolar epithelial cells (13).

In that study, the levels of *in situ* mRNA were found to be 1.5× higher in COPD patients compared with non-COPD patients. These data are in-line with our findings on sputum macrophage expression of CCL2 as shown herein, but with our approach we see a much stronger difference, that is, 22-fold. This difference may be due to the different technology (*in situ* hybridization versus RT-PCR) used in the two studies. The importance of CCL2 for recruitment of monocytes to the lung was shown by Maus *et al*. (28) in that blockade of the CCL2-CCR2 pathway strongly reduced monocyte recruitment to the lung. Given the strong increase of CCL2 mRNA in COPD in our study, these data suggest a possible role of this chemokine in the recruitment of additional monocytes into the airways. In studies by Traves *et al*. (2), an increase in CCL2 protein in induced sputum, but not in bronchoalveolar lavage fluid, was noted in COPD patients, consistent with our findings. In an earlier study, we reported an increase in small sputum macrophages in cystic fibrosis, similar to what is seen in COPD (9), and, in sputum supernatants, we found increased CCL2 protein (29). Therefore it is possible that small sputum macrophages are a source of sputum CCL2 in cystic fibrosis as well.

CCL7, previously called MCP-3 (monocyte chemoattractant protein-3), has an expression pattern that is more restricted compared with CCL2. mRNA expression of CCL7 is constitutive in platelets and has been induced by various inflammatory cytokines in monocytes, fibroblasts, endothelial cells, airway smooth muscle cells and in cells of the monocytic lineage (14). In experiments by Minty *et al*. (30) LPS stimulation of blood mononuclear leukocytes was not a consistent inducer of CCL7 mRNA. In some cases, LPS actually decreased levels of this mRNA, while concomitantly increasing IL-6 and TNF mRNA levels. By contrast, Polentarutti *et al*. (31) showed that both LPS and TNF can induce CCL7 transcripts. No information is available in the literature on CCL7 in COPD. The more than 10-fold induction of CCL7 mRNA seen for COPD sputum macrophages in our study indicates that this chemokine deserves further study in inflammatory disease.

CCL13, also called MCP-4 (monocyte chemoattractant protein-4), is a CC chemokine with potent eosinophil chemoattractant activity. According to Stellato *et al*. (32), CCL13 mRNA is found in cells collected in bronchoalveolar lavage of asthmatic and nonasthmatic subjects and was expressed in several human bronchial epithelial cell lines after cytokine stimulation. The chemotactic activity on eosinophils suggests that the induction of this chemokine might go along with eosinophilia in sputum samples. In the COPD samples studied herein, analysis of cytospins did not re-

veal eosinophils higher than 0.5% (data not shown).

In addition to its action on eosinophils via the CCR3 eotaxin receptor, CCL13 can have much broader activity owing to its interaction with CCR1 and CCR2. Since these receptors are expressed by lymphocytes and monocytes, the strong upregulation of CCL13 mRNA in our study (more than 100-fold in COPD) suggest that CCL13 may have a role in controlling various types of leukocytes in the disease.

CCL22, initially called MDC (macrophage-derived chemokine), is a chemokine mainly produced by macrophages and dendritic cells (16). It is upregulated by IL-4 and downregulated by IFNγ and is involved in chemotaxis of T_H2-cells, features that suggest a role of CCL22 in allergy. In the original description of CCL22, chemotactic activity for dendritic cells, natural killer cells and for monocytes was demonstrated (33), indicating a broader activity of CCL22 beyond allergy.

We noted in the present study a more than 10-fold increased expression of CCL22 in sputum macrophages in smokers and in COPD. Consistent with our findings, an increase of CCL22 mRNA in tissue and of protein in lavage fluid was reported for COPD (17).

Given the high levels of chemokine transcripts in the sputum macrophages, the chemokine protein detected in the sputum supernatant likely is produced by these cells, although a contribution by neutrophils or bronchial epithelial cells cannot be excluded. When looking at the strong induction of chemokine transcripts, the induction of protein is lower (compare Figures 1 and 4). The level of induction of protein as reported herein is consistent with what has been reported in other studies by Traves *et al*. (2) and Rao *et al*. (29). This can be explained by the ready diffusion of the protein into the tissue and by its binding to cell surface receptors, such that part of the protein secreted by the cells evades detection in sputum samples. As discussed earlier, interference with the CLL2-CCR2 axis strongly decreases recruitment of monocytes to the lung (28). This suggests that CLL2 protein is crucial to inflammation even when the increases of protein, which can be detected in induced sputum, are moderate.

Table 3. Comparison of chemokine expression patterns.

		Expression of chemokine mRNA[a]			
	% Small sputum MΦ	CCL2	CCL7	CCL13	CCL22
COPD (n = 11)	85.9	++	++	++++	++
Smokers (n = 4)	59.8	+	+	++	++
LPS inhalation (n = 4)	63.6[b]	+	+	−−	NC[c]

[a]Chemokine transcript expression is relative to healthy controls.
[b]After LPS inhalation.
[c]NC = no change.

When looking at smokers, we see a pattern similar to COPD, but the increase in percentage of small sputum macrophages and the strength of induction of chemokine transcripts was less pronounced (see Figure 2). This similar pattern suggests that cigarette smoke inhalation does already induce inflammatory mechanisms in the airways that persist in COPD, even after cessation of smoking, as was the case for 10 of the 11 COPD patients analyzed herein. The data also confirm that smoking is a potent inducer of inflammation in the airways and they suggest that cigarette smoke is able to recruit a broad spectrum of leukocytes to the lung via induction of chemokines.

LPS inhalation by healthy volunteers has led to a clear increase of small sputum macrophages indicating that the induction of these cells may represent a nonspecific response in inflammation. In studies on LPS inhalation by Alexis *et al*. (34), no increase of the percentage of small macrophages was seen—probably because samples were taken early at 6 hours after LPS, compared with 24 hours as done in our studies.

While induction of small macrophages was a consistent finding in our study, the gene expression pattern with respect to chemokines was clearly distinct in that, in COPD and smokers, CCL13 transcripts were increased, while after LPS inhalation, it was decreased.

These data show that, based on the chemokine expression pattern, the LPS-induced small sputum macrophages are distinct from the COPD-associated small sputum macrophages. Notably, CCL13 expression is reduced after LPS challenge as compared with the more than 100-fold induction in COPD. This differential gene expression may be explained by the fact that LPS is inhaled only once, while smokers inhale the smoke of several cigarettes per day. It may also indicate that different types of monocytes are recruited in COPD and smokers as compared with a single LPS inhalation. In any event because of the decrease of CCL13, chemotaxis will be more restricted after LPS inhalation as compared with cigarette smoking or smoke-triggered COPD. The CCR1-4 targeting chemokines CCL 3, 4, 5, 8, 11, 14, 15, 17, 23, 24 and 26 were deselected from the set because there was no informative difference between COPD and controls. We cannot exclude that one or more of these chemokines may be induced selectively with LPS inhalation.

The expression pattern for the four chemokines in the three groups of probands is compiled in Table 3. It demonstrates that the overall pattern is similar in COPD and smokers. The lower level of expression in smokers goes along with a lower percentage of small macrophages. By contrast, the pattern is distinct after LPS inhalation with the notable difference for CCL13, which is strongly decreased.

Taken together, we demonstrate herein an increase in small sputum macrophages in COPD patients, in asymptomatic smokers and after LPS inhalation in healthy volunteers. In COPD and healthy smokers, the gene expression

pattern for chemokines, which target CCR 1–4, shows a selective induction of only 4 out of 15 chemokines tested, while after LPS inhalation only 3 chemokines are induced. The data demonstrate that sputum macrophages can show different patterns of gene expression dependent on the type of induction.

DISCLOSURES

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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