

Characterization of a population of small macrophages in induced sputum of patients with chronic obstructive pulmonary disease and healthy volunteers

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SUMMARY

The inflammatory process in chronic obstructive pulmonary disease (COPD) is active mainly in the airways, but little is known about the properties of the inflammatory cells in this compartment. We have studied leucocytes in induced sputum of COPD patients compared to controls in order to uncover what types of macrophages might be involved in the disease. Sputum induction was performed by inhalation of nebulized sodium chloride solution. Leucocytes were isolated and stained with specific monoclonal antibodies for analysis in flow cytometry. Flow cytometry analysis revealed that a major portion of CD14⁺ macrophages in COPD has lower forward scatter, i.e. they are small macrophages. While in control donors these small macrophages accounted for 6.9% of all macrophages, the percentage of these cells in COPD was 45.7%. CD14 and HLA-DR expression was high on these small sputum macrophages while the large sputum macrophages expressed only low levels of these surface molecules, both in control donors and COPD patients. Small sputum macrophages of both control donors and COPD patients showed higher levels of constitutive tumour necrosis factor (TNF) compared to the large macrophages. TNF was inducible by lipopolysaccharide (LPS) preferentially in the small sputum macrophages in the control donors but there was no further induction in COPD patients. These data show that the small sputum macrophages are a major macrophage population in COPD and that these cells exhibit features of highly active inflammatory cells and may therefore be instrumental in airway inflammation in COPD.

Keywords COPD induced sputum macrophages

INTRODUCTION

COPD is an inflammatory disease of the airways that is induced by aerosols from cigarette smoke [1] and also by aerosol generated in insufficiently ventilated homes in underdeveloped areas of the world [2]. This inflammatory process leads to airway constriction and instability of the airways such that they can collapse during expiration [3].

The disease is characterized as a chronic inflammation of the airways that continues even when the primary stimulus is removed, i.e. when the patient discontinues smoking. Of the people exposed only 15% develop chronic obstructive pulmonary disease (COPD) and the reasons for this may lie in polymorphisms of genes involved in inflammation [4–6]. The chronic inflammation goes with obstruction of the airways, either by swelling and

mucus production or by fibrotic changes of the airways that lead to instability of bronchi and bronchioli. As a result, forced expiration is severely decreased such that the fixed (i.e. resistant to sympathicomimetics) reduction of forced expiratory volume in 1 second (FEV₁) is a hallmark of the disease. Lung function is impaired further in many patients by the additional destruction of alveolar septae leading to emphysema [7].

In order to study the inflammatory response in COPD we have analysed induced sputum, which provides cells and mediators that are present in the large airways [8,9]. Several reports show an increase in the number of granulocytes in COPD [10–13], but macrophages are also considered important cells in COPD. These cells can be recovered from bronchoalveolar lavage and from induced sputum. Here, Alexis *et al.* have provided data to indicate that sputum and alveolar lavage are similar when looking at various cell surface markers [14]. In the present report we have analysed induced sputum in COPD patients compared to control donors and we have analysed the macrophages using monoclonal antibodies and flow cytometry. Using this approach we have discovered a strong increase in COPD of a population of small macrophages which appear to have a proinflammatory function based

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on their level of cell surface receptors and their capacity to produce the cytokine tumour necrosis factor (TNF). Therefore, these cells may be important for the pathophysiology of airway inflammation.

MATERIALS AND METHODS

Subjects

The study population consisted of 20 healthy control individuals, 17 COPD patients who were treated with both oral and inhaled glucocorticoids (COPD GC⁺), six COPD patients who were treated with inhaled glucocorticoids alone (COPD GCi) and five COPD patients with no glucocorticoids at all (COPD GC⁻). Table 1 shows biometric data of the four study groups. None of the COPD patients had a history of asthma or allergic rhinitis. All subjects with COPD had fixed airway obstruction which was defined as FEV₁ < 80% predicted, with no reversibility (improvement of < 15% of baseline) after inhalation of 200 µg salbutamol. All patients had normal serum levels of α₁-antitrypsin and of IgG, IgA, except for two patients who had IgG levels of around 5 g/l. In

addition we examined a small control group of three healthy asymptomatic smokers.

COPD patients were receiving regular therapy with inhaled β-adrenergics and all of them were receiving either parenteral or twice-daily oral doses of theophylline. Furthermore, the COPD GC⁺ group received daily oral glucocorticoids, some of them in addition by inhalation. Patients who received inhalative glucocorticoids (GC) had either Budesonid (range 600–1600 µg/day), Fluticasone (range 500–600 µg/day) or Beclomethasone (400 µg/day). All the COPD patients were recruited for production of induced sputum during a stable state of disease at least 10 days after treatment of exacerbation.

Healthy volunteer donors were recruited from hospital personnel. All individuals in the healthy control group were non-smokers and had no history of lung diseases. None of them had an airway infection in the 4-week period of sputum induction.

Written informed consent was obtained from each individual. The protocol was approved by the Ethics Committee of the Medical School of the Ludwig-Maximilians-University (Munich, Germany).

Table 1. Study population

Patient no.	Diagnosis	Sex	Age	FEV ₁ (%)	GC		Pack-years	Ex-S since years	% small MΦ of total MΦ
					oral mg/day	inhal			
1	COPD GC ⁺	m	77	49	10	+	n.s.		50.6
2	COPD GC ⁺	m	79	40	15	+	90	14	14.4
3	COPD GC ⁺	m	75	57	20	+	40	37	21.4
4	COPD GC ⁺	f	77	62	10	+	50	3	12
5	COPD GC ⁺	m	67	44	28	+	25	–	29.7
6	COPD GC ⁺	f	58	64	17	+	20	–	22.2
7	COPD GC ⁺	m	65	34	15	+	50	–	47.4
8	COPD GC ⁺	m	66	28	12.5	+	50	3	42.3
9	COPD GC ⁺	m	70	42	15	+	25	0.5	32
10	COPD GC ⁺	m	71	56	10	+	25	31	48
11	COPD GC ⁺	m	72	73	25	+	20	26	61.2
12	COPD GC ⁺	f	56	50	10	+	n.s.		28.1
13	COPD GC ⁺	f	57	62	10	+	125	–	52.7
14	COPD GC ⁺	m	77	60	12.5	+	45	2	88.5
15	COPD GC ⁺	m	68	36	15	–	110	–	34.2
16	COPD GC ⁺	f	52	53	12	+	50	–	83.4
17	COPD GC ⁺	m	58	12	10	+	70	0.1	84.6
		m12/f5	67.3 ± 8.6	48.3 ± 15.3					44.3 ± 24.0
18	COPD GCi	f	61	61	–	+	30	–	33.2
19	COPD GCi	m	76	77	–	+	60	30	44.7
20	COPD GCi	m	58	35	–	+	80	1	32
21	COPD GCi	m	72	68	–	+	70	0.5	6
22	COPD GCi	m	73	62	–	+	45	3	43.2
23	COPD GCi	m	67	26	–	+	80	3	60
		m5/f1	67.8 ± 7.1	54.8 ± 19.9					36.5 ± 18.0
24	COPD GC ⁻	m	58	76	–	–	11	–	64.9
25	COPD GC ⁻	m	58	41	–	–	40	–	41.3
26	COPD GC ⁻	m	63	54	–	–	25	–	54.9
27	COPD GC ⁻	m	74	45	–	–	100	–	86
28	COPD GC ⁻	f	74	72	–	–	40	11	100
		m4/f1	65.4 ± 8.1	57.6 ± 15.8					69.4 ± 23.6
Controls		m15/f5	37.8 ± 12.9						6.9 ± 6.7

Ex-S = ex smoker; n.s. = non-smoker.

Sputum induction and processing

Sputum induction. Sputum induction was performed as described previously [15,16]. In brief, the subjects were instructed to mouthwash with water. Sputum induction was performed by stepwise inhalations of 10 min each with increasing concentrations of sodium chloride (0.9%, 3%, 4% and 5% in healthy individuals and 0.9% and 3.0% in COPD patients) nebulized by an ultrasonic nebulizer (Multisonic LS 290, Schill, Probstzella, Germany). This device generates 3–4 µm aerosols at a maximum saline output of 0.75 ml/min. After mouthwashing and blowing the nose again, individuals were encouraged to cough deeply. Sputum was coughed into Petri dishes (d = 13.5 cm) and processed immediately. The total nebulization time in the COPD patient groups did not exceed 10 min, whereas it was up to 40 min in the healthy control donors, dependent on the time-point when sputum was produced.

Sputum processing. After careful isolation of sputum solid phase, as described in Spanevello *et al.* [17], total weight was determined and recorded. To homogenize the solid phase of the sputum samples by cleavage of disulphide bonds of mucin glycoproteins, two volume parts of sputolysin reagent (Calbiochem-Novabiochem, La Jolla, CA, USA) containing 6.5 mM dithiothreitol and 100 mM phosphate buffer (pH 7.0) were added. After vortexing briefly, the mixture was incubated at 37°C and vortexed every 10 min until the sputum was homogenized, in total no longer than 60 min. The sputum samples were diluted with 1 volume phosphate buffered saline (Gibco, Karlsruhe, Germany) and cells were then pelleted by centrifugation for 10 min at 400 g and 4°C. The resulting sputum cells of the different inhalation steps (0.9%, 3%, 4% and 5% saline) were pooled.

Analysis of sputum cells by microscopy

The resulting cell pellet (see above) was dissolved in lipopolysaccharide (LPS)-free RPMI-1640 cell culture medium modified with 1% L-glutamine, 2000 U/ml penicillin, 2 mg/ml streptomycin, 1% non-essential amino acids, 10% low LPS fetal calf serum (all purchased from GIBCO, Karlsruhe, Germany) and 1% of oxalacetate, pyruvate and insulin supplement (OPI) (Sigma, Taufkirchen, Germany). Total cell number was determined using a Neubauer haemocytometer (Table 2). For differential cell counts, which were performed by an observer blind to the clinical characteristics of the subjects, slides were prepared using cytopsins and stained with DiffQuick (Dade Behring, Marburg, Germany). At least 300 leucocytes per slide were analysed. Contamination of squamous cells was less than 10% in all samples.

Characterization of sputum cells by flow cytometry

Surface markers. Isolated and washed sputum cells were stained with specific monoclonal antibodies or the corresponding

isotype control from the same manufacturer. The following antibodies were used: CD14, RMO52-PC5 (isotype: mouse IgG2a-PC5); and HLA-DR, Immu357-PC5 (isotype: mouse IgG1-PC5; all Beckman Coulter, Krefeld, Germany). At least 5000 macrophages were analysed and fluorescence intensity was expressed as specific mean channels (delta MnIX). Previous studies have found no substantial change of either CD14 or HLA-DR after incubation of leucocytes with DTT [18]. In order to reduce problems with autofluorescence we employed phycoerythrin-cyan 5 antibody conjugates which emit at a wavelength of >600 nm (red).

Intracellular proteins. Intracellular staining for TNF was performed using the Cytotfix/Cytoperm kit (no. 2075KK, Pharmingen, Heidelberg, Germany) according to the manufacturer's instructions. In brief, up to 1×10^6 sputum cells in 1 ml culture medium + 10% fetal calf serum (FCS) or 600 µl of heparinized blood from the same donor as control were incubated in the presence of 10 µg Brefeldin A/ml (Sigma B-7651; Taufkirchen, Germany) and either left untreated or stimulated with 1 µg LPS/ml (from *Salmonella minnesota*; Sigma L-6261; Taufkirchen, Germany) for 4 h at 37°C. Erythrocytes in whole blood samples were then first lysed with CoulterQ-Prep reagents (Beckman Coulter, Krefeld, Germany) and both samples stained with CD14-PC5. Sputum cells and blood samples were then processed equally while using anti-TNF-phycoerythrin (PE) (Becton Dickinson, Heidelberg, Germany) for intracellular staining. As a control for the specificity of the anti-TNF antibody we used a 10-fold molar excess (0.8 µg) of human rTNF (kindly provided by Dr D. Männel, Regensburg, Germany). After staining with CD14-PC5 cells were washed twice in phosphate-buffered saline (PBS)/2% FCS and then incubated 20 min with Cytotfix/Cytoperm at 4°C for permeabilization. After two washing steps with PBS/2% FCS, cells were incubated with anti-TNF-PE or with anti-TNF-PE plus recombinant TNF (rTNF) as control together with Perm/Wash and PBS/2% FCS. After a final wash cells were analysed in an EPICS XL (Beckman Coulter, Krefeld, Germany) flow cytometer while gating on CD14 positive cells for small macrophages and on forward *versus* side scatter for large macrophages.

Statistics

Statistical analysis was performed using Student's *T*-test. We compared each of the COPD patients group with the control group and the three different COPD groups against each other. A value $P < 0.05$ was considered significant. In addition we performed an analysis of variance (ANOVA) test for the figures that compared more than two groups (Figs 1 and 2). Significance to the level $P < 0.05$ was expressed as 'yes' or 'no'.

Table 2. Cellular composition of sputum samples

	Control donors <i>n</i> = 20	COPD GC ⁻ <i>n</i> = 5	COPD GCi <i>n</i> = 6	COPD GC ⁺ <i>n</i> = 17
Total cell count (count $\times 10^6$)	0.8 $\pm$ 1.0	29.8 $\pm$ 59.4	3.9 $\pm$ 5.7	11.4 $\pm$ 16.8
Granulocytes (%)	35.8 $\pm$ 21.6	79.0 $\pm$ 15.4	79.3 $\pm$ 9.5	86.9 $\pm$ 4.9
Macrophages (%) (microscopy)	56.6 $\pm$ 21.9	20.3 $\pm$ 14.3	20.3 $\pm$ 9.6	11.3 $\pm$ 4.7
Macrophages (%) (FACS analysis)	52.8 $\pm$ 13.9	24.9 $\pm$ 14.0	20.6 $\pm$ 8.8	18.9 $\pm$ 10.4

Total cell count GC⁻ *versus* GC⁺ not significant.

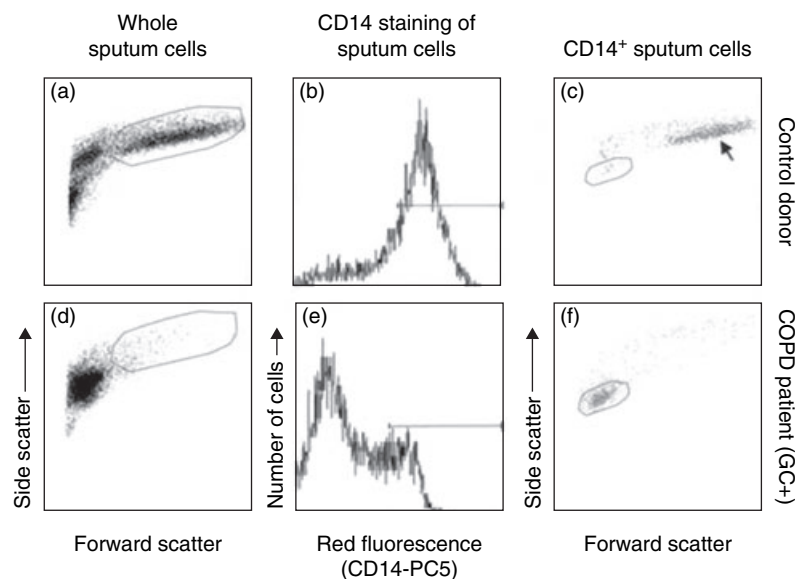


Fig. 1. Scatter analysis of induced sputum samples. Whole sputum samples of a control donor (a) and a COPD GC⁺ patient (d) in forward *versus* side scatter analysis. The cells within the large frame represent the large macrophages: 66% of total cells in the control donor and 1.2% for the COPD GC⁺ patient in the given example. (b,e) The fluorescence histogram for CD14-PC5 gated on all leucocytes. The horizontal bar marks the CD14 positive proportion of cells. Forward *versus* side scatter of these cells is shown in (c) and (f) for all CD14 positive cells. The arrow in (c) shows the scatter position of the large sputum macrophages. The small sputum macrophages are shown in the small frames in (c) (control donor, 4% of all cells, 6.7% of all macrophages) and (f) (COPD GC⁺, 8.3% of all cells, 29.7% of all macrophages).

RESULTS

Cellular composition of induced sputum samples by microscopy

We compared the cellular composition in induced sputum samples from 20 healthy volunteers with five COPD patients without steroid therapy (COPD GC⁻), six with inhalative glucocorticoids only (COPD GCi) and 17 COPD patients treated with both oral and inhalative glucocorticoids (COPD GC⁺). Table 2 shows the results from cell differentiation obtained from microscopic analysis of cytospin samples stained with DiffQuick dye. The total leucocyte counts obtained were 0.8×10^6 in controls, while in COPD numbers were increased to an average of 13.5×10^6 with a wide range in recovery. Lowest values were found in GCi patients (Table 2). The percentage of macrophages as assessed by light microscopy was $56.7 \pm 21.9\%$ in healthy volunteers compared to $14.9 \pm 9.0\%$ in COPD. The percentage of macrophages in COPD was decreased due to a relative increase of the granulocytes. However, in absolute numbers there was an average increase of macrophages by factor 2.5 when looking at all COPD patients studied. Among these the GCi patients showed macrophage numbers similar to controls (Table 2).

Characterization of sputum macrophages by flow cytometry

We next turned to flow cytometry, because in light microscopy it is often difficult to identify macrophages clearly. Initially we compared the differences in scatter profile of normal control sputum samples and COPD patients. As shown in Fig. 1, a major part of the cells in the control sample (Fig. 1a) represent large macrophages (marked with large frame), whereas almost no large macrophages can be detected in COPD patients (Fig. 1d, large frame). In order to focus on macrophages we then used staining with a PC5 conjugated mouse monoclonal antibody to CD14 (Fig. 1b,e). When we gated on the CD14 positive cells (marked with the bar in Fig. 1b and 1e) and analysed their light

scatter properties we detected the population of large sputum macrophages in control donors (Fig. 1c, arrow). By contrast, in COPD (Fig. 1f) a major population of CD14 positive macrophages show low forward–side scatter, indicating lower cell size. We therefore termed these cells ‘small sputum macrophages’. These small macrophages, as gated in Fig. 1f, account for 30% of all macrophages in this example. By comparison, in the control donor these macrophages, as gated in Fig. 1c, comprise 6.7% of all macrophages. The light scatter properties of the small sputum macrophages suggest that they are low in size. In order to confirm this we determined the size of macrophages in cytospin preparations by microscopy. The average size of small macrophages in control donors is 23.6 ± 4.4 ($n = 5$) and in COPD patients it is 10.1 ± 4.3 ($n = 5$). This confirms that the majority of macrophages in COPD sputum is smaller compared to the respective cells from healthy donors. In order to demonstrate that the small sputum macrophages are clearly distinct from granulocytes, we performed co-staining with CD14 and the granulocyte marker CD66b. As shown in Fig. 2, the CD14⁺ cells are clearly negative for CD66b. To further confirm that the CD14⁺ and HLA-DR⁺ cells are the same population, we demonstrated in a two-colour fluorescence analysis with CD14-PC5 and HLA-DR-FITC that the CD14⁺ small macrophages are also positive for HLA-DR (data not shown). This double-positive population is not contaminated with HLA-DR positive lymphocytes, because the whole sputum sample contains less than 0.005% CD3-positive cells.

The average percentage of small sputum macrophages among all macrophages based on scatter and CD14 expression is summarized in Fig. 3. For control individuals the small sputum macrophages account for $6.9 \pm 6.7\%$ of all macrophages, while the percentage of the cells in average of all COPD patients is $47.1 \pm 24.6\%$. For comparison, we examined three asymptomatic

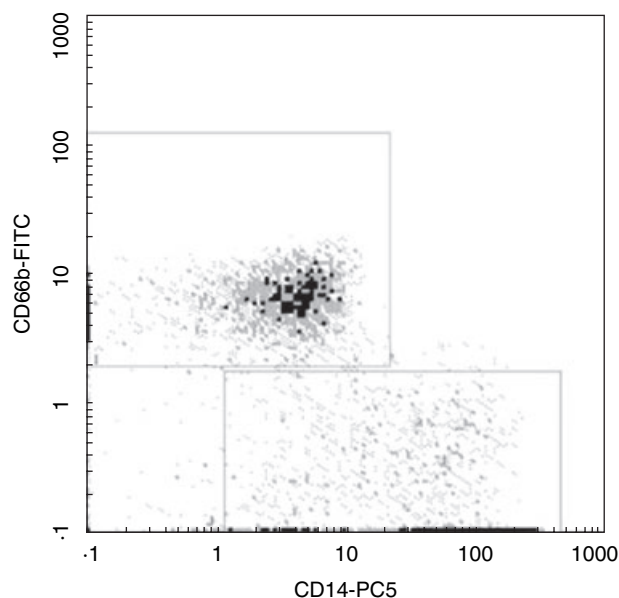


Fig. 2. Discrimination between granulocytes and macrophages. Whole sputum cells of a COPD patient were costained with CD14-PC5 and CD66b-FITC. The upper gate (a) shows the population of CD14-negative but CD66b-positive granulocytes whereas the lower gate (b) represents the CD14-positive, but CD66b-negative sputum macrophages (one representative out of five experiments).

smokers (average of 6 pack years) and also detected elevated levels of small macrophages with a percentage of $35 \pm 19\%$ among all macrophages, while the total leucocyte counts were equal as in the non-smoking controls ($0.47 \pm 0.3 \times 10^6$). In some patients (nos 14, 16, 17 and 28 in Table 1) the small macrophages accounted for more than 80% of all macrophages. GC⁻ patients had somewhat higher values compared to the GC-treated patients, but the differences were not significant (Fig. 3). On average the percentage of small sputum macrophages in all COPD patients was increased by a factor of 6.8 when compared to control donors. This demonstrates that the small sputum macrophages are a major macrophage population in COPD. Our cohort included out-patients in remission and these also showed high percentages of small sputum macrophages ($77.6 \pm 15.7\%$ of all macrophages; $n = 3$, patients 3, 22 and 27). We have also analysed a group of five healthy smokers. The mean percentage of small sputum macrophages of all macrophages accounted for $56.3 \pm 31.7\%$ in this group and was thus increased compared to the controls.

Having shown that there is an additional population of small macrophages we then determined the total number and percentage of all sputum macrophages by flow cytometry. These data show that in healthy control donors the percentage of macrophages accounted for $52.8 \pm 13.9\%$ of all sputum leucocytes compared to $20.4 \pm 10.6\%$ in all three COPD patient groups, which is comparable to the microscopy determination (Table 2).

CD14 and HLA-DR expression

Next we compared the level of expression of CD14, the LPS-receptor, and the expression of class II antigen HLA-DR on the two macrophage subsets by determining specific fluorescence intensity, i.e. by subtracting channels for isotype control from channels for CD14 and HLA-DR antibodies. Figure 4 reveals the CD14 expression on both macrophage subsets in a representative

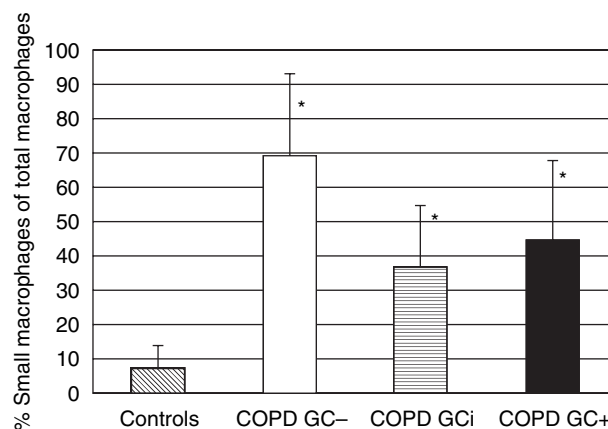


Fig. 3. Percentage of small sputum macrophages of total macrophages. Given is the average percentage of small sputum macrophages among the total number of macrophages: $6.9 \pm 6.7\%$ in control donors, $69.4 \pm 23.6\%$ in COPD GC⁻, $36.5 \pm 18.0\%$ in COPD GCⁱ and $44.2 \pm 23.9\%$ in COPD GC⁺ patients. $P < 0.05$ in controls compared to all COPD groups (asterisks). Using the ANOVA test with a P -level < 0.05 , all COPD groups are significantly different from controls; COPD GCⁱ and COPD GC⁺ are both different from COPD GC⁻, but no significant difference could be detected in the COPD GCⁱ from the COPD GC⁺ group.

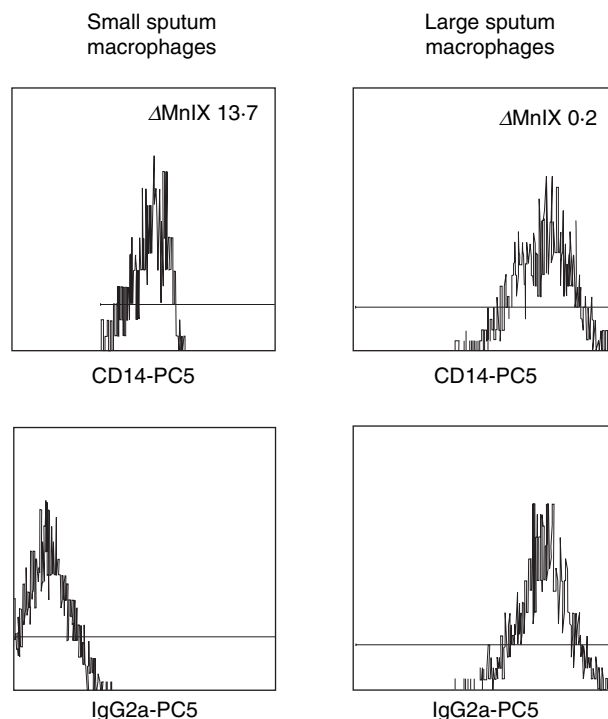


Fig. 4. CD14 expression on sputum macrophages. Whole sputum cells of a COPD GC⁺ patient were stained with either CD14-PC5 (upper panel) or the corresponding isotype control mouse IgG2a-PC5 (lower panel). Shown is the specific cell surface expression of CD14 in the small (left panel) and the large (right panel) sputum macrophages. The Δ MnIX value for the small sputum macrophages is 13.7, whereas it is 0.2 for the large sputum macrophages.

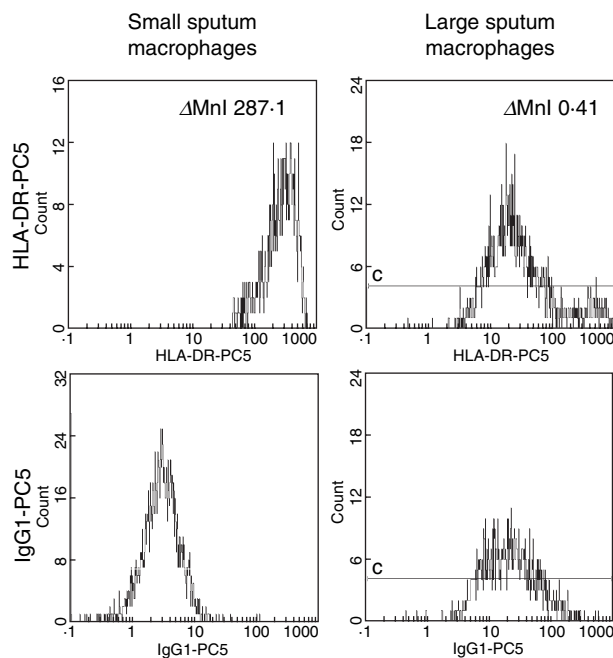


Fig. 5. HLA-DR expression on sputum macrophages. Whole sputum cells of a COPD GC⁺ patient were stained with either HLA-DR-PC5 (upper panel) or the corresponding isotype control mouse IgG1-PC5 (lower panel). Shown is the specific cell surface expression of HLA-DR in the small (left panel) and the large (right panel) sputum macrophages. The Δ MnIX value for the small sputum macrophages is 287.1, whereas it is 0.4 for the large sputum macrophages.

COPD GC⁺ patient. The upper panel shows the fluorescence intensity for CD14-PC5 on small (left histogram) and large (right histogram) sputum macrophages, whereas the fluorescence intensity of the specific isotype control is shown in the lower panel. In this example the resultant delta mean channel for CD14 is 13.7 for the small and 0.2 for the large macrophages, revealing a 68-fold stronger expression on the small macrophages. The expression of HLA-DR in Fig. 5 is generated from the same COPD GC⁺ patient as the data in Fig. 4. The fluorescence intensity calculated in delta mean channels for the small sputum macrophages (left panel) accounts for 287.1, whereas it is 0.41 for the large macrophages (right panel). Figure 6 summarizes the results of the four study groups. The expression for the large sputum macrophages was set to 1 for better comparison. Expression of CD14 (Fig. 6, upper panel) on small macrophages is significantly higher compared to the large macrophages, and this was found for controls (152-fold higher) and in all three COPD groups (in average 136-fold higher). Similar results were obtained for the HLA-DR antigen (Fig. 6, lower panel). It was 47-fold increased in healthy controls and 79-fold in COPD.

The increase in small sputum macrophages compared to the large macrophages in both CD14 and HLA-DR was statistically significant in all three groups. Antigen expression on the small macrophages in control and COPD groups were comparable (differences not significant). The data indicate that the small sputum macrophages may be functionally more competent cells.

Production of TNF

In order to examine the inflammatory potential of the small and large sputum macrophages we analysed TNF production after 4 h

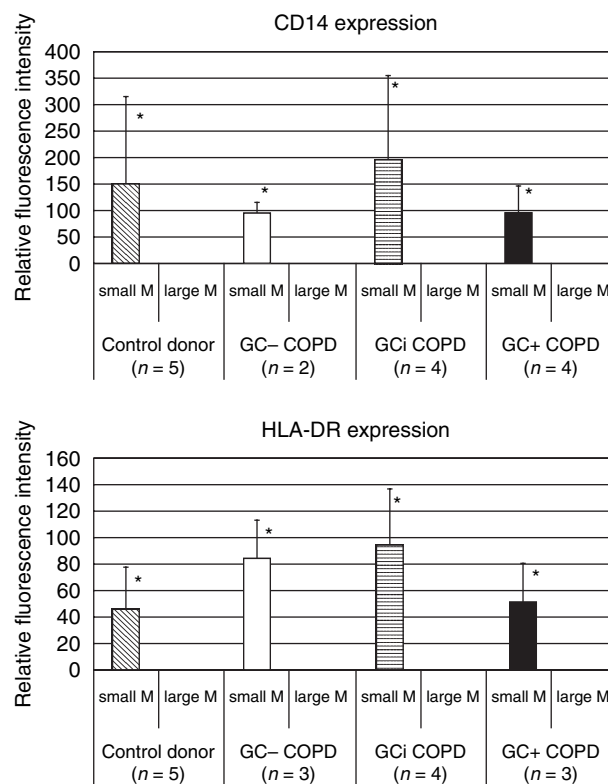


Fig. 6. CD14 and HLA-DR expression. Shown is the relative fluorescence intensity of the small sputum macrophages of the different study groups in relation to the large macrophages that was set at 1 for comparison. For CD14 (upper panel) the small macrophages in controls reveal a 152.1 ± 161.6 -fold stronger expression compared to large macrophages. The values were 95.2 ± 19.7 -fold higher in COPD GC⁻, 195.4 ± 154.6 -fold in COPD GCi and 96.4 ± 50.1 -fold higher in COPD GC⁺ patients. Results for HLA-DR expression (lower panel) on small macrophages: 47.2 ± 31.4 -fold stronger in controls, 85.5 ± 27.5 -fold in COPD GC⁻, 95.0 ± 42.5 in COPD GCi and 52.1 ± 29.6 in COPD GC⁺. $P < 0.05$ for small macrophages compared to large macrophages for CD14 and HLA-DR in all groups studied (asterisks). Using the ANOVA test with a P -level < 0.05 there are no significant differences neither between small macrophages of the control group and the three COPD groups nor between the three COPD groups, concerning CD14 and HLA-DR expression.

of culture without or with LPS. Figure 7 gives the data on intracellular TNF obtained in a healthy volunteer. The upper panel (Figs 7a,c,e,g) gives the fluorescence intensity for intracellular TNF, the lower panel (Figs 7b,d,f,h) shows the corresponding controls with addition of a 10-fold molar excess of rTNF. In the example given there is some constitutive TNF production in small sputum macrophages that were left untreated for 4 h (Fig. 7a,b, delta MnI (Δ MnI) 1.28), whereas these cells show a strong increase in LPS-induced TNF production after 4 h stimulation (Fig. 7e,f, Δ MnI 12.8). The large sputum macrophages exhibit almost no constitutive TNF production (Fig. 7c,d, Δ MnI 0.47) and they produce only small amounts of TNF after LPS stimulation (Fig. 7g,h, Δ MnI 2.17).

TNF data from control donors and COPD patients are summarized in Fig. 8. Panel (a) demonstrates constitutive TNF in small and large macrophages in control donors. LPS stimulation increased TNF by factor 6 in small macrophages but only by factor 2 in large macrophages. In COPD patients (panels b, c and d)

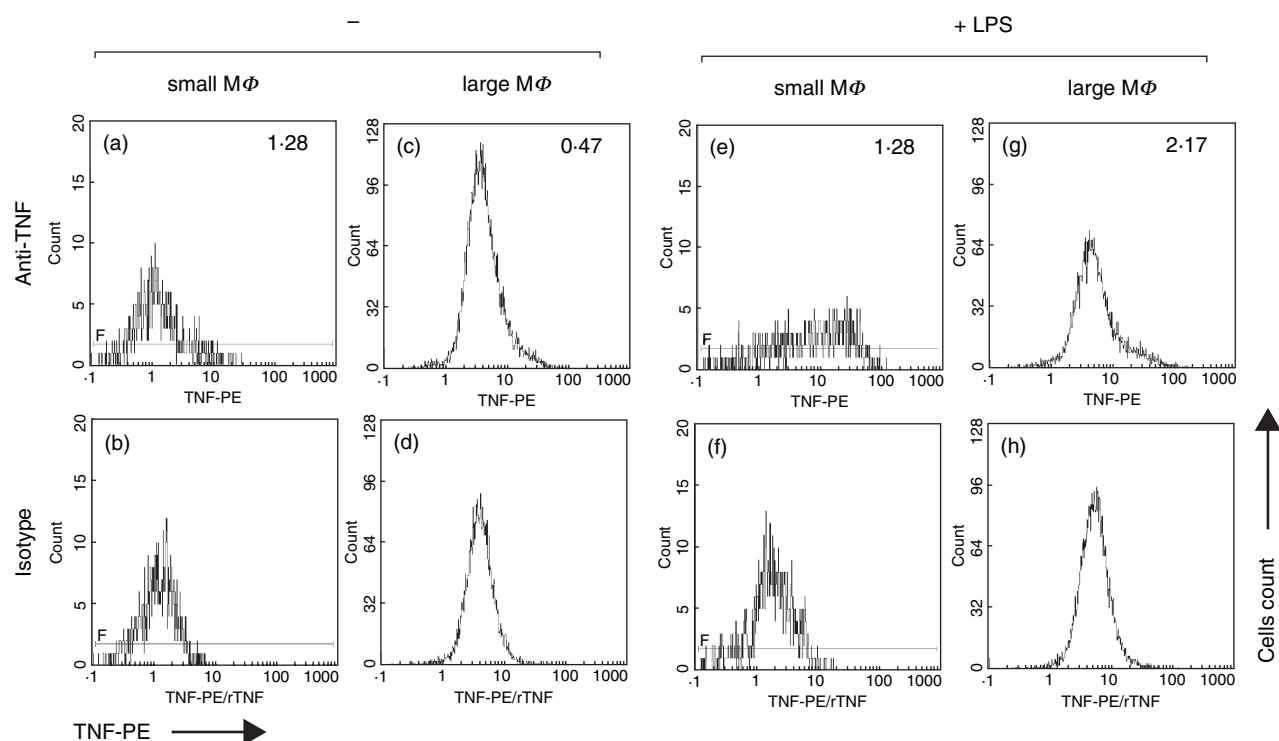


Fig. 7. TNF production in a control donor. Whole sputum cells were incubated for 4 h $\pm$ 1 μ g LPS/ml and counterstained with CD14-PC5. The upper panel (a, c, e, and g) shows the fluorescence intensity for TNF gated on small or large macrophages, the lower panel (b, d, f and h) the corresponding controls with 10-fold molar excess of rTNF.

the small macrophages produce constitutive TNF but there is no increase after LPS stimulation. The large macrophages of the COPD patients are incompetent for both constitutive and LPS-induced TNF production, except for the GC⁺ patients, where these cells show some activity (Fig. 8c). Taken together, these data show that the small sputum macrophages are functionally more competent and that in many COPD patients they are the exclusive TNF producers.

DISCUSSION

In the present study we describe a strong increase of a population of small macrophages in sputum of patients with COPD. Most of our COPD patients were admitted to the hospital for exacerbation and they were studied after the disease had been stabilized by therapy. The more advanced stage of disease in our cohort is reflected in the low mean of FEV₁ that was $51.4 \pm 16.2\%$ in average of all patients. The majority of patients was studied post-treatment of exacerbation shortly before discharge, but small sputum macrophages were also increased in out-patients with stable COPD. Nevertheless, exacerbation may contribute to enhance small sputum macrophages. The numbers and properties of small sputum macrophages still need to be studied in a larger group of patients with stable COPD. Longitudinal studies of COPD patients over the course of their disease may also help to define a possible influence of exacerbation on this parameter.

Consistent with earlier reports, we found in induced sputum that in apparently healthy control donors the majority of the cells were macrophages [19,20], and this was seen both by morphology and flow cytometry. Also, we can confirm that in COPD the

majority of cells are neutrophilic granulocytes as reported previously [10–13].

What was not noted previously is that in COPD there is a large proportion of small macrophages which in light scatter histograms overlap with the granulocytes (Fig. 1). These cells can be identified readily when monoclonal antibodies against antigens such as CD14 are used for identification of macrophages. Although overlapping in light scatter histograms with the granulocytes these cells were clearly of macrophage nature, based on the absence of CD66b (Fig. 2) and on the expression of CD14 and HLA-DR antigens. Also these CD14 positive cells were found to be positive for CD68 by intracellular immunofluorescence (data not shown) and they were negative for CD3. Hence, based on this pattern of markers (CD14⁺, CD68⁺, HLA-DR⁺, CD66b⁻, CD3⁻) these cells can be assigned to the monocyte/macrophage lineage.

We found small macrophages in sputum of healthy donors to account for 6–9% of all macrophages consistent with Alexis and Becker [14]. As shown herein these small sputum macrophages are dramatically expanded in COPD. One might speculate that the increase of small sputum macrophages could impact directly on airway obstruction. Statistical comparison of FEV₁ and percentage small sputum macrophages, however, showed no correlation ($P > 0.05$). We assume that these cells contribute to pathophysiology of the disease. Here, analysis of additional parameters of lung destruction is required. Preliminary data from asymptomatic smokers showed that the small macrophages are also elevated in the induced sputum samples, indicating that these cells may be involved in inflammatory processes caused by cigarette smoke.

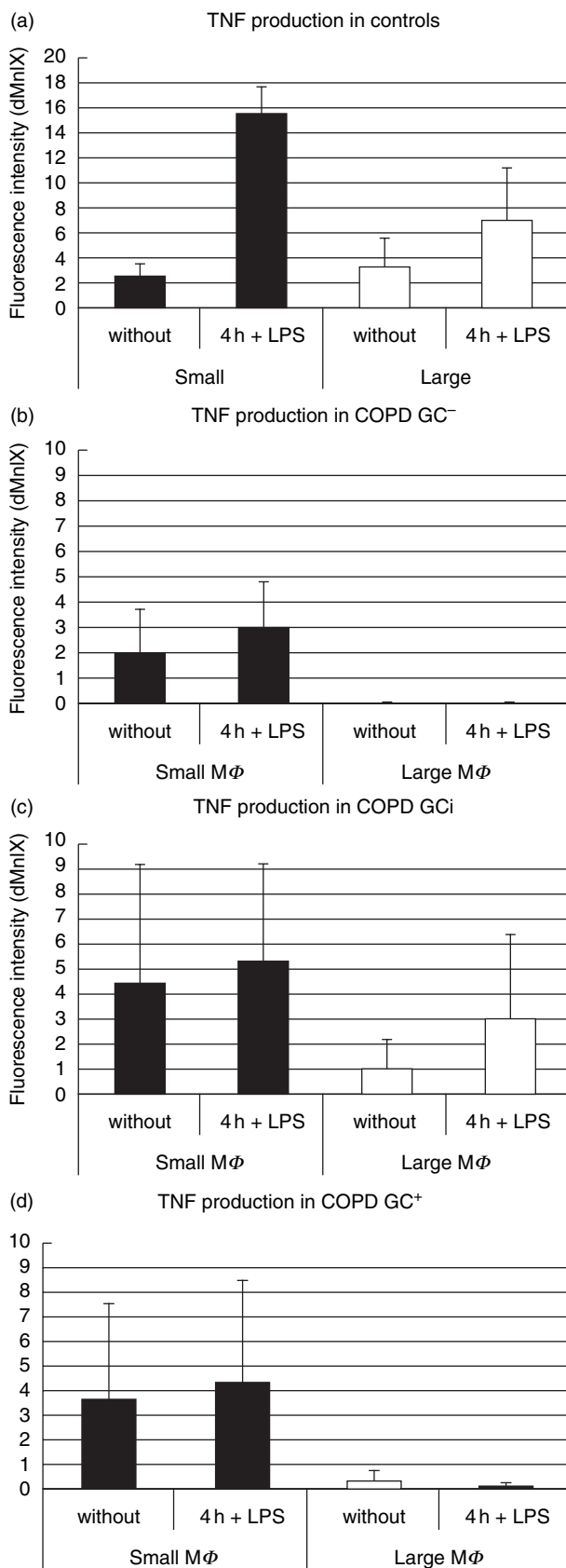


Fig. 8. TNF production of the study groups in comparison. Results are shown in Δ MnI for the two macrophage populations ($P < 0.05$ for small macrophages compared to large macrophages).

Because COPD patients in our cohort have an average age of 67.1 ± 7.9 years, it is difficult to generate a sputum control donor group of a similar age. One might argue that the higher average value of small sputum macrophages in COPD is an effect of age and not of disease. In our control group the four donors with an age of >50 years had small macrophages at $6.9\% \pm 3.4\%$, indicating that age as such does not lead to an increase of small sputum macrophages.

In both patients and controls we found that the large macrophages exhibit only minimal expression of HLA-DR and CD14, while the small macrophages showed very pronounced levels of these cell surface molecules (Figs 4, 5, 6). This suggests that the small macrophages may be the immunologically active cells and therefore they may be important for airway inflammation.

When looking at TNF production we noted a constitutive expression in both types of macrophages in the control donors and LPS stimulation led to a fivefold increase in the small macrophages. In COPD patients (with exception of the GCi patients) constitutive TNF was found only in the small but not in the large macrophages and there was no apparent increase by additional stimulation. The high amounts of TNF in unstimulated macrophages of the COPD patients may be due to an *in vivo* activation of the cells as part of the chronic inflammatory process. The lack of increase of TNF production after *in-vitro* stimulation of COPD sputum macrophages may be due to this preceding *in-vivo* stimulation that leads to tolerance. This tolerance has been observed in patients with various infectious diseases [21–24]. While the constitutive TNF produced by small macrophages is at a similar level in patients and controls the number of small sputum macrophages in COPD can, however, be increased dramatically, i.e. 10-fold for COPD GC⁻, fivefold for COPD GCi and about sixfold for COPD GC⁺. The total TNF produced by these cells in COPD is therefore substantially higher and this may drive inflammation in the airways.

The majority of patients in our cohort received glucocorticoid therapy for acute exacerbation. Earlier studies [25,26] indicated that inhaled GC had no effect on numbers of granulocytes and macrophages in patients with inflammatory airway disease. Based on the design of our study it is difficult to draw any conclusions about GC effects, because we have studied patients only at one point in time. Patients treated with inhalative GC had lower total leucocyte counts in sputum and the percentage of small sputum macrophages was also lower, suggesting a suppressive effect of the steroid treatment. Also, patients with oral GC therapy (COPD GC⁺) had lower total leucocyte counts compared to untreated patients, while the percentage of granulocytes was increased. Finally, HLA-DR expression on small macrophages was somewhat lower in the GC⁺ patients compared to GC⁻ and GCi. While this is consistent with reports that show lower DR expression after GC therapy in other types of patients [27], the number of patients in our study is low and differences are not significant.

With regard to TNF production, large macrophages showed some TNF production in GCi-treated patients while untreated and GC⁺ COPD patients showed no or only minimal TNF expression. We do not think that this indicates a GC-induced rebound of TNF production [28], but assume rather that these patients had high production of TNF to begin with and that this was not sufficiently suppressed by the inhalative GC therapy. In order to resolve the issue of GC effects on macrophages in induced sputum of COPD patients sequential measurements before and after GC therapy are required.

In our studies we have focused on the major proinflammatory cytokine TNF. Future studies will need to analyse other pro- and anti-inflammatory cytokines, chemokines, matrix metalloproteinases and reactive oxygen production in order to understand better the role of these cells in airway inflammation. The question is how these small macrophages get into the large airways. Given that the bronchial wall is heavily inflamed in the disease [29], we assume that these cells emigrate from the local tissue to the mucosal surface. Small macrophages have also been identified in bronchoalveolar lavage [30]. Rousseau *et al.* noted in a study on acute respiratory distress syndrome (ARDS) that the alveolar cells in these patients had a phenotype similar to blood monocytes [31]. Based on studies in the mouse, the same group demonstrated that these cells are newly emigrated blood monocytes [32]. Along these lines we suggest that the small sputum macrophages are newly emigrated cells coming from peripheral blood. In the mouse blockade of monocyte immigration also blocked influx of neutrophils [33], indicating that the newly recruited monocytes are central to the inflammatory process. Also, studies in the rat have demonstrated that depletion of monocytes but not of neutrophils prevent smoke-induced destruction of the lung [34]. While such studies, which selectively deplete monocytes/macrophages, have not been conducted in humans, we assume that these cells are also central players in patients with inflammatory lung disease.

Taken together, we have shown herein that a population of small macrophages with high CD14 and DR expression and with constitutive TNF production is increased dramatically in patients with COPD.

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