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Differences in local versus systemic TNFα production in COPD: inhibitory effect of hyaluronan on LPS induced blood cell TNFα release

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ABSTRACT

Background: COPD is characterized by both airway inflammation and systemic alterations. To elucidate the relationship between local and systemic inflammation, TNFα production by sputum cells and blood cells of COPD patients and controls was compared. Moreover, the effect of the extracellular matrix compound hyaluronan (HA) on TNFα release was studied.
Methods: Four study groups were included: 10 steroid-free COPD patients, 8 steroid-treated patients, 10 healthy smokers, and 11 healthy non-smokers. Sputum cells and blood were incubated for 24 hours with or without LPS, in absence or presence of HA (122 kDa or HMW fragment). TNFα was measured by ELISA.
Results: Sputum cells produced spontaneously high levels of TNFα, but were unresponsive to LPS. Sputum cells from COPD patients (both steroid-free and steroid-treated) produced significantly less TNFα than cells from healthy non-smoking subjects (p=0.017 and p=0.001 respectively). In contrast, blood cells produced TNFα only in response to LPS. No differences in TNFα production by blood cells of either patient group versus the control groups were observed. In addition, HA (both fragments tested) partially blocked LPS (1 ng/ml) induced TNFα release by blood cells from all study groups, whereas TNFα production by sputum cells was not influenced by HA.
Conclusion: These data indicate a difference in local versus systemic TNFα production. Sputum cells of COPD patients produced less TNFα as compared to controls which could contribute to impaired local defence. An inhibitory effect of the ECM compound HA on blood cell TNFα release was observed, which was comparable for patients and controls.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a complex heterogeneous respiratory disease, characterized by the progressive development of airflow limitation that is largely irreversible. Airway inflammation is a key feature of COPD and is reasoned to play a pathogenic role [1]. Influx of neutrophils and macrophages in airway wall was related to airway obstruction in patients with severe COPD [2]. Moreover, CD8+ lymphocytes infiltration was seen throughout the whole lung which was inversely related to forced expiratory volume in one second (FEV_1) [3, 4]. Progression of disease was also associated with presence of PMN, macrophages, CD4 cells and lymphocyte subtypes in the small airways [5]. These inflammatory cells may be an important source of inflammatory mediators and proteases, which can enhance inflammatory processes in COPD. In induced sputum of COPD patients elevated levels of the chemokine interleukin (IL)-8, the pro-inflammatory cytokine tumor necrosis factor (TNF)α and its soluble receptors (sTNF-R) were observed [6, 7]. Moreover, also in the circulation enhanced levels of inflammatory markers such as TNFα and the acute phase protein CRP, have been reported [7, 8], indicative of a systemic inflammatory reaction. A clear association between circulating inflammatory mediators and other systemic effects of COPD, such as tissue wasting and cardiovascular morbidity, has been demonstrated (reviewed in [9]). So far, very little is known about the origin of the systemic inflammation in COPD. In a previous study we demonstrated that levels of sputum and circulating inflammatory markers were not correlated, suggesting that the systemic inflammation in COPD is not due to an overflow of inflammatory mediators from the pulmonary compartment [7]. In order to further elucidate the relationship between local and systemic inflammation, the production of TNFα by sputum cells and blood cells of COPD patients (either steroid-free or steroid-treated) versus smoking and non-smoking control subjects was analysed.

Chronic pulmonary inflammation and lung injury are associated with damage, repair and remodelling of the extracellular matrix (ECM). Recently, we have demonstrated enhanced levels of the ECM compound hyaluronan (HA) in sputum supernatant of COPD patients [10]. Moreover, in circulation of inflammatory diseases such as rheumatoid arthritis elevated HA levels were reported [11]. HA is known to have pro-inflammatory potential when present in small fragments, and to be anti-inflammatory in high molecular size [12]. Therefore, we also analysed the effect of HA on sputum and blood cell TNFα production and compared responsiveness of COPD versus control subjects to this ECM compound.

METHODS

Study groups

COPD patients

Eighteen patients with moderate to severe COPD whose characteristics are given in table 1, were recruited from the outpatient clinic and rehabilitation centre CHU-Sart-Tilman. COPD was diagnosed according to he GOLD criteria i.e. a postbronchodilation (400 µg inhaled sabutamol) FEV1/FVC ratio < 70%. All the COPD subjects were life long heavy smokers (more than 15 pack years). They were all using long acting bronchodilator including tiotropium, formoterol or salmeterol. Eight out of the 18 patients were receiving inhaled corticoids (1000 µg fluticasone or 800 µg budesonide/day). The latter group was indicated as steroid-treated patients, whereas the other patients were referred to as steroid-free COPD patients.

Control subjects

Twenty one control subjects were recruited through advertisement from the hospital staff of CHU Sart Tilman. Care was taken to recruit patients who matched the COPD with respect to the age and sexe. Ten of our control subjects were current smokers with an average 23 pack years (table 1).The protocol was approved by the local ethical committee and volunteers gave their signed informed consent.

Sputum induction and processing

After pre-medication of the subjects with a 400-µg inhaled salbutamol, sputum was induced by inhalation of an hypertonic saline (NaCl 4.5%) when FEV_1 was > 65% predicted and by isotonic saline (NaCl 0.9%) when FEV_1 was < 65% predicted. In order to improve the bronchoprotection additional amounts of salbutamol were delivered during the saline nebulization itself as previously described [13]. Saline aerosols were delivered by an ultrasonic nebulizer (Ultra-Neb 2000, De Vilbiss, Somerset, USA) with an output set at 1.5 ml/minute. Each subject inhaled the aerosol for three consecutive periods of 5 minutes for a total time of 15 minutes. For safety reasons, the FEV_1 was monitored every 5 minutes and the induction stopped when FEV_1 fell by more than 20% from baseline.

The whole sputum was collected in a 50 ml polypropylene tube, weighed, and diluted 1:4 with phosphate buffered saline without Ca^{2+} and Mg^{2+} (DPBS) vortexed 30 seconds and centrifuged at 800 *g* for 10 minutes at 4°C. Supernatant and cell pellet diluted in 20 ml of DPBS were filtered separately on double thickness gauze. Supernatant was stored at –80°C until analyses for TNFα and IL-8. Cells were washed and suspended in 1 ml DPBS, for total cell counts using a manual haemocytometer. The differential cell count was performed on cytospins stained with Diff-Quick by counting 500 cells under a light microscope.

Sputum cell and whole blood cell stimulation

Sputum cells were obtained as reported above. In order to perform blood cell stimulation heparinized whole blood was collected and diluted 1:20. Both sputum cells ($4*10^5$ non-squamous cells/ml) and whole blood were dissolved in RPMI-1640, supplemented with penicillin and streptomycin and 5% of heat inactivated foetal calf serum (FCS) and incubated for 24 hours at 37 ˚C in plates coated with anti-TNFα antibody as described below. Stimulation was performed in the absence or presence of lipopolysaccharide (LPS E.coli, Sigma, St Louis, MO) and in the absence or presence of HA 122 kDa (Polytech, Trieste, Italy) or HA $>10^6$ Da (Ostenil; Chemedica AG, München, Germany) the latter being indicated as high molecular weight (HMW) HA. Both HA compounds were checked for endotoxin content using LAL assay (detection level of 1 pg/ml), which was not found.

TNFα assay

TNFα produced by sputum cells and blood cells was measured by a modified two-step sandwich type immuno assay (immunotrapping technique). The antibodies and standards were purchased from Biosource (Fleurus, Belgium). Cells were stimulated in apyrogen microwells (nunc maxisorp, VWR, Belgium), which have been coated with anti-TNFα antibodies (Ms X Hu TNFα Clone 68B 6A3, Ms IgG1 and 68B 2B3,Ms IgG2) and in parallel recombinant TNFα standards were added to wells. After 24 hours of incubation the wells were washed six times and incubated during 2 hours with a biotinylated anti-TNFα Ab (Ms X Hu TNFα biotin Clone 68B 3C5, Ms IgG1), followed by streptavidin-horseradish peroxidase. The substrate chromogen solution TMB was used and absorbance was measured at 450 nm. Sensitivity was around 10-15 pg/ml for the immunotrapping technique, applied to cell culture. The Biosource assay has been validated for conditioned medium but not for serum. This observation together with an improved sensitivity led us to use the Quantikine high sensitivity R&D TNFα immunoassay (detection level of 0.5 pg/ml) to measure TNFα in the serum samples. Moreover, the Pelikine compact human TNFα assay (Sanquin, Amsterdam, the Netherlands) was used to detect TNFα in sputum supernatant. Detection limit of the latter assay was 1 pg/ml.

IL-8 assay.

IL-8 levels were determined using specific sandwich ELISA as described by Bouma and colleagues [14]. Detection limit of the assay was 80 pg/ml.

Statistical analysis

Results are presented as median (range) in tables. Inter-group comparisons were performed using Kruskal-Wallis H test; in case that differences were present ($p<0.05$) further analysis of subsets of groups were performed using the Mann-Whitney U test or the Fishers exact test (to compare categorical variables). The intra-group comparisons, in order to test the effect of LPS on TNFα production, and to analyse the effect of HA on spontaneous and LPS induced TNFα release, were performed by Friedman test and if appropriate (at $p<0.05$) followed by a paired Wilcoxon-rank sum test. Correlations between parameters were evaluated using Spearman's rank correlation analysis. Statistical Package for the Social Sciences, version 12.0 for Windows (SPSS Inc., Chicago, IL) was used. A p value of less than 0.05 denotes the presence of a significant statistical difference.

RESULTS

Patient characteristics

The characteristics of the patients with COPD and the healthy control subjects are summarized in table 1. All four study groups consisted mostly out of men, and all COPD patients were current or ex-smokers. In both control groups of non-smokers and healthy smokers, all pulmonary function parameters were in the normal range, which were significantly reduced in the COPD study groups. The FVC was rather low in the COPD patients but this was explained by the importance of air trapping as reflected by high values of residual volume (RV), and total lung capacity (TLC). No differences in lung function between steroid-treated and steroid-free patients were found. Furthermore, all four study groups had comparable body mass index (BMI).

Table 1 Demographic and Functional Characteristic of Study Groups

	Control subjects		COPD patients	
	Non smoking (n=11)	Smoking (n=10)	Steroid-free (n=10)	Steroid-treated (n=8)
Gender (male/female)	6/5	7/3	8/2	6/2
Age	53 (42-69)	51 (41-63)	67 (38-74)* #	58 (45-78)
Smoking behaviour				
-non/ex/current	11/0/0	0/4/6*	0/2/8*	0/6/2*
-pack-years	0	23 (13-44)*	45 (24-56)* #	47 (15-60)* #
FEV_1 (ml)	3760 (2130-5670)	3465 (2480-4840)	1215 (890-3010) * #	1155 (600-1410)* #
FEV_1 (% predicted)	109 (94-133)	99 (87-121)	41 (29-75)* #	35 (23-62)* #
FVC (ml)	4690 (2620-6550)	4260 (2940-7030)	2525 (1530-5350) * #	1675 (1040-3090)* #
FVC (% predicted)	120 (91-127)	100 (89-143)	59 (44-107)* #	48 (29-72)* #
FEV_1/FVC (%)	80 (71-90)	79 (71-90)	53 (43-62)* #	60 (42-68)* #
RV (% predicted)	ND	ND	215 (141-242)	227 (189-242)
TCL (% predicted)	ND	ND	109 (104-146)	120 (100-131)
BMI	23.4 (21.2-31.6)	24.3 (21.2-31.6)	25.6 (21.0-29.4)	25.9 (16.5-25.9)

Values are expressed as median (range) or as absolute numbers.

FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; BMI, body mass index; ND, not determined.

Kruskal-WallisH was used to test for differences between groups, and if appropriate ($p<0.05$) subsequent analysis of subgroups was performed by Mann-Whitney U test or the Fishers exact test.

* $p<0.05$ as compared to non-smoking controls

# $p<0.05$ as compared to smoking controls

TNFα production from sputum cells

Sputum induction was performed successfully in the four different study groups. Although COPD patients tended to have higher median levels of total cell count and neutrophil number together with reduced macrophage amounts, differences between study groups were not significant (table 2). The chemokine IL-8 was significantly enhanced in sputum of COPD patients (table 2), and correlated significantly with neutrophil count, as analysed for whole group (n=36, r=0.399, p=0.021; data not shown). TNFα was detectable only in part of the sputum samples, and levels of TNFα were not different between study groups (table 2).

In order to analyse local TNFα production, sputum cells were cultured for 24 hours in absence and presence of LPS, a bacterial cell wall constituent, known to have strong inflammatory capacity.

Sputum cells spontaneously produced considerable amount of TNFα (fig 1). TNFα production by cells from COPD patients (both study groups) was significantly lower as compared to non-smoking control subjects, and tended to be different as compared to smoking controls (steroid-free COPD versus smoking controls p=0.08; steroid-treated COPD versus smoking controls p=0.1). No relationship between viability of sputum cells and spontaneous TNFα production was seen (n=36, r=0.124, p=0.47). The presence of LPS (either 1 ng/ml or 10 ng/ml) did not affect the spontaneous TNFα production, for all groups, with the exception of LPS 1 ng/ml in the non-smoking control subjects (p=0.022, see fig 1).

Table 2 Sputum Characteristics

	Control subjects		COPD patients	
	Non smoking (n=9)	Smoking (n=10)	Steroid-free (n=9)	Steroid-treated (n=8)
Weight sputum (g)	4.3 (2.5-7.5)	4.4 (1.0-7.4)	3.1 (1.7-9.1)	2.5 (1.4-4.9)
Squamous cells (%)	18 (6-30)	12.5 (3-54)	19 (9-47)	13 (0-45)
Viability (%)	59 (27-79)	61 (39-77)	75 (25-87)	74 (37-90)
10^6 cells/g [a]	0.5 (0.3-1.8)	0.6 (0.1-2.4)	0.9 (0.3-3.2)	1.2 (0.5-11.7)
Neutrophils				
-%	52.0 (16.0-77.6)	38.3 (4.6-87.0)	57.6 (12.0-91.4)	87.3 (5.2-95.0)
-10^6/g	0.29 (0.08-1.41)	0.19 (0.02-0.81)	0.47 (0.11-2.74)	1.03 (0.02-10.67)
Macrophages				
-%	35.8 (14.0-58.4)	35.4 (8.0-74.8)	33.8 (6.6-77.0)	8.3 (3.6-62.4)
-10^6/g	0.29 (0.07-.65)	0.35 (0.04-1.8)	0.13 (0.06-0.87)	0.23 (0.04-0.56)
Lymphocytes				
-%	2.8 (0.2-4.8)	1.9 (02-10.0)	1.4 (0.0-5.0)	1.2 (0.0-4.6)
-10^6/g	0.01 (0.00-0.04)	0.01 (0.00-0.24)	0.01 (0.00-0.05)	0.015 (0.00-0.12)
Eosinophils				
-%	0.0 (0.0-3.6)	0.1 (0.0-6.2)	0.0 (0.0-2.0)	0.3 (0.0-49.6)
-10^6/g	0.0 (0.0-0.02)	0.0 (0.0-0.09)	0.0 (0.0-0.02)	0.005 (0.0-0.35)
Epithelial cells				
-%	9.8 (0.8-28.2)	10.2 (1.4-66.4)	5.8 (1.0-39.4)	1.1 (0.0-31.4)
-10^6/g	0.06 (0.0-0.17)	0.11 (0.01-0.35)	0.06 (0.02-0.83)	0.035 (0.0-0.19)
IL-8 positive samples	5/9	8/10	9/9	8/8
IL-8 (ng/ml)[a]	0.11 (0.08-2.81)	0.42 (0.08-4.59)	1.17 (0.28-25.0) *	5.75 (0.91-14.40) *#
TNFα positive samples	2/9	4/10	4/10	5/8
TNFα (pg/ml)[a]	1.0 (1.0-3.3)	1.0 (1.0-8.9)	1.0 (1.0-25.8)	4.5 (1.0-307.8)

Values are expressed as median (range).

Kruskal-WallisH was used to test for differences between groups, and if appropriate ($p<0.05$) subsequent analysis of subgroups was performed by Mann-Whitney U test

* $p<0.05$ as compared to non-smoking controls

# $p<0.05$ as compared to smoking controls

[a] Statistical differences between study groups were calculated based on all samples. In case of non detectable samples, lower detection level of assay was used which was 80 pg/ml in case of IL-8 and 1 pg/ml for TNFα..

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TNFα production from blood cells

In blood significant enhanced leucocytes number were present in both COPD subgroups as compared to controls, which was mainly due to increased amounts of neutrophils (table 3). A negative correlation between blood neutrophil number and FEV_1 % predicted was found for whole study group (n=39, r= -0.656, p=0.000) which was still valid after analysis of subgroups of patients and controls (fig 2). In serum of all subjects low levels of TNFα could be detected, however, no discrimination between study groups could be made based on concentration of this cytokine (table 3).

In contrast to sputum, culture of blood cells did not result into spontaneous production of TNFα, whereas release of this cytokine was concentration dependently induced by LPS (fig 3). No differences were seen in LPS (1 ng and 10 ng/ml) induced TNF release between study groups. Since TNFα release was measured in 1:20 diluted whole blood, and leukocytes counts were different between groups, TNFα release per 20.000 leucocytes was calculated, which did not result into differences between study subjects (data not shown).

Table 3 Blood Differential Cell Count and TNFα level

	Control subjects		COPD patients	
	Non smoking (n=11)	Smoking (n=10)	Steroid-free (n=10)	Steroid-treated (n=8)
Leucos x 10^6/ml	5.7 (4.4-8.7)	7.2 (4.6-10.4)	9.1 (7.1-13.9) * #	8.7 (6.1-16.8) *#
Neutrophils				
-%	49.6 (44.4-73.0)	57.1 (45.4-70.8)	61.8 (49.9-68.4) *	69.9 (54.3-85.5) *#
-10^6/ml	3.3 (2.13-5.5)	4.1 (2.1-6.4)	5.9 (3.6-8.7) *	6.7 (3.7-13.6) * #
Lymphocytes				
-%	36.5 (17.8-42.7)	32.6 (18.8-39.9)	27.3 (19.8-39.9) *	20.4 (9.1-33.7) *#
-10^6/ml	2.1 (1.3-3.5)	2.0 (1.3-3.3)	2.6 (1.5-3.7)	1.8 (0.8-2.7)
Monocytes				
-%	5.7 (4.1-9.9)	6.3 (3.5-8.7)	6.4 (4.3-8.1)	5.6 (3.3-8.7)
-10^6/ml	0.4 (0.3-0.7)	0.4 (0.3-0.6)	0.6 (0.3-1.1) * #	0.5 (0.3-1.0)
Eosinophils				
-%	2.4 (0.9-4.2)	1.7 (0.6-4.1)	2.5 (0.8-7.6)	1.7 (0.0-7.8)
-10^6/ml	0.1 (0.1-0.3)	0.1 (0.1-0.3)	0.2 (0.1-0.5)	0.2 (0.0-0.9)
Basophils				
-%	0.7 (0.4-1.0)	0.7 (0.4-1.3)	0.6 (0.3-0.9)	0.4 (0.0-1.2)
-10^6/ml	0.04 (0.02-0.06)	0.05 (0.02-0.0)7	0.05 (0.03-0.1)	0.03 (0.0-0.14)
TNFα (pg/ml)	3.5 (1.7-9.6)	3.4 (2.6-3.7)	3.8 (2.5-6.1)	3.6 (2.5-4.9)

Values are expressed as median (range).

Kruskal-WallisH was used to test for differences between groups, and if appropriate ($p<0.05$) subsequent analysis of subgroups was performed by Mann-Whitney U test.

* $p<0.05$ as compared to non-smoking controls

# $p<0.05$ as compared to smoking controls

Effect of HA on TNFα production by sputum and blood cells.

Since the ECM compound HA was reported to modulate inflammatory reactions, next the effect of HA on TNFα production by blood and sputum cells, of patients and control subjects, was analysed. With respect to blood, no inducing effect of either LMW HA (122 kDa) or HMW HA (>10^3 kDa) on spontaneous TNFα production was observed (table 4). Interestingly, both fragments partially blocked LPS 1 ng/ml induced TNFα release by blood cells for all study groups, as shown in table

4. This inhibition was comparable for all three concentrations of HA tested namely, 1 μg/ml, 10 μg/ml and 100 μg/ml (data not shown). The reduction of TNFα release by HA was not longer present when blood cells were stimulated with LPS 10 ng/ml, with the exception of the HMW HA fragments lowering the TNFα release in the subgroup of steroid-free patients (table 4). No constitutive effect of both HA compounds on spontaneous or LPS induced TNFα release by sputum cells was observed (data not shown).

Table 4 Effect of HA on spontaneous and LPS stimulated blood cell TNFα release

		Control subjects		COPD patients	
		Non smoking (n=11)	Smoking (n=10)	Steroid-free (n=9)	Steroid-treated (n=8)
LPS 0	none	<10 (<10-21)	<10 (<10-22)	<10 (<10-57)	<10 (<10-47)
	HA 122 kDa	<10 (<10-19)	<10 (<10-14)	<10 (<10-27)	<10 (<10-25)
	HA >10^3 kDa	<10 (<10-15)	<10 (<10-29)	<10 (<10-37)	<10 (<10-105)
LPS 1 ng/ml	none	738 (125-3172)	533 (98-1607)	1117 (96-3057)	829 (86-2468)
	HA 122 kDa	496 (16-1916)*	189 (24-1547)*	859 (74-2684)*	404 (10-1433)*
	HA >10^3 kDa	466 (10-2305)*	122 (37-1784)*	928 (117-2840)*	558 (41-1783)*
LPS 10 ng/ml	none	1677 (625-3681)	1902 (1110-2654)	2619 (1694-3386)	2405 (794-3543)
	HA 122 kDa	1501 (520-3513)	1654 (540-2696)	2534 (1151-3760)	2296 (559-3080)
	HA >10^3 kDa	1386 (421-3214)	1708 (908-2606)	2545 (1428-3225)¶	2477 (828-3264)

Values are expressed as median (range). TNFα was measured by ELISA and expressed in pg/ml. Detection level of assay was 10 pg/ml, therefore samples that were not measurable are indicated as <10 pg/ml. The effect of 10 μg/ml HA 122 kDa and 10 μg/ml HA >10^3 kDa was indicated in the table. Intra-group comparisons were performed by Friedman test and if appropriate (at $p<0.05$) followed by a paired Wilcoxon-rank sum test.

* $p<0.05$ as compared to TNFα production in response to LPS 1 ng/ml without HA added.

¶ $p<0.05$ as compared to TNFα production in response to LPS 10 ng/ml without HA added.

DISCUSSION

This study shows a clear difference in sputum cell versus blood cell TNFα production in COPD patients and control subjects. Sputum cells produced constitutively high levels of TNFα, which was significantly lower in COPD patients as compared to controls. No effect of LPS on sputum cell TNFα production was seen. In contrast, blood cells produced no TNFα spontaneously, and mounted a significant TNFα production in response to LPS, which was not different between study groups. Further indications for discrepancies between local versus systemic inflammation were obtained by the observation that the ECM compound HA partially blocked LPS induced TNFα release by blood cells, but did not affect TNFα production by sputum cells. No indication for altered responsiveness of cells of COPD patients towards HA was obtained.

Steroids are known to have anti-inflammatory effects ex vivo, however, the effect of these drugs on COPD inflammation are not so clear. Inhaled steroid have been shown to reduce neutrophil cell count in sputum [15] but other studies could not confirm this effect on airway inflammation [16]. In the present study, no differences in lung function and in local and systemic TNFα release between subgroups of patients treated with inhaled steroids versus steroid-free patients were found, indicating that steroids may not have a dramatic effect on TNFα production ex vivo. Nevertheless, since the performed study is cross-sectional, intervention studies are required to strengthen this conclusion.

Sputum neutrophilia is generally accepted as a characteristic feature in COPD [6, 17]. Although in this study a tendency towards enhanced neutrophil numbers and reduced macrophage count in patients was seen, no significant differences in differential cell counts between COPD versus control subjects were found. The absence of significance could be due to the relative small size of the study groups analysed. In addition, care has been taken to match control subjects with respect to age, since Thomas et al. demonstrated that the induced sputum neutrophil count increased significantly with age [18]. Therefore, the relatively high neutrophil number observed in controls in this study, as compared to other studies [6, 17], may be due to the older age of the controls, and could be the reason for the absence of significantly enhanced neutrophilia in COPD sputum. Nonetheless, raised sputum IL-8 levels, another characteristic of COPD sputum [6, 7] was found in our patient group, and correlated with neutrophil counts. Sputum TNFα was detectable only in part of the samples and did not discriminate study groups, similarly as published previously [7, 19] although some authors also found raised TNFα levels in COPD [6]. This discrepancy could be due to differences in severity of COPD patients studied or to technical aspects such as the way of sputum processing or the TNFα assay used.

In order to study local TNF production we used here ex vivo sputum cell culture, which previously has been demonstrated by us to be a suitable model to look at cytokine airway production [20]. A high constitutive TNFα production by sputum cells was demonstrated, which was strongly reduced in COPD patients as compared to control subjects. The contrast between high TNFα production ex vivo versus low TNFα sputum levels observed in this study, suggest either that the in vivo produced TNFα is immediately captured or consumed or alternatively that in vivo factors are present that modulate local TNFα production which are absent in the in vitro cell culture conditions. Moreover, since this study was performed on a total cell population, further studies have now to be performed to elucidate which sputum cells are the main TNFα producers during culture ex vivo and to get more insight into underlying mechanisms causing the observed differences between COPD versus control subjects.

Opposed to our data, Profita et al, showed enhanced TNFα production by sputum cells of COPD patients [21]. A difference in macrophage and neutrophil count between patient and controls was present in the latter study unlike our study, which could contribute to the discrepancy in results.

Instead, our data suggesting suppression of inflammatory potential in COPD, are supported by the observation of a reduced cytokine production by primary bronchial epithelial cells of COPD patients [22]. The inflammatory response is critical to control the growth of pathogenic micro-organisms, a process in which TNFα can play a central role by its direct activating effects on macrophages and neutrophils. Reduced production of TNFα by sputum cells could therefore contribute to impaired local defence and thus to enhanced bacterial colonisation, as seen in COPD [23]. However, attention also has to be paid to the potential detrimental role of TNFα in COPD by its contribution to the destruction of lung parenchyma [24]. Therefore, further studies are required to determine the implication of the reduced TNFα production by sputum cells ex vivo in COPD patients.

Increasing doses of LPS did not affect sputum cell TNFα release, suggesting that these cells are unresponsive to LPS, in contrast to blood cells. Reactivity of sputum cells to PHA and FMLP has been reported [25, 26], indicating that this unresponsiveness is specific for LPS. It is well known that macrophages exposed to suboptimal doses of LPS are rendered tolerant to subsequent exposure to LPS [27]. Therefore we speculate that continuous exposure to bacteria known to colonize airways has made cells tolerant for LPS. Moreover, the discrepancy in viability of sputum cells, which was approximately 60-70%, versus blood cells previously demonstrated to be above 95% (data not shown) could contribute to some of the observed differences and will be subject of further study.

Significantly enhanced circulating leukocyte numbers were present in COPD patients, as reported previously [28], indicative for a systemic inflammatory response. Moreover a correlation between neutrophil count and FEV_1 was demonstrated. In this study no enhancement in circulating TNFα levels was detected in the COPD patients, who all had normal BMI. These data are therefore in line with other studies showing unaltered TNFα in serum of weight stable patients, whereas enhanced TNFα levels and TNFα production has been demonstrated in weight losing patients [29, 30]. No difference in spontaneous and LPS induced TNFα release by whole blood was observed between the study groups. Similarly, Aldonyte et al, showed no difference in basal and LPS stimulated TNFα release by monocytes of COPD versus controls. However, in the latter study different production of IL-8 and MMP-9 was demonstrated [31], indicating alterations in production of a specific subset of mediators.

Until now, the origin of the systemic inflammation, which is considered to have a potential pathogenic role in other systemic effects of COPD such as nutritional abnormalities and weight loss or skeletal muscle dysfunction [9] remains poorly understood. This study shows that TNFα production by sputum cells and by blood cells are regulated differently, suggesting that the inflammatory processes in the airways and the circulation are independent processes. This hypothesis is confirmed by studies showing no correlation between levels of inflammatory mediators in sputum and plasma [7, 32]. In addition, a recent short-term study on the effect of the anti-TNFα drug infliximab administered by infusion did not reveal beneficial effects on local inflammatory indices [33], further indicating that the local and systemic compartment have to be considered two separate entities.

Studies on alveolar septal wall remodelling in mild to moderate emphysema show a loss of total tissue, interstitial thickening and increased number of interstitial fibroblasts and macrophages [34]. One interesting compound of ECM is HA a pleiotropic glycosaminoglycan, composed of repeating disaccharide units of N-acetyl-D-glucosamine-ß (1→4)-D-glucuronic acid-ß (1→3). Accumulating evidence suggests that HA contributes to both homeostasis and disease [35]. Enhanced circulating levels of HA were seen in inflammatory disorders [11]. Involvement in pathogenesis of COPD has been suggested by the observation of enhanced levels of HA in BALF and sputum of these patients [10, 36]. Evidence indicates that biological effects of HA are dependent upon its molecular weight,

as LMW HA was shown to be pro-inflammatory and HMW HA to have anti-inflammatory capacity [12]. Opposed to our expectations, the 122 kDa HA fragment used in this study did not induce TNFα release by blood or sputum cells. Stimulating effects of HA with comparable size has been reported for macrophages [12], eosinophils [37] and renal epithelial cells [38]. Possibly the difference in cell types analysed or the presence of endogenous proteins in the diluted whole blood such as enzymes degrading HA or proteins contaminants such as lysozyme able to bind HA, could account for this contradiction. Moreover, both the HMW HA and the 122 kDa HA fragment reduced the LPS induced TNFα release by blood cells. Since HA is reported to bind to the LPS receptor TLR4 on dendritic cells [39], we speculate that interaction of HA with TLR4 on blood cells did not lead to cellular activation, but hindered the LPS induced cellular activation. HA had no effect on sputum cell TNFα production. This lack of HA influence could not be due to action of endogenous proteins which were washed away during isolation of cells. Our results did not provide an indication that cells derived from COPD patients show altered sensitivity towards HA as compared to control subjects.

Overall, this study suggests an independent regulation of local versus systemic inflammation in COPD. TNFα production by sputum cells seems to be impaired, which could result into defective local defense. Further studies have to be performed to further elucidate these processes. Moreover, we showed an inhibitory effect of the extracellular matrix compound HA on LPS induced blood cell TNFα release, which was not different between patients and controls.

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COMPETING INTEREST STATEMENT
E.F.M. Wouters serves as a consultant to GlaxoSmithKline (GSK) and is a member of scientific advisory boards for GSK, Boehringer iIngelheim, Astra Zeneca, Centocor and Numico and received lecture fees from GSK, Astra Zeneca, Boehringer Ingelheim, Pfizer and Numico. He received research grants between 2001 and 2004 from GSK, Astra Zeneca, Boehringer Ingelheim, Centocor and Numico.

LEGENDS

Figure 1 TNFα production by sputum cells of COPD patients and control subjects.
TNFα production by sputum cells (20.000 cells /well) was measured by a dynamic immunoassay during a 24-h culture period. Cells were incubated in RPMI 5% FCS, without LPS (blanco) or with LPS 1 ng/ml or LPS 10 ng/ml. Data are presented as median with interquartile range (box) and range (whiskers) of n=10 non-smoking controls, n=10 smoking controls, n=9 steroid-free COPD patients, and n=8 steroid treated COPD patients. P-value indicates a difference as compared to spontaneous TNFα production by sputum cells from non-smoking controls

Figure 2 Relationship between number of neutrophils in blood and FEV_1 % predicted.
A significant negative relationship was seen between absolute amount of neutrophils in peripheral blood and severity of airway obstruction as assessed by FEV_1 % predicted both for A. COPD patients n=18, r= -0.489, p=0.039 and B. control subjects n=21, r= -0.438, p=0.047.

Figure 3 TNFα production by blood cells of COPD patients and control subjects.
Heparinized whole blood was 1:20 diluted in RPMI 5% FCS, and TNFα production was measured by a dynamic immunoassay during a 24-h culture period. Cells were incubated without LPS (blanco) or with LPS 1 ng/ml or LPS 10 ng/ml. Data are presented as median with interquartile range (box) and range (whiskers) of n=11 non-smoking controls, n=10 smoking controls, n=9 steroid-free COPD patients, and n=8 steroid-treated COPD patients.

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