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Local and systemic cellular inflammation and cytokine release in chronic obstructive pulmonary disease

C. Moermans [a], V. Heinen [b], M. Nguyen [b], M. Henket [b], J. Sele [b], M. Manise [a], J.L. Corhay [b], R. Louis [b]

[a] *Université de Liège, GIGA I³ Research Croup, Department of Pneumology, Dr. R. Louis Tour GIGA-CHU Liège (B34, +2), Avenue de l'hopital, 13 4000 Liège, Sart Tilman, Belgium*

[b] *Department of Pneumology, CHU Liège, Bâtiment Central, +3, Local 154, Avenue de l'hopital, 13 4000 Liège, Sart Tilman, Belgium*

ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is a chronic airway inflammatory disease caused by repeated exposure to noxious gases or particles. It is now recognized that the disease also features systemic inflammation. The purpose of our study was to compare airway and systemic inflammation in COPD to that seen in healthy subjects and to relate the inflammation with the disease severity.
Methods: Ninety-five COPD patients, encompassing the whole severity spectrum of the disease, were recruited from our outpatient clinic and rehabilitation center and compared to 33 healthy subjects. Induced sputum and blood samples were obtained for measurement of inflammatory cell count. Interleukin (IL)-4, IL-6, IL-10, TNF-α and IFN-γ produced by 24 h sputum and blood cell cultures were measured.
Results: Compared to healthy subjects, COPD exhibited a prominent airway neutrophilic inflammation associated with a marked IL-10, IL-6 and TNF-α release deficiency that contrasted with a raised IFN-γ production. Neutrophilic inflammation was also prominent at blood level together with raised production of IFN-γ, IL-10 and TNF-α. Furthermore, sputum neutrophilia correlated with disease severity assessed by GOLD stages. Likewise the extent of TNF-α release from blood cells also positively correlated with the disease severity but negatively with that of sputum cell culture. Blood release of TNF-α and IL-6 negatively correlated with body mass index. Altogether, our results showed a significant relationship between cellular marker in blood and sputum but poor relationship between local and systemic release of cytokines.
Conclusions: COPD is characterized by prominent neutrophilic inflammation and raised IFN-γ production at both bronchial and systemic level. Overproduction of TNF-α at systemic level correlates with disease severity and inversely with body mass index.

Keywords : COPD ; Sputum ; Blood ; Cell count ; Cytokine release

1. Introduction

Chronic obstructive pulmonary disease (COPD) causes a significant health burden worldwide, in terms of morbidity and mortality. COPD is characterized by progressive and not fully reversible airflow limitation. It is a chronic airway inflammatory disease with a systemic component related to repeated inhalation of noxious gas and particles, in particular tobacco smoke [28].

The principal abnormalities in airways are the presence of a persistent inflammatory response as well as a structural remodelling that thickens the airway wall. A destruction of alveoli is also present and leads to the occurrence of emphysema [7]. The epithelial cells are damaged by the inhalation of noxious particles and there is an activation of innate (mainly neutrophils and macrophages) and adaptative immune cells (mainly CD8 cells). These cells are responsible for the release of proteases, cytokines/chemokines and mediators, which lead to inflammation and remodelling. COPD has been seen as a Th1 disease but some data suggest that Th2 cytokine may also play a role [1,2]. On the other hand some data have suggested that COPD may be favoured by a lack of anti-inflammatory cytokine such as IL-10 [38].

COPD is not only associated with an abnormal inflammatory response in the lung but also with systemic inflammation, including systemic oxidative stress, activation of circulating inflammatory cells and increased levels of circulating inflammatory cytokines [16].

Induced sputum is a recognized non-invasive technique to assess the cellular composition in chronic airway disease including COPD [29]. In addition, induced sputum cell culture has been shown to be a valid model to investigate cytokine production from airway cells in asthma [3,22,27,32].

The purpose of our study was twofold. First, to compare airway and systemic inflammation between COPD and healthy subject and secondly, to assess how this inflammation relates to disease severity in COPD. Here, we evaluated the sputum and blood cell composition as well as the sputum and blood cell cytokine production in 95 COPD and 33 healthy subjects. As for cytokine, we decided to analyse the level of interleukin-4 (IL-4) and interferon-γ (IFN-γ) as markers of the Th2/Th1 balance, TNF-α and IL-10 as pro- and anti-inflammatory cytokines, respectively, and IL-6 as a cytokine playing a role in the transition from innate toward adaptive immunity [18].

2. Materials and methods

2.1. Study design and subject characteristics

The demographic and functional characteristics of patients are given in Table 1. COPD patients (n = 95) were recruited from our outpatient clinic and rehabilitation centre CHU-Sart Tilman. Diagnosis of COPD was made according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (post-bronchilator-400 µg salbutamol-ratio FEV1/FVC < 70%). We divided COPD patients in three groups according to GOLD classification of severity (GOLD II: FEV1 < 80% and ≥ 50%; GOLD III: FEV1 < 50% and ≥ 30% and GOLD IV: FEV1 < 30%. All COPD patients were in stable condition at the time of blood and sputum collection and no patients were studied within 8 weeks of having an upper tract infection or any exacerbations requiring change in maintenance treatment or oral steroid and antibiotic prescription. Healthy subjects (n = 33) were recruited by advertisement among the hospital. None of them exhibited respiratory symptoms and all had normal spirometric results and airways responsiveness (provocative concentration of metacholine causing a fall in FEV1 of 20% > 16 mg/ml). Atopy was not formally studied but no healthy subject was reporting overt allergy symptoms by the time of sampling. According to the medical file, the large majority of COPD patients (87 out of 95) had not reported clinical history of allergy in the past nor were they taking regular anti-allergic drugs such as HI - antagonists. All participants gave informed consent and the study was approved by the local Ethic's Committee.

Table 1 *Demographic and functional characteristics.*

	Healthy N = 33	COPD N = 95	p-value
Age (years)	40 ± 12	62 ± 12	<0.0001
Sex (m/f)	24/10	73/21	0.55
Tobacco status (ns/es/cs)	22/4/8	0/64/30	<0.0001
BMI (kg/m^2)	24.1 ± 3.3	26.0 ± 5.59	0.07
Inhaled CS	0	64	
Oral CS	0	9	
LABA	0	59	
SABA	0	52	
LAMA	0	30	
SAA	0	39	
FEV1 (L)	3.93 ± 1.23	1.22 ± 0.44	<0.0001
FEV1 (% predicted)	110 ± 16	43 ± 15	<0.0001
FVC (L)	4.69 ± 1.50	2.47 ± 1.86	<0.0001
FVC (% predicted)	110± 18	61 ± 17	<0.0001
FEV1/FVC (%)	87 ± 13	55 ± 17	<0.0001
GOLD II	0	24	
GOLD III	0	51	
GOLD IV	0	19	

Results are expressed as mean ± SD. FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; BMI, body mass index; CS, corticosteroids; LABA, long acting beta 2 agonist; SABA short acting beta 2 agonist, LAA, long acting muscarinic antagonist; SAA, short acting anticholinergic.

2.2. Peripheral blood sampling and cell count

Peripheral blood samples were collected in apyrogenic, heparinised tubes (Venosafe; TERUMO®, Leuven, Belgium). The total and differential blood cell counts were obtained with an Advia 120 automatic counter (Siemens, Erlangen, Germany). Counting and cell typing were based on flow cytometry with bidimensional volume distribution, peroxydase concentration, and lobularity of leucocytes as parameters.

2.3. Sputum induction and processing

After premedication with 400 µg inhaled salbutamol, sputum was induced by inhalation of hypertonic (NaCl 5%) or isotonic (NaCl 0.9%) saline according to the FEV1 value (> or < than 65% predicted). Saline was combined with additional salbutamol delivered by an ultrasonic nebulizer (Ultra-Neb 2000; Devilbiss, Somerset, PA, USA) with an output set at 0.9 ml/min as previously described [9]. Each subject inhaled the aerosol for three consecutive periods of 5 min for a total of 15 min. For safety reasons, FEV1 was monitored throughout the induction and this one was stopped if FEV1 fell by more than 20% from baseline.

The whole sputum was collected in a plastic container, weighed, and homogenized by adding three volumes of phosphate-buffered saline (PBS), vortexed for 30 s, and centrifuged at 800g for 10 min at 4 °C. Supernatant was separated from cell pellet, which was washed twice in Roswell park memorial institute medium (RPMI) 1640 supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml at 400g for 10 min at 4 °C. Squamous cells, total cell counts and cell viability checked by trypan blue exclusion were performed with a manual hemocytometer. The differential leukocyte count was performed on cytospins stained with May-Grunwald-Giemsa on 500 cells. A determined volume of RPMI+ antibiotics was then added to the cell suspension to obtain a concentration of 2×10^6 nonsquamous cell/ml.

2.4. Blood and sputum cell culture and cytokine assay

Cytokines (IL-4, IL-6, IL-10, TNF-α and IFN-γ) were measured by immunotrapping assay which is based on early capturing of cytokines secreted during cell culture. The antibodies and standards were purchased from Biosource (Cytosets; Biosource, Invitrogen, Merelbeke, Belgium). Fifty microliters from standards or whole blood (diluted twice) or sputum cell suspension (2×10^6 cells/ ml) was incubated at 37 °C with 200 µl RPMI 1640 supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml (Cambrex, Verviers, Belgium), and 2% of inactivated foetal calf serum (Cambrex) in apyrogen microwells, which were coated previously with specific antibodies directed toward the chosen cytokines. After 24 h, the wells were washed, and 150 µl of a solution containing biotinylated detection antibodies specific to the cytokines was added for 2 h at room temperature. The wells were washed again and filled with a solution containing streptavidin horse-radish peroxidise for 45 min at room temperature. Then, 100 µl tetramethylbenzidine chromogen solution was added for 10-20 min in the dark. The reaction was stopped by adding 50 µl H2S04 1 M. The amount of substrate converted to products was thereafter detected as optical densities at 450 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Multiscan Ascent; Thermo Labsystems, Helsinki, Finland). The sensitivity of the ELISAs was determined by running a set of ten blanks and was calculated as the mean response plus 2 standard deviations. The sensitivities of our assays were 6 pg/ml for IL-4, 6 pg/ml for IL-6, 4 pg/ml for IL-10, 6 pg/ml for TNF-α, and 7 pg/ml for IFN-γ.

2.5. Statistical analysis

The demographic and functional characteristics were expressed as mean ± SD and comparisons between groups were performed by unpaired student "t" test for continuous variables and Chi-square test for categorical analyses.

Blood and sputum cell counts as well as cytokine levels were expressed as median (IQR).

Comparisons between groups were performed by a Mann-Whitney test and correlations were analysed with a Spearman rank test. Regarding production according to the age, differences between tertiles were analysed by a Kruskal-Wallis test. A p-value < 0.05 was considered as statistically significant.

3. Theory

COPD prevalence and morbidity continue to increase throughout the world and it represents a significant burden on the healthcare system. It is the fourth leading cause of death in most industrialized countries and it is projected to be the third worldwide by 2020.

Therefore, the identification of biomarkers involved in the immunopathogenesis of COPD is crucial and may provide myriad opportunities for possible intervention. Indeed, such biomarkers may be useful for monitoring disease progression and evaluating the effects of therapeutics or providing targets for therapies.

In addition, the lack in the understanding of the relationships between plasma and airway secretions in COPD highlights the importance of studying immune cells and mediators regulation at local and systemic level.

4. Results

4.1. Patient characteristics

Patients in both groups consisted mostly of men and COPD patients were all current or ex-smokers. Other demographic and treatment characteristics are given in Table 1. The pulmonary function parameters of COPD patients were significantly reduced compared to the healthy volunteers.

4.2. Sputum and blood cell counts

Detailed cell counts are given in Table 2. As for sputum COPD were characterized by a raised total sputum cell number ($p < 0.01$). The proportion of neutrophils ($p < 0.0001$) and, to a lesser extent, that of eosinophils ($p < 0.01$) were increased compared to healthy subjects while the proportions of macrophages, lymphocytes and epithelial cells were decreased in COPD. When expressed as absolute cell counts per gram of sputum, both neutrophils and eosinophils were clearly raised in COPD but no difference was detected with respect to other cell types.

At blood level, COPD displayed raised total leucocyte counts ($p < 0.0001$) as well as a raised proportion of neutrophils ($p < 0.01$) whereas we observed a lower proportion of lymphocytes ($p < 0.01$) and monocytes ($p < 0.05$) as compared to healthy subjects. Absolute neutrophil number per blood volume was twice as great in COPD as in healthy subjects ($p < 0.0001$). Likewise, absolute lymphocyte and monocyte numbers were slightly greater in COPD ($p < 0.05$ and $p < 0.01$ respectively).

Table 2 *Sputum and blood cell counts.*

	Healthy $N = 33$	COPD $N = 95$	p-value
Sputum			
Squamous (%)	18 (12-33)	22 (8-36)	0.99
Total non squamous (10^6/g)	0.53 (0.33-1.33)	1.23 (0.49-5.22)	0.001
Viability (%)	61 (54-74)	62 (50-75)	0.99
Macrophages (%)	42.8 (29.8-61.3)	16 (6.7-34.6)	<0.0001
Macrophages (10^3/g)	245 (100-600)	220 (100-520)	0.83
Neutrophils (%)	32.6 (14.6-53.9)	72.6 (39.2-83.3)	<0.0001
Neutrophils (10^3/g)	195 (35-485)	615 (245-4035)	<0.0001
Lymphocytes (%)	2 (0.9-3.7)	1.2 (0.5-2.2)	<0.05
Lymphocytes (10^3/g)	10 (0-30)	30 (10-310)	0.74
Eosinophils (%)	0 (0-0.5)	0.6 (0-2.5)	<0.01
Eosinophils (10^3/g)	0 (0-5)	35 (10-1030)	<0.001
Epithelial cells (%)	9.6 (2.7-19.8)	3.6 (0.8-13)	<0.05
Epithelial cells (10^3/g)	45 (15-125)	40 (15-95)	0.80
Blood			
Leucocytes (1/µl)	6700 (5405-7650)	9150 (7420-11,080)	<0.0001
Neutrophils (%)	54.5 (49-61.6)	62.9 (56.1-69.5)	<0.01
Neutrophils (1/µl)	3665 (2695-4535)	5910 (4405-6865)	<0.0001
Lymphocytes (%)	33.7 (26.6-37.5)	25.7 (20.3-31.6)	<0.01
Lymphocytes (1/µl)	1875 (1445-2510)	2305 (1760-2885)	<0.05
Monocytes (%)	6.1 (5.3-7.8)	5.8 (4.9-6.8)	<0.05
Monocytes (1/µl)	415 (335-495)	500 (415-645)	<0.01
Eosinophils (%)	1.8 (1.2-3.2)	2 (0.9-3.4)	0.94
Eosinophils (1/µl)	130 (85-185)	160 (100-325)	0.12
Basophils (%)	0.7 (0.4-0.8)	0.5 (0.3-0.7)	0.06
Basophils (1/µl)	40 (30-65)	50 (30-60)	0.84

Results are expressed as median (IQR). Differences were calculated by a Mann-Whitney test.

4.3. Cytokine release from sputum and blood cell culture

Sputum cytokine levels are shown in Table 3. Sputum cells from COPD strikingly released less IL-10 ($p < 0.001$), TNF-α ($p < 0.0001$) and to a lesser extent IL-6 ($p < 0.01$) as compared to those of healthy subjects. IL-6 was detectable in sputum cell culture of 76% of healthy subject while it was only detectable in 52% of COPD. By contrast, IFN-γ Fig. 1a) was more often detectable in COPD (29% of subjects) than in healthy subjects (6%) ($p < 0.05$). IL-4 was rarely detectable in both COPD and healthy subjects (less than 10% of subjects).

Blood leukocytes from COPD released more IFN-γ Fig. 1b) and TNF-α as compared to those of healthy subjects ($p < 0.01$ for both). IFN-γ was detectable in 61% of COPD and only 27% of healthy subjects. Although undetectable in the majority of the subjects, IL-10 was more frequently detected in COPD (46%) than in healthy subjects (7%) ($p < 0.01$). No difference was observed for IL-4 and IL-6 which were undetectable in the majority of both COPD (80% and 57% respectively) and healthy subjects (70% for both cytokines). The differences for TNF-α, IL-10 and IFN-γ remained significant when cytokine levels were normalized according to blood cell count (data not showed).

4.4. Relationship between sputum and blood cells and cytokines released in sputum and blood

The percentage of sputum neutrophils, lymphocytes and eosinophils were weakly but significantly correlated with their blood counterparts in COPD patients ($p < 0.0001$ and $r = 0.44$; $p < 0.01$ and $r = 0.33$; $p < 0.0001$ and $r = 0.43$ respectively). As for cytokines, we found a weak negative correlation between the TNF-α released from sputum and blood cells in the COPD group ($p < 0.05$ and $r = -0.27$). For other cytokines, no correlation was found between cytokine released at sputum and blood levels in any group of subjects.

4.5. Relationship between cells, cytokine release and disease severity

In COPD, the% predicted post-bronchodilator FEV1 was inversely related to the total sputum cell number ($r = -0.28$; $p < 0.01$) and the proportion of neutrophils ($r = -0.33$, $p < 0.01$) but not to the proportion of eosinophils ($r = -0.08$, $p > 0.05$). Accordingly there was a significant increase in sputum neutrophil proportion with the GOLD stages (Fig. 2). Similarly, we found a relationship between blood cell TNF-α production and disease severity as reflected by the GOLD stages (Fig. 3). Indeed, GOLD stage IV patients exhibited a greater level of TNF-α than GOLD III (542 pg/ml (104-486) versus 172 pg/ml (28-414), $p < 0.05$) and tended to have a greater level than GOLD II (542 pg/ml (104-486) versus 42 pg/ml (13-913), $p = 0.07$).

Table 3 *Sputum and blood cytokine release.*

	Healthy $N = 33$	COPD $N = 95$	p-value
Sputum (10^5 cells/well) standardized cell concentration			
IL-4 (pg/ml)	0 (0-0)	0 (0-0)	0.31
IL-6 (pg/ml)	29 (5-207)	7 (0-49)	<0.01
IL-10 (pg/ml)	50 (34-77)	22 (8-57)	<0.001
IFN-γ (pg/ml)	0 (0-0)	0 (0-11)	<0.05
TNF-α (pg/ml)	2248 (1501-3923)	1116 (544-2042)	<0.0001
Blood (50 µl diluted twice) Standardized blood volume			
IL-4 (pg/ml)	0 (0-7)	0 (0-0)	0.72
IL-6 (pg/ml)	0 (0-10)	0 (0-23)	0.12
IL-10 (pg/ml)	0 (0-0)	4 (0-14)	<0.01
IFN-γ (pg/ml)	0 (0-7)	11 (0-36)	<0.01
TNF-α (pg/ml)	31 (0-86)	146 (22-585)	<0.01

Results are expressed as median (IQR). Differences were calculated by a Mann-Whitney test.

4.6. Relationship between cytokine release and demographic and treatment characteristics

To take into account the impact of age, we divided our two groups in tertiles (Table 4). In healthy subjects, there was no correlation between age and cytokine release either from sputum or blood cells. In COPD, the production of IL-4, IL-6, IL-10 and IFN-γ was slightly greater in the oldest tertile but there was no impact of age on cytokine production from blood cells. In the COPD group, the body mass index (BMI) appeared to be negatively

correlated with TNF-α and IL-6 released from blood cells (r = -0.3 and r = -0.27 respectively; $p < 0.01$ for both). BMI did not correlate with any cytokine produced from sputum cells. In order to exclude the influence of current smoking on cytokine release, we compared the results in ex or non smokers healthy subjects (n = 26) with those seen in ex smokers COPD (n = 64). The differences in cytokine release persisted as for the whole groups of COPD and healthy subjects (Table 5). In COPD, TNF-α release from sputum cells was lower in current smokers (n = 30) than in ex-smokers (n = 64) (678 (483-1119) pg/ml vs 1434 (874-2202) pg/ml respectively; $p < 0.01$).

In COPD no difference in cytokine released either from sputum or blood cells was observed between those receiving inhaled corticosteroids (ICS) and those who were not treated by ICS.

Fig. 1a. *IFN-γ released from sputum cells of COPD vs healthy subjects.*

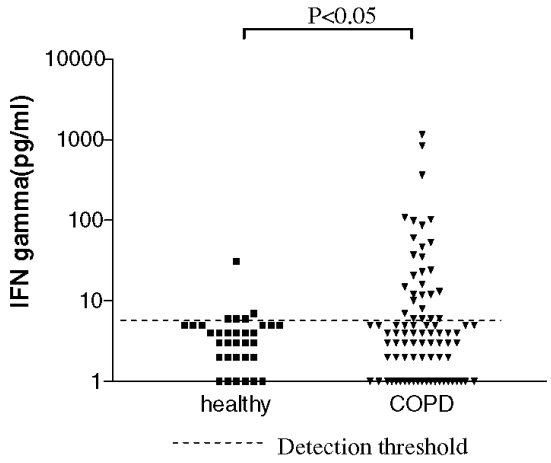

Fig. 1b. *IFN-γ released in blood of COPD vs healthy patients.*

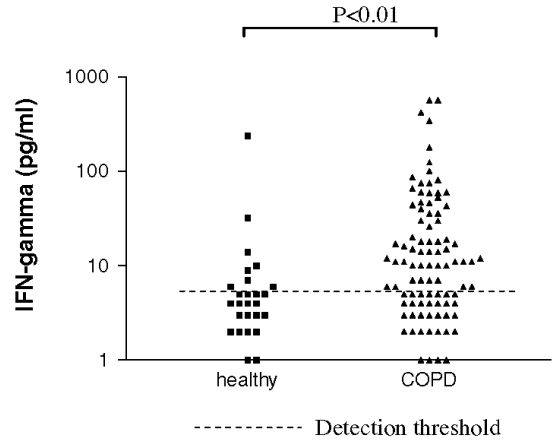

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Table 4 *Cytokine release according to age in healthy and COPD patients.*

	Tertile 1 Healthy subjects: *N* = 11 COPD patients: *N* = 32	Tertile 2 Healthy subjects: *N* = 11 COPD patients: *N* = 32	Tertile 3 Healthy subjects: *N* = 11 COPD patients: *N* = 31	*p*-value
Age (years)	Healthy: 26 (21-32) COPD: 51 (37-59)	Healthy: 41 (34-44) COPD: 62 (59-67)	Healthy: 52 (45-63) COPD: 73 (67-82)	<0.0001 <0.0001
Sputum (10e5 cells/well)				
IL-4 (pg/ml) Healthy	0 (0; 0)	0 (0; 0)	0 (0; 0)	0.64
IL-4 (pg/ml) COPD	0 (0; 0)	0 (0; 0)	0 (0; 22)	<0.05
IL-6 (pg/ml) Healthy	44 (0; 479)	31 (23; 461)	22 (0; 216)	0.84
IL-6 (pg/ml) COPD	0 (0; 54)	0 (0; 28)	24 (0; 91)	<0.05
IL-10 (pg/ml) Healthy	47 (22; 63)	85 (48; 167)	44 (37; 52)	0.13
IL-10 (pg/ml) COPD	22 (8; 53)	11 (7; 30)	51(8; 80)	<0.05
IFN-γ (pg/ml) Healthy	0 (0; 0)	0 (0; 0)	0 (0; 0)	0.16
IFN-γ (pg/ml) COPD	0 (0; 0)	0 (0; 4)	0 (0; 36)	<0.05
TNF-α (pg/ml) Healthy	2238 (1303; 3633)	2942 (1091; 4084)	2221 (1574; 4033)	0.75
TNF-α (pg/ml) COPD	1132 (577; 2576)	1106 (567; 1769)	1088 (362; 2124)	0.94
Blood (50 µl diluted twice)				
IL-4 (pg/ml) healthy	0 (0; 90)	0 (0; 4)	0 (0; 4)	0.56
IL-4 (pg/ml) COPD	0 (0; 0)	0 (0; 9)	0 (0; 0)	0.31
IL-6 (pg/ml) healthy	0 (0; 17)	0 (0; 14)	0 (0; 10)	0.7
IL-6 (pg/ml) COPD	0 (0; 23)	3 (0; 22)	0 (0; 31)	0.99
IL-10 (pg/ml) healthy	0 (0; 3)	0 (0; 0)	0 (0; 0)	0.51
IL-10 (pg/ml) COPD	5 (0; 14)	2 (0; 20)	2 (0; 14)	0.85
IFN-γ (pg/ml) healthy	0 (0; 0)	0 (0; 20)	0 (0; 9)	0.14
IFN-γ (pg/ml) COPD	12 (0; 45)	3 (0; 19)	11 (0; 43)	0.27
TNF-α (pg/ml) healthy	0 (0; 130)	42 (6; 427)	42 (7; 90)	0.47
TNF-α (pg/ml) COPD	347 (17; 745)	135 (27; 606)	113 (22; 386)	0.75

Patients were divided in tertiles according to the age. Age is expressed as mean (range) and other results are expressed as median (IQR).
Differences were calculated by a Kruskal-Wallis test.

Fig. 2. *Sputum neutrophil count according to the disease severity (—: median value).*

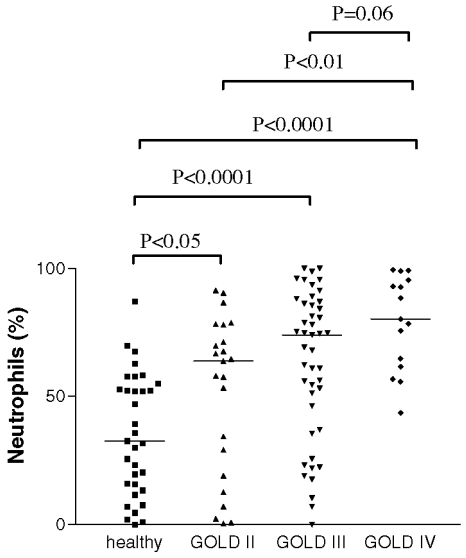

Fig. 3. *Blood cell TNF-α production according to disease severity (—: median value).*

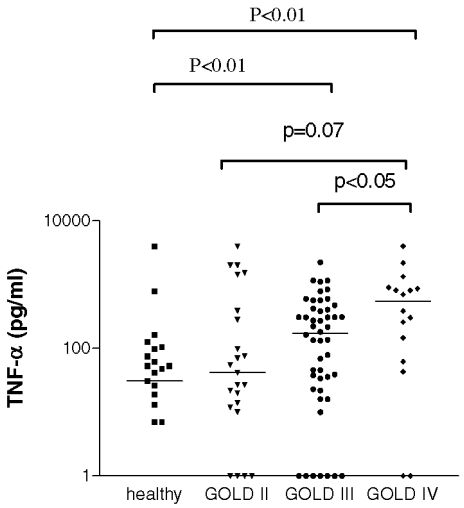

Table 5 *Cytokine released in non or ex-smokers healthy subjects versus ex-smokers COPD patients.*

	Healthy *N* = 26	COPD *N* = 64	*p*-value
Sputum (10e5 cells/well)			
IL-4 (pg/ml)	0 (0-0)	0 (0-0)	NS
IL-6 (pg/ml)	60 (14-500)	7 (0-53)	<0.01
IL-10 (pg/ml)	50 (32-74)	25 (8-64)	<0.05
IFN-γ (pg/ml)	0 (0-0)	0 (0-12)	0.07
TNF-α (pg/ml)	2644 (1818-4000)	1328 (788-2202)	<0.001
Blood (50 µl diluted twice)			
IL-4 (pg/ml)	0 (0-8)	0 (0-0)	NS
IL-6 (pg/ml)	0 (0-10)	0 (0-23)	NS
IL-10 (pg/ml)	0 (0-0)	0 (0-14)	<0.01
IFN-γ (pg/ml)	0 (0-0)	10 (0-41)	0.0001
TNF-α (pg/ml)	25 (0-101)	155 (24-650)	<0.01

Results are expressed as median (IQR). Differences were calculated by a Mann-Whitney test.

5. Discussion

COPD exhibited a raised airway neutrophilic inflammation associated with a marked IL-10, IL-6 and TNF-α release deficiency that contrasted with a raised IFN-γ production. Neutrophilic inflammation was also prominent at blood level together with raised production of IFN-γ, IL-10 and TNF-α. Furthermore, we found a significant correlation between the sputum neutrophil count and the disease severity reflected by the GOLD stages. At the systemic level, production of TNF-α also correlated to GOLD stages and inversely to BMI.

Here we used here an original model of cell culture previously validated [13,26]. Our study showed a raised production of IFN-γ from sputum cells of COPD while there was no evidence of overproduction of IL-4, which remained rarely detectable in both groups. Our finding highlights the Th1 profile that prevails in the airway inflammation of COPD. The reason for this IFN-γ production is not clear at present but is likely to reflect ongoing immune response towards microbial agents although arguments also exist to support auto-immune mechanisms [7]. Our results are in keeping with those of Di Stefano et al. who reported greater IFN-γ expression in bronchial biopsies of COPD patients [11].

In contrast to what was found for IFN-γ, the production of TNF-α, IL-6 and IL-10 from sputum cells were reduced in COPD patients as compared to healthy subjects. The reduced production of TNF-α confirmed our previous results [10] and those of reduced IL-6 and IL-10 are novel. We postulate that the lower release of TNF-α might impair local immune defence and could make the subjects more prone to chronic infection while the deficient IL-6 release might make them unable to properly resolve acute airway inflammation following

infectious assault [18]. On the other hand, reduced IL-10 production extends the finding of a previous study looking at sputum supernatant [38] and may be seen as a deficient anti-inflammatory mechanism.

There may be, however, some discrepancy between the results found in sputum supernatant and those reported here from sputum cell culture. Indeed, there are some, but not all [10,40] reports showing a significant increase of TNF-α in sputum supernatant [19] or BAL fluid [36]. Likewise, some studies reported greater IL-6 levels in sputum supernatant from COPD [4,14] while their sputum failed to release more IL-6 in our study. As for IFN-γ it was reported to be normal in sputum supernatant of COPD compared to healthy subjects [14,42]. It highlights the fact that sputum supernatant and sputum cell culture supernatant are not the same milieu. The difference could be explained by the fact that cellular sources that contribute to the supernatant content are more varied than those contributing in the sputum cell culture model. Supernatant content not only depends on the secretion from airway cells lying within the lumen but also from resident structural cells and in particular from the epithelial layer, which is not strongly represented in the sputum cell culture. In addition, plasma exudation may also contribute to a rise in mediator/cytokine concentration found in the supernatant whereas it does not influence the level measured in sputum cell culture [34]. Finally, another factor that has to be taken into account in the interpretation of our results is the fact that COPD patients display a greater number of cells per gram of sputum which may counterbalance in vivo the reduced production of cytokines per standardized cell number.

Regarding cytokine release from blood cells, there was a clear overproduction of IFN-γ, IL-10 and TNF-α in COPD. The enhanced release of IFN-γ extents the Th1 concept at the systemic level. The majority of COPD patients (61%) produced INF-γ from their blood cells, which was not the case in healthy subjects. Interestingly, we observed that 75% of COPD patients producing IFN-γ from their sputum cells also released this cytokine from their blood cells. Neither the sputum nor the blood production of IFN-γ appeared to be related to disease severity reflected by the GOLD stages. The IFN-γ producers did not distinguish from their counterparts according to their demographic and treatment features. The raised IL-10 and TNF-α release at the blood level contrasts with what was found in sputum. Our result of raised TNF-α production in COPD is in keeping with results from Schols et al. [33]. These cytokine over-productions seen in COPD persisted even after normalization per cell number, which reinforces the finding.

Because we found a relationship between TNF-α release and GOLD stages as well as an inverse relationship between TNF-α and the BMI, our data support an important role for systemic TNF-α in the disease progression and the cachexia often observed in the advanced stages of the disease. It is line with studies showing relationship between losing weight and serum TNF-α in COPD [8,12,15,37]. Alteration of FEV1 values might not be strictly related to change in airway calibre but may also depend on pulmonary elastolysis and reduced respiratory skeletal muscle forces, both processes likely to be influenced by TNF-α [6,15]. Surprisingly, the production of IL-6, which is the most important inducer of acute-phase protein synthesis, was not different from that in healthy volunteers. This might be explained by the fact that our patients were in stable conditions when sampled as there are reports of increased blood IL-6 levels during exacerbations [41].

By contrast to smoking, taking inhaled corticosteroids (ICS) did not seem to change the cytokine release from sputum or blood cell culture as there was no difference between those who were or were not on ICS.

The main limitation of our study is the age difference between our COPD and our healthy controls. Although age may influence the neutrophil cell counts [39], our results showed that the reduced release of IL-6 and IL-10 from sputum cells in COPD can not be accounted for by the age as the youngest were those in whom this reduction was the most apparent. However, we can not rule out a role of age in raised IFN-γ production from sputum cells in COPD even if it was not observed in healthy subjects. Furthermore, no relationship between age and cytokine release from blood cells was found in any of the two groups. This makes us confident that, overall, differences in cytokine release between COPD and healthy subjects are unlikely to be accounted for by the age difference between the two groups.

Another limitation of the study is the mismatch in tobacco habits between healthy subjects and COPD. However, it clearly appeared from our data that ex smokers COPD still markedly distinguished from non or ex-smokers healthy subjects. This highlights the fact that COPD by itself, irrespective of the current smoking status, is characterized by a disturbed cytokine production both at systemic and local level.

As for the cell component of inflammation, our results showed a raise in total sputum cell number linked to higher proportion of neutrophils in COPD patients which is keeping with previous studies [25,30,31]. In addition, we confirmed that the% predicted post bronchodilator FEV1 was inversely correlated to the proportion of neutrophils [23,25,35]. It suggests that neutrophilic inflammation is an important factor in the pathogenesis of

irreversible airflow limitation in COPD [5]. On the other hand, high sputum neutrophil count may reflect constant activation of local innate immunity in response to bacterial colonization developing in remodelled and altered airways [20]. Interestingly, although sputum eosinophil count was also increased in COPD but not related to the magnitude of airway obstruction. In previous studies, eosinophilic COPD was found to be associated with asthmatic features such as greater reversibility to β2 agonist, raised exhaled NO [24] as well as sign of mast cell activation [21]. Raised circulating number of leucocytes and mainly of neutrophils was also seen in blood of COPD patients. This observation is in line with previous studies and supports the concept of systemic inflammation [17]. In our study, COPD patients also show raised circulating number of lymphocytes and monocytes per blood volume even if this was less conspicuous than for neutrophils. The raised number of monocytes and lymphocytes is likely to partly contribute to the enhanced cytokine release from whole blood sample of COPD.

6. Conclusions

Our study confirms the neutrophilic inflammation in COPD both at the local and the systemic level. It shows raised local and systemic IFN-γ production highlighting the pertinence of Th1 concept in COPD. A systemic enhanced release of TNF-α is also an important feature of COPD which is related to disease severity and BMI.

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