TLR4 up-regulation and reduced Foxp3 expression in mechanically ventilated smokers with obstructive chronic bronchitis.

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1   **TITLE: TLR4 up-regulation and reduced Foxp3 expression in mechanically ventilated**

2   **smokers with obstructive chronic bronchitis.**

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22   **RUNNING HEAD: Innate immunity and immune regulation in acute respiratory failure.**

23

24   **KEY WORDS**: toll like receptors, Foxp3, chemokines, smokers, respiratory failure.

25

26   **LIST OF ABBREVIATIONS**

27   COPD=Chronic obstructive pulmonary disease; C= Controls; CB= chronic bronchitis; S= smokers;

28   Mini-BAL= mini-bronchoalveolar lavage; TLR= Toll like receptor; Foxp3=f forkhead box P3; IP-

29   10= interferon gamma induced protein 10; IL-8= interleukin 8.

30

31 **CONFLICTS OF INTEREST**

32 Elisabetta Pace- Competing interests: None declared.

33 Maria Ferraro - Competing interests: None declared.

34 Antonino Giarratano- Competing interests: None declared.

35 Chiara Cipollina-Competing interests: None declared.

36 Mark Gjomarkaj- Competing interests: None declared.

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SUMMARY

Background. Chronic bronchitis (CB) is a risk factor in chronic obstructive pulmonary disease (COPD) for accelerated lung function decline and increased mortality. The lung and systemic inflammatory and immunological profile of COPD patients with CB which acutely experience respiratory failure upon a disease exacerbation is unknown.

Methods. In this study, we explored the expression of Foxp3 by western blot analysis, TLR4 by immunocytochemistry and the concentrations of IP-10 and IL-8 by ELISA in the mini-bronchoalveolar lavages (mini-BAL) and in the peripheral blood of patients with respiratory failure requiring intubation and mechanical ventilation. The recruited subjects were separated into three different groups: smokers with CB and COPD (COPD, n=18), smokers with CB but without COPD (S, n=8) and patients without CB and without COPD (C, n=10).

Results. In mini-BAL of COPD group, Foxp3 and IP-10 were significantly reduced while TLR4 was significantly increased in comparison to C. TLR4 was also increased in mini-BAL of S. In COPD peripheral blood, Foxp3 was reduced in comparison to C but no significant differences were observed for TLR4 and for IP-10. No significant differences were observed for IL-8 concentrations in the mini-BAL and in the blood of the recruited patients. The mini-BAL TLR4 expression correlated with the Clinical Infective Pulmonary Score.

Conclusions. In exacerbated COPD patients with respiratory failure, lung and systemic reduced immune regulatory events (low Foxp3 expression) and lung increased innate immunity responses (high TLR4 expression) occur. These events may contribute to the increased inflammatory events leading to respiratory failure.

62   **INTRODUCTION**

63   Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease, is associated with

64   pulmonary and extra-pulmonary clinical manifestations and includes different clinically relevant

65   subtypes (1). One of the COPD subtypes is characterized by chronic bronchitis (CB). CB is a risk

66   factor for accelerated lung function decline in COPD, increased hospitalization, and increased

67   mortality (2).

68   Cigarette smoke represents the most important risk factor for CB (3). Chronic oxidative stress of

69   cigarette smoking induces mucus secretion and the increased mucus viscosity renders the airways

70   susceptible to bacterial infections, a hallmark of CB (3).

71   A key component of the innate immunity and of the innate defence mechanisms against infections

72   is represented by the toll like receptor (TLR) family (4) .

73   A recent hypothesis regarding COPD pathogenesis suggests as "step 1 of the disease" the activation

74   of innate responses by injured tissue components (5). Products derived from epithelial cell injury

75   can act as ligands for TLR4 and TLR2, thus amplifying inflammatory responses within the airways.

76   Cigarette smoke is able to increase the expression of TLR4 and to orientate the activation of TLR4

77   toward an increased release of IL-8 and a reduced release of interferon gamma-induced protein 10

78   (IP-10) in bronchial epithelial cells (6).

79   In the airways of COPD patients mechanically ventilated due to acute respiratory failure, there is an

80   increased expression of TLR4 and an increased chemotactic activity toward neutrophils but a

81   reduced concentration of IP-10 with a reduced chemotactic activity toward lymphocytes (7). The

82   increased airway inflammation in the airways of COPD patients may also be sustained by the

83   impairment of immune regulatory events and in particular may be linked to the reduced expression

84   of the forkhead box P3 (Foxp3), a transcription factor crucially involved in T regulatory activities.

85   COPD patients have, in small airways, decreased numbers of Foxp3 positive cells that negatively

86   correlate with airflow obstruction (8) (9). Although COPD is associated with lung and systemic

87 inflammation, it is unknown whether the alteration in the innate and immune regulatory events

88 observed in the airways of COPD patients are also present in the systemic compartment.

89 The objectives of this study were to investigate the immune regulatory events and the inflammatory

90 and the host defence responses in the airways and in the systemic compartment of COPD patients

91 with CB. Exacerbated COPD patients who experienced an acute respiratory failure requiring

92 endotracheal intubation and mechanical ventilation were compared to patients requiring also

93 intubation and mechanical ventilation but without COPD and without CB. In the recruited patients

94 the expression of TLR4, the expression of Foxp3 and the release of specific chemokines (IP-10 and

95 IL-8) were investigated.

96

97   **MATERIALS AND METHODS**

98   **Patient population**

99   This study was conducted at the ICU of the Department of Anestesiology, Reanimation and

100   Emergency of the University of Palermo, Italy. Local ethic committee permission and informed

101   written consent from either the patient or closest relatives were obtained. The patients were

102   classified into the following groups: 1) subjects without smoking history, without history of

103   previous CB or chronic pulmonary diseases including asthma and COPD and with acute respiratory

104   failure upon surgery for abdominal or thoracic aneurysm, (Controls =C; n=10); 2) smoking subjects

105   (>15 pack-year) with CB, without COPD and with acute respiratory failure upon surgery for

106   abdominal or thoracic aneurysm (S; n=8); 3) smoking subjects (>15 pack-year) with CB with

107   COPD (GOLD-1-2) and with acute respiratory failure upon treatment failure of an acute

108   exacerbation (COPD; n=18). The COPD patients fulfilled the diagnostic criteria of COPD (10) with

109   a post-bronchodilator obstruction (FEV_1 < 80% predicted, and FEV_1/FVC ratio < 70%). Patients

110   with x-ray or clinical evidence of sepsis or pneumonia at the time of mini-bronchoalveolar lavage

111   (mini-BAL) collection were not included. CB were defined on the basis of symptoms and in

112   particular productive cough lasting more than three months in more than two years. All recruited

113   subjects required mechanical ventilation and underwent therapy with antibiotics and systemic

114   corticosteroids (no significantly different doses among the patients included in the three groups).

115   The antibiotics were adjusted to cover any identified pathogens on the basis of antibiograms. COPD

116   patients, before COPD exacerbation, were undergoing therapy with bronchodilators but not with

117   corticosteroids. Exacerbations were defined as previously reported (10) and were treated with

118   antibiotics and systemic corticosteroids. At the ICU admission, data for Clinical Pulmonary

119   Infective Score (CPIS), the simplified Acute Physiology Score (SAPS II) and sepsis-related organ

120   failure assessment (SOFA) were collected from each recruited patient. Paired mini-BAL and blood

121   samples were collected from all participants. Microbiology of mini-BAL was also assessed.

mini-BAL collection and processing

123   Distal lung fluid samples (mini-BAL) were obtained using BAL Cath system (by Kimberly Clark)

124   within 1 h from the intubation. The protected catheter was blindly advanced through the

125   endotracheal tube until it was wedged into a distal airway and aliquots of 10 ml of sterile 0.9%

126   NaCl were instilled and gently suctioned (recovered volume about 70% of the instilled volume).

127   Mini-BAL samples were filtered through a sterile gauze and then centrifuged at 1300 rpm for 10

128   min to separate cells from supernatants. Total and differential (diff-quick staining) cell counts were

129   assessed. The cell fraction was used for immunocytochemistry and western blot experiments. The

130   supernatants were assessed for cytokine levels.

Blood samples

132   Blood samples (10 ml) were collected from C, S, COPD subjects and then processed for obtaining

133   plasma and peripheral blood mononuclear cells (PBMC). PBMC were isolated from blood s by

134   Ficoll-Hypaque (Pharmacia) gradient centrifugation. The cells were suspended in RPMI 1640 tissue

135   culture medium (Invitrogen Life Technologies) supplemented with 1% heat-inactivated FCS

136   (Invitrogen Life Technologies), 2 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml

137   streptomycin, 5 x 10^{-5} M 2-ME and 85 µg/ml gentamicin. Purity and viability were tested using

138   trypan blue exclusion.

Immunocytochemistry

140   The expression of TLR4 was evaluated using a rabbit polyclonal antibody (Santa Cruz

141   Biotechnology)**.** Immunocytochemistry was performed using AP-LSAB2 (DAKO, Glostrup,

142   Denmark) kit following the manufacturer's instructions and new Fuchsin as chromogenic substrate

143   (DAKO) (cytoplasmic red staining). Negative controls were performed using rabbit or mouse

144   negative control immunoglobulins (DAKO). Data are expressed as percentage of positive cells.

Western blot analysis

146   The expression of Foxp3 was evaluated by western blot analysis as previously described (11) with

147   minor modifications. 40 µg of total protein were loaded in the gel. All blots were probed using a

148   goat polyclonal antibody anti-Foxp3 (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA).

149   Membranes were then stripped and incubated with goat polyclonal anti–ß-actin (Sigma) as

150   housekeeping protein to normalize differences in protein loading. Revelation was performed with an

151   enhanced chemioluminescence system (GE Healthcare, Chalfont St. Giles, UK) followed by

152   autoradiography. Negative controls were performed in the absence of primary antibody or including

153   an isotype control antibody. Data are expressed as densitometric arbitrary units by correction with

154   the density of the bands obtained for beta-actin.

155   **Measurement of IL-8 and IP-10**

156   The concentrations of IL-8 and IP-10 in plasma from C, S, CB-COPD subjects were determined by

157   an enzyme-linked immunosorbent assays (ELISA) following the manufacturer's instructions

158   (Quantikine; R&D Systems, Minneapolis, MN).

159   **Statistics**

160   Data are expressed as median (25-75 percentiles). Kruskal Wallis test was performed for

161   comparisons among patient groups. A non-parametric Mann Whitney test was then applied as the

162   initial Kruskal Wallis test was significant. The Wilcoxon test was used for comparisons between

163   mini-BAL and autologous peripheral blood in each recruited patient. The Spearman test was used

164   for correlations. P< 0.05 was accepted as statistically significant.

165

166   **RESULTS**

167   **Demographic characteristics of the subjects.**

168   The demographic characteristics of the three study groups are shown in Table 1. SAPS II and SOFA

169   scores revealed no significant differences in the recruited patients. The CPIS score was significantly

170   higher in COPD than in C (Table 1). No significant differences for CPIS score were shown in S in

171   comparison to C and to COPD. The total and the differential cell counts of mini-BAL are shown in

172   Table 2. Significantly higher numbers of total cells and of neutrophils were present in COPD

173   patients. Microbiology of mini-BAL was shown in table 3.

174

175   **Expression of TLR4 in cells from mini BAL and from peripheral blood cells.**

176   The percentage of TLR4 positive cells was significantly higher in mini-BAL cells from COPD and

177   from S in comparison to mini-BAL from C. The percentage of TLR4 positive cells was significantly

178   higher in mini-BAL cells from COPD in comparison to mini-BAL from S and to autologous

179   peripheral blood (figure 1) (table 4). No significant differences in TLR4 expression were observed

180   in peripheral blood cells among C, S and COPD (figure 1) (table 4).

181   **Concentrations of IL-8 and of IP-10 in mini BAL and in peripheral blood cells.**

182   The concentrations of IL-8 were significantly higher in mini-BAL of all the recruited patients (C, S

183   and COPD patients) in comparison to autologous peripheral blood (figure 2). No significantly

184   different concentrations of IL-8 were observed in mini-BAL and in peripheral blood among C, S

185   and COPD (figure 2). The IP-10 concentrations were significantly reduced in mini-BAL from

186   COPD in comparison to C and S and in comparison to autologous peripheral blood (figure 3). The

187   IP-10 concentrations were significantly increased in peripheral blood from COPD in comparison to

188   mini-BAL from C. No significantly different concentrations of IP-10 were observed in peripheral

189   blood between S and C (figure 3).

190   **Expression of Foxp3 in mini BAL and in peripheral blood cells.**

191   In COPD and in S the expression of Foxp3 in mini-BAL and in peripheral blood was significantly

192   lower than in C (figure 4 A-B) (table 4).. In COPD the expression of Foxp3 in mini-BAL and in

193   peripheral blood was significantly lower than in S (figure 4 A-B) (table 4).. No significant

194   differences were observed in the expression of Foxp3 in mini-BAL and in autologous peripheral

195   blood in all the recruited patients.

196   **Correlations**

197   Finally, we tested whether the observed alterations in TLR4, Foxp3 and IP-10 in both lung and

198   systemic compartments correlate with clinical scores of severity. CPIS correlates with mini-BAL

199   TLR4 expression (figure 5) but not with the other markers (Foxp3 and IP-10) (data not shown). No

200   significant correlations were observed between SOFA or SAPS II score with any of the tested

201   markers (data not shown).

202

DISCUSSION

204 COPD is a heterogeneous disease and includes different clinically relevant subtypes. A divergent

205 distribution of parenchymal (emphysema) and bronchial airway (chronic bronchitis) (CB) disease

206 contributes to the phenotypic heterogeneity in COPD (12). COPD is associated not only with an

207 abnormal inflammatory response in the lung but also with systemic inflammation, including

208 systemic oxidative stress, activation of circulating inflammatory cells and increased circulating

209 levels of inflammatory cytokines (12). The low–grade systemic inflammation present in COPD

210 patients may be responsible for the systemic clinical manifestations of the disease including

211 malnutrition, muscle wasting, osteoporosis, cardiovascular diseases, type II diabetes, anaemia and

212 depression (13).

213 This study explored whether lung and systemic inflammation occur concurrently and similarly in

214 patients with acute respiratory failure with and without CB or COPD. We demonstrated for the first

215 time that patients with both CB and COPD who underwent an acute exacerbation have a different

216 inflammatory profile (IP-10 levels) and a different alteration in the innate immune responses (TLR4

217 expression) in the airways and in the systemic compartment. Differently, in these patients a similar

218 alteration in the immune regulatory events (low Foxp3 expression) was observed in the airways and

219 in the systemic compartment. The novel aspects underlined by the present study are related to the

220 accurate selection of patients with a similar phenotype (CB) and a similar exposure to risk factor

221 (cigarette smoke) in order to limit the variability of the obtained results and to better identify the

222 role of the observed alterations in airway obstruction. Most of the studies report data from smoker

223 or COPD patients with different phenotypes.

224 CB is defined on the basis of chronic cough and sputum due to mucus hypersecretion and

225 histologically, it is characterised by airway inflammation, hypertrophy of submucosal mucus

226 secreting glands, and goblet cell hyperplasia (14). CB is a risk factor in COPD for accelerated lung

227 function decline, increased hospitalization, and increased mortality (2). In this regard it has been

228 demonstrated that the presence of CB may compromise the sterility of distal airway supporting the

229 hypothesis of natural progression from the simple mucus hypersecretion to purulent hypersecretion

230 and obstructive bronchitis (Hogg, Lancet 2004). Here, it is showed that the expression of TLR4 is

231 increased in COPD patients with CB and acute exacerbation in the airways but not in the systemic

232 compartment and this alteration is observed at lower extent also in smokers with CB but without

233 COPD and without acute exacerbation. TLR4 expressed is associated in these patients with the

234 presence of neutrophils which represent the predominant cell type in COPD patients. The data

235 provided extend and integrate previous results from our group showing that in the airways of COPD

236 patients with acute respiratory failure, there is an increased expression of TLR4 (7). Our data

237 demonstrate that TLR4 expression is higher in mini-BAL cells in comparison to blood compartment

238 suggesting an up-regulation of TLR4 at the transit from blood into the airway compartment. This

239 phenomenon seems to be specific for TLR4 since it has been previously demonstrated that in COPD

240 patients the expression of TLR2 is lower on sputum neutrophils in comparison to blood

241 compartment indicating a down-regulation of TLR2 at the transit from blood into the airway

242 compartment (16).

243 TLR4 signaling, through MyD88 and IRAK1, plays a predominant role as a regulator of smoke-

244 induced protease production (17). Furthermore, CSE increase the expression of TLR4 but not of

245 TLR2 and modify the functional activation of TLR4 generating an imbalance between cytokines

246 with opposite functions such as IL-8 and IP-10 (6). IP-10 concentrations in mini-BAL of smoker

247 COPD who are mechanically ventilated for acute exacerbation are reduced in comparison to another

248 group of patients mechanically ventilated but not smokers and without COPD (7). We confirm here

249 the presence of reduced concentrations of IP-10 within the airways of mechanically ventilated and

250 exacerbated COPD patients and demonstrate for the first time that within the systemic compartment

251 in the same patients an increased concentration of IP-10 is observed in comparison to patients with

252 acute respiratory failure but without CB and COPD. Nasal epithelial cells obtained from smokers

253 create an overall cytokine microenvironment that after infection with influenza suppresses the

254 concentrations of IP-10 (18). When the bronchial epithelial cells were exposed to CSE, the release

255 of IP-10 decreases while the release of IL-8 increases (6). Although no significantly different

256 concentrations of IL-8 were observed in mini-BAL and in peripheral blood between COPD, S and C

257 within the airways the elevated concentrations of IL-8 are not balanced by elevated IP-10

258 concentrations. The prevalence of IL-8 may in turn sustain the influx of neutrophils into the airways

259 thus triggering innate immunity responses, while IP-10 attracts monocytes and lymphocytes (19)

260 thus promoting activation of adaptative responses to efficiently and specifically limit microbial

261 invasion and to restrain the harmful effects of prolonged neutrophil activation. The findings that

262 mini-BAL from COPD had reduced IP-10 concentrations and reduced chemotactic activitites

263 toward lymphocytes (7) might contribute to explain why the differential cell counts of mini-BAL

264 from COPD failed to have lymphocytes and prompted us to explore whether lymphocyte regulatory

265 activities were altered in these patients. CD4$^+$ Foxp3$^+$ regulatory T lymphocytes (Treg) are a

266 subclass of CD4$^+$ T cell receptor (TCR) $\alpha\beta^+$ T cells that are essential to preserve immune

267 homeostasis (20), (21). Stable expression of the transcription factor Foxp3 is a prerequisite for the

268 maintenance of suppressive properties in CD4+ regulatory T cells. Foxp3 mRNA expression is not

269 itself sufficient for stable Foxp3 protein expression (22). The epigenetic modifications, such as

270 histone modification or DNA methylation, control regulatory T cells by controlling Foxp3 gene

271 expression through altering the accessibility of the Foxp3 locus and by acetylating or deacetylating

272 Foxp3 protein, thereby enabling the epigenetic regulation of Foxp3 target genes. Absence of the

273 Foxp3 transcription factor at systemic level leads to the rapid development of fulminant multiorgan

274 autoimmunity. A decreased Foxp3 expression in COPD patients and smokers parallels the

275 aggravation of the disease (23). Patients with moderate or severe COPD upon fluticasone and

276 salmeterol combination therapy show an increased proportion of Foxp3+Tregs in the total

277 peripheral blood CD4+T cell population (24). Smokers with normal lung function and COPD

278 patients have increased numbers of Foxp3-positive cells in large airways but they have decreased

279 numbers of Foxp3-positive cells in small airways (25). In the patients with acute respiratory failure

280 recruited in the present study, the decreased expression of Foxp3 is present in smokers and in

281 COPD in the distal airways and in the systemic compartment suggesting that the alterations in the

282 immune regulatory activities are early events in the disease and may contribute to the systemic

283 effects of the disease. It is conceivable that cigarette smoke may contribute to induce epigenetic

284 modifications leading to the reduced expression of Foxp3 in smokers and in COPD smokers at both

285 the airways and systemic compartment. No study has already specifically addressed this point yet.

286 Finally, the relevance of the observed alterations in the severity of the recruited patients was

287 assessed. Several commonly used scoring systems exist assessing the severity of the disease in

288 critically ill patients by predicting mortality. In the present study the recruited patients were

289 classified on the basis of their SOFA, SAPS II and CPIS score. The mortality in elderly patients

290 was higher than that of the younger patients and SAPS II (26) was an independent predictor of

291 mortality in elderly patients with sepsis (27). The SOFA score, widely used in many cardiac

292 surgical intensive units, is used for grading organ dysfunction or failing organ system (28) .

293 Prognostic relevance of the SOFA score in combination with inflammatory parameters was also

294 found in a recent study conducted by Zügel *et al*. (29). The CPIS score is calculated on the basis of

295 points assigned for various signs and symptoms of pneumonia (eg, fever and extent of oxygenation

296 impairment) and a CPIS >6 may serve as a surrogate tool to facilitate the diagnosis of ventilator-

297 associated pneumonia (30). In the present study, recruited patients have no significant different

298 SOFA or SAPS II scores and these scores did not correlate with any of the tested markers. The only

299 differences among the recruited patients were related to the CPIS score and CPIS score correlates

300 with TLR4 mini-BAL expression. Future studies on a larger cohort of patients are needed to clarify

301 whether the assessment of TLR4 expression in miniBAL may improve the predictive value of CPIS

302 to early identify patients with ventilator-associated pneumonia.

303 In conclusion, patients with both CB and COPD who underwent an acute exacerbation have a

304 different alteration in the levels of IP-10 and in TLR4 expression in the airways and in the systemic

305   compartment while they have a similar alteration in Foxp3 expression in the airways and in the

306   systemic compartment. The inflammatory profile of the COPD patients in the present study may be

307   the result of the failure to properly clear the inflammatory cells after the exacerbation of the disease.

308

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413   **LEGENDS TO THE FIGURES**

414   **Figure1. Increased expression of TLR4 in mini-BAL but not in the peripheral blood of S and**

415   **of COPD.** Mini-BAL cells and paired blood samples were recovered from C (n=10), from S (n=8)

416   and from COPD (n=18) patients. The expression of TLR4 was assessed by immunocytochemistry

417   using an anti-TLR4 polyclonal antibody; * $p<0.05$ vs C; ** $p<0.05$ vs S; # $p<0.05$ vs autologous

418   peripheral blood. Data are expressed as median (25-75 percentiles).

419   **Figure 2. Absence of differences in the concentrations of IL-8 in mini-BAL and in the**

420   **peripheral blood of S and of COPD.** Mini-BAL supernatants and paired blood samples were

421   recovered from C (n=10), from S (n=8) and from COPD (n=18) patients. IL-8 concentrations were

422   measured by ELISA as described in "materials and methods" and are expressed as pg/ml. Data are

423   expressed as median (25-75 percentiles).

424   **Figure 3. Reduced concentrations of IP-10 in mini-BAL but not in the peripheral blood of**

425   **COPD.** Mini-BAL supernatants and paired blood samples were recovered from C (n=10), from S

426   (n=8) and from COPD (n=18) patients. IP-10 concentrations were measured by ELISA as

427   described in "materials and methods" and are expressed as pg/ml. * $p<0.05$ vs C; ** $p<0.05$ vs S; #

428    $p<0.05$ vs autologous peripheral blood. Data are expressed as median (25-75 percentiles).

429   **Figure 4. Reduced expression of Foxp3 in mini-BAL and in the peripheral blood of S and of**

430   **COPD.** Mini-BAL cells and paired blood samples were recovered from C (n=10), from S (n=8) and

431   from COPD (n=18) patients. Total proteins were extracted and analysed for Foxp3 expression by

432   western blot analysis. Membranes were then stripped and incubated with goat polyclonal anti–ß-

433   actin.**. A.** Densitometric analysis of Foxp3 expression. Signals corresponding to Foxp3 on the

434   various western blots were semiquantified by densitometric scanning, normalized and expressed

435   after correction with the density of the band obtained for beta-actin (mean±SD). * $p < 0.05$. **B**.

436   Representative western blot analysis for Foxp3 expression from C, S and COPD subjects. Data are

437   expressed as median (25-75 percentiles).

438   **Figure 5. Correlations between the expression of TLR4 in miniBAL and CPIS score.** The

439   expression of TLR4 in mini-BAL of C (n=10), S (n=8) and COPD (n=18) was correlated with

440   CPIS score by Spearman Correlation test.

Table 1: Demographic and clinical characteristics of the subjects:

Characteristic	C	S	COPD	P value
No. of subjects	**10**	**8**	**18**	
Age (yr)	73 (72-78)	79.5 (76-82)	76 (71-81)	n.s.
Male/Female	7/3	5/3	11/7	n.s.
Cigarette smoke Packs/years	-	45±20	50±21	n.s.
FEV1(% of predicted normal)	85±2.5	87±3.9	67±5*	*p<0.05 COPD vs C and S
FEV1/FVC (% of predicted normal)	77±3	76±2	65±2*	*p<0.05 COPD vs C and S
CPIS	2.3±1.1	3.3±1.4	3.7±0.9*	*P<0.05 COPD vs C
SOFA	9.1±1.2	8.7±1.3	8.9±1	n.s.
SAPS II	53.6±7.8	59.5±11	56±7	n.s.

Data are expressed as percentiles (age) or as mean ± SD.

1   **Table 2: Total and differential cell counts of mini-BAL**

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	C =10	S=8	COPD=18	P value
Mini-BAL cells Total number (X1000)/ml	536±269	919±167*	3,2±2,400*	* p<0.05 COPD and S vs C
% Neutrophils	46±30	48±17	83±13*	*p<0.05 COPD vs C and S
% Macrophages	51±29	52±16	17±13*	*p<0.05 COPD vs C and S
% Lymphocytes	2.7±1.3	0*	0*	*p<0.05 COPD and S vs C

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5   Results are expressed as mean ± SD.

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Table 2: Microbiology of miniBAL

	C with MiniBAL cultures Positive (n=4)	C with Mini BAL cultures Negative (n=6)	CFU
Patient#1		X	
Patient#2		X	
Patient#3	Candida		$\geq 10^3$/ml
Patient#4	Candida		$\geq 10^3$/ml
Patient#5		X	
Patient#6		X	
Patient#7	Candida		$\geq 10^3$/ml
Patient#8		X	
Patient#9		X	
Patient#10	Candida		$\geq 10^3$/ml
	S with MiniBAL cultures Positive (n=3)	S with Mini BAL cultures Negative (n=5)	CFU
Patient#1		X	
Patient#2		X	
Patient#3	Candida		$\geq 10^3$/ml
Patient#4	Candida		$\geq 10^3$/ml
Patient#5		X	
Patient#6		X	
Patient#7		X	
Patient#8	Acinetobacter Baumannii		$\geq 10^3$/ml
	COPD with MiniBAL cultures positive (n=7)	COPD with Mini BAL cultures negative (n=11)	CFU
Patient#1	Candida		$\geq 10^3$/ml
Patient#2		X	
Patient#3		X	
Patient#4	Acinetobacter Baumannii		$\geq 10^3$/ml
Patient#5		X	
Patient#6	Candida		$\geq 10^3$/ml.
Patient#7		X	
Patient#8	Pseudomonas Aeruginosa		$\geq 10^3$/ml
Patient#9		X	
Patient#10	Acinetobacter Baumannii		$\geq 10^3$/ml
Patient#11		X	
Patient#12		X	
Patient#13	Candida		$\geq 10^3$/ml
Patient#14		X	
Patient#15		X	
Patient#16	Pseudomonas Aeruginosa		$\geq 10^3$/ml
Patient#17		X	
Patient#18		X	

1   **Table 2: Total and differential cell counts of mini-BAL**

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	C =10	S=8	COPD=18	P value
TLR4 expression in Mini-BAL cells (% of positive cells)	21.9±6.4	39.8±3.8	64.88±4.8	*p<0.05 COPD and S vs C
TLR4 expression in peripheral blood cells (% of positive cells)	25.4±6.5	17.5±1.76	26.8±6.37	n.s.
Foxp3 expression in Mini-BAL cells (arbitrary units)	2.5±0.24	1.83±0.12*	1.39±0.41*	*p<0.05 COPD and S vs C
Foxp3 expression in peripheral blood cells (arbitrary units)	3.79±0.3	2.36±0.36	1.75±0.53	*p<0.05 COPD and S vs C

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5   Results are expressed as mean ± SE.
6   * Mann Whitney test
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Figure 1

TLR4 expression in miniBAL and in Peripheral blood
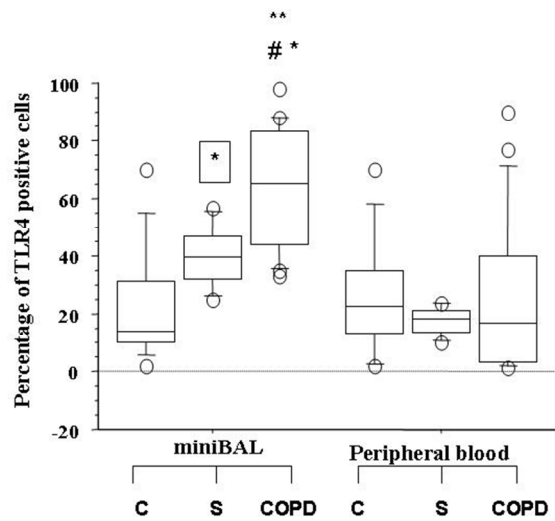
254x190mm (96 x 96 DPI)

Figure 2
IL-8 Concentrations in miniBAL and in Peripheral blood
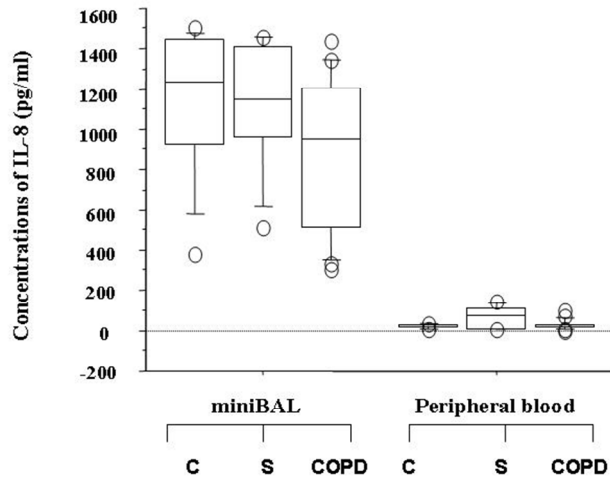
254x190mm (96 x 96 DPI)

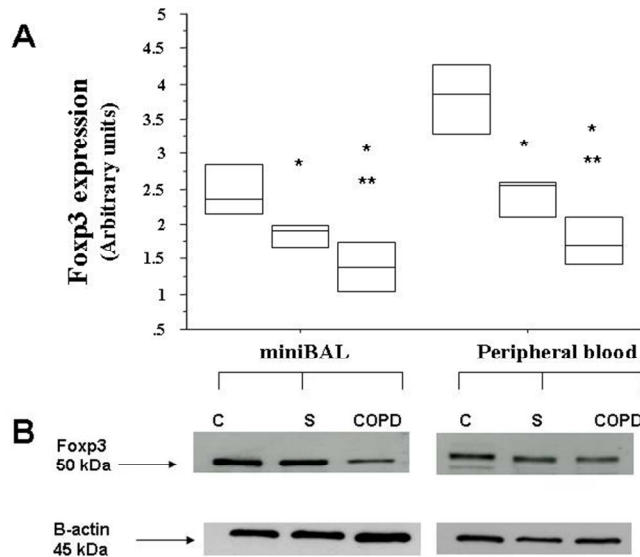# Figure 3
IP-10 Concentrations in miniBAL and in Peripheral blood
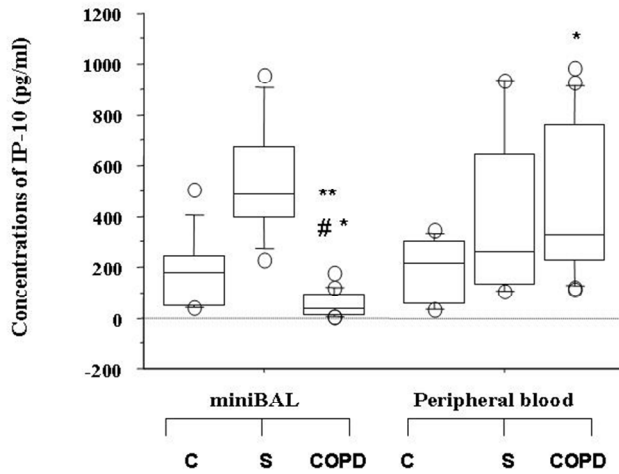
254x190mm (96 x 96 DPI)

Figure 4
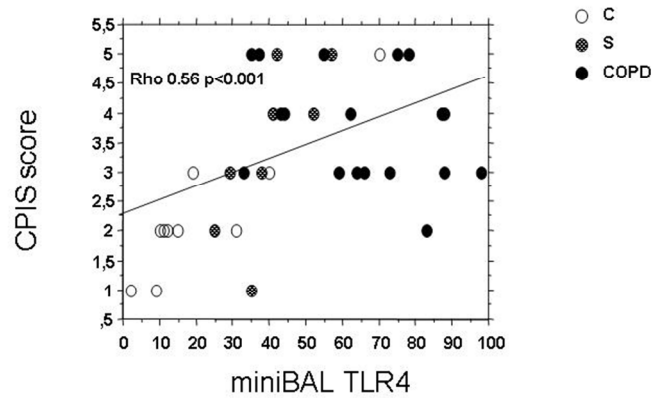## Foxp3 expression in miniBAL and in Peripheral blood

254x190mm (96 x 96 DPI)

Figure 5
TLR4 expression in miniBAL correlates with CPIS score

254x190mm (96 x 96 DPI)