

Macrophage activation in exacerbated chronic obstructive pulmonary disease with and without community-acquired pneumonia

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ABSTRACT:

In large series of nonresponding community-acquired pneumonia (CAP) patients, chronic obstructive pulmonary disease (COPD) was observed to be a protective factor for nonresponse to initial antibiotics. This intriguing fact may be linked to changes in the phenotype of inflammatory cells and, in particular, to the induction of classical-M1 or alternative-M2 activation of macrophages, which result in different inflammatory profiles.

We evaluated the effect of sputum obtained from patients with acute exacerbation of COPD (AECOPD), CAP, and COPD plus CAP on the phenotypic changes in macrophages. Human THP1 cells differentiated to macrophages were incubated with sputum from patients with AECOPD, CAP or COPD+CAP, and expression of TNF α , IL-6, mannose receptor and arginase was measured to evaluate the phenotype acquired by macrophages. We found that sputum from CAP patients induced the M1 phenotype and that from AECOPD patients induced an M2-like phenotype. Sputum from CAP+COPD patients did not present a clear M1 or M2 phenotype.

These results indicate that microenvironment in the lung modulates the activation of macrophages, resulting in different phenotypes in AECOPD, CAP and COPD+CAP patients. This different type of activation induces different inflammatory responses and may be involved in the different outcome observed when COPD and CAP present simultaneously.

Key words: COPD, CAP, Macrophages, Inflammation, Sputum

INTRODUCTION

Community-acquired pneumonia (CAP) is a common cause of acute illness in adults and one of the most frequent causes of hospital admission associated with significant mortality rates. The mortality rate is very high among hospitalized patients, ranging from 5% to 15%, and represents the most frequent infectious cause of death among patients of all ages [1].

In these patients, the principal risk factors for acquiring CAP are old age and pulmonary comorbidities such as chronic obstructive pulmonary disease (COPD). Indeed, CAP is a frequent event in the course of COPD, a fact explained mainly by the abnormal pulmonary defence mechanisms. For this reason, a number of clinical and epidemiological studies have been carried out in order to determine whether COPD was a predictor of increased mortality in patients hospitalized due to pneumonia.

Surprisingly, some studies did not find differences in 30-day mortality when comparing COPD patients to non-COPD patients with CAP [2]. This is an intriguing finding since one would expect a higher mortality in such a severe population, and is a finding that has generated debate in clinical practice [3-5]. Furthermore, in a recent study of patients with antibiotic treatment failure, Menendez et al [6] found that, in large series of nonresponding CAP patients, COPD was a protective factor for nonresponse to initial antibiotics. In this line, it has been recently reported that, in a series including thousands of hospitalized CAP patients, the mortality of COPD was the lowest (10%) compared with a series of comorbidities that even reached 25% mortality [7].

There may be several explanations for this. First, it is known that in up to 50% of COPD patients, the lower airways have an increased inflammatory status linked to the colonization of the lower airways by pulmonary pathogens [8]. This status is related to changes in cytokines such as TNF α or IL-6 and other inflammatory mediators, and also with the cellular stress response and the generation of anti-inflammatory and protective mediators. This different microenvironment may produce protective effect against bacterial attack.

Another possible factor is the acute administration of steroids that also inhibits the inflammatory response, thereby modifying the interactions between COPD and CAP [9]. We do not have clinical data regarding the percentage of hospitalized COPD patients who receive steroids in clinical practice. However, in a study designed to evaluate the effect of steroids, Confalioni et al. observed that hydrocortisone infusion

in severe CAP attenuates systemic inflammation and leads to earlier resolution of pneumonia and a reduction in sepsis-related complications and mortality [10].

In the regulation of inflammation, macrophages play a central role in modifying the duration and magnitude of the inflammatory response. These cells may adopt different phenotypes induced by different stimuli. The classically activated (M1) macrophages promote inflammation, extracellular matrix destruction and bactericidal activities. In contrast, the alternatively activated (M2) macrophages promote tissue regeneration, angiogenesis, cell proliferation and inhibition of the inflammatory response [11].

In CAP patients, the classical M1 macrophage phenotype is induced by bacterial products and the cytokines involved in the initial inflammatory response. In contrast, alveolar macrophage activation in COPD patients appears to be more complex. This is because inflammation, airway remodelling, and parenchymal destruction are established features of COPD and include both M1 (inflammation) and M2 (remodelling) stimuli. However, some reports indicate that a down-regulation of M1 genes and a gradual up-regulation of some M2-related genes can be observed in COPD patients [12].

This polarized response between CAP and COPD in terms of macrophage activation is the consequence of different microenvironmental conditions in these pathologic conditions. It could thus be suspected that the combination of COPD and CAP will result in a particular lung environment and in a particular stimulus for macrophage activation. Since this different inflammatory status may be an explanation for the protective effect of COPD it may be important to evaluate the effect of mediators present in the airways of this particular group of patients on the activation of macrophages. Despite the differences with respect to bronchoalveolar lavage, [13] induced sputum has been shown to be a reproducible noninvasive approach for determining the presence of inflammatory cells and mediators in the airways of the lungs [14].

In this study, we designed a series of experiments to evaluate the effect of the microenvironment in the lungs on modulating the inflammatory response. For this purpose, we measured the effect of sputum obtained from patients with acute exacerbation of COPD (AECOPD), CAP and COPD plus CAP on the phenotypic changes on macrophages *in vitro*.

MATERIAL AND METHODS

Study design

In the initial experiment, we evaluated the effect of mediators present in the lung microenvironment of patients with CAP + COPD on the activation of macrophages. We compared this activation with that obtained from patients with only COPD or CAP. For this purpose, cultured macrophages from the human macrophage cell line THP1 were incubated with sputum obtained from patients corresponding to groups CAP + COPD, CAP, COPD or Control. Sputum was filtered in order to remove bacteria, viruses or cell debris, thus ensuring that the phenotypic changes observed were induced only by soluble mediators. An unstimulated cell line was used instead of alveolar macrophages from patients, in order to avoid possible interference by previous activations due to pharmacological treatments or environmental pollutants.

In the next experiment, we evaluated how the bacterial products modify the response of macrophages to COPD mediators. THP1 cells incubated with sputum from COPD patients were also treated also LPS, and the resulting phenotypes were compared with that obtained in the absence of LPS. Finally, the HL-60 macrophage cell line, which does not express CD14 (a co-receptor along with TLR 4 for the detection of LPS in leukocytes), was used to evaluate the phenotypic changes induced not by bacterial products but by other mediators present in sputum.

Patients and controls

We included three types of patients:

- 1-COPD patients with Community-acquired pneumonia requiring hospitalisation
- 2-Exacerbated COPD patients without community-acquired pneumonia
- 3- Community-acquired hospitalized patients without COPD

Controls were defined as healthy individuals that did not have any of the above conditions

Community-acquired pneumonia (CAP) was defined as a new infiltrate in chest x-ray and at least two compatible clinical symptoms (fever, cough, expectoration, pleuritic chest pain) [15]. Exclusion criteria for enrolling patients were patients admitted in the previous 15 days, patients undergoing treatment with cytostatic and/or other immunosuppressive therapy and HIV-positive patients.

COPD was defined according to a functional respiratory criteria (FEV1 <70%, FEV1 PD test <60%, and FEV1/FVC <70% measured before hospitalization), together with an age of more than 20 years, a history of smoking at least of 20 pack-years and a compatible clinical history [16]. Acute exacerbation was defined according to the criteria of Anthonisen et al [17], which includes increased dyspnea, sputum volume and purulence (Anthonisen type I exacerbation). All patients from the three groups were hospitalized on the decision of the attending physician. All patients (COPD with pneumonia, exacerbated COPD without pneumonia and pneumonia without COPD) were treated with antibiotics according to local guidelines. All patients with exacerbated COPD without pneumonia received intravenous steroids for between three and five days and dosages were then gradually reduced (initial dosage, 60 mg/12 h). All samples were obtained during the first 24 h after admission and before the administration of steroids. We studied a total of 50 cases, of which 16 had an acute exacerbation of COPD without pneumonia, 11 had COPD and CAP, 15 had CAP without COPD, and 7 were control patients (Table 1).

Sample collection

Sputum from patients was obtained from spontaneous cough within the first 24 hours. Gram stain and sputum culture was processed only if the sample was adequate. Sputum was considered valid if the sample contained more than 25 leukocytes and fewer than 10 epithelial cells per field (100).

Induced sputum in control group:

Sputum production was induced by inhalation of a 5% hypertonic saline solution for between 5 and 10 minutes. Hypertonic saline was delivered by a nebulizer device (Ultraneb 2000; DeVilbiss Healthcare Inc, Somerset, PS, USA) attached to an oxygen supply. Subjects were then encouraged to cough and expectorate in a sterile plastic container.

Macroscopic saliva was removed and samples were collected in an Eppendorf tube. The volume of the sputum was measured and a volume of Sputolysin (Calbiochem-Behring, USA) was added to the sputum in proportion of 1:4, the sample was shaken for 15 minutes at room temperature and then filtered through a 48- μ m nylon mesh to remove mucus and squamous cells. The suspension was then centrifuged at 2000 rpm for 10 minutes. The supernatant was collected and 500 μ L were filtered in a Vivaspin 100 K spin concentrator in order to remove bacteria, viruses and cell debris. Finally, the sample was stored at -70°C for subsequent analysis.

Cell culture

Human monocyte THP1 and HL60 myeloid leukaemia cell lines were cultured in suspension in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 95% air, 5% CO₂ at 37°C. Cells were differentiated to macrophages by a first incubation with 0.16 mM phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, Mo, USA) for 48 hours at 37°C in plastic Petri dishes (Nunclon; Nunc Inc., Naperville, IL, USA). PMA was removed 24 hours before the experiments. This treatment results in the induction of a macrophage-like phenotype for these cell lines [18-19].

In initial experiments sputum from patients with COPD or CAP was added in duplicate to the media at a concentration of 20%, and THP1 or HL60 cells were incubated for 24 hours. After incubation, RNA was obtained and the expression of markers for M1 and M2 phenotypes was analyzed. In subsequent experiments, the effect of sputum from patients with COD plus CAP was also evaluated. Saline solution treated with Sputolysin and processed as sputum was used for the control samples.

In some experiments, LPS (*E.coli* 0111:B4) (Sigma Chemical Co., St. Louis, Mo, USA) was added to a final concentration of 100 ng/mL.

RNA extraction and RT-PCR:

Total RNA from cells was extracted using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). One µg total RNA was used for amplification using the Invitrogen One Step RT-PCR System according to the manufacturer's instructions. The following primers were used: TNF-α forward 5'-cttctccttctgatcgtgg-3' and reverse 5'-gctggttatctctcagctcca-3'; IL-6 forward 5'-aaagaggcactggcagaaaa-3' and reverse 5'-gaggtgccccatgctacattt-3'; Mannose Receptor forward 5'-tgacacacttttggggatca-3' and reverse 5'-aaactgaacgggaatgcac-3'. Fragments were amplified using 30-35 cycles of PCR, each cycle consisting of 15 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C. The resulting RT-PCR products were electrophoresed on 2% agarose gels with DNA markers, stained with ethidium bromide, and viewed under UV light. GAPDH was used as an internal control for stable expression (housekeeping gene): the forward primer was 5'-tcctctgacttcaacagcgacacc-3' and the reverse primer was 5'-tctctcttctcttctgtctcttgc-3'. The public-domain software ImageJ version 1.32 was used to quantify the intensities of the bands obtained.

Cell viability:

The cytotoxic effects of different sputums on macrophages were quantified using a 3-(4,5-dimethylthiazole 2-yl)-2,5 diphenyl-tetrazolium bromide (MTT) cellular proliferation assay (Chemicon International, Inc, Temecula, CA). Cells were seeded at 1×10^4 cells/well (0.1 ml) on a 96-well plate and incubated with sputum for 24 hours at 37°C. Then, the cells were incubated for a further 2 hours with 0.02 ml MTT reagent at 37°C. After incubation, absorbance was measured by spectrophotometry at 570 nm.

Statistical analysis:

Data have been expressed as mean \pm SEM. Means of different groups were compared using a one-way analysis of variance. The Tukey multiple comparison test was performed for to evaluate significant between-group differences. Differences were assumed to be significant when $p < 0.05$.

RESULTS

Patients

We studied 50 cases of which 16 had AECOPD without pneumonia, 11 had COPD and CAP, 15 had CAP without COPD and 7 control patients. All AECOPD patients were Class I or II according to the Anthonisen [16] criteria.

The main general characteristics of these patients are shown in Table 1

Patients with CAP (with and without COPD) were treated with levofloxacin or with the combination of a beta-lactam + a macrolide (usually ceftriaxone+ azithromycin) All patients with exacerbated COPD without pneumonia received intravenous steroids for three to five days and dosages were then gradually reduced (initial dosages: 60 mg/12 h). All patients in all the three categories survived

Cell viability

Macrophages treated with increasing concentrations (to 40%) of sputum do not show changes in cell viability. Consequently, a 20% sputum concentration in cell culture media was used in all the experiments.

Changes in macrophage phenotype

Macrophage differentiated THP1 cells treated with AECOPD sputum showed an increased expression on the mannose receptor as well as Arginase mRNA (Figure 1). However, despite the absence of an observed increase in TNF- α expression, IL-6 showed significantly increased induction. This result is similar, but not identical, to what would be expected during an M2 differentiation. Moreover, CAP sputum induces an M1 response in these cells, characterized by the lack of changes in mannose receptor or arginase and significant increases in both TNF- α and IL-6 expression. Finally, in cells treated with sputum obtained from patients with CAP plus COPD a significant increase was only observed in IL-6. Increases in mannose receptor, TNF- α and arginase were observed but were not significant.

Effect of LPS on cytokine expression

The induction of IL-6 in macrophages treated with AECOPD sputum may be linked to the presence of low levels of bacterial products. To evaluate this possibility, we analyzed the effect of LPS in cells treated with AECOPD sputum. As expected, LPS induces the expression of IL-6 and TNF- α in control cells (Figure 2). No additional IL-6 induction was observed in CAP-treated cells but a significant increase was found in AECOPD-treated cells, suggesting the involvement of different mechanisms between COPD and CAP activation.

Activation in the presence of CAP sputum may be linked to the presence of microbial products in the medium. Since CD14 acts as a co-receptor, along with TLR4, to detect LPS in leukocytes, we evaluated the effects on HL60 cells, a cell line similar to THP1 but which, when differentiated to macrophages does not express CD14 (figure 3A). When the same experiment was carried out in HL60 cells (Figure 3B), no additional increases were observed in AECOPD treated cells.

DISCUSSION

The main finding of this study was the observation that microenvironment present in the lung may modify the progression of the inflammatory response by inducing a different macrophage-activation phenotype in AECOPD, CAP or COPD+CAP patients. The progression of CAP and COPD generates different pathophysiological situations. Consequently, the response induced by the defence mechanisms in the lung could be expected to be highly different, resulting in clearly differentiated lung microenvironments. However, when these two pathological processes occur simultaneously, the resulting cell environment in the lung may potentiate or inhibit several homeostatic mechanisms involved in the tissue response to aggression [5]. The resulting response may then be deleterious and in severe CAP patients with previous COPD, it may result in a higher mortality rate than in non-COPD patients. For this reason, the finding that, in a particular group of patients with antibiotic failure, COPD is a protective factor is highly surprising [6].

In this case, changes in the lung microenvironment may result in a different cell response to the aggression. In particular, inflammatory and immune cells are highly sensitive to mediators present in the lung, and the particular activation profile has a profound effect on the activation of inflammatory cells in response to the aggression [11].

We tested this possibility by treating macrophage-differentiated THP1 cells with sputum obtained from AECOPD or CAP patients and comparing the activation profile triggered with that resulting from the treatment of these cells with sputum from COPD + CAP patients. Induced sputum has been used as a non-invasive tool for investigating the airways. It provides the mediators present in the large airways and several reports show a correlation between inflammatory cells and cytokines present in sputum and in bronchoalveolar lavage fluid. In our experimental conditions, the sputum was filtered to avoid the presence of inflammatory cells or microorganisms. Hence, the observed effects are linked only to the presence of soluble mediators in the pulmonary microenvironment. In addition, the use of an unstimulated cell line avoids the interfering effect of previous corticosteroid therapy or other factors that might modify the response of alveolar macrophages from patients.

As expected, macrophages incubated with sputum obtained from CAP patients showed an increased expression of the inflammatory cytokines TNF- α and IL-6. This may be

associated with the classical M1 activation [20] associated with the antimicrobial activity of macrophages (Figure1). In contrast, no changes were observed in arginase or mannose receptors associated with the alternative M2 activation of macrophages. These results indicate that there are mediators in the pulmonary milieu that promote the M1 antimicrobial activation of macrophages. Clearly, bacterial products may be involved in this effect, together with different cytokines or lipid mediators that also promote and maintain the M1 response, including interferon- γ and other macrophage-derived cytokines that result in a positive pro-inflammatory feedback.

Furthermore, treating the macrophages with sputum from AECOPD patients results in the induction of a clearly different phenotype. In this case, the induction of a response similar to M2 seems to be induced. Macrophages showed an induction of arginase and mannose receptor, and no induction was observed for TNF- α , suggesting activation of the alternative phenotype [20]. However, IL-6 is induced in macrophages treated with AECOPD sputum, and IL-6 is inhibited during the characteristic M2 activation. In fact, COPD is considered a Th1 disease, but it is known that some Th2 cytokines, such as IL-4, are also present in greater amounts in bronchoalveolar lavage fluid from AECOPD patients [21]. It is therefore not surprising that the cell response to the sputum was not a theoretic, ideal, M1 or M2 profile.

More interesting are the differences observed in macrophages treated with sputum from COPD+CAP patients. In this case, only a moderate, non-significant, increase was observed in arginase, mannose receptor and TNF- α expression and only induction of IL-6 was maintained. This activation indicates the absence of a clear M1 or M2 response in the macrophages.

Of note is the fact that the sputum from COPD+CAP patients induces a different activation of macrophages than the sputum from CAP or AECOPD patients. This is because macrophages play a central role in the regulation of the inflammatory response as well as in the repair processes. Situations that modify the macrophage activation may have a strong effect on the final resolution of the disease process. In the case of COPD+CAP patients, a protective effect may exist due to reduced tissue damage associated with the less intense inflammatory response.

These changes in induction may be related to the presence of bacterial products in combination with the cytokines generated during AECOPD. As indicated, sputum was filtered in order to avoid the presence of cells or bacteria when macrophages are

incubated, but the presence of LPS or other bacteria-derived products of low molecular weight may be expected.

To evaluate the effect on macrophage activation of bacterial products in an AECOPD-derived microenvironment, we incubated macrophages with sputum from COPD or CAP patients, and a dose of LPS was added to the cell culture medium. In these conditions (Figure 2), the expression of TNF- α was strongly increased in all experimental groups, indicating that TNF- α expression induced by CAP sputum does not achieve maximum cell capacity. In contrast, adding LPS to the medium increased the expression of IL-6 induced in macrophages treated with control sputum, but had no effect when the sputum was obtained from CAP patients, reflecting the different regulation between TNF- α and IL-6. The strong response of TNF- α agrees with that reported by Hackett et al [22] using lung tissue explants incubated with LPS. More interesting is the fact that sputum from AECOPD patients clearly induces the expression of IL-6, but the addition of LPS results in an additional induction. This fact indicates that IL-6 induction associated with AECOPD involves mechanisms different from that associated with CAP. It may be that IL-6 expression in macrophages is directly induced by LPS in CAP and by inflammatory cytokines in COPD.

This was confirmed in additional experiments by using the HL60 cell line, that could also be differentiated to macrophages by treatment with TPA, but that do not express CD14 [18] when differentiated (Figure 3A). Since CD14 plays a role in the detection of bacterial LPS, together with TLR [23], the activation observed in these cells must be related mainly to cytokines present in sputum, with bacterial products playing a far less important role. In these conditions, the induction of IL-6 in response to AECOPD sputum was maintained but the additional response to LPS was lost (Figure 3B).

This suggests that the microenvironment present in the lung in these pathological conditions is able to modulate the activation of macrophages, resulting in different phenotypes. In patients with COPD plus CAP, this phenotype is different from that induced by mediators present in the lungs of patients with AECOPD or CAP. This different activation may result in a different inflammatory response and may be involved in the different outcome observed when these two entities (COPD and CAP) present simultaneously.

From a clinical point of view, the results of this study reopen the debate on short-term mortality of COPD patients with CAP. Since steroids did not affect the results of the

study we can speculate that a downregulation in the inflammatory response occurs in COPD patients when they are challenged by a new, acute aggression, such as pneumonia

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Table I: General characteristics of patients studied.

	CONTROLS	AECOPD	CAP+	COPD + CAP
N	7	16	15	11
Age (y)± SD	30.86 ±5.49	70.29 ± 10.76	69.79 ±13.08	65.4± 13.43
Sex (M/F)	4/3	14/2	10/5	9/2
Non smokers (%)	71.4	0	28.6	11.1
Smokers (%)	28.6	35.7	28.6	11.1
Ex-smokers (%)	0	64.3	42.9	77.7
FEV1 % of predicted values (± SD)	-	33.85 (± 12.7)	-	44.37 (± 24.97)
FVC/FEV1 (± SD)	-	53.78 (± 15.07)	-	66.12 (± 26.63)

Abbreviations: AECOPD, Acute exacerbation of COPD Anthonyssen type I; CAP, community-acquired pneumonia; COPD+ CAP, chronic obstructive pulmonary disease + community-acquired pneumonia.

Figure 1: Changes in expression of M1- (TNF- α and IL-6) and M2- (mannose receptor and arginase) related mediators in macrophage-differentiated THP1 cells after incubation with 20% sputum for 24 hours. Results are expressed as increases with respect to the control group. AECOPD indicates acute exacerbation of chronic obstructive pulmonary disease; CAP, community-acquired pneumonia.

*=p<0.05 with respect to Control

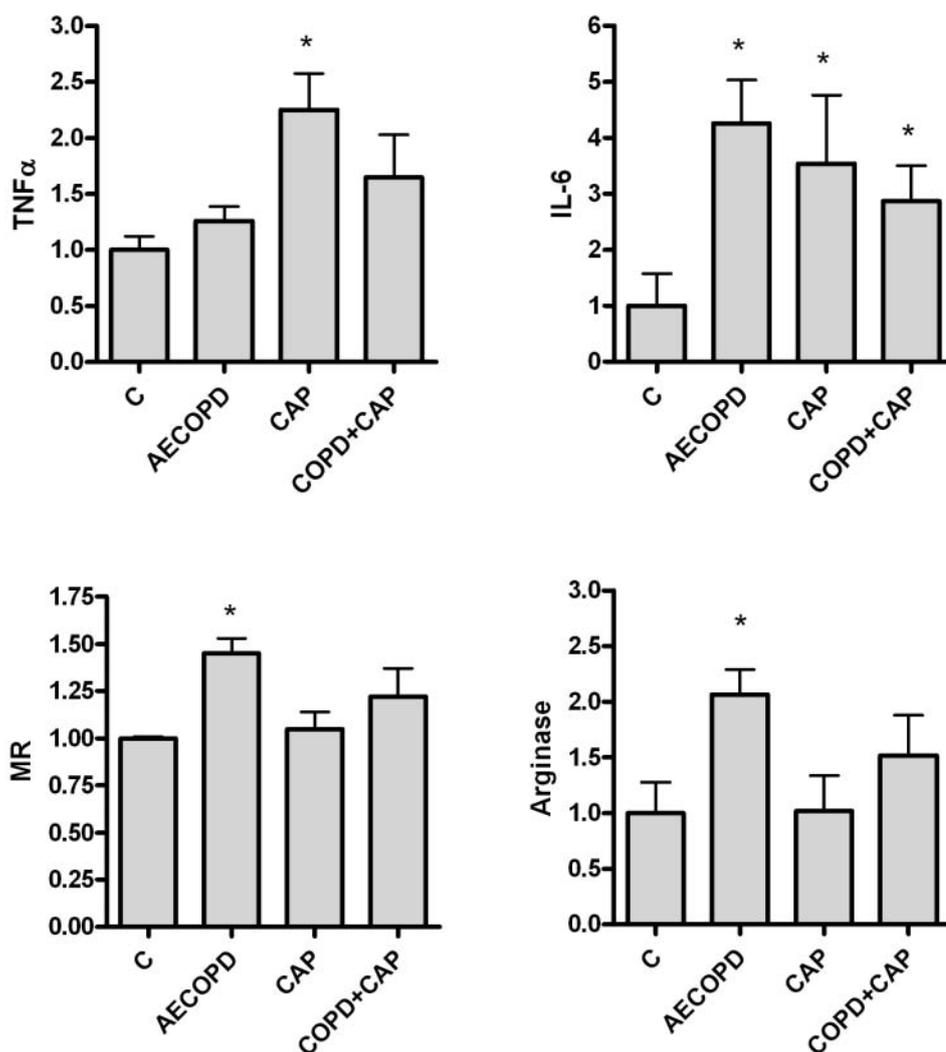


Figure 2: Effect of LPS (100 ng/mL) treatment on macrophage-differentiated THP1 cells incubated with 20% sputum for 24 hours. Results are expressed as increases with respect to the

control group. AECOPD indicates acute exacerbation of chronic obstructive pulmonary disease; CAP, community-acquired pneumonia.

* = $p < 0.05$ with respect to Control; + = $p < 0.05$ with respect to Non-treated

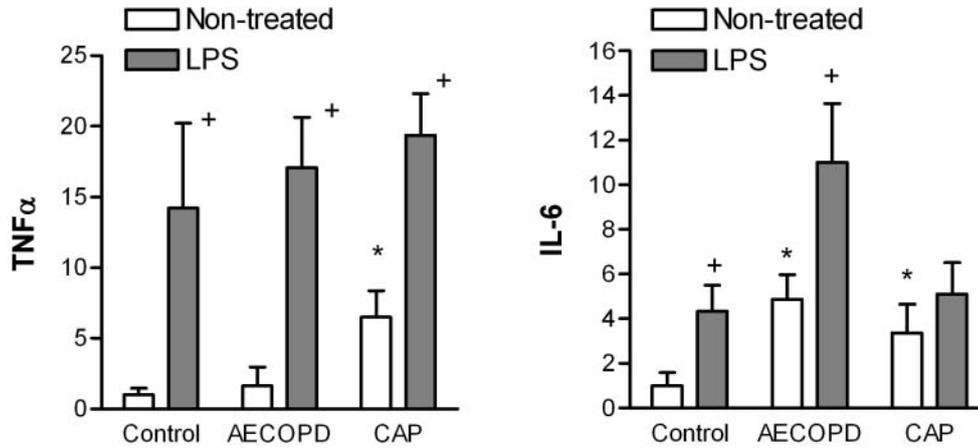


Figure 3: (A) Lack of CD14 expression in macrophage-differentiated HL60 cells. After TPA treatment, both THP1 and HL60 cells acquire a macrophage-like phenotype and express CD11b. In contrast, CD14 was expressed by only in THP1 cells. (B) Effect of LPS (100 ng/ml) treatment on macrophage-differentiated HL60 cells incubated with 20% sputum for 24 hours. Results are expressed as increases with respect to the control group. AECOPD indicates acute exacerbation of chronic obstructive pulmonary disease; CAP: community-acquired pneumonia.

* = $p < 0.05$ with respect to Control; + = $p < 0.05$ with respect to Non-treated

