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Increased lung neutrophil apoptosis and inflammation resolution in nonresponding pneumonia

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ABSTRACT: Neutrophil activation state and its relationship with an inflammatory environment in community-acquired pneumonia (CAP) remain insufficiently elucidated. We aimed to evaluate the neutrophil apoptosis and cytokine pattern in CAP patients after 72 h of treatment, and their impact on infection resolution.

Apoptosis of blood and bronchoalveolar lavage (BAL) neutrophils was measured in nonresponding CAP (NCAP), in responding CAP (blood only) and in patients without infection (control). Proinflammatory (interleukin (IL)-6, IL-8) and anti-inflammatory (IL-10) cytokines were measured. Main outcomes were clinical stability and days of hospitalisation.

Basal neutrophil apoptosis was higher in the BAL and blood of NCAP, whereas spontaneous apoptosis (after 24 h culture) was lower. Cytokines in NCAP were higher than in responding CAP and control: IL-6 was increased in BAL and blood, IL-8 in BAL and IL-10 in blood. An increased basal apoptosis ($\geqslant$20%) in BAL of NCAP was associated with lower systemic IL-10 ($p<0.01$), earlier clinical stability ($p=0.05$) and shorter hospital stay ($p=0.02$). A significant correlation was found for systemic IL-6 and IL-10 with days to reach stability and length of stay.

After 72 h of treatment, an increased basal alveolar neutrophil apoptosis might contribute to downregulation of inflammation and to faster clinical stability.

KEYWORDS: Bronchoalveolar lavage, cytokines, length of stay, neutrophil apoptosis, nonresponding community-acquired pneumonia

Community-acquired pneumonia (CAP) has an incidence of five per 1,000 adults [1] and its mortality ranges between 5% and 15% of hospitalised patients. An adequate host response facilitates microbial killing while limiting excessive inflammation and tissue damage [2]. However, ~10% of CAP patients develop nonresponding CAP (NCAP), which leads to delayed resolution and poor outcome [3].

In CAP, activated neutrophils in the blood migrate and accumulate in the lungs, ingest and kill bacteria and, finally, undergo apoptosis. Macrophages remove apoptotic neutrophils, precluding subsequent release of inflammatory cytokines and promoting resolution of inflammation [4, 5]. Although neutrophils are usually short-lived immune cells, their lifespan prolongation is critical in their efficacy. However, as neutrophils' lytic enzymes can also induce organ damage, their activation and survival must be tightly regulated [5–9].

In NCAP, a persistent lung and systemic inflammation has been reported [10–12]. However, the state of neutrophil activation remains to be elucidated. We hypothesised that the persistence of activated neutrophils in the lungs in NCAP might be contributing to the maintenance of inflammation and to a delayed resolution.

We aimed primarily to evaluate the neutrophil apoptosis and the cytokine pattern (interleukin (IL)-6, IL-8 and IL-10), both in the lung and in blood, in hospitalised patients with NCAP. Secondarily, we investigated their impact on the clinical parameters of stability and length of stay (LOS).

METHODS AND MATERIALS

Study design

A prospective study was performed in admitted patients with CAP. Pneumonia was defined as the presence of a new infiltrate with concordant symptoms and laboratory results (fever $\geqslant$38°C, purulent sputum and leukocyte count $\geqslant$12,000 mm^{-3}). Exclusion criteria were admission in the previous 15 days and immunosuppressive treatment.

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The inclusion criteria in the NCAP group were: fulfilment of clinical conditions of NCAP after 72 h of treatment (see definition below) and performance of bronchoscopy indicated by the physician in charge. Patients were included in the study on the day when they underwent bronchoscopy.

Two control groups matched by age (±10 yrs) and comorbid conditions were included: 1) the CAP group comprised patients with CAP who reached clinical stability (definition below); 2) the control group comprised patients without infection referred to bronchoscopy for peripheral lung nodules or minor haemoptysis.

We collected data on age, sex, smoking habits, comorbidities, clinical signs and symptoms, Pneumonia Severity Index [13], chest radiograph, biochemical analyses, microbiological findings, and previous and current antibiotic treatment.

The study was approved by the ethics committee of the University and Politecnic Hospital La Fe (Valencia, Spain) and written informed consent was obtained. No bronchoalveolar lavage (BAL) fluid was obtained in the CAP group because the ethics committee would not approve bronchoscopy in patients with adequate response.

Definitions

NCAP was defined as persistence of a high temperature (≥38°C) and/or clinical symptoms after 72 h of antibiotic treatment, chest radiographic progression of pneumonia (>50% increase of infiltrates along with persistence of high temperature and/or clinical symptoms), empyema, septic shock and/or the need for mechanical ventilation [12].

The CAP group comprised patients who fulfilled clinical stability criteria [14, 15]. Stability was defined as the achievement of all the following parameters: temperature ≤37.2°C, heart rate ≤100 beats per min, respiratory rate ≤24 breaths per min, systolic blood pressure ≥90 mmHg, and oxygen saturation ≥90% or arterial oxygen tension ≥60 mmHg without supplemental oxygen.

LOS was calculated as the number of days from admission until discharge.

Obtaining and processing of BAL

BAL was performed according to recommended guidelines [16]. In the NCAP group, BAL was performed in the involved lobe, and in those patients with diffuse pulmonary infiltrates or in the control group, in the middle lobe or lingula. Five aliquots of sterile saline solution were instilled and immediately aspirated. The first aliquot (20 mL) was discarded. The remaining four aliquots (30 mL each) were pooled together in a single siliconised sterile glass: half of these four aliquots were sent to a microbiology laboratory and the other half to a biochemistry laboratory. The mean±SEM volume of BAL fluid obtained was 39±3 mL.

Neutrophil purification from BAL samples

The BAL samples were immediately pelleted by centrifugation at 353×*g* for 5 min at 4°C. The supernatant volume was measured and frozen. The pellet was resuspended with culture medium (RPMI 1640 without phenol red; Invitrogen, Carlsbad, CA, USA), passed through two sheets of gauze, and centrifuged at 353×*g* for 5 min at 4°C. The cell pellet was washed twice with PBS and resuspended with hypotonic solution (NH_4Cl 155 mM, $NaHCO_3$ 2.46 mM, EDTA·$4H_2O$ 3.72 mM; pH 7.4) for 5 min on ice. After centrifugation at 304×*g* for 5 min at 4°C, the pellet was resuspended in PBS. The neutrophil amount and viability were determined using a trypan blue exclusion method [17, 18]. For light microscopy studies, cytospin slides were stained by the Panoptic method (Panreac Quimica SA, Barcelona, Spain).

Preparation of blood samples

In NCAP, blood samples were obtained before bronchoscopy, and in the CAP group, after reaching stability. Plasma aliquots were withdrawn before neutrophil isolation for cytokine determination by ELISA. The isolation of neutrophils was performed as previously described [19]. The pellet was resuspended with a hypotonic solution for 5 min on ice, and spun down with PBS at 304×*g* for 5 min to pellet the neutrophils (similar to BAL samples). Cells were resuspended in PBS and counted.

Detection of apoptosis by flow cytometry

Neutrophils were stained with Annexin V detection kit (TACS™ Annexin V-fluorescein isothiocyanate (FITC); R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Samples were analysed using an EPICS XL (Beckman-Coulter, Hialea, FL, USA) [20]. Neutrophils were gated according to their forward and side scatter characteristics with a minimum of 10,000 gated events from each sample. Controls were included to set up compensation with no stained cells and cells stained with Annexin V or propidium iodide alone. Apoptotic cells were distinguished from normal and necrotic cells by labelling with Annexin V and exclusion of propidium iodide. Each subpopulation was expressed as a percentage of the total neutrophil population.

In addition, staining with CD16 FITC (IOTest; Beckman-Coulter) in separate aliquots of BAL and blood samples was applied to detect the positive neutrophil population.

For detection of apoptosis increments (spontaneous apoptosis) after 24 h of *in vitro* culture, neutrophils were seeded in RPMI supplemented with 10% FBS, L-glutamine and antibiotics (penicillin and gentaminicin; Invitrogen) and cultured at 37°C for 24 h.

Measurement of cytokines IL-6, IL-8 and IL-10 in BAL and plasma

The determination of IL-6, IL-8 and IL-10 was performed with a commercial enzymoimmunoassay technique (Pharmingen, BD Biociencias, Madrid, Spain) following the instructions of the manufacturer. The limits of detection were 4.7 $pg \cdot mL^{-1}$ for IL-6, 3.1 $pg \cdot mL^{-1}$ for IL-8, and 7.8 $pg \cdot mL^{-1}$ for IL-10.

Statistical analysis

Statistical analysis was performed using the SPSS 12.0 and GraphPad software (San Diego, CA, USA). The Chi-squared test was used for categorical variables, and the Mann–Whitney U-test for continuous variables. Correlation was analysed using Spearman's rho correlation analysis. Data are shown as mean±SEM unless otherwise indicated. Cytokine levels and LOS are shown as medians and interquartile ranges (IQRs). A value of $p<0.05$ was considered statistically significant. The end-point variables were analysed with regard to the basal

neutrophil apoptosis in BAL stratified as high (⩾20%, 75th percentile of basal apoptosis) or low (<20%) apoptosis rates.

RESULTS

General characteristics of groups

60 patients (29 NCAP, 15 CAP and 16 controls without infection) were recruited. Six NCAP patients were excluded due to an alternative diagnosis: one patient with tuberculosis, three with lung cancer, one with bronchiolitis obliterans and organising pneumonia, and one with invasive pulmonary aspergillosis, leaving 23 in the study group. Features of NCAP were: 11 patients with persistent fever and clinical worsening, nine with progression of infiltrates in chest radiograph (two with pleural effusion), and three with respiratory insufficiency requiring invasive ventilation; one of these patients died. The control group without infection included 16 adults, seven with minor haemoptysis and nine with peripheral lung nodules, whose diagnosis ruled out infection and whose comorbidities, sex and age were similar to those in the NCAP group and CAP group (table 1).

The median (IQR) interval since admission to the day in which samples were taken in the NCAP group was 6 (4–8) days, and was 5 (4–5) days in the CAP group.

Aetiological diagnosis in the NCAP group was established in 14 (60.9%) cases: seven *Streptococcus pneumoniae*, one *Escherichia coli*, two methicillin-resistant *Staphylococcus aureus* (MRSA), one *Pseudomonas aeruginosa* and five mixed aetiology (three *S. pneumoniae* and other micro-organisms, two MRSA and other). In BAL fluid, the following micro-organisms were isolated: one *S. pneumoniae*, two *Streptococcus* spp., one *Enterococcus* spp., one *P. aeruginosa*, two MRSA, one *Acinetobacter baumannii*, 10 potentially nonpathogenic micro-organisms, and none in three cases. Aetiology in the CAP group was: four *S. pneumoniae* (27%), one *P. aeruginosa* (7%) and unidentified in 10 cases.

TABLE 1 Characteristics of nonresponding community-acquired pneumonia (NCAP) and control groups

	NCAP	CAP	Control	p-value#
Subjects n	23	15	16	
Age yrs	61±3	66±4	60±2	NS
Male/female n	16/7	12/3	14/2	NS
Smoker/nonsmoker n	6/17	13/2	9/7	NS
Comorbidities				
Heart disease	6 (26)	3 (20)	2 (12)	NS
COPD	7 (30)	7 (46)	5 (31)	NS
Chronic renal disease	2 (9)	0	1 (6)	NS
Cerebrovascular disease	3 (13)	3 (20)	1 (6)	NS
PSI score	90±6	102±7	0	
Blood leukocyte count mm^{-3}	12752±1268	9877±1065	8217±730	0.02
PMN %	82±11	76±3	62±4	0.001
BAL fluid				
Total cell count mm^{-3}	7.5±2.2		0.5±0.2	0.01
PMN %	38±4.5		8±3	<0.001

Results are expressed as mean±SEM or n (%), unless otherwise stated. CAP: community-acquired pneumonia; COPD: chronic obstructive pulmonary disease; PSI: Pneumonia Severity Index (FINE score); PMN: polymorphonuclear neutrophils; BAL: bronchoalveolar lavage; NS: nonsignificant. #: Mann–Whitney U-test and Chi-squared test for continuous and categorical variables, respectively.

Apoptosis and cytokine results in BAL and blood

Leukocyte counts in blood (table 1) and BAL fluid were significantly higher in the NCAP group. Apoptosis values from flow cytometry (fig. 1a–c) were compared with data obtained by judging the nuclear morphology by light microscopy (fig. 1d) and similar results were obtained.

The basal apoptosis in BAL (fig. 2) was higher (13±3%) in NCAP compared with controls (6±3%), although significance was not reached; no data were available for CAP (as described in Methods and materials section). A similar pattern in blood was observed in NCAP compared with control and CAP (15±4%, 4±1% and 6±2%, respectively) (fig. 2c). No significant correlation was found between apoptotic neutrophils in BAL and in blood in the NCAP group. Spontaneous apoptosis after 24 h of culture was studied in a subset of patients (n=5) to observe neutrophil apoptosis *in vitro* (fig. 2b and d). Spontaneous apoptosis was lower in blood in NCAP (compared with control and CAP; $p<0.05$). The same pattern appeared in BAL (compared with control, no data for CAP) although significance was not reached.

The median levels of systemic cytokines are depicted in figure 3. IL-6 was significantly higher in BAL of the NCAP patients (compared with controls), and in blood (compared with controls and a trend compared with CAP). IL-8 was higher in BAL in NCAP (compared with control), although no differences were found in blood (compared with controls and CAP). Conversely, IL-10 showed significantly higher levels in blood (compared with controls and CAP), without differences in BAL (compared with controls).

In the NCAP group, cytokine levels in BAL compared with blood showed significantly higher median (IQR) IL-8 BAL levels, 4,358 (868–12,117) *versus* 38 (24–78) pg·mL^{-1}, and significantly higher IL-10 in blood, 22 (9–60) *versus* 3 (2–9) pg·mL^{-1}, whereas no differences were found concerning IL-6.

In the NCAP group, a statistically significant positive correlation was found between IL-6 in blood and BAL fluid ($r=0.7$, $p=0.01$). IL-6 levels in BAL were also found to be significantly correlated to IL-10 levels in blood ($r=0.7$, $p=0.02$). A significant correlation of the percentage and absolute count of neutrophils in BAL was found with IL-8 in BAL ($r=0.6$, $p=0.01$ and $r=0.7$, $p<0.01$, respectively).

Clinical resolution and outcome

One of the NCAP patients died. The median (IQR) number of days to reach clinical stability was 12 (7–18) days, and the median LOS was 20 (12–34) days. Basal high neutrophil apoptosis in BAL (⩾20%) showed a significant negative correlation with LOS ($r= -0.5$, $p=0.02$) and with days needed to reach clinical stability ($r= -0.4$, $p=0.05$). Patients with high apoptosis in BAL had shorter LOS (median 10 *versus* 21 days) and needed fewer days for stability (median 7 *versus* 13 days). Neutrophil apoptosis in blood did not correlate either with clinical stability or with LOS ($r= -0.2$, $p=0.4$ and $r= -0.1$, $p=0.7$, respectively).

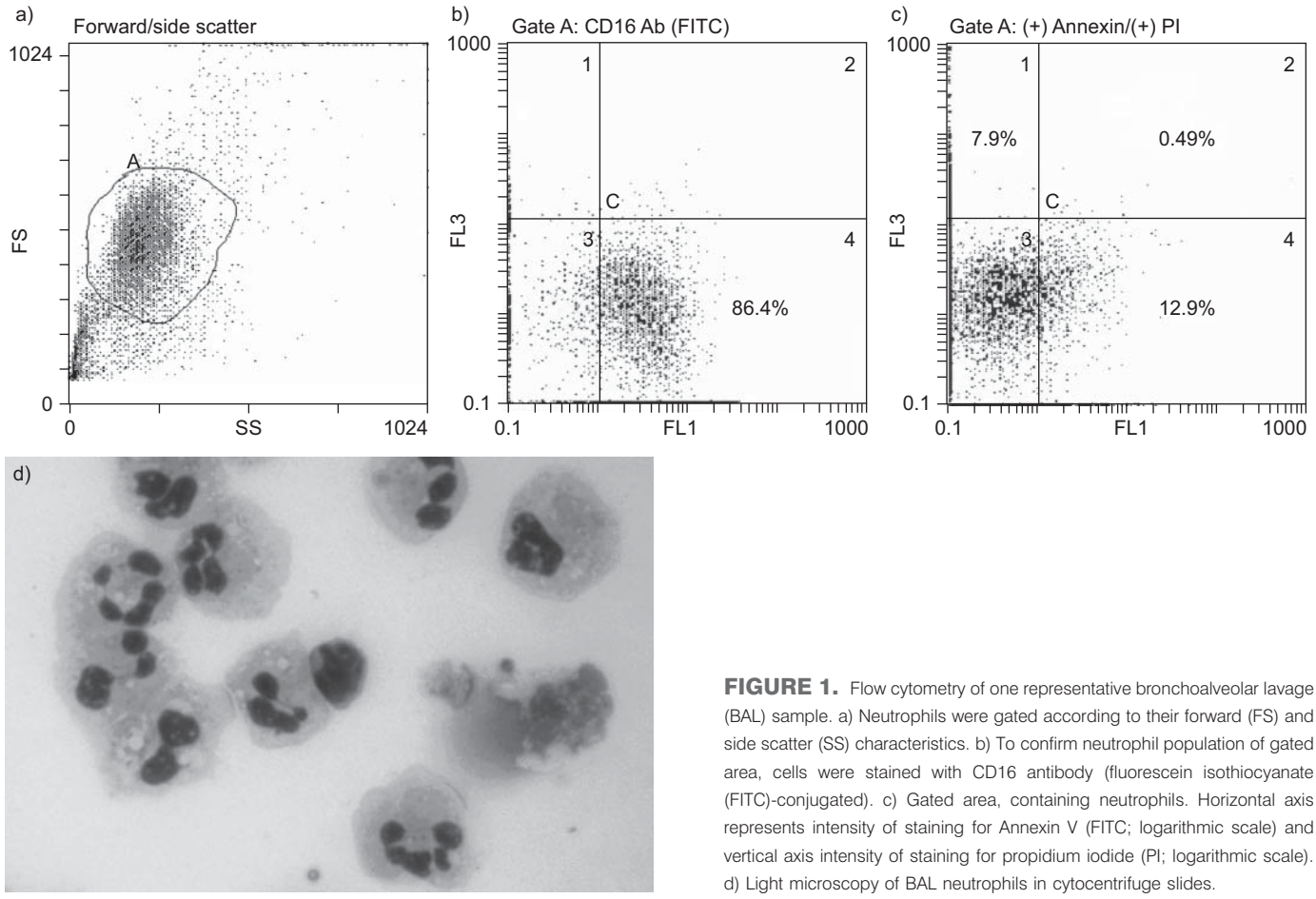

FIGURE 1. Flow cytometry of one representative bronchoalveolar lavage (BAL) sample. a) Neutrophils were gated according to their forward (FS) and side scatter (SS) characteristics. b) To confirm neutrophil population of gated area, cells were stained with CD16 antibody (fluorescein isothiocyanate (FITC)-conjugated). c) Gated area, containing neutrophils. Horizontal axis represents intensity of staining for Annexin V (FITC; logarithmic scale) and vertical axis intensity of staining for propidium iodide (PI; logarithmic scale). d) Light microscopy of BAL neutrophils in cytocentrifuge slides.

Cytokine levels in NCAP stratified according to high ($\geqslant$20%) or low (<20%) basal BAL neutrophil apoptosis are depicted in figure 4. Systemic IL-10 was significantly lower in those with high apoptosis. A trend to lower IL-6 and IL-8 was found in those with high apoptosis both in BAL and blood. IL-6 and IL-10 levels in blood showed a positive significant correlation with the number of days to reach clinical stability ($r=0.7$, $p<0.01$ and $r=0.7$, $p<0.01$, respectively), and IL-10 with LOS ($r=0.7$, $p=0.02$). In the CAP group, a significant positive correlation was found for blood IL-6 and IL-10 with number of days to reach clinical stability ($r=0.4$, $p=0.02$ and $r=0.7$, $p<0.001$, respectively), and with LOS ($r=0.4$, $p=0.03$ and $r=0.7$, $p<0.001$, respectively).

DISCUSSION

The most important findings of this study are: 1) systemic basal neutrophil apoptosis was significantly higher in the NCAP group than in responding CAP and controls, while spontaneous apoptosis at 24 h was lower; 2) the inflammatory cytokine pattern showed higher pro-inflammatory IL-6 and IL-8 in BAL, and higher IL-6 along with higher IL-10 in blood; 3) patients with NCAP who had increased basal apoptosis in BAL ($\geqslant$20%) reached clinical stability earlier and their LOS was shorter; and 4) low basal alveolar apoptosis was associated with higher IL-10 in blood and a trend for higher IL-8 and IL-6 in BAL and blood.

The apoptosis of neutrophils after killing micro-organisms is considered essential for the downregulation of inflammation while precluding the release of their cytotoxic components. The relevance of high initial and persistent inflammation in CAP has been thoroughly studied. Initial raised inflammatory cytokines in NCAP, and at 72 h, have been associated with slower clinical stability and poorer outcome [12, 21–23]. As expected, our findings showed an increase in cytokine levels and alveolar neutrophils, which reflects an ongoing inflammation along with certain degree of apoptosis. In fact, we found higher basal neutrophil apoptosis in BAL (12%) than DROEMANN *et al.* [24] (1%), probably because they included patients at an earlier stage. Interestingly, we also found a slightly higher neutrophil apoptosis in blood, reflecting the functional continuum of the infection.

Although absolute basal apoptosis was increased, neutrophil survival was also prolonged; that is, their functional longevity was enhanced. Spontaneous apoptosis at 24 h was higher in controls than in NCAP, as reported elsewhere [4]. This finding illustrates neutrophil fate during infection, especially because BAL neutrophils were not completely purified from the rest of the BAL cells, and cytokines released by co-cultured cells would reflect the *in vivo* inflammation process. Interestingly, in patients with clinical stability, both basal and spontaneous apoptosis mimics the pattern of controls without infection. The lower systemic basal apoptosis in CAP represents a phase in

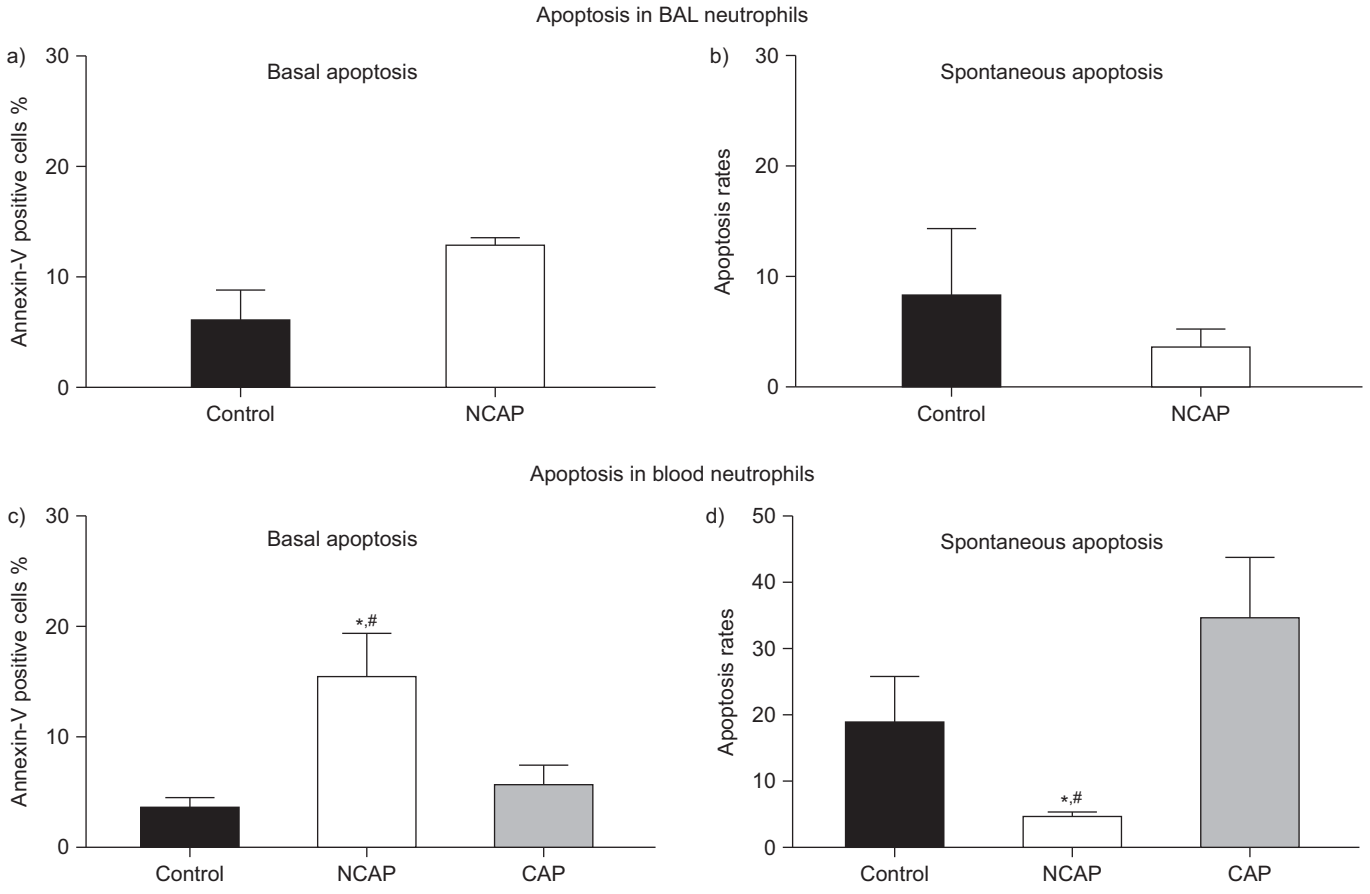

FIGURE 2. Apoptosis of neutrophils in bronchoalveolar lavage (BAL) of nonresponding community-acquired pneumonia (NCAP) and control (a and b) and peripheral blood of NCAP, control and community-acquired pneumonia (CAP) patients (c and d). Apoptosis was determined after neutrophil purification (basal apoptosis, a and c) and after *in vitro* culture for 24 h (spontaneous apoptosis, b and d). Apoptosis rate is defined as percentage of cells in apoptosis at 24 h compared with that at 0 h. Data are presented as mean±SEM. *: $p<0.05$, NCAP *versus* control; #: $p<0.05$, NCAP *versus* CAP.

which leukocytes are declining, as it corresponds to a resolving infection with decreasing cytokines (IL-6 and IL-10). These results indicate that a different neutrophil apoptosis kinetic is a feature of NCAP patients.

Activation of neutrophils is necessary to clear infection, as well as their apoptosis to adequately resolve inflammation [25]. During the initial phases of infection, cytokines may enhance neutrophil activation, particularly IL-8 prime neutrophils, and prolong their lifespan [26]. We have confirmed the higher pro-inflammatory cytokine levels of IL-6 and IL-8 in BAL fluid after 72 h of treatment, as expected in NCAP patients. Interestingly, the predominant inflammatory cytokines in the pulmonary and systemic compartments revealed different patterns: higher IL-8 in BAL, higher IL-10 in blood and similar IL-6 levels in both compartments. SCHÜTTE *et al.* [27] have also reported similar findings with IL-8 and IL-6 in patients with severe pneumonia. The higher IL-8 levels in BAL compared with blood highlight the active phase of infection and the upregulation of neutrophils. In fact, a positive correlation between IL-8 and the absolute count of neutrophils in BAL was confirmed. Concerning IL-6, we found a positive correlation between levels in BAL and in blood, which indicates that an inflammatory process originating in the lung is continued in the systemic compartment. Conversely, IL-10 levels in blood indicate a predominant anti-inflammatory pattern. In fact, significantly lower systemic IL-10 and a trend for lower IL-6 was found in patients with clinical stability. Taking all these results together, a different speed of inflammatory resolution is suggested: slower in the lung than systemically. Moreover, we found a correlation between systemic inflammatory resolution and clinical resolution, *i.e.* among systemic IL-6 and IL-10 and stability and LOS.

The interaction between alveolar neutrophils, cytokines and adequate restoration of homeostasis is far from completely understood. Our results indicate that, when alveolar apoptosis was low in BAL, systemic IL-10 and IL-8 in BAL fluid were raised. This association may have several explanations. First, a reduced apoptosis may reflect an ongoing inflammation with the potential for prolonging the tissue damage. Secondly, bacterial load, or the pathogens themselves, might alter apoptosis thus evading their efficient killing [26]. Thirdly, an exuberant cytokine environment might delay apoptosis, as reported in acute lung injury, where it is associated with a dysregulation of apoptosis [28]. In sepsis, a delayed apoptosis due to prolonged activation of nuclear factor-κB causes neutrophils to remain active [29, 30]. Enhancement of neutrophil apoptosis, however, has

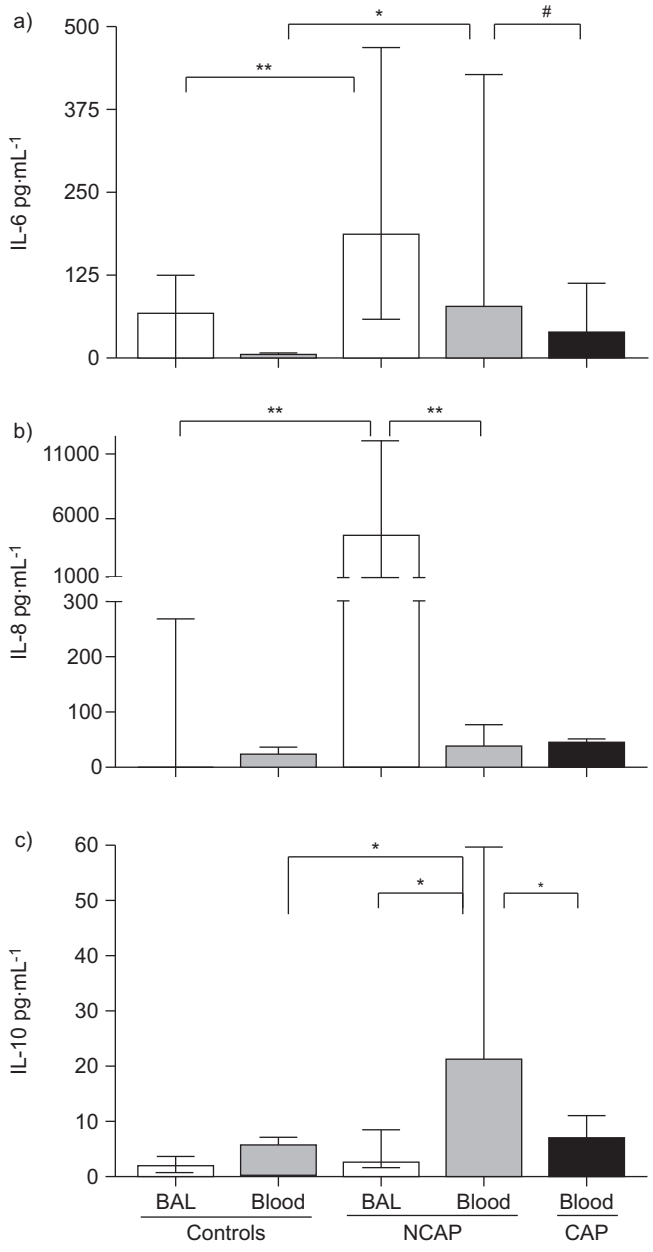

FIGURE 3. Cytokine levels from nonresponding community-acquired pneumonia (NCAP), community-acquired pneumonia (CAP) and control groups in bronchoalveolar lavage (BAL) and peripheral blood. Data are presented as median and interquartile range. *: $p<0.05$; **: $p<0.01$; #: $p=0.06$.

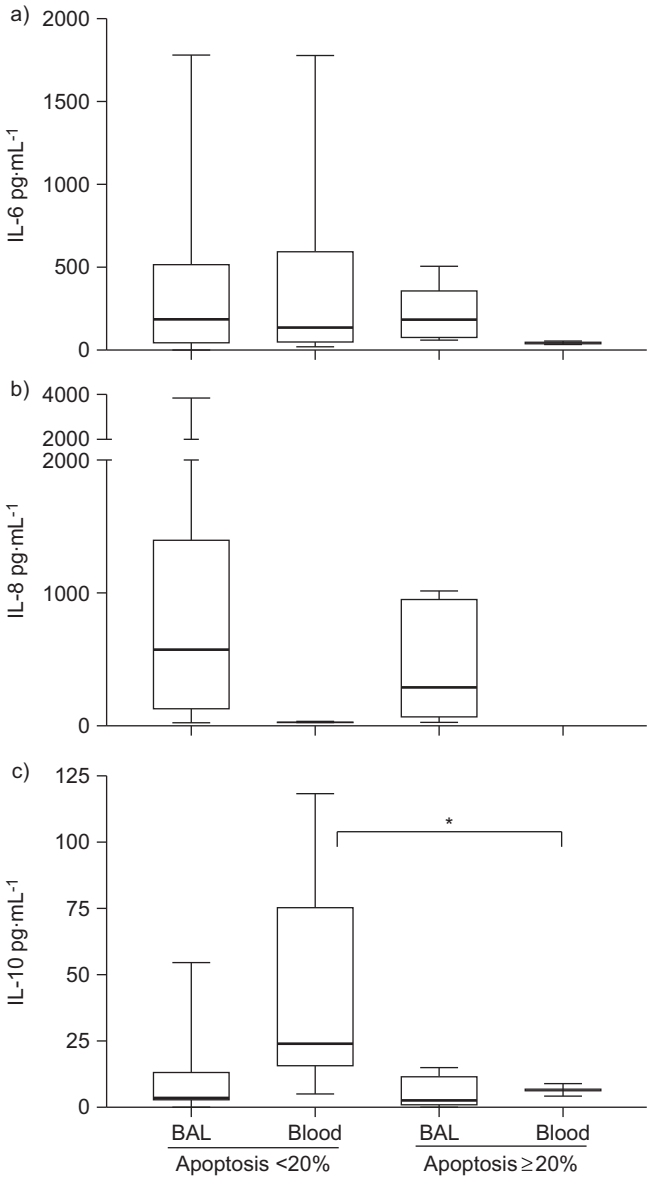

FIGURE 4. Cytokine levels in bronchoalveolar lavage (BAL) and blood of nonresponding community-acquired pneumonia patients classified as low (<20%) or high (≥20%) BAL apoptosis. Data are presented as median and interquartile range. *: $p<0.05$.

been associated with less lung damage and mortality [31]. In an animal model, localised apoptosis was found to be a feature in the resolution of pneumonia [32].

Clinical relevance of alveolar apoptosis was proved when analysing resolution of infectious parameters. We found that a higher alveolar apoptosis was significantly associated with faster clinical stability and shorter LOS. A similar significant correlation was found between systemic levels, both in NCAP and responding CAP, of cytokines, clinical stability and LOS.

To our knowledge, this is the first study to investigate the role of neutrophil apoptosis, both in the lung and systemically, in NCAP. With regard to potential limitations, we have to point out a possible beta error in statistical analyses due to the limited number of cases included and the lack of BAL in the responding CAP group.

In summary, basal apoptosis in NCAP is high both in lung and in blood while spontaneous apoptosis at 24 h is lower than in responding CAP. High IL-8 levels in BAL fluid were associated with accumulation of neutrophils in the lung. Systemic inflammation with raised IL-6 and IL-10 is associated with a delayed clinical stability and LOS. In NCAP, a greater basal alveolar neutrophil apoptosis was associated with earlier clinical stability and lower LOS, which suggests that neutrophil apoptosis precludes persistent local inflammation, thereby contributing to a faster recovery of normal homeostasis.

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STATEMENT OF INTEREST

None declared.

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