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Brief Conclusive Report

Impaired innate immune response of leukocytes from ascitic fluid of patients with spontaneous bacterial peritonitis

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ABSTRACT

An ascitic microenvironment can condition the immune response of cells from cirrhotic patients with spontaneous bacterial peritonitis. To characterize this response, we determined the cytokine concentrations in ascitic fluid and analyzed the phenotype and function of ascitic leukocytes at diagnosis and after antibiotic-induced resolution in sterile ascites and ascitic fluid of 2 spontaneous bacterial peritonitis variants: positive and negative bacteriological culture. At diagnosis, a high concentration was found of IL-6 and IL-10 in the ascitic fluid from negative and positive bacteriological culture. The IL-6 concentration correlated with the percentage of neutrophils ($R = 0.686$, $P < 0.001$). In this context, positive and negative culture neutrophils had an impaired oxidative burst, and, after the antibiotic, the negative culture spontaneous bacterial peritonitis burst was fully recovered. Higher concentrations of IL-6 and IL-10 correlated with the presence of low granular CD 14^{low} macrophages ($R = -0.436$, $P = 0.005$ and $R = 0.414$, $P = 0.007$, respectively). Positive culture spontaneous bacterial peritonitis macrophages expressed the lowest levels of CD16, CD86, CD11b and CD206, and HLA-DR, suggesting an impaired global function. Treatment increased all markers on the positive culture macrophages and CD11b and CD86 on negative culture macrophages. In negative culture spontaneous bacterial peritonitis, this increase was accompanied by phagocytic function recovery. The antibiotics then reverted the marker levels on positive and negative culture macrophages to the levels on sterile ascitis macrophages and restored ascitic negative culture cell function. *J. Leukoc. Biol.* 98: 819–825; 2015.

Abbreviations: CD = cluster of differentiation, HCV = hepatitis C virus, MFI = mean fluorescence intensity, NegC-SBP = negative culture spontaneous bacterial peritonitis, PMN = polymorphonuclear leukocyte, PosC-SBP = positive culture spontaneous bacterial peritonitis, SA = sterile ascitis, SBP = spontaneous bacterial peritonitis, SSC = side scatter channel

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

SBP is among the most severe complications arising in patients with cirrhosis [1]. SBP originates when viable bacteria cross the intestinal wall in a process known as bacterial translocation. After crossing the intestinal wall, bacteria reach the mesenteric lymph nodes and enter the systemic circulation and ascitic fluid [2].

SBP is an infection of initially sterile ascitic fluid. SA is defined when the neutrophil counts are <250 cells/mm^3 and the bacteriological culture is negative at the moment of paracentesis. Peritoneal infection causes an inflammatory reaction that increases the number of PMNs in ascitic fluid. Two criteria are required for a PosC-SBP diagnosis: the number of PMNs in the ascitic fluid must exceed 250 cells/mm^3 and a germ must be isolated from the bacteriological cultures [3]. Approximately 50% of patients with >250 PMNs/mm^3 will have a negative culture [4]. This situation is known as NegC-SBP. Because NegC-SBP and PosC-SBP have similar clinical and prognostic characteristics, the current treatments for both diagnoses are identical [1].

SBP resolution is associated with the disappearance of systemic and local symptoms and signs of infection: a reduction of the PMN count in the ascitic fluid (<250 cells/mm^3) and a negative ascitic fluid culture. When the infection is not resolved, the mortality rate is high [5, 6]. An assessment of the immune response during the course of the infection will help recognize treatment failure as early as possible.

We hypothesized that the immune response during SBP is created by soluble inflammatory mediators present in the ascitic fluid of patients with cirrhosis. Altered soluble mediators could then be associated with a characteristic inflammatory response induced by ascitic neutrophils and macrophages. This response is initiated after the recognition of pathogens through TLRs and mostly consists of phagocytosis and an oxidative burst. TLR

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activation then triggers the release of inflammatory mediators, including IL-1, IL-6, IL-12, IL-10, and TNF-α [7, 8]. Accordingly, the potential role of innate immune cells in SBP can be determined by examining the cellular phenotype, function capability, and cytokine response profile at the moment of bacterial infection. It has been reported that in the presence of bacterial DNA, peritoneal macrophages synthesize higher amounts of NO than macrophages without bacterial DNA. In addition, the presence of bacterial DNA has been associated with a marked activation of cytokine synthesis implicated in the inflammatory immune response and poor clinical outcomes [9, 10].

In the present study, we compared the inflammatory content in the ascites of patients with SA, PosC-SBP, and NegC-SBP. For this purpose, we first analyzed TNF-α, IL-6, and IL-10 levels. We then studied the association of each cytokine level with the cellular composition and function. Finally, we analyzed the expression of surface markers on macrophages in the ascitic fluid. Macrophage markers were selected according to their contribution to innate immune mechanisms such as antigen presentation (HLA-DR), phagocytosis (CD64, CD16), scavenger function (CD36, CD163), and activation (CD86, CD11b) [11, 12]. Because the immune response of patients with SBP after treatment is still poorly understood, we compared the phagocytosis, oxidative burst, and marker expression of ascitic monocytes in PosC-SBP and NegC-SBP before and after antibiotic treatment.

MATERIALS AND METHODS

Patients

We collected ascitic fluid samples by paracentesis from 19 cirrhotic patients with SBP. SA fluid was collected from 9 patients who had cirrhosis and refractory ascites requiring paracentesis. SA was diagnosed when the ascitic PMN count was <250 cells/mm^3 and the bacterial culture was negative. SBP was diagnosed when the ascitic PMN count was ≥ 250 cells/mm^3 and the bacterial culture was positive or negative (PosC-SBP or NegC-SBP, respectively). The ascitic fluid from SBP patients was collected at admission and on the fifth day of antibiotic treatment. The ethics committee at the institution approved the study protocol, and all patients provided written informed consent to be included in the present study.

Cell isolation from ascitic fluid samples and flow cytometry analysis

The ascitic fluid samples (10 ml) from patients with SA, NegC-SBP, or PosC-SBP were centrifuged at 2000 rpm for 10 min, and the supernatants were frozen for cytokine detection. The cells were collected from the pellet, washed with PBS, and counted for the phenotyping and functional assays. For flow cytometry analysis, 0.5×10^6 cells were stained in 100 μl with anti-CD14 PEDy647 and anti-CD16 FITC (ImmunoTools, Friesoythe, Germany), anti-CD11b, anti-CD86, anti-CD36 PE, anti-CD163 PE, anti-CD206, and anti-CD64 (BD Biosciences, San Jose, CA, USA) or the respective isotype control. The red blood cells were then lysed, and the white blood cells were fixed using the TQ-Prep System (Coulter Corp., Miami, FL, USA) to be analyzed by flow cytometry. The granularity (SSC) and the surface expression of the markers were analyzed on ascitic CD14$^+$ macrophages. Analyses were performed on a Beckman Coulter FC500 cytometer (Beckman Coulter, Jersey City, NJ, USA). The percentage of positive CD16, CD163, and CD206 cells (percentage of cells) and the MFI of CD14, CD64, CD86, CD11b, HLA-DR, and CD36 were calculated using CXP Analysis software, version 2.2 (Beckman Coulter).

Production of reactive oxidants

To determine the percentage of neutrophils or monocytes that produce reactive oxidants, 10^5 ascitic fluid cells were incubated for 20 min either with 200 ng/ml PMA (Sigma-Aldrich, St. Louis, MO, USA) or without stimulus at 37°C. The formation of reactive oxidants was monitored by the oxidation of dihydrorhodamine 123 (Sigma-Aldrich) to rhodamine by flow cytometry (MFI).

Phagocytosis assay

Phagocytosis was measured using FITC-labeled *Escherichia coli* bacteria (K-12 strain) BioParticles, Alexa Fluor 488 conjugate (Invitrogen, Eugene, OR, USA). In brief, 2×10^5 cells from ascitic fluid were mixed with 20 μl of FITC-labeled *E. coli* bacteria (1×10^7) at 37°C for 2 h. To identify the macrophages, the cells were stained with anti-CD14-PEDy647. Phagocytosis was expressed as the MFI of FITC, corresponding to the number of labeled bacteria engulfed by a single cell.

Cytokine and total NO levels

Ascitic fluid samples were tested for TNF-α, IL-10 (BD Pharmingen, San Diego, CA, USA), IL-6 (ImmunoTools, Friesoythe, Germany), and total NO concentrations (R&D Systems, Minneapolis, MN, USA) using specific ELISAs. The limits of detection were 30 pg/ml for TNF-α and IL-10, 10 pg/ml for IL-6, and 3.2 μM for total NO.

Statistical analysis

Comparisons between groups were performed using the Mann-Whitney *U* test for unpaired data and the Wilcoxon test for paired data. The correlations were analyzed using the Spearman test. $P < 0.05$ was considered statistically significant. The data are expressed as the mean ± standard error the mean.

RESULTS AND DISCUSSION

Cytokine and NO levels in ascitic fluid from PosC-SBP, NegC-SBP, and SA patients

We analyzed the concentration of IL-10, IL-6, and TNF-α in the ascitic fluid of PosC-SBP and NegC-SBP patients before and after treatment and SA patients (**Fig. 1**). No significant differences were seen in the TNF-α levels among the 3 groups of patients at diagnosis (PosC-SBP 39.65 ± 6.05 pg/ml; NegC-SBP 35.40 ± 7.81 pg/ml; SA 37.87 ± 7.46 pg/ml). After treatment, the TNF-α levels tended to decrease in NegC-SBP and were significantly reduced in PosC-SBP (PosC-SBP 25.27 ± 7.25 pg/ml, $P = 0.02$; NegC-SBP 30.05 ± 7.85 pg/ml). Before treatment, the IL-6 levels in PosC-SBP and NegC-SBP were expectedly higher than those in SA (PosC-SBP 17,608 ± 3123 pg/ml; NegC-SBP 10,165 ± 2084; SA 2068 ± 586 pg/ml; PosC-SBP vs. SA, $P < 0.001$; NegC-SBP vs. SA, $P = 0.002$). SBP has already been associated with an elevated production of inflammatory mediators in several reports [13]. Consequently, the elevated levels of IL-6 and its mediators have been considered markers for the diagnosis of SBP and the outcome of the disease [14, 15]. After treatment, the IL-6 levels had diminished in PosC-SBP and NegC-SBP, reaching levels similar to those of SA (after treatment PosC-SBP 2547 ± 738 pg/ml; after treatment NegC-SBP 3764 ± 1871 pg/ml; before vs. after treatment PosC-SBP, $P = 0.001$ and NegC-SBP, $P = 0.01$).

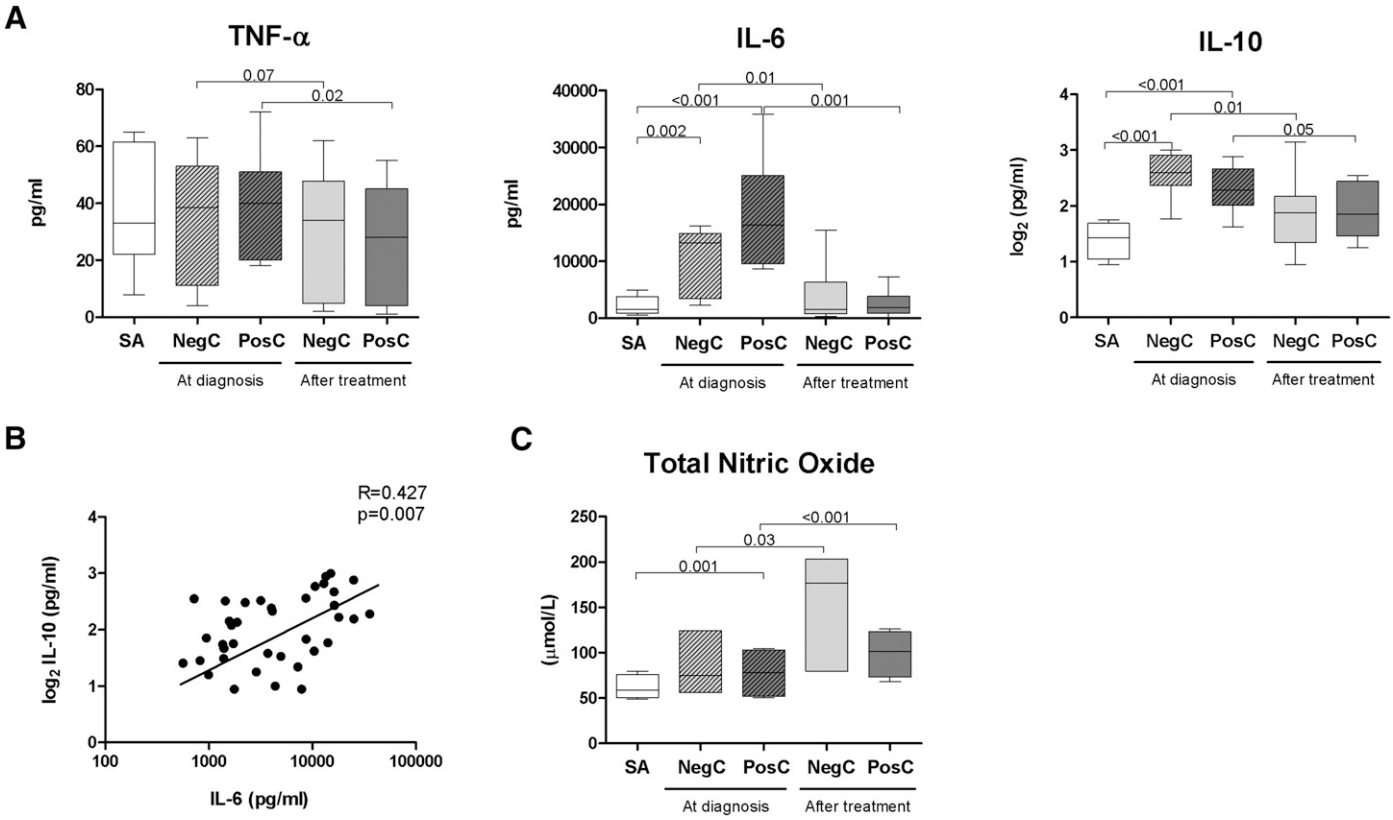


Figure 1. Cytokine levels and NO in the ascitic fluid. The levels of TNF-α, IL-6, IL-10, and total NO in ascitic fluid of cirrhotic patients with sterile ascites (SA, $n = 9$), NegC-SBP ($n = 8$), and PosC-SBP ($n = 11$) at diagnosis and after antibiotic treatment were quantified by ELISA. The Mann-Whitney U test was used for comparisons between the SA and 2 SBP groups and the Wilcoxon test for the comparisons between the same group before and after treatment. Correlation between cytokine levels was analyzed using the Spearman test.

At diagnosis, the IL-10 levels were significantly higher in PosC-SBP and NegC-SBP than in SA [PosC-SBP 2.30 ± 0.13, NegC-SBP 2.56 ± 0.14, SA 1.37 ± 0.10 log(pg/ml); PosC-SBP vs. SA, $P < 0.001$; NegC-SBP vs. SA, $P < 0.001$]. They had diminished in both groups after treatment [PosC-SBP 1.91 ± 0.16, NegC-SBP 1.87 ± 0.23 log(pg/ml); before vs. after treatment PosC-SBP, $P = 0.05$; before vs. after treatment NegC-SBP, $P = 0.01$].

The IL-10 and IL-6 levels correlated positively ($R = 0.427$, $P = 0.007$; Fig. 1B) This correlation could be the consequence of the IL-10 induction by IL-6 [16]. Elevated levels of both cytokines have been previously described in patients with noninfected ascites and with SBP [17]. In patients with chronic hepatitis C, the serum levels of both cytokines have been correlated with the histopathological damage to the liver [18].

As expected, the NO levels in the ascitic fluid were significantly higher in PosC-SBP than in SA at diagnosis (SA 62.29 ± 5.86 vs. PosC-SBP 77.8 ± 9.1 μM; $P = 0.001$; NegC-SBP 85.2 ± 12.8 μM). After antibiotic treatment, the NO concentration had increased significantly in both PosC-SBP and NegC-SBP (PosC-SBP 99.37 ± 8.5 μM; NegC-SBP 153 ± 23.7 μM; $P < 0.001$ and $P = 0.03$, respectively; Fig. 1C).

Association of IL-6 levels with neutrophil presence

The IL-6 levels correlated significantly with the percentage of neutrophils in the ascitic fluid ($R = 0.686$, $P < 0.001$; **Fig. 2A**). As expected, the percentage of neutrophils was significantly higher in PosC-SBP and NegC-SBP than in SA (PosC-SBP 75.44 ± 6.14%, NegC-SBP 61.25 ± 5.20%, SA 8.42 ± 2.09%; PosC-SBP vs. SA, $P < 0.001$; NegC-SBP vs. SA, $P < 0.001$; Fig. 2B). This finding can be explained by the IL-6 production of recently recruited neutrophils in PosC-SBP [19]. In recent studies, IL-6 was also shown to suppress neutrophil apoptosis via a platelet-activating factor-like mechanism [20, 21]. Other reports showed that IL-6 represents a regulator of neutrophil trafficking during the inflammatory response, because it orchestrates chemokine production [22]. After antibiotic treatment, the percentage of neutrophils decreased significantly in PosC-SBP (27 ± 88%, $P < 0.001$) and NegC-SBP (15.67 ± 5.63%, $P = 0.002$; Fig. 2B). Despite the higher percentage of neutrophils in PosC-SBP and NegC-SBP, these cells in both groups had an impaired oxidative burst (MFI 12.08 ± 3.75 and 11.10 ± 4.2, respectively) compared with SA neutrophils (33.65 ± 7.35; $P = 0.04$ and $P = 0.03$, respectively; Fig. 2C and D). After treatment, the oxidative burst of neutrophils from NegC-SBP, but not from PosC-SBP, increased significantly (35.85 ± 7.27), reaching levels similar to SA.

Association of cytokine levels with macrophage phenotype and function

The influence of the elevated cytokine concentrations was not limited to neutrophils. The phenotype and function of

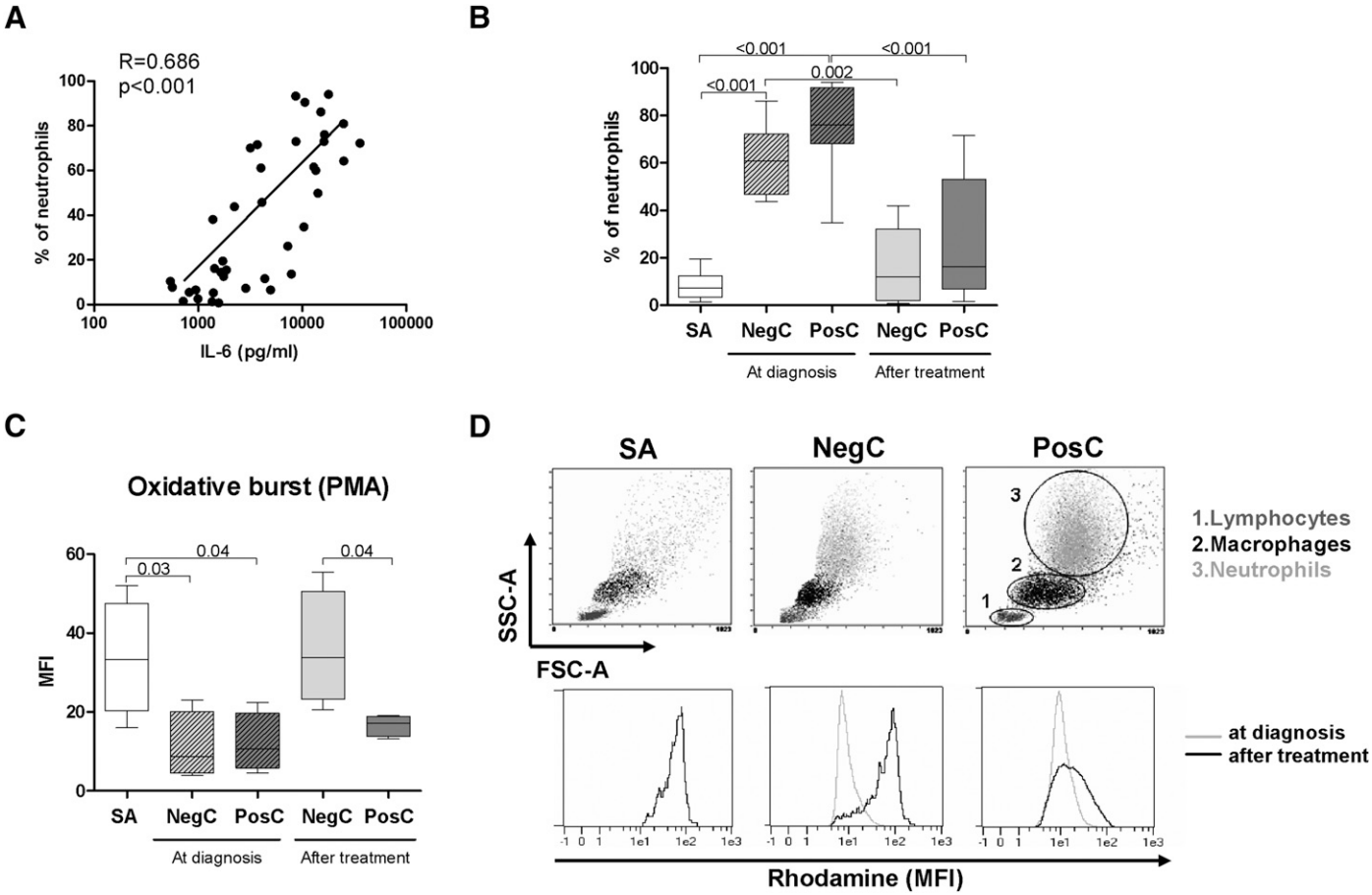


Figure 2. Neutrophil percentages and function in SA ($n = 9$), NegC-SBP ($n = 8$), and PosC-SBP ($n = 11$). (A) Correlation between IL-6 concentration and neutrophil percentages in SA and SBP (Spearman correlation). (B) Neutrophil percentages in ascitic fluid from patients with SA, NegC-SBP, and PosC-SBP at diagnosis and after antibiotic treatment. (C) Oxidative burst activity in neutrophils of the ascitic fluid from patients with SA, NegC-SBP, and PosC-SBP. The Mann-Whitney *U* test was used for the comparisons between the SA and 2 SBP groups and the Wilcoxon test for the comparison between the same group before and after treatment. (D) Flow cytometry dot plots with the cellular morphology (SSC-A and forward light scatter [FSC]-A) identifying the 3 major subpopulations of leukocytes and histograms with rhodamine. MFI expression (oxidative burst measure) of gated neutrophils from 3 representative patients with SA, NegC-SBP, and PosC-SBP at diagnosis.

macrophages of the ascitic fluid from patients with cirrhosis was also associated with the IL-6 and IL-10 levels. The IL-10 levels correlated with the presence of ascitic macrophages with low granularity (low SSC; $R = -0.414$, $P = 0.007$; **Fig. 3A**). The ascites from the NegC-SBP and PosC-SBP patients had significantly more macrophages with low SSC than did those from SA patients (percentage of PosC-SBP, 79.70 ± 6.07%; percentage of NegC-SBP, 79.81 ± 4.84%, and percentage of SA, 58.10 ± 6.87%; PosC-SBP vs. SA, $P = 0.03$; NegC-SBP vs. SA, $P = 0.02$; Fig. 3B). It is possible that the production of IL-6 by resident macrophages precedes the production of IL-10 and the recruitment of peripheral blood neutrophils and monocytes with low granularity [23]. After treatment, the percentage of low SSC macrophages in PosC-SBP and NegC-SBP was similar to that of SA (PosC-SBP 68.68 ± 5.85%, NegC-SBP 59.66 ± 7.48%). Macrophages from NegC-SBP and PosC-SBP also had a lower expression of CD14 on the cell surface (measured as MFI) than did macrophages from SA (Fig. 3B). PosC-SBP macrophages in our study showed the lowest expression of CD14 and cellular complexity, comparable in expression to peripheral blood monocytes. According to the previously reported events during infection in PosC-SBP, it is possible that induced chemokines and hemodynamic derangement facilitate the recruitment of circulating monocytes to ascitis [13, 24, 25]. This CD14 expression on the macrophages of ascitic fluid correlated inversely with the IL-6 levels ($R = -0.436$, $P = 0.005$; Fig. 3C). We next found that differences in CD14 expression were accompanied by changes in macrophage function. The particular ascitic fluid environment then induced a specific phenotype—and consequent function—of macrophages. A strong correlation was observed between CD14 expression and phagocytosis ($R = 0.841$, $P < 0.001$). Macrophages from PosC-SBP and NegC-SBP ascites, predominantly with $CD14^{low}$ expression, had an impaired phagocytosis (MFI 26.67 ± 10.75 and 31.81 ± 4.26, respectively). A direct consequence of this phenotype is the poor bactericidal capacity of PosC-SBP macrophages [26, 27] and the tolerant state of the macrophages. After treatment, phagocytosis of macrophages from NegC-SBP increased, reaching levels similar to that of SA macrophages (NegC-SBP 84.73 ± 25.04, PosC-SBP 36.41 ± 13.89, SA 74.27 ± 2.79; Fig. 3D). However, the phagocytosis of macrophages from PosC-SBP

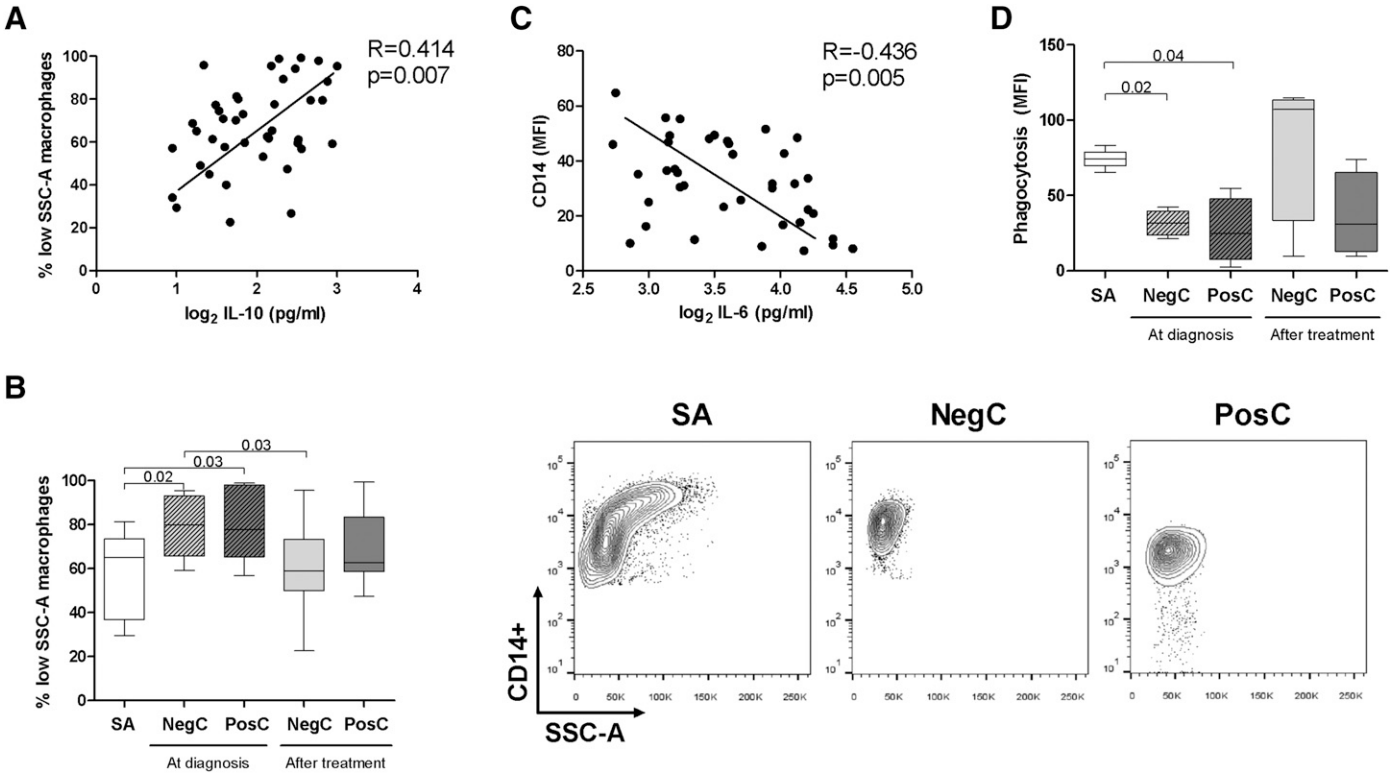


Figure 3. Macrophage phenotype, morphology, and function in SA (n = 9), NegC-SBP (n = 8), PosC-SBP (n = 11). (A) Correlation between IL-10 concentration and morphology (SSC) of macrophages (Spearman correlation). (B) Percentages of macrophages with low granularity (measured as SSC) in ascitic fluid of patients at diagnosis and after antibiotic treatment. The Mann-Whitney U test was used for the comparisons between SA and the 2 SBP groups and the Wilcoxon test for the comparisons between the same group before and after treatment. Representative flow cytometry contour plot of ascitic macrophages from patients with SA, NegC-SBP, and PosC-SBP. Plot shows SSC on the X-axis (morphology parameter corresponding to cellular granularity) and CD14 expression on the Y-axis. (C) Correlation between IL-6 and CD14 expression (MFI) on macrophages of ascitic fluid. (D) Phagocytic activity expressed as MFI in the macrophages of ascitic fluid from patients. The Mann-Whitney U test was used for the comparisons between the SA and 2 SBP groups and the Wilcoxon test for the comparisons between the same group before and after treatment.

did not increase. In this context, bacteria in the ascitic fluid cannot be easily destroyed, and PosC-SBP patients have a high predisposition to ascitic fluid infection recurrence [28].

Phenotypic profile of ascitic macrophages

To fully understand the capability of ascitic macrophages from PosC-SBP, NegC-SBP, and SA, we analyzed the surface markers involved in activation (CD64, CD16, and CD86), adhesion (CD11b), antigen presentation (HLA-DR), and scavenger receptors (CD36, CD163, and CD206) by flow cytometry (**Fig. 4** and Supplemental Figs. 1 and 2). Macrophages were gated according to $CD14^+$ expression (Supplemental Fig. 1). The levels of CD11b and CD86 were significantly lower in NegC-SBP and PosC-SBP than in SA macrophages (for MFI of CD11b, PosC-SBP 5.05 ± 0.75 and NegC-SBP 5.76 ± 1.19 vs. SA 9.76 ± 1.22; P = 0.005 and P = 0.04, respectively; for MFI of CD86, PosC-SBP 1.34 ± 0.16 and NegC-SBP 1.59 ± 0.31 vs. SA 3.06 ± 0.32; P = 0.003 and P = 0.01, respectively). The levels of HLA-DR, CD16, and CD206 were significantly lower in PosC-SBP than in SA (for MFI of HLA-DR, PosC-SBP 6.35 ± 0.84 vs. SA 16.46 ± 1.85; $P < 0.001$; for the percentage of $CD16^+$ cells: PosC-SBP 52.57 ± 8.06 vs. SA 81.85 ± 6.20; P = 0.01; for the percentage of $CD206^+$ cells, PosC-SBP 32.37 ± 8.59 vs. SA 66.58 ± 6.52; P = 0.01). The levels of CD64, CD36, and CD163 were similar in the 3 groups of patients at diagnosis. The expression of surface markers on macrophages from SA was compatible with the phenotype of resident macrophages of the peritoneal cavity. High HLA-DR expression confirmed that these macrophages are fully competent APCs. The marker expression of PosC-SBP, with low levels of HLA-DR, CD11b, CD16, and CD86, suggests an impaired ability for antigen presentation, phagocytosis, and adhesion [29]. A similar low HLA-DR expression has recently been described on ascitic macrophages from patients with NegC-SBP, and it has been associated with a high bacterial DNA burden [9].

After treatment, the levels of CD86 increased significantly (PosC-SBP 2.50 ± 0.36 and NegC-SBP 2.74 ± 0.33; P = 0.01 and P = 0.03, respectively), reaching levels similar to those of SA. Treatment significantly increased the levels of HLA-DR, CD16, and CD206 on PosC-SBP macrophages (MFI 23.98 ± 4.45%, P = 0.01; 70.34 ± 4.79%, P = 0.01; 76.23 ± 9.90%, P = 0.05, respectively). In PosC-SBP, the CD64 and CD36 had significantly increased after antibiotic treatment (CD64 before 2.44 ± 0.31 and after 3.82 ± 0.61, P = 0.03; CD36 before 3.04 ± 0.39 and after 5.34 ± 0.49, P = 0.01). We showed that by eliminating bacteria with antibiotics, the phenotype, but not the function, of ascitic macrophages in PosC-SBP can be switched to a steady state. Many

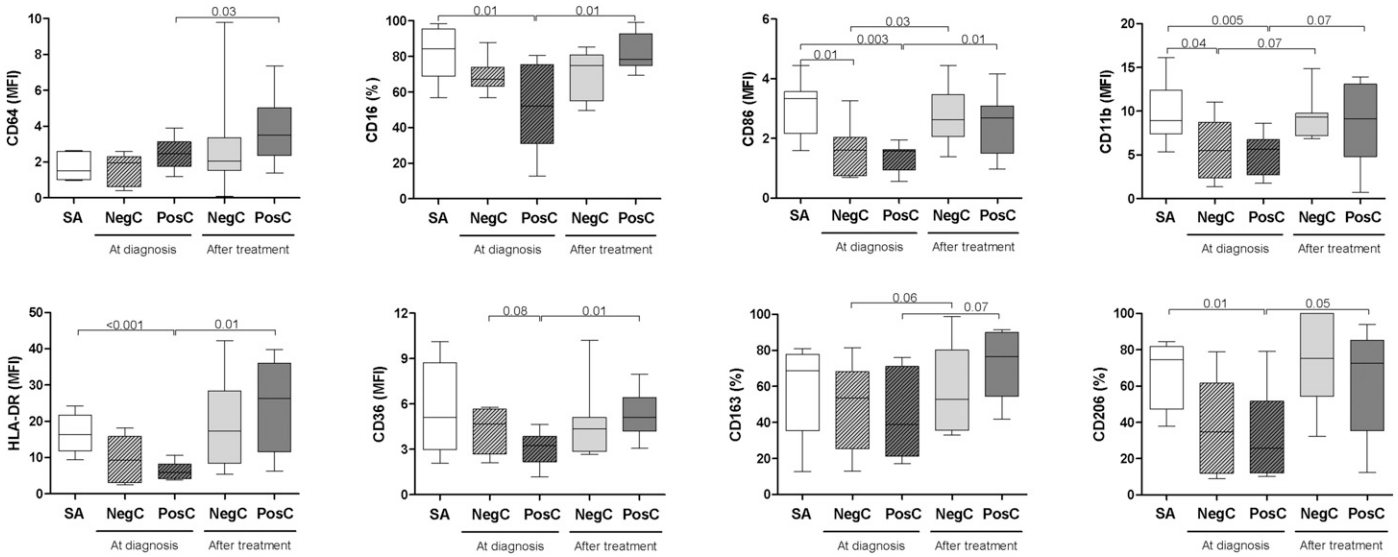


Figure 4. Phenotype of macrophages in SA ($n = 9$), NegC-SBP ($n = 8$), and PosC-SBP ($n = 11$) by flow cytometry. Ascitic macrophages were gated based on $CD14^+$ expression. They were also stained with monoclonal antibodies anti-CD16, anti-CD64, anti-CD86, anti–HLA-DR, anti-CD11b, anti-CD206, anti-CD36, and anti-CD163. The results are expressed as the percentage of cells for CD16, CD163, and CD206 and as the MFI for CD64, CD86, CD11b, HLA-DR, and CD36. The Mann-Whitney *U* test was used for the comparisons between the SA and SBP groups and the Wilcoxon test for the comparisons between the same group before and after treatment.

changes can be induced by this antibiotic therapy. Antibiotics decrease the circulating endotoxin levels and the presence of viable bacteria, regulate the production of inflammatory mediators [30], and prevent episodes of recurrent SBP and other bacterial infections [31–33]. Many of these changes might influence the phenotype of ascitic macrophages. Our findings, along with those of others, suggest that antibiotics have different consequences depending on the presence or absence of viable bacteria in the SBP. This treatment can fully restore the functional status of neutrophils and macrophages from NegC-SBP but not from PosC-SBP. Several nonexclusive models could explain the different behavior between NegC-SBP and PosC-SBP. One possibility is that the stage of macrophages in NegC-SBP and PosC-SBP differs at SBP diagnosis. If left untreated, one third of NegC-SBP patients can have positive microbial test findings, suggesting that NegC-SBP macrophages at diagnosis are at an earlier infection phase [34]. A second possibility is that the presence of more viable bacteria in PosC-SBP than in NegC-SBP requires longer antibiotic treatment to completely clear the infection. A recent study observed that infectious stimuli induce a functional switch in the macrophages of the peritoneum [35]. Despite the lack of viable bacteria in NegC-SBP, ascites from these patients could contain bacterial fragments. One recent study detected bacterial DNA by RT-PCR in NegC-SBP [36, 37], and this DNA can effectively activate cells [10]. However, bacterial fragments and viable bacteria are not necessarily equivalent at the level of macrophage activation. A third possibility is that, even in the absence of viable bacteria or their products, the presence of particular inflammatory mediators in the ascitic fluid of NegC-SBP and PosC-SBP modulate macrophage phenotype distinctly [38]. We are currently studying other soluble factors that could contribute to the differences between NegC-SBP and PosC-SBP at the end of treatment.

Our results suggest that regular phenotyping and an extensive analysis of the immune state of macrophages and neutrophils from the ascitic fluid could help to further understand the pathophysiological events in ascitic fluid infection and to optimize the treatment of these patients.

AUTHORSHIP

J.C.N. performed ELISA, functional assays, and flow cytometry experiments, analyzed results, and wrote the manuscript. E.S. performed flow cytometry experiments and analyzed results. C.R., E.R., and M.P. included patients, collected samples, and determined the clinical characterization of the patients. C.G. and C.J. analyzed the results and supervised the experiments. G.S. analyzed the results, included patients, collected samples, and wrote the manuscript. S.V. performed the experimental design, analyzed the results, and wrote the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

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