Ascitic fluid regulates the local innate immune response of patients with cirrhosis

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Abbreviations

CRP, C-reactive protein; CS, conditioned supernatant; FBS, fetal bovine serum; HN, healthy neutrophils; IL, interleukin; LPS, lipopolysaccharide; MELD, model for end-stage liver disease; MFI, mean fluorescence intensity; NET, neutrophil extracellular trap; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear cell; SA, sterile ascites; SBP, spontaneous bacterial peritonitis; Dx, diagnosis; Post, post-antibiotic treatment; TNF-α, tumor necrosis factor α

1. SUMMARY

Background & Aims: Ascitic neutrophils from cirrhotic patients with spontaneous bacterial peritonitis (SBP) exhibit an impaired oxidative burst that could facilitate bacterial infection. However, the influence of the cell-free ascitic fluid of these patients on neutrophil function has not been investigated. To analyze this influence, we determined the ascitic levels of cytokines, resistin and lactoferrin and their association with neutrophil function, disease severity score and SBP resolution. ***Methods:*** We analyzed NETosis induction by microscopy and oxidative burst by the flow cytometry of healthy neutrophils cultured in ascitic fluid from cirrhotic patients with sterile ascites (SA) and with SBP before and after antibiotic treatment. Resistin, IL-6, IL-1 receptor antagonist, IL-1β and lactoferrin levels were measured in ascitic fluids and supernatants of cultured neutrophils and peripheral blood mononuclear cells (PBMCs) by ELISA. ***Results:*** Upon stimulation, healthy neutrophils cultured in SBP ascitic fluid produced lower NETosis and oxidative burst than those cultured in SA. Ascitic resistin levels were negatively correlated with NETosis, oxidative burst and ascitic glucose levels; and positively correlated with the model for end-stage liver disease score. After an *E.coli* or TNF-α stimulus, neutrophils were the major resistin producers. Resistin indirectly reduced the oxidative burst of neutrophils and directly reduced the inflammatory phenotype of monocytes and TNF-α production. ***Conclusions:*** Bacterial-induced resistin production can downregulate the inflammatory response of macrophages and neutrophil function in ascitic fluid. Consequently, this downregulation may jeopardize the elimination of bacteria that translocate to ascitic fluid in patients with cirrhosis.

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2. INTRODUCTION

Ascites formation is associated with poor prognosis, decreased survival and the favoring of the development of other complications in patients with cirrhosis.[1] It is caused by portal hypertension, leading to the accumulation of ascitic fluid in the peritoneal cavity.[2] At the first stage, ascitic fluid is known as sterile ascites (SA). SA is characterized by negative bacterial culture and a low number of polymorphonuclear cells (PMN, <250cells/mm^3).[3] A severe complication of ascites is the bacterial infection of the ascitic fluid, which is known as spontaneous bacterial peritonitis (SBP). SBP occurs by bacterial translocation due to gut dysbiosis and the increased intestinal permeability described in cirrhosis.[4] Gut bacteria translocate to the mesenteric lymph nodes, enter the bloodstream and finally reach the ascitic fluid.[5] Ascitic fluid in SBP is characterized by an increased number of PMN (≥250/mm^3) and the bacterial culture can be either positive (culture-positive SBP) or negative (culture-negative SBP). Patients with SBP require antibiotic treatment until the number of ascitic PMN decreases below 250/mm^3 and the culture is negative, in which case infection has been resolved.[3]

We have previously demonstrated that ascitic neutrophils exhibited an impaired oxidative burst and ascitic macrophages expressed lower levels of CD16, CD86, CD11b, CD206 and HLA-DR in patients with cirrhosis and SBP, compared to patients with SA.[6] A recent study has also reported that circulating neutrophils from patients with decompensated cirrhosis produced impaired neutrophil extracellular traps (NETs).[7] An accepted explanation for this impairment is that circulating neutrophils in patients with cirrhosis are exhausted due to systemic inflammation. This acquired impairment reduces bactericidal capacity, phagocytosis and chemotaxis, thereby facilitating infection.[8]

This circulating neutrophil dysfunction of patients with alcoholic hepatitis has been reproduced *in vitro* by incubating healthy neutrophils (HN) with patients' plasma.[9] However, the influence of cell-free ascitic fluid on the neutrophil function has not been investigated. We have previously found a high concentration of IL-6 and IL-10 in the ascitic fluid of patients with SBP. Another protein that is upregulated in the ascitic fluid of patients with peritonitis is resistin.[10] Since this protein has been associated with liver function impairment and poor prognosis,[11] and is stored in neutrophil granules,[12] resistin may have an effect on ascitic neutrophil function.

We propose that the ascitic fluid from patients with decompensated cirrhosis contributes to local neutrophil dysfunction. Moreover, the resolution of infection in these patients with antibiotic treatment may regulate their ascitic fluid content, thereby restoring neutrophil function. To evaluate this hypothesis, we firstly determined the oxidative burst and NETosis of healthy neutrophils cultured in the ascitic fluids of patients with SA and patients with SBP before and after antibiotic treatment. Secondly, we analyzed the ascitic levels of cytokines, resistin and lactoferrin and their association with neutrophil function. Since resistin showed the best association with neutrophil dysfunction, we determined its cellular origin and induction mechanisms. Finally, we analyzed the association of resistin with the disease severity score and SBP resolution.

3. MATERIAL AND METHODS

3.1. Patients and sample collection

Ascitic fluid samples were obtained from 10 SA patients (<250 PMNs/mm^3 and negative bacterial culture) and 16 patients with culture-positive SBP at diagnosis (Dx) (≥250 PMNs/mm^3 and positive bacterial culture), after antibiotic treatment (Post, *n*=6) (<250 PMNs/mm^3 and negative bacteria). Only culture-

positive SBP patients were included because culture-negative SBP is a more heterogeneous entity.[13] The protocol was approved by the ethics committee of Hospital S.Creu I S.Pau and patients gave their written consent.

3.2. Healthy neutrophil (HN) isolation

Neutrophils were isolated by density gradient (Lymphoprep, AXIS-SHIELD PoCAs, Oslo, Norway), dextran-sacarose (Sigma-Aldrich, St. Louis, MO, USA) sedimentation and red blood cell lysis (RBC Lysis Buffer, BioLegend, San Diego, CA, USA).

3.3. Oxidative burst

HN ($2x10^4$) were cultured in medium containing either 10% fetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel) as control, 10% SA, 10% SBP ascitic fluid or 50% conditioned supernatant (CS, obtained as indicated below). Next, 200 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) or medium was added for 30 min. Reactive oxidants were monitored by the oxidation of dihydrorhodamine 123 (Sigma-Aldrich) to rhodamine by flow cytometry (MFI) using MACSQuant Analyzer.

3.4. NET induction, staining and quantification

HN ($15x10^4$) were seeded on coverslips with medium supplemented with 10% FBS, 10% SA or 10% SBP ascitic fluid. NET formation was induced with 125 ng/ml PMA for 2 h. Coverslips were paraformaldehyde fixed, incubated with anti-CD66b antibody (BD Biosciences, San Diego, CA, USA) and with 100 nM Sytox Green (Invitrogen, Carlsbad, CA, USA) and, 30 min later, with anti-mouse IgG AlexaFluor-594 (Life Technologies, Rockford, IL, USA). We calculated the NET-rate by dividing the total area of NETs by the CD66+ neutrophil counts.

3.5. Levels of soluble factors

Soluble factors were tested by ELISAs: IL-6 (ImmunoTools, Friesoythe, Germany), resistin (R&D Systems, Minneapolis, MN), IL-1Ra, IL-1β (PeproTech, London, UK), lactoferrin (Assaypro, St Charles, MO, USA) and TNF-α (BD Biosciences). Limits of detection were: 10 pg/ml for IL-6, 30 pg/ml for resistin, IL-1Ra, IL-1β and TNF-α and 625 pg/ml for lactoferrin. Resistin levels in the medium and fetal bovine serum were undetectable.

3.6. Stimulation of cells with heat-killed bacteria

Bacterial pellets from cultured *Escherichia coli* (ATCC 25922) and *Enterococcus faecium* (ATCC 19434) were diluted in saline solution, counted and heat-killed at 100ºC for 20 min. HN ($2x10^4$) or PBMCs ($2x10^4$ monocytes) were 20h cultured at 25 bacteria/cell ratio or 10 ng/ml TNF-α (BD Bioscience). Supernatants were collected to determine the TNF-α concentration or to be used as conditioned supernatant (CS). Cells were stained with anti-CD14 APC, anti-CD16 FITC and anti-CD11b APC-Cy7 (ImmunoTools) and analyzed by flow cytometry.

3.7. Whole blood cultures

One mL of blood was 20h-stimulated with: 10 ng/ml TNF-α (BD Bioscience), 100 ng/ml lipopolysaccharide (LPS), 50 ng/ml IL-6, 500 ng/ml IL-1Ra (ImmunoTools), 500 ng/ml resistin and 40 ng/ml IL-10 (PeproTech).

3.8. Statistical analysis

Groups were compared with the Mann-Whitney *U* test and paired data with the Wilcoxon test. The Pearson test was used for correlations and Fisher's exact test for frequency comparisons. Significance was established at $P<0.05$. Values are expressed as mean±standard error.

4. RESULTS and DISCUSSION

4.1. Characteristics and clinical evolution of patients

Patients with SBP presented higher Child-Pugh and MELD scores and a higher ascitic fluid neutrophil count than patients with SA. *E.coli* was the most frequently isolated bacteria in the ascitic fluid cultures of patients with SBP (Table 1). Patients with SBP were treated according to current guidelines.[14,15] and mean antibiotic treatment was 4 days. Nine patients achieved resolution of SBP within the first 5 days of treatment (4 patients in 2 days, 2 patients in 3 days and 3 patients in 4 days), 6 patients needed more than 5 days of treatment (2 patients needed 5 days, 1 patient 6 days, 1 patient 7 days and 2 patients 8 days) and 1 patient died before the end of the treatment period.

4.2. Effect of ascitic fluid on healthy neutrophil functions

We have previously shown that neutrophils from the ascitic fluid of SBP-DX patients had impaired function.[6] To decipher whether this impairment was due to ascitic fluid content, we stimulated healthy donor neutrophils (HN) in the presence of ascitic fluid from SBP and SA patients. Upon PMA-stimulation, HN cultured in SBP-Dx ascitic fluid produced a lower oxidative burst and fewer NETs than HN cultured in SA and in the control medium (oxidative burst MFI: SBP-Dx 41.5±6; SA 118.6±17.6; control 70.1±10.1; P=0.001 and P=0.036, respectively; NET area/number of neutrophils: SBP-Dx 7.4±1.7; SA 285.6±88.8; control 819.4±383.9*;* P<0.001 and P=0.001, respectively; Figure 1A-C).There was a positive correlation between the oxidative burst of PMA-stimulated HN cultured in ascitic fluid and the previously calculated PMA-stimulated oxidative burst of ascitic neutrophils of the respective patient [6] *(*r=0.707, P=0.05; data not shown). Tritto et al. have already shown that plasma samples from patients with stable cirrhosis induced phagocytic impairment in healthy neutrophils.[16] These results, together with our findings, suggest that neutrophil dysfunction is partially due to the molecules present in the ascites or plasma of these patients. We

could speculate that these molecules are a surrogate marker of advancing disease or a consequence of chronic endotoxemia and immune exhaustion.[17, 18]

This induced dysfunction was partially reversible since antibiotic treatment restored NET production in SBP samples (SBP-Post 134.9±54.1, *P*=0.031; Figure 1B-C). However, after antibiotic treatment, no changes were observed in the oxidative burst (MFI: SBP-Post 33.1±7.4; Figure 1A). There was a positive correlation between the oxidative burst and the NET production of HN cultured in SA and in SBP-Dx samples *(r*=0.483, *P*=0.031; data not shown), though not in SBP-Post samples. Our findings suggest that antibiotic treatment can only restore the ROS-independent pathway of NETosis.[19]

4.3. Distinctive soluble factors in ascitic fluid

We then analyzed the concentration of ascitic proteins, which may have an impact on the function of neutrophils. Ascitic resistin, IL-1Ra, IL-6 and lactoferrin levels in SBP patients at diagnosis were higher than in SA patients. After antibiotic treatment, the resistin, IL-1Ra, IL-6 and lactoferrin levels of patients with SBP tended to decrease without reaching SA levels (pg/ml resistin: SA 3512±738.9; SBP-Dx 19059±5968; *P<0*.001; SBP-Post 12660±4273; IL-1Ra: SA 43.96±6.4; SBP-Dx 1834±904.9; *P<0*.001; SBP-Post 78.9±16; IL-6: SA 4483 ± 1014; SBP-Dx 58025±15468; *P*= 0.002; SBP-Post 15467±8091; ng/ml lactoferrin: SA 178±36; SBP-Dx 4355±1201; SBP-Post 1006±392; P<0.001; Figure 2A). IL-1β levels were below the limit of detection in 90% of SA, 76.5% of SBP-Dx and 90% of SBP-Post samples (data not shown). We did not observe any relationship between the number of days of treatment and the level of decrease of each ascitic protein. There was a positive correlation between resistin and IL-6 levels *(r*=0.723, *P<0*.0001), between resistin and IL-1Ra levels *(r*=0.653, *P<0*.001), between IL-1Ra and IL-6 levels *(r*=0.807, *P*<0.0001),

between lactoferrin and resistin levels (r=0.645, P<0.001) and between lactoferrin and IL-1Ra levels (r=0.787, P<0.001; Supplementary Figure 1). In ascites, we observed that resistin was positively correlated with cytokines, and neutrophil-derived molecules, such as lactoferrin. However, only resistin levels were negatively correlated with the oxidative burst and NET production at diagnosis *(*r=-0.678, P=0.006*;* r=-0.639, P=0.004; respectively; Figure 2B).

4.4. Association between ascitic resistin and SBP characteristics, liver function and clinical evolution

Next, we analyzed the association of ascitic resistin levels with bacterial infection. We found that resistin levels tended to be higher in patients infected by Gram-negative bacteria than those infected by Gram-positive bacteria (Gram-Neg pg/ml: 33238±10857; Gram-Pos pg/ml: 9891±1617; P=0.09). However, Snall et al. have observed *in vitro* that resistin levels were higher in the supernatants of neutrophils cultured with Gram-positive than with Gram-negative bacteria.[20] One explanation for this discrepancy with our findings is that the resistin levels in the *in vitro* report were the direct result of neutrophil stimulation, whereas the resistin levels we observed in the ascitic fluid were the final outcome of several *in vivo* processes, such as production after bacterial stimulation, consumption by local cells and the regulation of resistin production by signals in the ascitic environment.

The ascitic resistin levels of SBP patients with an infection resolution in the first 5 days of antibiotic treatment were lower than those of patients who needed more than 5 days or who died during the first 5 days of treatment (≤5 days pg/ml: 8161±1394; >5 days or exitus pg/ml: 31349±10328; P=0.002; Figure 3A). Therefore high resistin levels were associated with a delayed resolution of infection in these patients.

Resistin has also been associated with insulin resistance, and liver cirrhosis is often characterized by hyperinsulinemia and insulin resistance.[21] We found that resistin levels correlated with ascitic glucose levels *(r*=-0.543, *P*=0.044; Figure 3B) and tended to correlate with the number of PMN/mm^3 *(r*=0.437, *P*=0.118) in our patients. Lin et al suggested that TNF-α could upregulate the adipose resistin gene in experimental cirrhosis.[22] The elevated levels of resistin then antagonize hepatic insulin action and raise plasma glucose levels.[23,24]

We found that ascitic resistin and IL-6 levels correlated with the MELD score *(r*=0.49, *P*=0.013*; r*=0.549, *P*=0.006, respectively; Figure 3C and Supplementary Figure 2). Similarly, plasma resistin levels in patients with cirrhosis have been associated with a poor prognosis and several complications and increased with the Child-Pugh and MELD scores and with markers of inflammation such as TNF-α and CRP.[11,25] In addition, it has been shown by other authors that cirrhotic patients with elevated plasma resistin levels had an increased mortality during follow-up.[11, 26]

4.5. Induction of resistin and IL-6

To identify the resistin source, isolated HN and PBMCs were cultured with inflammatory factors. Unstimulated and stimulated HN with *E.coli* and TNF-α produced more resistin than unstimulated and stimulated PBMCs. (HN pg/ml: Unstimulated 3360±253; *E.coli* 5117±726.3; TNF-α 5482±856.8; PBMCs pg/ml: Unstimulated 165±101.5; *E.coli* 1558±1056; TNF-α 353.4±100; Unstimulated *P*=0.0001; *E.coli P*=0.01; TNF-α *P*=0.003; Figure 4A). To identify the factors responsible for the resistin production, we analyzed whole blood cultures stimulated with different inflammatory cytokines. LPS and TNF-α increased resistin levels (pg/ml, Unstimulated 8519±2124; LPS 14283±3537; *P*=0.072; TNF-α 15761±2550; *P*=0.001; Figure 4B). Resistin was first identified as an

adipocyte-secreted hormone in rodents, but it was almost undetectable in human adipose tissue [27] and highly expressed in circulating leukocytes and bone marrow. We detected resistin secretion by neutrophils, but not by monocytes,[27, 28] and this secretion increased upon activation. In line with our findings, resistin has been shown to be secreted by circulating neutrophils in patients with Graves' disease and sepsis.[29, 30]

To identify whether the factors responsible for resistin production were also responsible for IL-6 production, we analyzed whole blood cultures stimulated with different inflammatory cytokines. LPS, resistin and IL-1Ra increased IL-6 levels (pg/ml, Unstimulated 74.9±74.9; LPS 19259±3478; resistin 1080±373.2; IL-1Ra 3365±707.2; *P*=0.005, *P*=0.03 and *P*=0.0075, respectively; Figure 4C). The oxidative burst of PMA-stimulated HN from whole blood was not affected by culture with inflammatory factors (MFI: Unstimulated 117.6±13.7; LPS 109.8±13.5; IL-6 101.1±9.7; resistin 118.3±9.3; TNF-α 109.7±8.2; IL-10 122.7±12.8; IL-1Ra 113.8±13.1).

4.6. Resistin influence on neutrophils and monocytes

To analyze whether resistin had a direct negative effect on neutrophil function, we stimulated healthy donor neutrophils in the presence of resistin. We did not observe any difference in the oxidative burst of HN incubated with different concentrations of resistin (10, 20, 40, 500 and 1000 ng/ml) and without resistin (MFI: Unstimulated 27±8.1; PMA 135.6±16.6; 10 ng/ml resistin+PMA 139.2±23.4; 20 ng/ml resistin+PMA 144.9±23.5; 40 ng/ml resistin+PMA 155.5±17.2; Figure 5A). This finding suggests that there was no direct effect of resistin on isolated or whole blood cultured neutrophils. This result is in discrepancy with Cohen et al who reported that resistin inhibited the oxidative burst of PMA-stimulated neutrophils.[31] Even though we tested different

concentrations of PMA and resistin and different stimulation times in an effort to replicate their findings, we were not able to detect the regulation of neutrophil function by resistin. We cannot rule out the possibility that this discrepancy is due to unknown culture factors.

Another possibility is that resistin has an indirect negative effect on neutrophil function, through its effect on PBMCs. In line with this, we found that the oxidative burst of HN in the presence of resistin-conditioned supernatant from *E.coli*-stimulated PBMCs pre-incubated with resistin (r-E-CS) was significantly lower than in the presence of supernatant from stimulated PBMCs without pre-incubation resistin (E-CS) (MFI: 137.5±25.54 vs 170.5±35.54, P=0.04; Figure 5B). These findings suggest that resistin has an indirect effect on neutrophils through resistin-induced molecules in conditioned supernatants.

Monocytes in PBMCs are a potential target of resistin. In fact, we have seen a negative correlation between ascitic resistin levels and the previously determined expression of markers on ascitic monocytes [6] (MFI: CD11b r=-0.727, P=0.01; CD86 r=-0.739, P=0.01; CD14 r=-0.729, P=0.04; Supplementary Figure 3) When healthy monocytes were pre-incubated with resistin before *E.coli* stimulation, the percentage and absolute numbers of CD14+CD16+ decreased (% CD14+CD16+: resistin+*E.coli* 33.9±3.8; *E.coli* 46±2; P=0.05; numbers CD14+CD16+: resistin+*E.coli* 3314±1209; *E.coli* 5906±1672; P=0.045; Figure 5C). We found an inverse result in the CD14+CD16- subpopulation, the percentage and absolute numbers of which increased when stimulated monocytes were pre-incubated with resistin (data not shown, P=0.02). Our findings suggest that resistin induces phenotype changes on monocytes. In addition, we observed decreased TNF-α production when monocytes were stimulated in the presence of resistin (TNF-α pg/ml: resistin+*E.coli*: 4586±599.1; *E.coli* 5725±712.5; P=0.01; Figure 5D).

Here, we propose a model to explain the origin and role of resistin in the ascitic fluid of patients with SBP. According to our results, the presence of Gram-negative bacteria stimulates ascitic macrophages. Thus, LPS induces the production of inflammatory cytokines, such as TNF-α, which induces the production and secretion of resistin. Resistin then activates a negative feedback loop with neutrophils and macrophages to regulate their excessive inflammatory response. As a result, neutrophil function is turned down and the macrophage phenotype becomes less inflammatory. The consequence of this downregulation is an impaired innate immune system that jeopardizes an effective anti-microbial response, facilitating the spread of infection and the development of peritonitis. In this context, treatment with antibiotics, and the consequent elimination of bacteria, reduces the bacterial load and with it, the inflammatory signals induced by resistin. The final outcome is a return to the status before bacterial infection. Since there is no available neutralizing antibody against resistin, we cannot rule out the possibility that resistin is the major contributor to neutrophil impairment or that a more complex combination of ascitic factors is regulating the neutrophil function. Our future work will be focused on exploring these factors in order to perform a multivariant analysis on a larger cohort of patients.

Future studies should focus on considering immunomodulatory agents as an adjuvant therapeutic approach, in addition to the current standard of care, in order to improve the prognosis of patients with cirrhosis and SBP.[32, 33]

5. AUTORSHIP

J.C.N., L.P., A.M.: ELISA, functional assays, flow cytometry, results analysis, and manuscript writing. C.Z., E.C., E.S., G.J.: flow cytometry and results analysis. F.N.: selection and culture of bacteria. G.S., M.P., E.R., C.G., C.Gu, E.A.: inclusion of patients and samples and clinical characterization of patients. C.G. and C.J.: results analysis and supervision. G.J., J.N., L.P.: results analysis, processing of samples and manuscript writing. S.V.: experimental design, results analysis, and manuscript writing.

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7. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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9. FIGURE LEGENDS

Figure 1. Effect of ascitic fluid from SBP and SA on healthy neutrophils. (A) Oxidative burst capacity of PMA-stimulated HN cultured in 10% FBS (Control, *n* = 6), 10% SA (*n* = 8), 10% SBP at diagnosis (Dx, *n* = 9) or 10% SBP after treatment (Post, *n* = 6) for 40 min. No oxidative burst was observed in neutrophils without stimulation. (B) Quantification of NET production of PMA-stimulated HN cultured in 10% FBS (Control, *n* = 6), 10% SA (*n* = 9), 10% SBP at diagnosis (*n* = 11) or 10% SBP after treatment (*n* = 6) for 2 h. No NETosis was produced by neutrophils without stimulation. (C) Visualization of NET production of one representative experiment at 10X. NETs are visualized in green (Sytox Green) and HN in red (anti-CD66b). The Mann-Whitney *U* test was used for comparisons between Control, SA and 2 SBP groups. The Wilcoxon test was used for the comparisons between SBP at Dx and SBP after treatment.

Figure 2. Distinctive concentration of soluble factors in ascitic fluid. (A) Measurement of resistin, IL-1Ra, IL-6 and lactoferrin levels in SA (*n* = 10), SBP at diagnosis (Dx, *n* = 16) and SBP after treatment (Post, *n* = 6). (B) Correlations between resistin levels and oxidative burst and NET production of HN cultured in 10% SA (white) and in 10% SBP at diagnosis (grey). The Mann-Whitney *U* test was used for comparisons between SA and 2 SBP groups. The Wilcoxon test was used for the comparisons between SBP at Dx and SBP after treatment. Correlations were analyzed using the Pearson test.

Figure 3. Association between resistin levels in ascitic fluid and analytical characteristics of SBP. (A) Comparison of the ascitic fluid resistin levels of SBP patients with infection resolution during the first 5 or less days of antibiotic treatment and patients who needed more than 5 days to resolve SBP or died

565 during the first 5 days of treatment. (B) Correlation between resistin levels and
566 glucose levels (mg/dl) measured in SBP at diagnosis. The black dot shows a
567 patient with high glucose levels in both ascites and plasma, raising suspicions
568 that glucose levels in ascites were not entirely due to infection. (C) Correlations
569 between resistin levels and the MELD score in patients with SA (white) and
570 SBP at diagnosis (grey).

571 **Figure 4**. Resistin and IL-6 production by stimulated cells. (A) Resistin levels of
572 HN (n = 5) and PBMCs cultured separately without stimulation (∅) or stimulated
573 with *E.coli* or TNF-α. (B) Resistin and (C) IL-6 production of healthy whole blood
574 (n = 5) without stimulation (∅) or stimulated with LPS, IL-6, Resistin, TNF-α, IL-
575 10 and IL-1Ra. The paired t-test was used for comparisons between stimuli.
576 Mann-Whitney *U* and Wilcoxon tests were used for unpaired and paired
577 comparisons of 2 groups respectively. .

578 **Figure 5.** Effect of priming with resistin (R) healthy PBMCs stimulated with
579 bacteria. (A) Representative experiment and results of the effect of resistin on
580 the oxidative burst capacity of HN. (B) PMA-induced oxidative burst of HN
581 cultured with resistin-conditioned supernatant (CS). HN were cultured with 50%
582 of supernatants from unstimulated or *E.coli*-stimulated PBMCs pre-incubated
583 with resistin (50 ng/ml) (r-CS, r-E-CS) or with media (CS and E-CS). (C)
584 Representative flow cytometry dot plots of monocyte subpopulations and
585 CD14+CD16+ monocytes counts of primed PBMCs gated on monocytes. The
586 total counts of monocytes did not change after 20h of culture conditions. (D)
587 TNF-α levels (*n*=5) of primed and unprimed PBMCs stimulated with *E.coli* or
588 *E.faecium*. The Wilcoxon test was used for comparisons between stimuli

Supplementary Figure 1. Associations between soluble factors measured in ascitic fluid. (A) Correlations between resistin and IL-6, resistin and IL-1Ra and IL-1Ra and IL-6, lactoferrin and resistin, lactoferrin and IL-1Ra and lactoferrin and IL-6 levels in SA (white) and SBP at diagnosis (grey). Correlations were analyzed using the Pearson test.

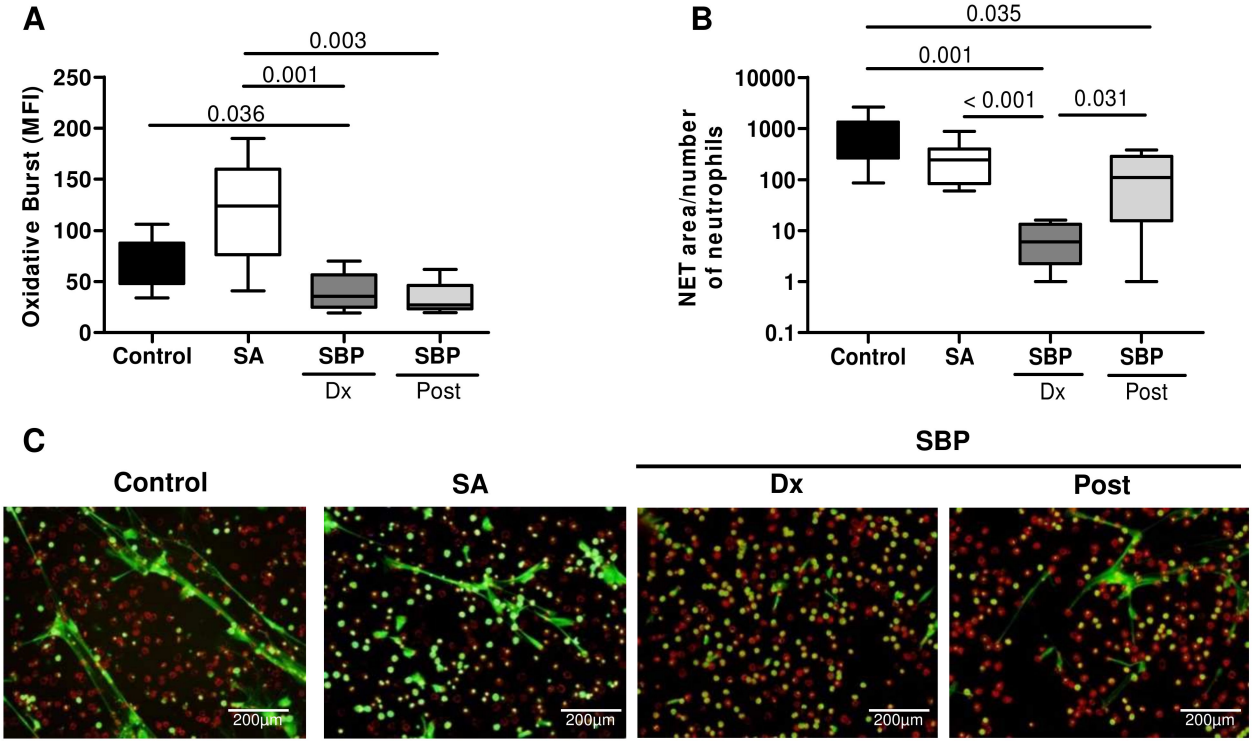
Supplementary Figure 2. Association between IL-6 levels in ascitic fluid and the MELD score of patients with SA (white) and SBP at diagnosis (grey). Correlations were analyzed using the Pearson test.

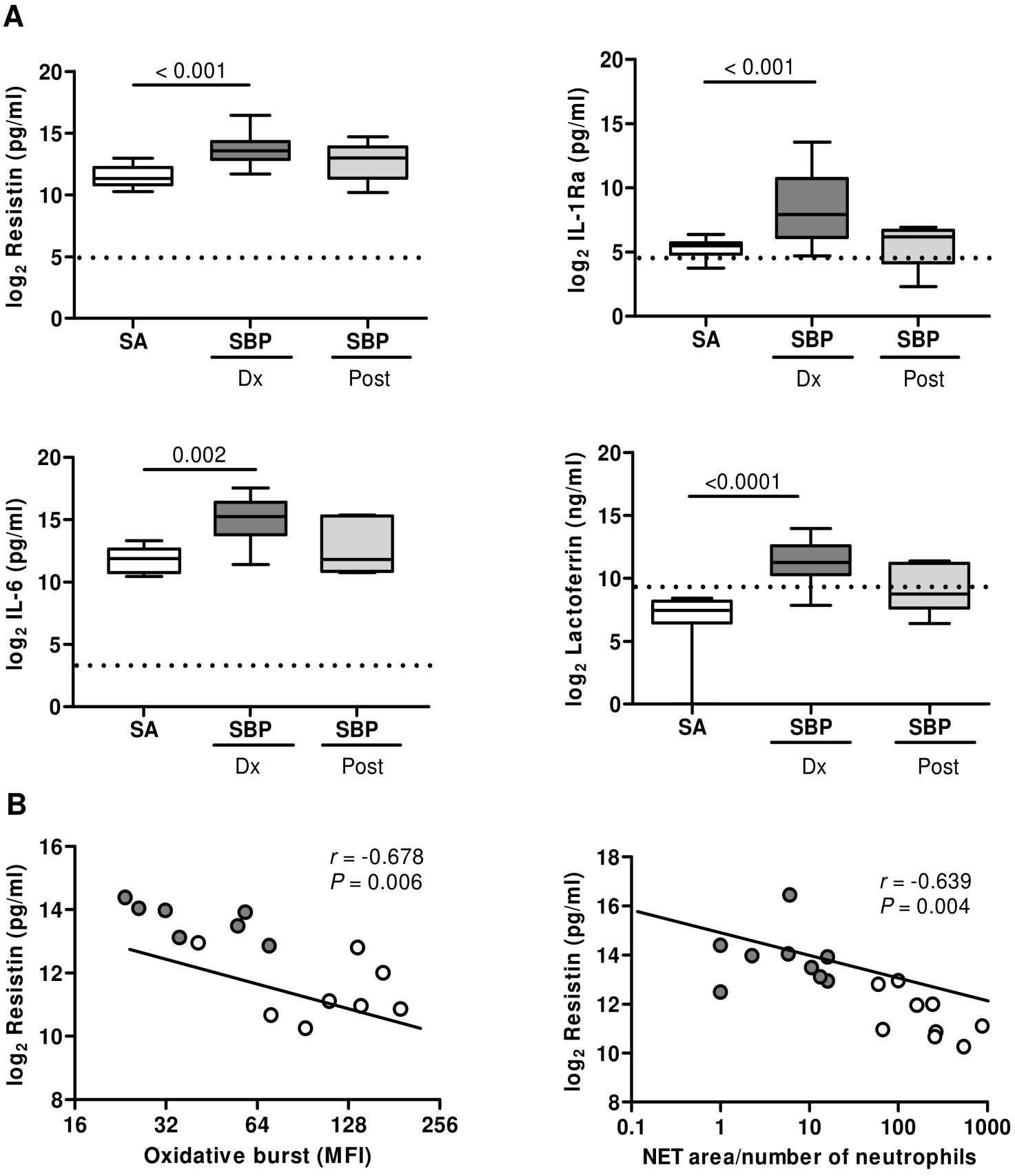
Supplementary Figure 3. Association between resistin levels in ascitic fluid and markers of ascitic macrophages in SA (white) and SBP at diagnosis (grey). Correlations between resistin levels in ascitic fluid and MFI expression of CD11b, CD86 and CD14 of ascitic macrophages. Correlations were analyzed using the Pearson test.

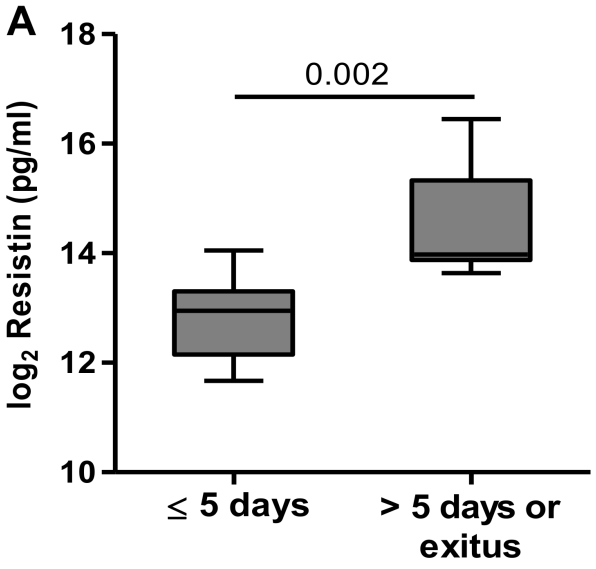
TABLE I. Baseline characteristics of patients with SA or SBP.

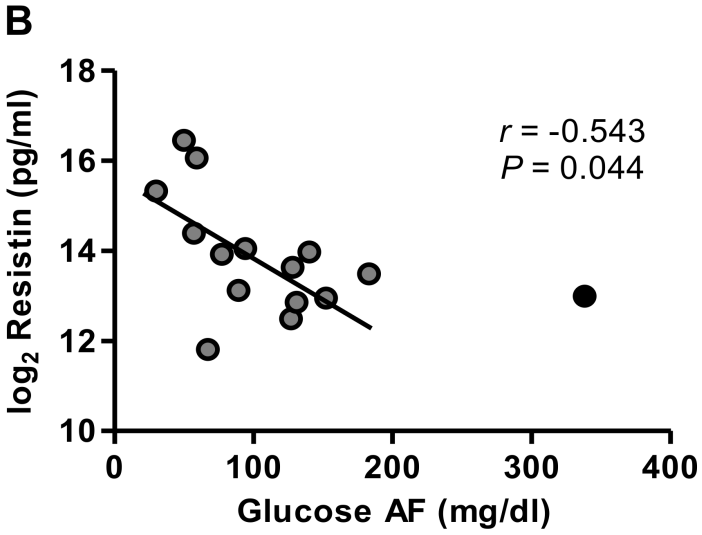
	SA ***n* = 10**	**SBP** ***n* = 16**	***P*** **value**
Age (years)	63.5 ± 2.9	72.0 ± 2.9	0.06
Gender (Male/Female) (%)	8 (80) / 2 (20)	7 (43.8) / 9 (56.3)	0.11
Etiology (%) -Alcohol / Hepatitis C virus/ Hepatitis B virus / Alcohol-Virus/ Cryptogenic	 5 (50) / 3 (30)/ 0 (0) / 1 (10)/ 1 (10)	 7 (43.8) / 6 (37.5) 1 (6.3) / 1 (6.3)/ 1(6.3)	0.91
Active alcohol intake (%)	2 (20)	2 (12.5)	0.63
Diabetes mellitus (%)	7 (70)	7 (43.8)	0.25
Child-Pugh score	8.1 ± 0.4	9.9 ± 0.4	**0.004**
MELD score	12.5 ± 1.1	18.0 ± 1.7	**0.01**
Previous decompensations (%)	10 (100)	15 (93.8)	1.00
- Previous ascites (%)	10 (100)	15 (93.8)	1.00
- Previous SBP (%)	2 (20)	7 (43.8)	0.39
- Previous encephalopathy (%)	2 (20)	11 (68.8)	**0.04**
- Previous variceal bleeding (%)	2 (20)	3 (18.8)	1.00
Previous hepatocelullar carcinoma (%)	0 (0)	1 (6.3)	1.00
Serum sodium (mmol/L)	135.1 ± 0.6	132.7 ± 1.4	0.14
Serum urea (mmol/L)	13.2 ± 1.6	13.9 ± 2.1	0.82
Serum creatinine (µmol/L)	116 ± 13.1	105.4 ± 12.5	0.43
Serum bilirubin (µmol/L)	31.0 ± 7.0	75.6 ± 14.4	**0.02**
Serum albumin (g/L)	28.4 ± 3.0	26.4 ± 1.3	0.50
Prothrombin time ratio	1.2 ± 0.04	1.6 ± 0.1	**<0.001**
Platelet count ($x10^9$/L)	121 ± 20.2	82.6 ± 9.6	0.16
Diuretics (%)	10 (100)	13 (81.3)	0.32
Norfloxacin prophylaxis (%)	4 (40)	7 (43.8)	0.89
Ascitic fluid neutrophil count (/mm^3)	16 ± 9.6	6143.5 ± 2122.4	**<0.001**
Positive ascitic fluid culture, n (%)	0 (0)	16 (100)	**<0.001**
- *Escherichia coli* - Streptococcus viridans group - *Streptococcus pneumoniae* - *Enterococcus faecium* - *Pseudomonas aeruginosa*		8 (50) 3 (18.8) 3 (18.8) 1 (6.3) 1 (6.3)	

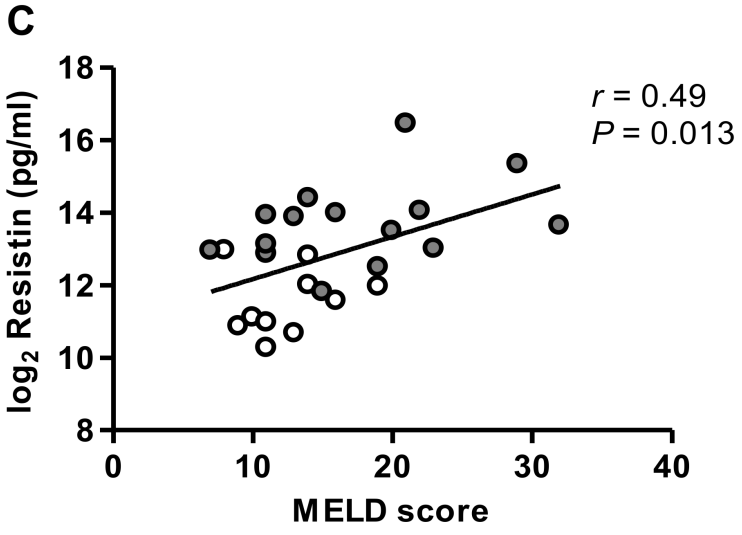
Values are expressed as mean ± standard error of the mean

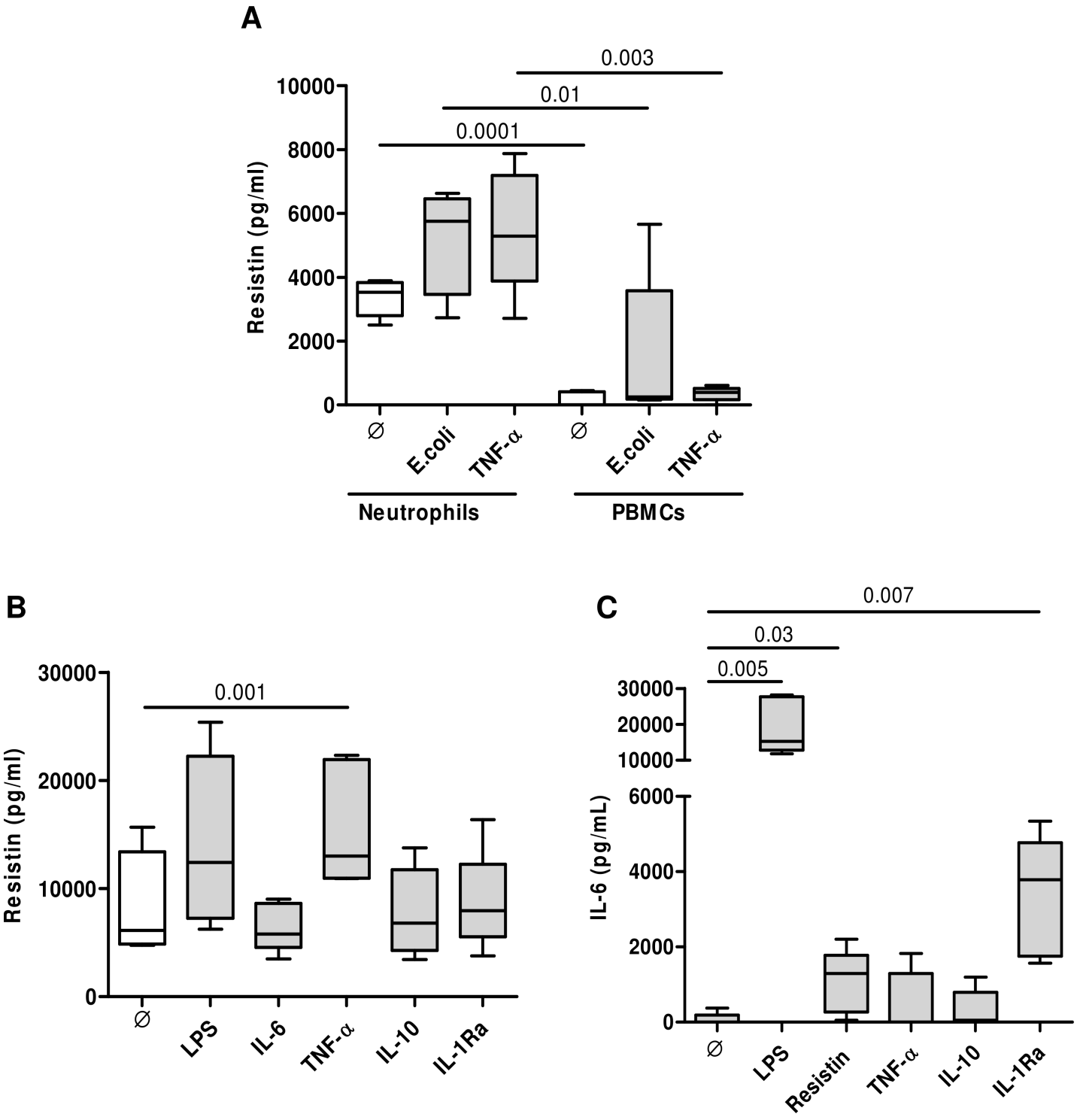

A
Oxidative Burst (MFI)
250
200
150
100
50
0
0.003
0.001
0.036
Control
SA
SBP
Dx
SBP
Post
B
NET area/number of neutrophils
10000
1000
100
10
1
0.1
0.035
0.001
< 0.001
0.031
Control
SA
SBP
Dx
SBP
Post
C
Control
SA
SBP
Dx
Post
200µm
200µm
200µm
200µm

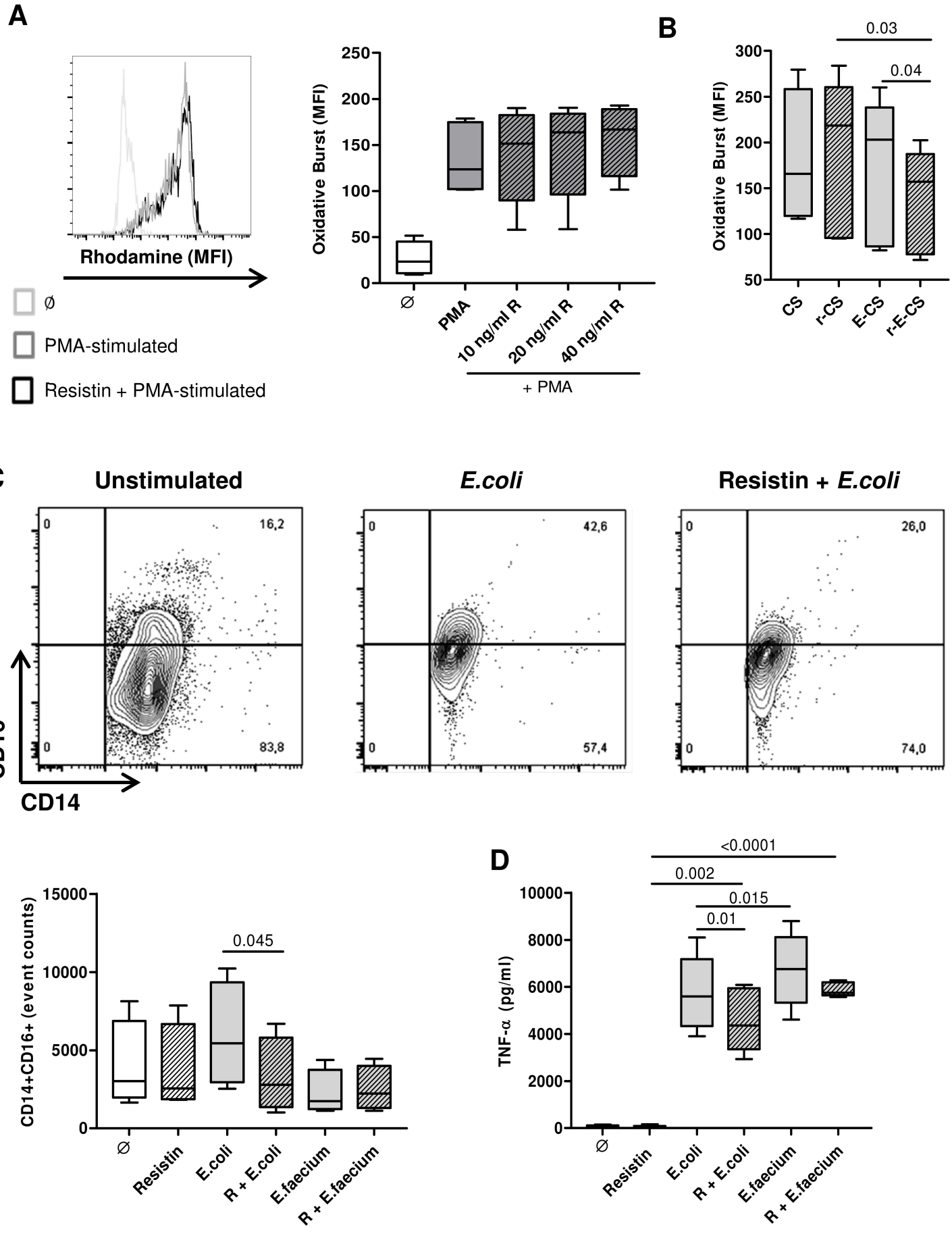

A
< 0.001
log2 Resistin (pg/ml)
20
15
10
5
0
SA
SBP
Dx
SBP
Post
< 0.001
log2 IL-1Ra (pg/ml)
0.002
log2 IL-6 (pg/ml)
<0.0001
log2 Lactoferrin (ng/ml)
B
r = -0.678
P = 0.006
log2 Resistin (pg/ml)
16
14
12
10
8
16
32
64
128
256
Oxidative burst (MFI)
r = -0.639
P = 0.004
18
0.1
1
10
100
1000
NET area/number of neutrophils


A
18
16
14
12
10
log2 Resistin (pg/ml)
0.002
≤ 5 days
> 5 days or exitus

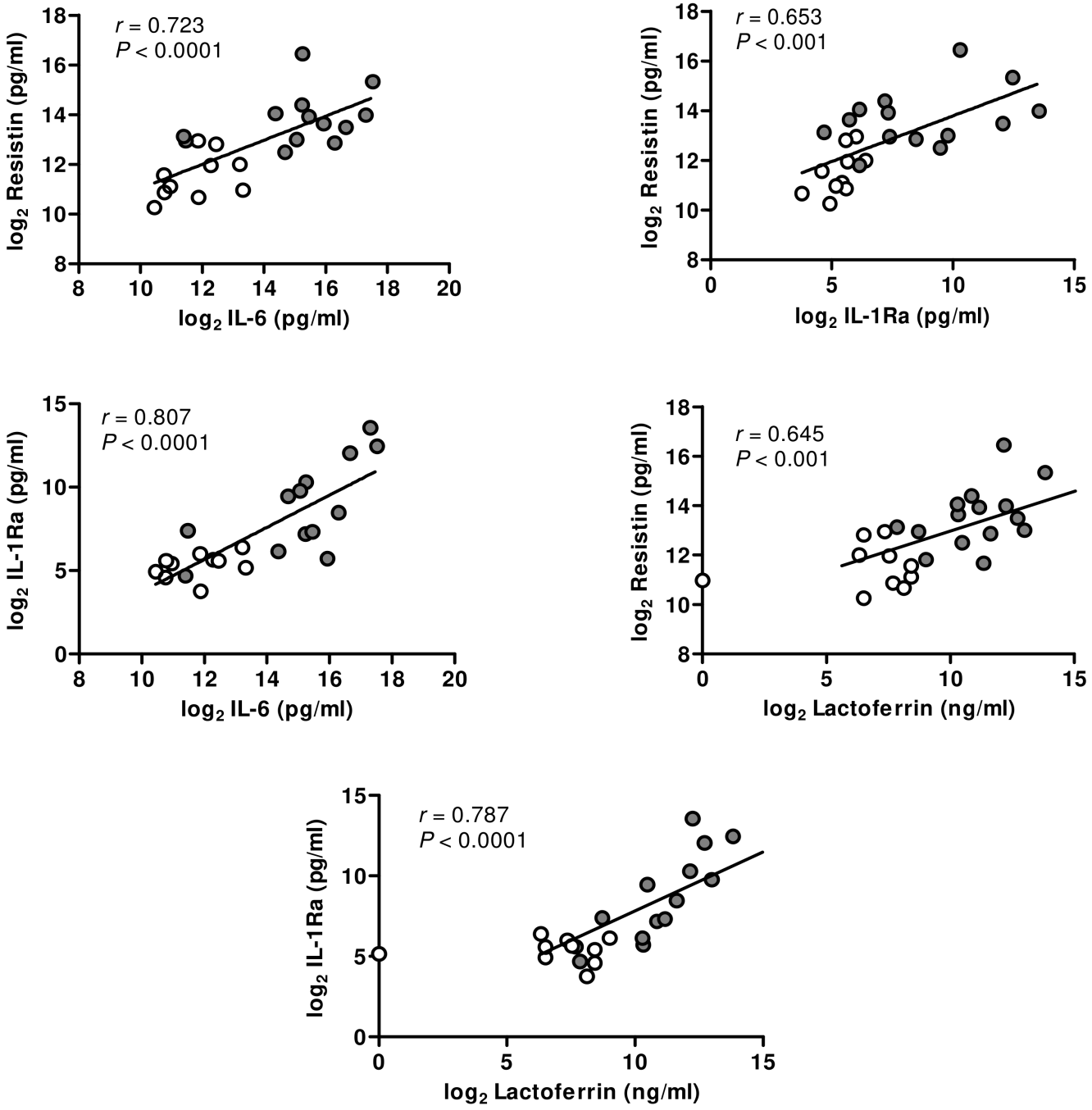

B
18
16
14
12
10
log2 Resistin (pg/ml)
r = -0.543
P = 0.044
0
100
200
300
400
Glucose AF (mg/dl)

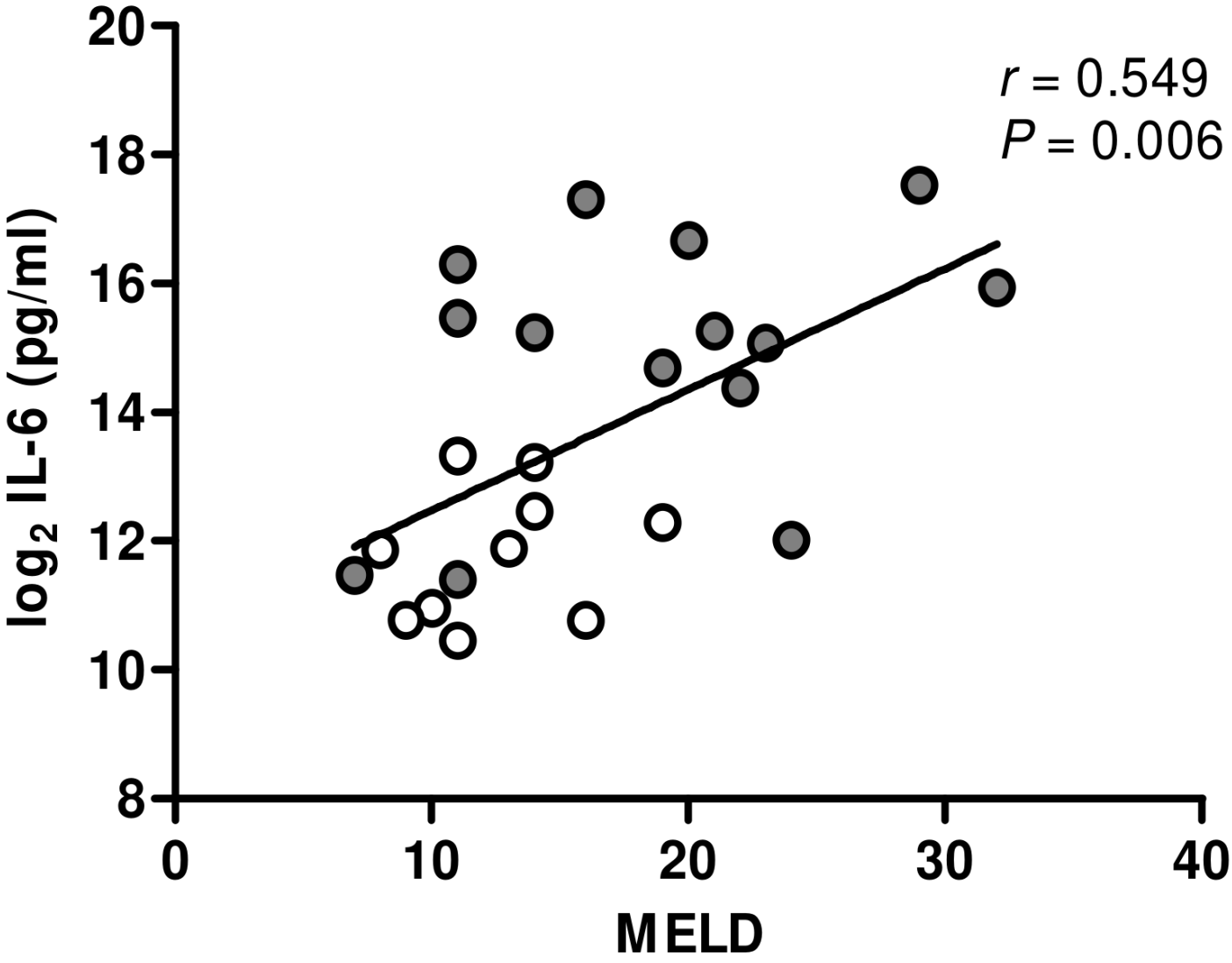

C
18
16
14
12
10
8
log2 Resistin (pg/ml)
r = 0.49
P = 0.013
0
10
20
30
40
MELD score

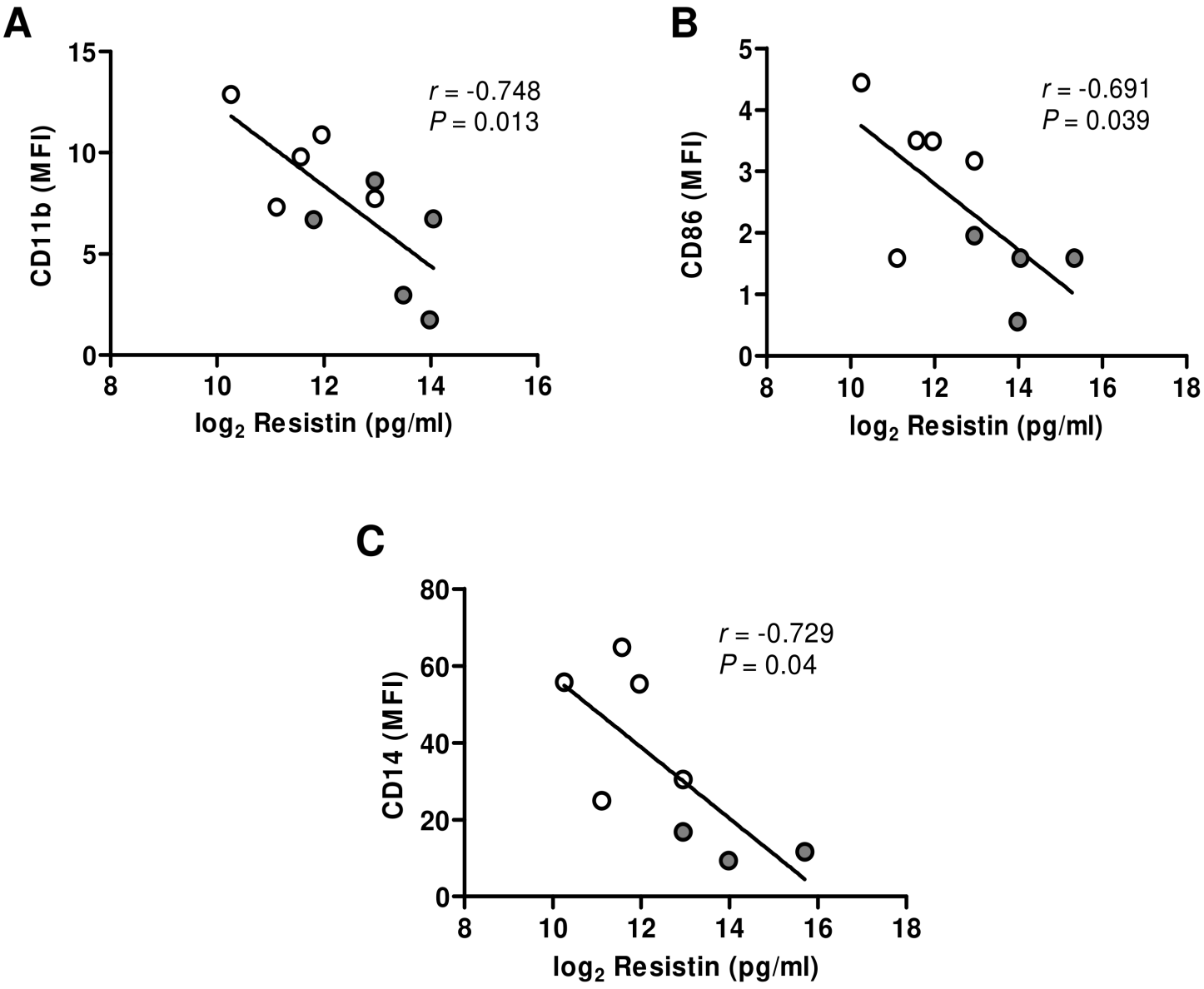

A
0.003
0.01
0.0001
10000
8000
6000
4000
2000
0
Resistin (pg/ml)
∅
E.coli
TNF-α
∅
E.coli
TNF-α
Neutrophils
PBMCs
B
0.001
30000
20000
10000
0
Resistin (pg/ml)
∅
LPS
IL-6
TNF-α
IL-10
IL-1Ra
C
0.007
0.03
0.005
30000
20000
10000
6000
4000
2000
0
IL-6 (pg/mL)
∅
LPS
Resistin
TNF-α
IL-10
IL-1Ra


A
Rhodamine (MFI)
Ø
PMA-stimulated
Resistin + PMA-stimulated
Oxidative Burst (MFI)
250
200
150
100
50
0
Ø
PMA
10 ng/ml R
20 ng/ml R
40 ng/ml R
+ PMA
B
0.03
0.04
Oxidative Burst (MFI)
300
250
200
150
100
50
CS
r-CS
E-CS
r-E-CS
C
Unstimulated
E.coli
Resistin + E.coli
CD16
CD14
16,2
83,8
42,6
57,4
26,0
74,0
CD14+CD16+ (event counts)
15000
10000
5000
0
0.045
Ø
Resistin
E.coli
R + E.coli
E.faecium
R + E.faecium
D
<0.0001
0.002
0.015
0.01
TNF-α (pg/ml)
10000
8000
6000
4000
2000
0
Ø
Resistin
E.coli
R + E.coli
E.faecium
R + E.faecium

A


r = 0.723
P < 0.0001
log2 Resistin (pg/ml)
log2 IL-6 (pg/ml)
r = 0.653
P < 0.001
log2 Resistin (pg/ml)
log2 IL-1Ra (pg/ml)
r = 0.807
P < 0.0001
log2 IL-1Ra (pg/ml)
log2 IL-6 (pg/ml)
r = 0.645
P < 0.001
log2 Resistin (pg/ml)
log2 Lactoferrin (ng/ml)
r = 0.787
P < 0.0001
log2 IL-1Ra (pg/ml)
log2 Lactoferrin (ng/ml)


20
18
16
14
12
10
8
log2 IL-6 (pg/ml)
r = 0.549
P = 0.006
0
10
20
30
40
MELD


A
CD11b (MFI)
15
10
5
0
8
10
12
14
16
log2 Resistin (pg/ml)
r = -0.748
P = 0.013
B
CD86 (MFI)
5
4
3
2
1
0
8
10
12
14
16
18
log2 Resistin (pg/ml)
r = -0.691
P = 0.039
C
CD14 (MFI)
80
60
40
20
0
8
10
12
14
16
18
log2 Resistin (pg/ml)
r = -0.729
P = 0.04