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Abnormal Plasma Microparticles Impair Vasoconstrictor Responses in Patients With Cirrhosis

PIERRE–EMMANUEL RAUTOU,[*,‡,§] JULIE BRESSON,[*,‡] YANNIS SAINTE–MARIE,[||] ANNE–CLEMENCE VION,[*,‡] VALERIE PARADIS,[¶,#,**] JEAN–MARIE RENARD,[*] CECILE DEVUE,[*] CHRISTOPHE HEYMES,[||] PHILIPPE LETTERON,[¶,#] LAURE ELKRIEF,[§,¶,#] DIDIER LEBREC,[§,¶,#] DOMINIQUE VALLA,[§,¶,#] ALAIN TEDGUI,[*,‡] RICHARD MOREAU,[§,¶,#] and CHANTAL M. BOULANGER[*,‡]

*INSERM Unité 970, Paris Cardiovascular Research Center - PARCC, Paris; ‡Université Paris Descartes, Sorbonne Paris Cité, UMR-S970, Paris; §Service d'Hépatologie, Hôpital Beaujon, Assistance Publique-Hôpitaux de Paris, Clichy; ||INSERM Unité 1048, Institut des Maladies Métaboliques et Cardiovasculaires, Toulouse; ¶INSERM Unité 773, Centre de Recherche Biomédicale Bichat-Beaujon CRB3, Clichy; #Université Denis Diderot-Paris 7, Paris; and **Service d'Anatomie Pathologique, Hôpital Beaujon, Clichy, France

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BACKGROUND & AIMS: Circulating membrane-shed microparticles (MPs) participate in regulation of vascular tone. We investigated the cellular origins of MPs in plasma from patients with cirrhosis and assessed the contribution of MPs to arterial vasodilation, a mechanism that contributes to portal hypertension. **METHODS:** We analyzed MPs from blood samples of 91 patients with cirrhosis and 30 healthy individuals (controls) using flow cytometry; their effects on the vascular response to vasoconstrictors were examined in vitro and in vivo. **RESULTS:** Circulating levels of leuko-endothelial ($CD31^+/41^-$), pan-leukocyte ($CD11a^+$), lymphocyte ($CD4^+$), and erythrocyte ($CD235a^+$) MPs were higher in patients with cirrhosis than in controls. Plasma of patients with cirrhosis contained hepatocyte-derived MPs (cytokeratin-18^+), whereas plasma from controls did not. The severity of cirrhosis and systemic inflammation were major determinants of the levels of leuko-endothelial and hepatocyte MPs. MPs from patients with advanced cirrhosis significantly impaired contraction of vessels in response to phenylephrine, whereas MPs from healthy controls or from patients of Child–Pugh class A did not. This effect depended on cyclooxygenase type 1 and required phosphatidylserine on the surface of MPs. Intravenous injection of MPs from patients with cirrhosis into BALB/C mice decreased mean arterial blood pressure. **CONCLUSIONS: Cirrhosis is associated with increases in circulating subpopulations of MPs, likely resulting from systemic inflammation and liver cell damage. The overall pool of circulating MPs from patients with advanced cirrhosis impairs vasoconstrictor responses and decreases blood pressure, contributing to the arterial vasodilation associated with portal hypertension.**

Keywords: Microvesicle; COX-1; Hypocontractility; Hepatitis.

In patients with cirrhosis, abnormal persistent vasodilation of arterial vessels leads to increased portal venous inflow and contributes to portal hypertension. Indeed, portal hypertension is determined by both the intrahepatic resistance to portal blood flow, related to liver architecture changes, and the extent of inflow into the portal venous system from the splanchnic bed. Portal hypertension is a major factor in the development of complications of cirrhosis, and because arterial vasodilation associated with portal hypertension is potentially reversible, understanding its mechanisms is of great therapeutic interest.[1]

Vasodilation is associated with both enhanced formation of vasodilators and vascular hyporesponsiveness to vasoconstrictors, also referred as to "vascular hypocontractility." Overproduction of nitric oxide cannot fully account for this effect, and several studies, including those in endothelial nitric oxide synthase (NOS-3) knockout mice, have shown that other factors are involved in the pathogenesis of arterial vasodilation. However, little is known about these factors, and more importantly, the mechanisms leading to vascular hypocontractility have not yet been elucidated.[1]

Microparticles (MPs) are membrane vesicles with a diameter ranging from 0.1 to 1 μm, released in extracellular space following cell activation or apoptosis.[2] MPs harbor at their surface most of the membrane-associated proteins of the cells they stem from and are characterized by the loss of plasma membrane asymmetry resulting in the exposure of phosphatidylserine on their outer leaflet.[2] MPs are present in the blood of healthy subjects, and their levels are increased in pa-

Abbreviations used in this paper: CFSE, carboxyfluorescein succinimidyl ester; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle medium; HUVEC, human umbilical vein endothelial cell; MELD, Model of End-Stage Liver Disease; MP, microparticle; NOS, nitric oxide synthase.

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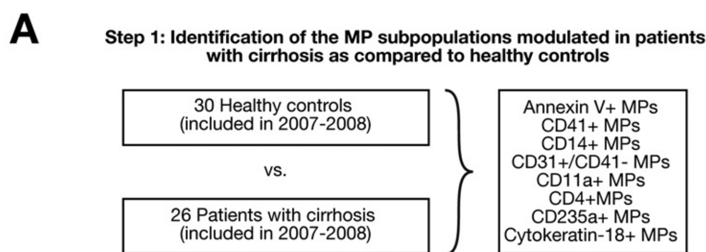

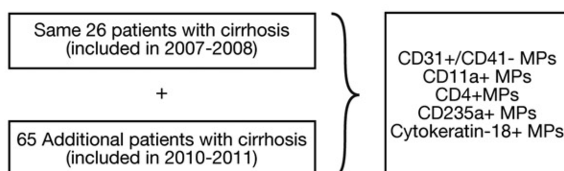

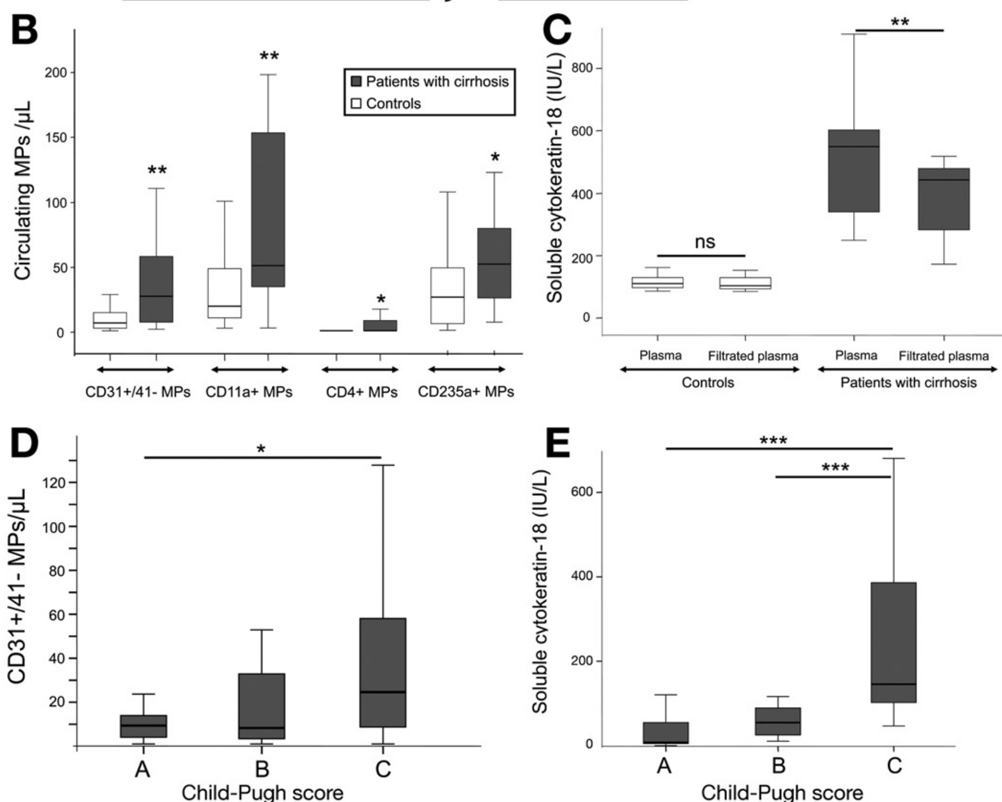

Figure 1. Cellular origin of circulating MPs in patients with cirrhosis and in healthy controls. (*A*) Study flow diagram. (*B*) $CD31^+/41^-$, $CD11a^+$, $CD4^+$, and $CD235a^+$ MPs are of leuko-endothelial, pan-leukocyte, lymphocyte, and erythrocyte origin, respectively (flow cytometry analysis; 26 patients with cirrhosis, 30 healthy controls). (*C*) Soluble native cytokeratin-18 levels (M65 antigen), a marker of hepatocytes, in native and filtrated (to remove MPs) plasmas (enzyme-linked immunosorbent assay; 9 patients with cirrhosis vs 5 controls). The difference between native and filtrated M65 antigen level reflects the level of MPs derived from hepatocytes. (*D*) Circulating levels of $CD31^+/41^-$ in 91 patients with cirrhosis according to Child–Pugh score (A, n = 16; B, n = 18; C, n = 55). (*E*) Levels of cytokeratin-18 (M65 antigen) bound to MPs (obtained by the difference between native and filtrated M65 antigen level) according to Child–Pugh score (A, n = 9; B, n = 9; C, n = 22). Data are given as median (*horizontal bar*), 25th and 75th percentile (*boxes*), and extreme values, which are less than 3 box lengths from either end of the box (*error bar*). ns, not significant; $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

tients with high atherothrombotic risk.[2] MPs are not inert by-products. A number of studies point out that MPs can affect several cellular functions, including vascular tone and vascular reactivity.[2] However, these studies were performed using MPs generated in vitro or isolated from patients with cardiovascular diseases or sepsis.[2] Because lipid and protein fractions of MPs, as well as their biological effects, greatly vary depending on the stimulus initiating cell blebbing and MP release,[3–5] we aimed to determine the level and cellular origins of circulating MPs in patients with cirrhosis and test the hypothesis that MPs of patients with cirrhosis contribute to vascular hypocontractility.

Patients and Methods

Patients and Controls

We included 91 patients admitted to the Liver Unit (Hôpital Beaujon, Clichy, France) for alcoholic and/or hepatitis C virus–related cirrhosis during 2 periods: 26 patients between 2007 and 2008 (the pilot cohort) and 65 additional patients between 2010 and 2011 (Figure 1*A*). None of the patients had severe sepsis, hepatocellular carcinoma assessed using serum α-fetoprotein level and computed tomographic scan or ultrasonography, or portal vein thrombosis. The pilot cohort was compared with a group of 30 healthy volunteers. The effect on vascular reactivity of MPs from 6 patients with end-stage renal failure included in a previous study was also analyzed.[6] All patients and controls gave their informed

consent to participate in the study. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Isolation and Characterization of Circulating MPs

Circulating MPs were isolated from platelet-free plasma obtained by successive centrifugations of venous blood as reported earlier.[5] Briefly, citrated venous blood (15 mL) was centrifuged at 500*g* for 15 minutes to remove cells. The supernatant of the first centrifugation was then centrifuged at 15,200*g* for 5 minutes to remove cell debris and apoptotic bodies (+18°C).[5,7] The resulting platelet-free plasma contained almost no apoptotic bodies (Supplementary Figure 1). H-Gly-Pro-Arg-Pro-OH (5 μmol/L; Calbiochem, La Jolla, CA) was then added, and the samples were stored frozen at −80°C until use. Flow cytometry analysis of MPs was performed on this platelet-free plasma, as described in Supplementary Materials and Methods.

Enzyme-Linked Immunosorbent Assays

To assess hepatocyte-derived MP levels, native soluble cytokeratin-18 levels (M65 antigen) were determined using commercially available immunoassays (M65 ELISA kit; Peviva AB, Bromma, Sweden) in the platelet-free plasma of controls and of patients both before and after 2 successive 0.2-μm filtrations (Ceveron MFU 500; Technoclone, Vienna, Austria). The difference between soluble cytokeratin-18 levels in initial and in filtrated platelet-free plasma reflected the concentration in hepatocyte MPs.

The stable metabolite of PGI2 (ie, 6-keto PGF1α) was measured by enzyme immunoassays (Cayman Chemical Co, Ann Arbor, MI) in the medium of rat aortic rings incubated for 1 hour with MPs or the corresponding supernatant.

Organ Chamber Experiments

To obtain, after dilution in aortic ring medium, concentrations in MPs similar to those present in the plasma of the patients, circulating MPs were concentrated from platelet-free plasma (obtained as described previously) using centrifugation at 20,500*g* for 150 minutes (+4°C). The MP pellet thus obtained in a minimal volume of supernatant was then resuspended in Dulbecco's modified Eagle medium (DMEM) to reach a final volume corresponding to 1/10 of the initial volume of plasma. The effect of MPs was compared with either the effect of the same volume of platelet-free plasma devoid of MPs (ie, the supernatant obtained after the 20,500*g* centrifugation and then filtered on 0.22- and 0.1-μm filters; thereafter referred to as "supernatant") also resuspended in DMEM or the effect of DMEM alone. This supernatant thus contains the same soluble factors as those present in the MP fraction but does not hold MPs.

A detailed description of organ chamber experiments is provided in Supplementary Materials and Methods. Briefly, rings from rat thoracic aortas were incubated with circulating MPs from patients or from controls at their circulating concentration or with the 20,500*g* supernatant or with DMEM alone. After 24 hours, the rings were mounted in organ chambers filled with modified Krebs–Ringer solution to study relaxation to acetylcholine chloride and contraction to phenylephrine, as previously reported.[6,8] Pilot experiments indicated that the plasma MP effect was similar at 2 and 24 hours of exposure (data not shown); therefore, unless otherwise stated, all experiments were performed after 24 hours of incubation.

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Induction of Cirrhosis by Bile Duct Ligation

Cirrhosis was induced in 12-week-old male Wistar rats (Charles River Laboratories, Saint-Aubin-les-Elbeuf, France) by bile duct ligation as described[9] (see Supplementary Materials and Methods for details).

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were plated in 24- or 6-well plates and incubated at 37°C in a 5% CO_2 incubator. HUVECs (2 different donors) were obtained from Promocell (Heidelberg, Germany) and cultured in endothelial cell basal medium supplemented with serum and growth factors (Promocell). All experiments were performed between passage 2 and 6.

Assessment of the Transfer of MPs to Endothelial Cells

To test the hypothesis of a transfer of MPs to endothelial cells, HUVECs were incubated with fluorescent circulating MPs from patients with cirrhosis. After 24 hours, the percentage of fluorescent HUVECs was analyzed on a LSR II flow cytometer (BD Biosciences, San Jose, CA) (see Supplementary Materials and Methods for details).

Western Blotting

Detection of cyclooxygenase (COX)-1, COX-2, NOS-2, NOS-3, and prostacyclin synthase on rat aortic rings and HUVECs incubated for 24 hours with MPs from patients with cirrhosis at their circulating concentration, supernatant, or DMEM was performed by Western blot analysis (see Supplementary Materials and Methods for details).

In Vivo Blood Pressure Measurement

Male C57Bl/6 (wild-type) mice weighing 27 to 33 g and male BALB/C nude mice weighing 30 to 36 g were injected retro-orbitally with circulating MPs from patients with cirrhosis or the same volume of the corresponding 20,500*g* supernatant. Two hours later, arterial blood pressure was measured before and after phenylephrine injection (see Supplementary Materials and Methods for details).

Statistics

Quantitative variables were expressed as median (range) and categorical variables as absolute and relative frequencies. Comparisons between groups of independent quantitative variables were performed using Mann–Whitney test. Wilcoxon test was used for paired variables (eg, effect of MPs and supernatant from the same patient). Comparisons between groups of qualitative variables were performed using χ^2 test and Fisher exact test, as appropriate. An analysis of variance for repeated measures was used to evaluate the concentration-response curves to phenylephrine and acetylcholine. Spearman's correlation coefficient was used to test the correlation between circulating MP levels and clinical, laboratory, and hemodynamic variables. Cumulative transplantation-free survival curves were calculated by Kaplan–Meier method. Patients who underwent liver transplantation were counted as censored. The potential relation of circulating MP level to the risk of death was analyzed by Cox regression univariate analysis. To determine if circulating MP levels were related to survival independently of Child–Pugh or Model of End-Stage Liver Disease (MELD) scores, we fitted 4 Cox models, including circulating MP level achieving a P value

<.05 at univariate analysis and each score successively, as previously reported.[10] All tests were 2 sided and used a significance level of .05. Data handling and analysis were performed with SPSS 17.0 (SPSS Inc, Chicago, IL).

Results

Cellular Origin of MPs From Patients With Cirrhosis

We first compared circulating MP levels and cellular origins in the 26 patients with cirrhosis from the pilot cohort and in 30 healthy controls (Figure 1*A*). Age and prevalence of cardiovascular risk factors were not different between patients and controls except for a lower prevalence of dyslipidemia and a lower body mass index in the former group (Supplementary Table 1). Despite this lower prevalence of dyslipidemia and a lower body mass index (dyslipidemia and obesity being known to be associated with increased $CD31^+$ MPs),[11] circulating levels of leuko-endothelial ($CD31^+/41^-$), pan-leukocyte ($CD11a^+$), lymphocyte ($CD4^+$), and erythrocyte ($CD235a^+$) MPs were higher in patients with cirrhosis than in controls (Figure 1*B*). Because circulating MPs harboring cytokeratin-18, a hepatocyte marker, could not be detected using flow cytometry analysis, enzyme-linked immunosorbent assays were conducted. As shown in Figure 1*C*, plasma from patients with cirrhosis contained hepatocyte-derived MPs, whereas plasma from controls did not. No differences were observed in circulating annexin V^+ MPs and MPs from other cellular origin (Supplementary Table 2). There was also no difference in annexin V^+ MPs between Child–Pugh A and Child–Pugh B or C patients (data not shown).

To identify the factors responsible for the increase in circulating $CD31^+/41^-$, $CD11a^+$, $CD4^+$, $CD235a^+$, and cytokeratin-18^+ MP levels in patients with cirrhosis, we included 65 additional patients with cirrhosis (Figure 1*A*). Their characteristics at inclusion were not different from those of the 26 patients from the pilot cohort (data not shown). The features of all 91 patients (ie, the 26 patients from the pilot cohort and the 65 additional patients) are presented in Table 1. Of these 91 patients, 31 underwent liver transplantation and 16 died. Cause of death was infection, alcoholic hepatitis, bleeding, encephalopathy, and unknown in 9, 2, 2, 1, and 2 patients, respectively. Median follow-up was 7.1 (range, 0–46.5) months. Six- and 12-month survival rates were 84% and 77%, respectively.

The circulating levels of MPs of leuko-endothelial ($CD31^+/41^-$) and hepatocyte origin (cytokeratin-18^+) increased with severity of cirrhosis, as attested by the strong correlations with MELD score, Child–Pugh score (Figure 1*D* and *E*) and serum bilirubin level and the inverse correlation with prothrombin index and serum albumin levels (Table 2). Moreover, patients with hepatic encephalopathy had 3.5-fold more MPs of leuko-endothelial origin than those without (39 [6–438] vs 11 [0–259] $CD31^+/41^-$ MPs/μL; n = 18 vs 71; P = .007) and patients with ascites had 4-fold more MPs of hepatocyte origin than those without (126 [10–680] vs 31 [0–147] IU/L soluble cytokeratin-18 bound to MPs; n= 30 vs 10; P = .008). Moreover, $CD31^+/41^-$ MP levels were significantly associated with survival (Supplementary Table 3) independently of Child–Pugh score but not of MELD score (Supplementary Table 4). Circulating CD11a MP levels were also associated with survival

Table 1. Baseline Characteristics of the 91 Patients With Cirrhosis

Characteristics	
Age (*y*)	56 (36–82)
Male sex, n (*%*)	68 (75)
Cause of cirrhosis, n (*%*)	Alcohol: 71 (78) Hepatitis C: 20 (22)
Body mass index (*kg/m²*)	24.1 (18.0–38.9)
Ascites, n (*%*)	69 (76)
Encephalopathy, n (*%*)	18 (20)
Esophageal varices (available in 77), n (*%*)	59 (77)
Child–Pugh score (A, B, C)	16 (18), 18 (20), 57 (62)
MELD score	14 (0–44)
Cardiovascular risk factors, n (*%*)	
Hypertension	18 (20)
Smoking	44 (48)
Diabetes	20 (22)
Dyslipidemia	6 (7)
Treatments, n (*%*)	
Beta-blockers	36 (40)
Diuretics	37 (41)
Antibiotics	23 (26)
Serum levels	
Leukocytes ($\times 10^9/L$)	6.0 (1.1–22.5)
Hemoglobin (*g/L*)	111 (69–165)
Platelet count ($\times 10^9/L$)	106 (28–356)
Prothrombin index (*%*)	43 (9–103)
Creatinine (*μmol/L*)	65 (39–150)
Albumin (*g/L*)	26 (9–53)
Aspartate aminotransferase (×*ULN*)	2.4 (0.9–7.3)
Alanine aminotransferase (×*ULN*)	1.0 (0.3–6.4)
Bilirubin (*μmol/L*)	58 (6–798)
C-reactive protein (available in 75) (*mg/L*)	13 (0–115)
Hemodynamic data (*mm Hg*)	
Hepatic venous pressure gradient (available in 47)	18 (7–36)
Central venous pressure (available in 47)	5 (0–17)
Mean arterial pressure	86 (63–113)
Circulating MP levels	
$CD31^+41^-$ (*MPs/μL*)	16 (0–438)
$CD11a^+$ (*MPs/μL*)	41 (0–2484)
$CD4^+$ (*MPs/μL*)	1 (0–83)
$CD235a^+$ (*MPs/μL*)	69 (2–1814)
Soluble cytokeratin-18 in native plasma (available in 40)	480 (141–4603)
Soluble cytokeratin-18 in filtrated plasma (available in 40)	402 (136–3951)

NOTE. Data are expressed as median (range) or frequency (%). Unless otherwise stated, data were available for 88 patients or more out of the 91 patients. In patients with hemodynamic assessment, liver catheterization was performed within 48 hours of blood sample collection. The upper limit of normal for aspartate aminotransferase was 31 IU/L in women and 35 IU/L in men. The upper limit of normal for alanine aminotransferase was 34 IU/L in women and 45 IU/L in men. ULN, upper limit of normal.

Table 2. Correlations Between Circulating MP Levels and Clinical or Laboratory Hemodynamics

	CD31⁺/41⁻ MPs	CD11a⁺ MPs	CD4⁺ MPs	CD235a⁺ MPs	Cytokeratin-18 in native plasma	Cytokeratin-18 in filtrated plasma	MP-bound cytokeratin-18
Child–Pugh score	***r* = 0.29** ***P* = .01**	*r* = 0.12 *P* = .27	*r* = 0.16 *P* = .14	***r* = 0.21** ***P* = .05**	***r* = 0.32** ***P* = .04**	*r* = 0.20 *P* = .22	***r* = 0.65** ***P* < .001**
MELD score	***r* = 0.34** ***P* = .001**	*r* = 0.16 *P* = .15	*r* = 0.16 *P* = .14	*r* = 0.13 *P* = .24	***r* = 0.37** ***P* = .02**	*r* = 0.30 *P* = .06	***r* = 0.58** ***P* < .001**
Prothrombin index (%)	***r* = −0.26** ***P* = .01**	*r* = −0.14 *P* = .20	*r* = −0.10 *P* = .34	*r* = −0.19 *P* = .07	***r* = −0.31** ***P* = .05**	*r* = −0.19 *P* = .23	***r* = −0.64** ***P* < .001**
Serum creatinine (*μmol/L*)	*r* = −0.08 *P* = .46	*r* = −0.20 *P* = .06	*r* = 0.00 *P* = .96	*r* = −0.18 *P* = .10	*r* = −0.23 *P* = .15	*r* = −0.13 *P* = .43	***r* = −0.38** ***P* = .01**
Serum albumin (*g/L*)	***r* = −0.24** ***P* = .02**	*r* = −0.11 *P* = .30	*r* = −0.15 *P* = .16	*r* = −0.17 *P* = .11	*r* = −0.16 *P* = .34	*r* = −0.05 *P* = .77	***r* = −0.49** ***P* = .002**
Serum aspartate aminotransferase (×*ULN*)	*r* = 0.29 *P* = .01	***r* = 0.26** ***P* = .02**	***r* = 0.28** ***P* = .01**	*r* = 0.17 *P* = .10	***r* = 0.66** ***P* < .001**	***r* = 0.60** ***P* < .001**	***r* = 0.48** ***P* = .002**
Serum alanine aminotransferase (×*ULN*)	*r* = 0.05 *P* = .62	*r* = 0.02 *P* = .86	***r* = 0.25** ***P* = .02**	*r* = 0.11 *P* = .32	***r* = 0.34** ***P* = .03**	***r* = 0.34** ***P* = .03**	*r* = 0.05 *P* = .76
Serum bilirubin (*μmol/L*)	***r* = 0.45** ***P* < .001**	***r* = 0.23** ***P* = .04**	***r* = 0.23** ***P* = .03**	***r* = 0.21** ***P* = .05**	***r* = 0.48** ***P* = .002**	***r* = 0.39** ***P* = .01**	***r* = 0.69** ***P* < .001**
Leukocytes (×10^9/*L*)	***r* = 0.34** ***P* = .001**	*r* = 0.11 *P* = .29	***r* = 0.21** ***P* = .05**	*r* = 0.09 *P* = .41	***r* = 0.38** ***P* = .02**	***r* = 0.33** ***P* = .04**	***r* = 0.37** ***P* = .02**
Hemoglobin (*g/L*)	*r* = −0.21 *P* = .05	*r* = −0.15 *P* = .17	*r* = −0.03 *P* = .75	*r* = −0.09 *P* = .43	*r* = −0.25 *P* = .11	*r* = −0.18 *P* = .26	***r* = −0.52** ***P* < .001**
Platelet count (×10^9/*L*)	*r* = 0.03 *P* = .76	*r* = 0.05 *P* = .65	*r* = −0.01 *P* = .96	*r* = −0.15 *P* = .16	*r* = −0.17 *P* = .30	*r* = −0.10 *P* = .56	*r* = −0.26 *P* = .11
C-reactive protein (*mg/L*)	***r* = 0.38** ***P* = .001**	*r* = 0.00 *P* = .97	***r* = 0.23** ***P* = .05**	*r* = 0.11 *P* = .37	*r* = 0.34 *P* = .05	*r* = 0.25 *P* = .15	***r* = 0.53** ***P* = .001**

NOTE. r, Spearman correlation coefficient. Correlations were assessed in 91 patients for CD11a⁺, CD31⁺/41⁻, and CD4⁺ MPs and in 40 patients for soluble cytokeratin-18. **Bold** text indicates significant correlations between MP levels and clinical, or laboratory parameters. ULN, upper limit of normal.

but without adding significant prognostic information to Child–Pugh or MELD score (Supplementary Tables 3 and 4).

The circulating levels of MPs of leuko-endothelial origin and of hepatocyte origin also correlated with markers of systemic inflammation, that is, C-reactive protein and leukocyte count and with markers of hepatocyte damage such as aspartate aminotransferase level (Table 2).

MPs of leuko-endothelial origin or of hepatocyte origin did not correlate with hepatic venous pressure gradient, likely because these parameters were not available for all stages of the disease, and only for the most seriously ill patients; 2 of the 47 patients with available hepatic venous pressure gradient measurement were Child–Pugh A. Indeed, such hemodynamic measurements were performed at the time of transjugular liver biopsy, a route restricted in our center to patients with hemostatic defects and/or ascites, as recommended earlier.[12]

There was no influence of age, body mass index, hypertension, smoking, diabetes, cause of cirrhosis, beta-blocker use, diuretic intake, and platelet count on circulating CD31⁺/CD41⁻, CD11a⁺, CD4⁺, CD235a⁺, and cytokeratin-18⁺ MP levels in the 91 patients with cirrhosis (data not shown). Antibiotic intake and female sex were only weakly associated with an increase in hepatocyte MPs (142 [83–680] vs 83 [0–587] IU/L soluble cytokeratin-18⁺ bound to MPs; n = 9 vs 30; *P* = .028) and in pan-leukocyte MP levels (55 [0–2484] vs 35 [2–1445] CD11a⁺ MP/μL; n = 23 vs 65; *P* = .047), respectively, and dyslipidemia with a decrease in erythrocyte MP level (32 [10–50] vs 73 [2–1814] CD235a⁺ MP/μL; n = 6 vs 83; *P* = .01).

MPs From Patients With Cirrhosis Impair Response to Vasoconstrictive Agents

Because systemic vasodilation plays a major role in the pathogenesis of the complications of chronic liver diseases,[13] and because MPs modulate vascular reactivity in several vascular pathologies,[2] we then assessed the potential role of MPs from patients with cirrhosis on systemic vasodilation using conventional organ chamber assays. Exposure of rat aortic rings for 24 hours to plasma MPs from patients with advanced cirrhosis (Child–Pugh B or C) at their circulating concentration impaired response to phenylephrine, whereas MPs from patients with compensated cirrhosis (Child–Pugh A) or from healthy controls had no effect (Figure 2*A*–*C*). MPs from patients with end-stage renal failure, analyzed in a previous study using the same protocol,[6] had also no effect on vascular response to phenylephrine; response to phenylephrine 10^{-4} mol/L was 12.5 (10.0–15.1) mN/mg for aortic rings incubated with MPs versus 12.3 (6.5–13.5) mN/mg with supernatant (n = 6 in each group; *P* = .249). The effect of MPs was not dependent on the cause of the liver disease, because it was induced with MPs from patients with alcoholic as well as hepatitis C virus–related cirrhosis

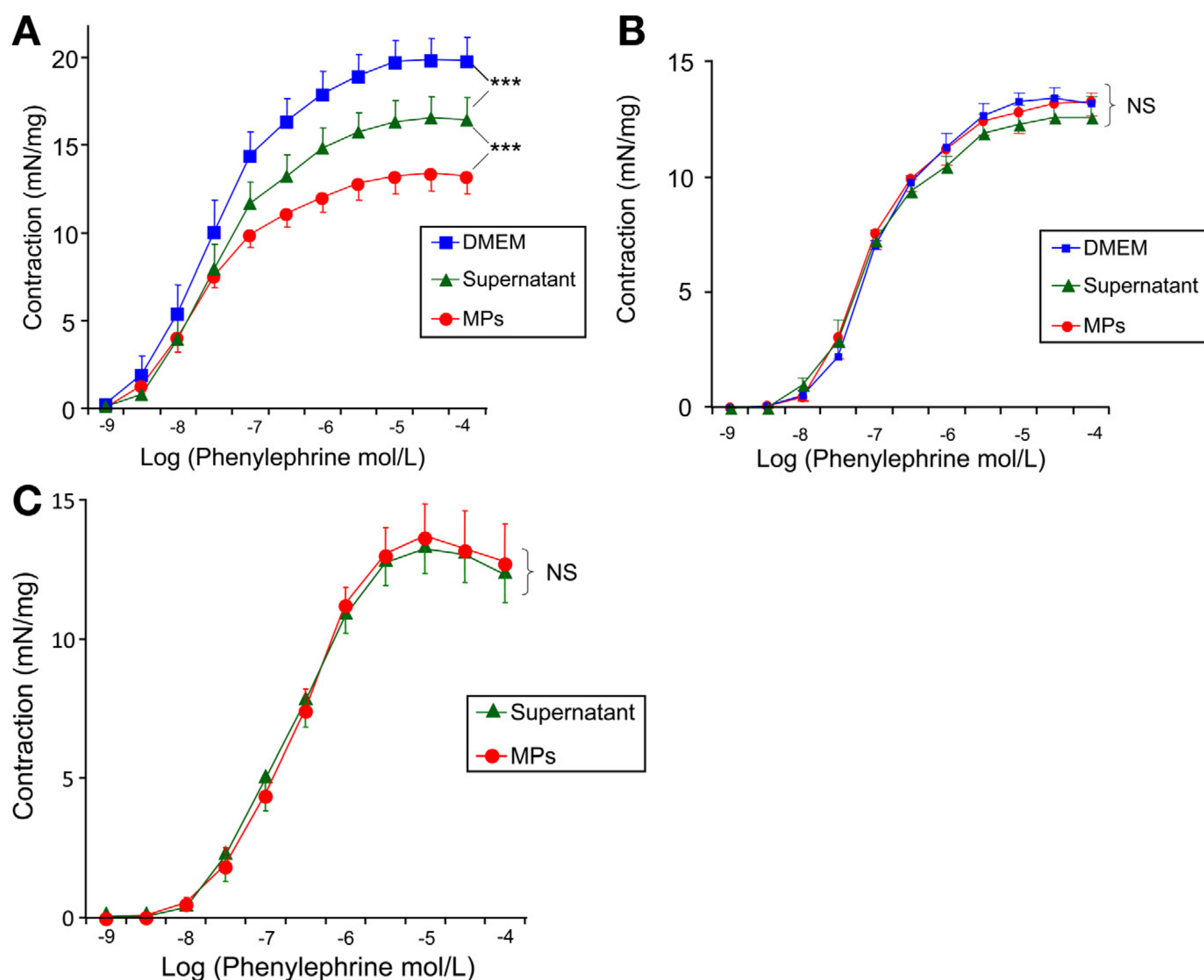

Figure 2. Circulating MPs from patients with advanced cirrhosis induce arterial hyporeactivity to vasoconstrictors. (*A–C*) Concentration-response curves to phenylephrine of healthy rat aortic rings previously incubated for 24 hours with plasma MPs at their circulating concentration or with controls (ie, plasma supernatant or DMEM). MPs from patients with Child–Pugh B or C cirrhosis impair contraction to phenylephrine (n = 9) (*A*), while MPs from healthy controls (n = 4) (*B*) or MPs from Child–Pugh A patients (n = 7) (*C*) have no effect. NS, no significant difference; $^{***}P < .001$.

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(data not shown). The effect of MPs was also observed with aortas from rats with cirrhosis (Supplementary Figure 2*A*). Unless otherwise stated, the following experiments were performed using circulating MPs from Child–Pugh B or C patients incubated for 24 hours with aortic rings from healthy rats. As shown in Supplementary Figure 2*B* and *C*, neither MPs from patients with cirrhosis nor from healthy controls modified endothelium-dependent relaxation to acetylcholine.

MPs From Patients With Cirrhosis Impair Response to Phenylephrine in a COX-1–Dependent Manner

We then sought to evaluate the molecular mechanisms leading to the impaired response to vasoconstrictive agents following exposure to circulating MPs from patients with cirrhosis. As shown in Figure 3*A*, mechanically removing endothelium from aortic rings restored contraction to phenylephrine, showing that MP treatment-induced vascular hyporeactivity results from the release of vasodilatory factors from endothelial cells. To investigate the potential role of NO and COX metabolites, the effect on response to phenylephrine of a NOS inhibitor (L-nitro arginine), a nonselective COX inhibitor (diclofenac), and selective inhibitors of COX-1 (SC-560) and COX-2 (NS-398) was examined. Contractions to phenylephrine were significantly increased following exposure to diclofenac and SC-560, whereas L-nitro arginine and NS-398 had no effect, suggesting a COX-1–dependent and NO synthase–independent mechanism (Figure 3*B–D*). In accordance with these results, the end-metabolite of the vasodilator prostacyclin (ie, 6-keto PGF1α) was increased following exposure of aortic rings to MPs (210 [207–1137] vs 136 [65–238] pg/mL in the medium of aortic rings incubated with MPs and supernatant, respectively; n = 3). MPs from patients with cirrhosis at their circulating concentra-

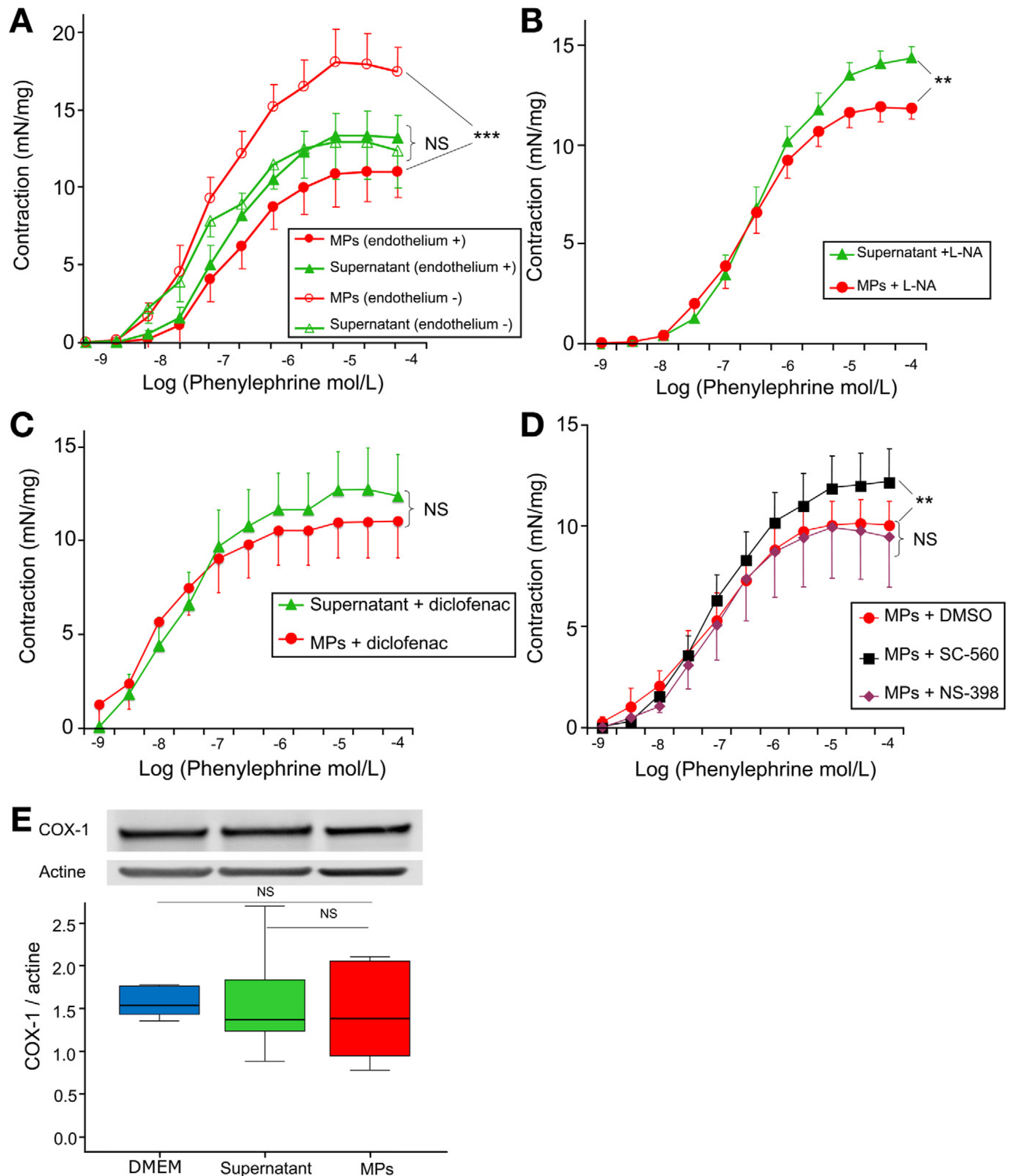

Figure 3. Arterial hyporeactivity to vasoconstrictors induced by circulating MPs from patients with cirrhosis is endothelium and COX-1 dependent. (*A–E*) Healthy rat aortic rings were incubated for 24 hours with plasma MPs from patients with Child–Pugh B or C cirrhosis at their circulating concentration or with supernatant. (*A*) Gently mechanically removing endothelium restores contraction to phenylephrine of aortic rings incubated with MPs but had no effect on rings exposed to supernatant (n = 4). (*B*) Exposure for 45 minutes of aortic rings to an inhibitor of NOS (L-nitro arginine, L-NA; 10^{-4} mol/L) did not restore contraction to phenylephrine (n = 6). (*C*) Exposure for 45 minutes of aortic rings to an inhibitor of COX (diclofenac; 10^{-6} mol/L) suppressed the effect of MPs (n = 6). (*D*) Exposure for 45 minutes of aortic rings to an inhibitor of COX-1 (SC-560; 10^{-6} mol/L) improved response to phenylephrine, whereas an inhibitor of COX-2 (NS-398; 10^{-6} mol/L) had no effect (n = 5). Data are expressed as mean ± SEM. (*E*) COX-1 level, assessed using Western blot, was not increased in aortic rings incubated for 24 hours with circulating MPs from patients with cirrhosis at their circulating concentration as compared with control conditions (DMEM or supernatant) (n = 6). Data are median (*horizontal bar*), 25th and 75th percentile (*boxes*), and extreme values, which are less than 3 box lengths from either end of the box (*error bar*). NS, no significant difference; $^{**}P < .01$; $^{***}P < .001$.

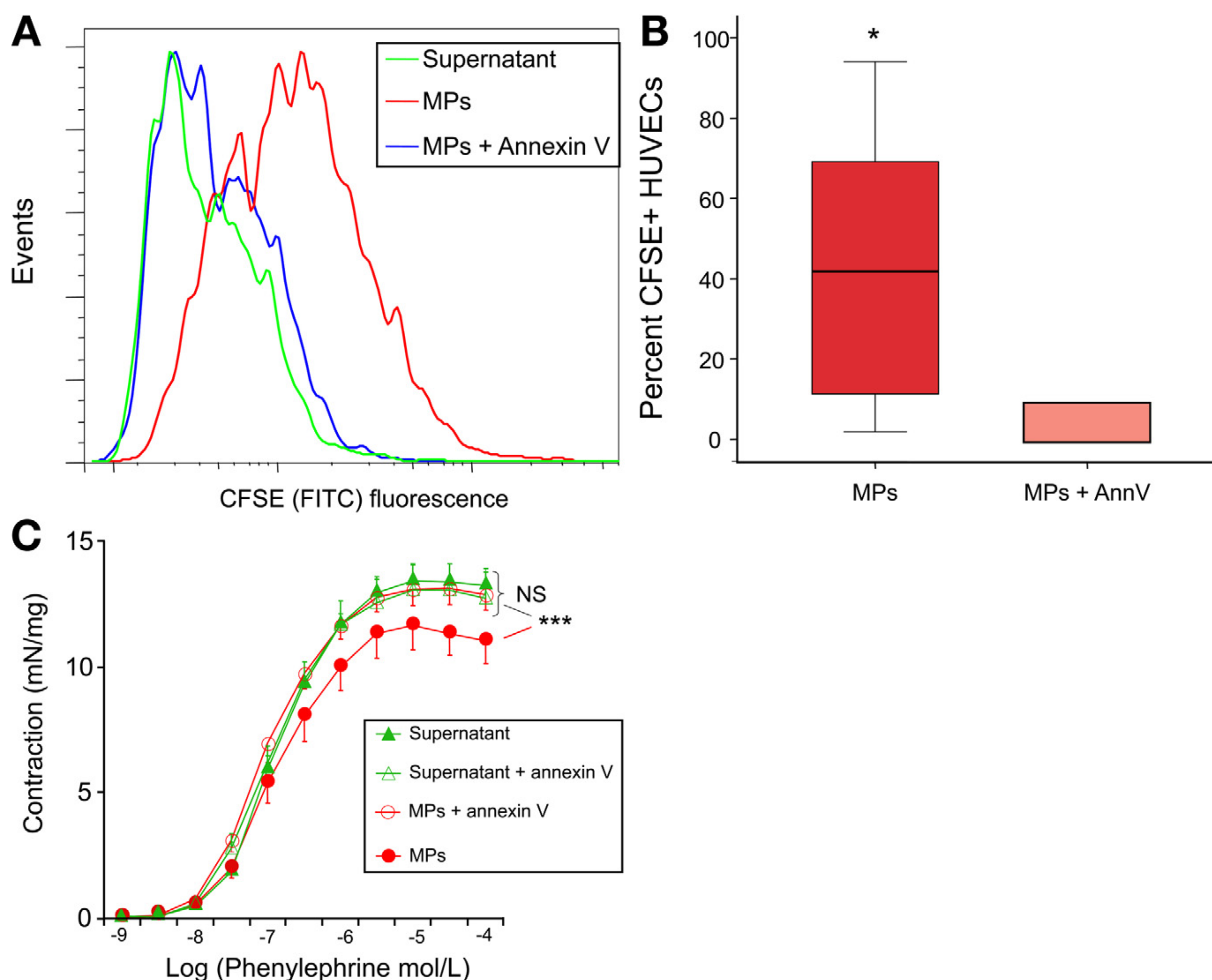

Figure 4. Cirrhotic MP effect depends on a transfer of MPs to endothelial cells. (*A* and *B*) Circulating MPs from cirrhotic patients were labeled with the fluorescent dye CFSE, washed to remove unbound dye, incubated for 30 minutes with $CaCl_2$ (5 mmol/L final concentration) with or without annexin V (4 μg/mL), and then exposed, at their circulating concentration, for 24 hours to HUVECs. As compared with HUVECs exposed to the washing supernatant (*green line*), those incubated with fluorescent MPs became fluorescent (*red line*). Preincubation with annexin V decreased the uptake of fluorescent MPs by HUVECs (*blue line*). (*A*) Representative flow cytometry fluorescence histogram. (*B*) Data are median (*horizontal bar*), 25th and 75th percentile (*boxes*), and extreme values, which are less than 3 box lengths from either end of the box (*error bar*) and given as the difference in $CFSE^+$ HUVECs per well between MP-exposed cells and control conditions (supernatant with or without annexin V). (*C*) Healthy rat aortic rings were incubated for 24 hours with plasma MPs from patients with Child–Pugh B or C cirrhosis at their circulating concentration or with supernatant. Response to phenylephrine was then assessed. Preincubation for 30 minutes of MPs with annexin V (4 μg/mL) in the presence of $CaCl_2$ (5 mmol/L) suppressed the cirrhotic MP effect (*C*, n = 6). Data are mean ± SEM. NS, no significant difference; *$P < .05$; ***$P < .001$.

tion did not change the expression of COX-2, NOS-2, and NOS-3 protein levels in rat aortic rings and in HUVECs (Western blot analysis; 24 hours; n = 6–8; data not shown). MPs from patients with cirrhosis also did not modify the COX-1 level in aortic rings (Figure 3*E*) or HUVECs (COX-1/actine: MPs 0.97 [0.48–1.79] vs supernatant 1.02 [0.74–1.43]; *P* = .866; n = 7; 24 hours). Prostacyclin synthase protein levels in rat aortic rings or in HUVECs were also not different between MP and supernatant-treated aortic rings or HUVECs (Western blot analysis; 24 hours; n = 8; data not shown). To confirm this lack of implication of protein synthesis, response to phenylephrine was assessed after incubation of aortic rings with actinomycin D (2 μg/mL). This inhibitor of transcription did not modify the effect of MPs on the response to phenylephrine; response to phenylephrine 10^{-4} mol/L was 11.1 (8.9–11.8) mN/mg for aortic rings incubated with MPs versus 13.8 (11.3–16.2) mN/mg with supernatant (*P* = .046; n = 6).

MPs From Patients With Cirrhosis Impair Response to Phenylephrine by Transferring Substrate for the Arachidonic Acid Metabolism Pathway

Given the absence of increase in COX-1 protein level and the lack of effect of protein synthesis inhibitor, we hypothesized that MPs from patients with cirrhosis transfer substrate for the arachidonic acid metabolism

pathway to endothelial cells. The first hint came from experiments showing that arachidonic acid (10^{-5} mol/L for 2 hours) impaired the maximal response to phenylephrine, therefore mimicking the effect of cirrhotic MPs on vascular reactivity (data not shown; n = 4; P = .009). To test this hypothesis, MPs were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) and incubated with HUVECs. After 24 hours, 44% (4%–97%) of HUVECs exposed to MPs were CFSE positive as compared with only 2% (0–4%) of those incubated with supernatant (P = .028; n = 6) (Figure 4*A*). Because anionic phospholipids such as phosphatidylserine have been implicated in such an uptake of MPs by endothelial cells,[5,14] we then examined the effect of annexin V that binds to phosphatidylserine. Preincubation of MPs with annexin V (in the presence of 5 mmol/L $CaCl_2$) decreased endothelial fluorescence resulting from cirrhotic MP exposure by 100% (34%–100%) (Figure 4*A* and *B*) and restored the maximal contractile response to phenylephrine (Figure 4*C*). Similar findings were obtained using diannexin, an annexin V homodimer (Supplementary Figure 3).[15] Quinacrine, an inhibitor of phospholipase A_2, also suppressed the effect of cirrhotic MPs on the contractile response to phenylephrine; after preincubation with quinacrine dihydrochloride (3×10^{-5} mol/L), response to phenylephrine 10^{-4} mol/L was 12.1 (10.5–14.8) mN/mg for aortic rings incubated with MPs versus 12.7 (4.6–19) mN/mg with supernatant (P = .767; n = 9). Taken altogether, these results suggest that membrane phospholipids of MPs are transferred to endothelial cells and act as substrate for phospholipase A_2 production of arachidonic acid metabolites.

MPs From Patients With Cirrhosis Decrease Arterial Blood Pressure In Vivo

We next assessed the in vivo relevance of these findings. Given the rapid clearance of MPs in vivo, a time point of 2 hours was chosen.[16,17] As shown in Figure 5, intravenous injection of MPs from patients with cirrhosis decreased arterial blood pressure both in wild-type and in BALB/C nude mice under basal conditions. Mice receiving MPs had also lower mean arterial pressure after phenylephrine injection than those receiving supernatant from the same patients (112 [65–128] vs 126 [83–140] mm Hg, respectively; P = .043; n= 5 wild-type mice in each group; phenylephrine 20 μg/kg body weight injected intravenously).

Discussion

The present study shows that the plasma of patients with cirrhosis contains high levels of MPs of leuko-endothelial, lymphocyte, erythrocyte, and hepatocyte origin. Circulating MPs from patients with advanced cirrhosis contribute to systemic vasodilation of cirrhosis. Indeed, they are taken up by endothelial cells and induce arterial hyporeactivity to vasoconstrictor agents in a phosphatidylserine- and COX-1–dependent manner.

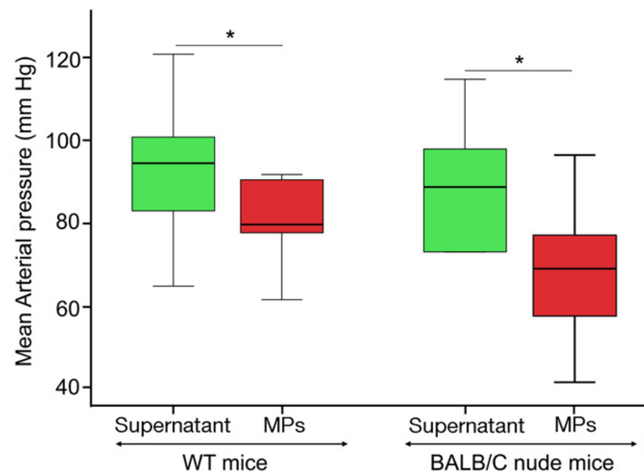

Figure 5. MPs from patients with cirrhosis induce arterial hypotension in vivo. Healthy C57Bl/6 (wild-type [WT]) mice and immunodeficient BALB/C nude mice were injected intravenously with MPs from patients with cirrhosis (to reach in mice blood a concentration in cirrhotic MPs corresponding to 60% of the circulating concentration in patients) or the corresponding supernatant. Two hours later, mice were anesthetized and tail arterial blood pressure was measured spontaneously. Data are given as median (*horizontal bar*), 25th and 75th percentile (*boxes*), and extreme values, which are less than 3 box lengths from either end of the box (*error bar*). *$P < .05$.

One strength of this study is the procedure to isolate MPs and the controls used, making highly unlikely a contamination by larger vesicles (ie, apoptotic bodies) or smaller vesicles (ie, exosomes) or a bias related to soluble factors.[7] First, the second centrifugation we used (15,200*g* for 5 minutes) spun down apoptotic bodies (Supplementary Figure 1).[7] Second, to evaluate the biological effects of circulating MPs on vascular reactivity, circulating MPs were spun down (20,500*g* for 150 minutes). We have previously shown that this 20,500*g* centrifugation step does not pellet exosomes.[18] Third, in all experiments, the potential contribution of soluble factors and proteins not bound to MPs has been assessed by the systematic use of the so-called "supernatant," that is, plasma from the same patients but devoid of MPs.

We observed here that levels of circulating MPs derived from leukocytes, endothelial cells, and hepatocytes were greater in patients with cirrhosis than in healthy controls. Interestingly, plasma levels of these specific MP subtypes augmented with severity of cirrhosis. This increase likely results from an overproduction of MPs. MPs are known to be released following cell activation or apoptosis,[19] and hepatocyte apoptosis occurs in chronic liver diseases such as viral hepatitis C and alcoholic hepatitis.[20] Monocytes from patients with cirrhosis are activated in basal conditions.[21] Moreover, the plasma of patients with cirrhosis contains endotoxin, a stimulus known to induce MP release from monocytes and macrophages.[22,23] The increase in proinflammatory cytokines in the plasma of patients with cirrhosis could induce the release of MPs by endothelial cells.[24,25] The hypothesis of a role of systemic inflammation in the formation of MPs in cirrhosis is

reinforced by the correlation observed here between C-reactive protein on the one hand and MPs of leuko-endothelial and hepatocyte origin levels on the other hand. An impaired MP clearance may also occur in cirrhosis because the liver, with other organs, contributes to MP clearance.[16,17,26]

No influence of cardiovascular risk factors (ie, age, body mass index, hypertension, smoking, diabetes, dyslipidemia, serum creatinine level) on circulating MP level was observed in patients with cirrhosis, contrary to what happens in patients without liver disease.[2] This suggests that the effect of cirrhosis on MP levels may mask other factors.

This study also shows for the first time that a proportion of the so-called "soluble cytokeratin-18," a promising tool for differentiating bland steatosis from steatohepatitis in nonalcoholic patients, is in fact carried by MPs.[27] Interestingly, this MP-bound cytokeratin-18 correlates with severity of liver disease and systemic inflammation much better than total soluble cytokeratin-18 (Table 2). This suggests that MP-bound cytokeratin-18 could be a useful marker of severity of cirrhosis.

Because MPs are not only markers of cell damage, but have also been proven as crucial effectors in several important biological functions, including vascular reactivity, we then assessed the potential contribution of MPs to systemic vasodilation. To test this hypothesis, we exposed rat aortic rings to the circulating concentration of annexin V^+ MPs, which was not different between controls and cirrhotic patients and between Child–Pugh A and Child–Pugh B or C patients. We observed that circulating MPs from patients with advanced cirrhosis impair ex vivo arterial contraction to phenylephrine, whereas MPs from Child–Pugh A cirrhotic patients, healthy controls, or patients with end-stage renal failure did not. This effect was obtained not only on aortic rings from healthy but also from cirrhotic rats. The significant decrease in arterial blood pressure observed in wild-type and BALB/C nude mice injected with cirrhotic MPs strongly suggests that these MPs induce vascular hypocontractility in vivo and indicates that circulating MPs might contribute to systemic vasodilation occurring in liver diseases.[13]

Our study shows that the effect of MPs from patients with advanced cirrhosis depends on their uptake by endothelial cells in a phosphatidylserine-, phospholipase A_2-, and COX-1–dependent manner, without COX1 protein overexpression. MPs deliver membrane phospholipids to endothelial cells, which are subsequently metabolized by several enzymes including phospholipase A_2 and COX-1, leading to the formation of vasodilatory prostaglandins and hyporeactivity to vasoconstrictors. The cell origin of the MPs responsible for this effect is so far unknown. Because there is no difference in annexin$^+$ MP levels between patients with cirrhosis and controls, other characteristics of MPs besides their exposure of phosphatidylserine should be involved. An implication in this effect of one or several MP subpopulations elevated in patients with cirrhosis would sound logical. However, establishing this would require sorting out each subpopulation of MPs and testing their respective effects on vascular reactivity, at concentrations matching those found in control plasma and in plasma from cirrhotic patients. This is not possible, because a too large volume of plasma would be requested.

This deleterious effect of MPs on systemic vasodilation through the COX-1 pathway is reminiscent of the studies on intrahepatic resistance by the group of Bosch and Garcia-Pagan. These investigators have shown that COX-1–derived prostanoids induce intrahepatic vasoconstriction, favoring the increase in resistance to portal blood flow in cirrhotic livers.[28] Two hypotheses could explain these differences (ie, a COX-1–dependent intrahepatic vasoconstriction and a systemic hyporesponsiveness to vasoconstrictors). First, circulating MPs may not have the same concentration and lipid composition in portal vein and in systemic circulation. Indeed, the liver is a major determinant of MP clearance.[17] Second, the phenotypic heterogeneity of sinusoidal and systemic endothelial cells is likely to affect the metabolism of arachidonic acid.[29]

In conclusion, cirrhosis is associated with an increase in several circulating MP subpopulations, likely resulting from systemic inflammation and liver cell damage. Our results show that MPs circulating in the blood of patients with advanced cirrhosis induce arterial hypocontractility and thus must contribute to systemic vasodilation.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.03.040.

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Reprint requests

Address requests for reprints to: Chantal M. Boulanger, PhD, Paris - Centre de Recherche Cardiovasculaire à l'HEGP, INSERM Unité 970, 56, rue Leblanc, 75737 Paris Cedex 15, France. e-mail: chantal.boulanger@inserm.fr; fax: (33) 1-53-98-79-52.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Flow Cytometry Analysis of MPs

MPs were analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter, Villepinte, France) as previously described.[1] Regions corresponding to MPs were identified in forward light scatter and side-angle light scatter intensity dot plot representation set at logarithmic gain. MP gate was defined, using calibration beads (0.1-, 1-, 4-, and 10-μm beads; Invitrogen, Eugene, OR), as events with a 0.1- to 1-μm diameter and then plotted on a fluorescence/forward light scatter fluorescence dot plot to determine MP counts positively labeled by specific antibodies (Supplementary Figure 1*A*). A known amount of Flowcount calibrator beads (20 μL; Beckman Coulter, Fullerton, CA) was added to each sample just before performing flow cytometry analysis. MP concentration was assessed by comparison to these Flowcount calibrator beads. The presence of phosphatidylserine at the surface of plasma MPs was assessed using fluoroisothiocyanate-conjugated Annexin V (Roche Diagnostics, Mannheim, Germany) diluted in appropriate buffer (140 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4) (Roche Diagnostics) in the presence or absence of $CaCl_2$ (5 mmol/L) as a negative control.[1]

As previously described,[1] the cellular origin of plasma MPs was determined using selective fluorochrome-labeled antibodies or their corresponding isotype-matched immunoglobulin, incubated at room temperature for 30 minutes in the dark with 20 μL of platelet-free plasma. Anti–CD11a-fluoroisothiocyanate was provided by Immunotech (Marseille, France). Anti–CD31-phycoerythrin, anti–CD41-phycoerythrin-cyanin5, and anti–CD235a-fluoroisothiocyanate were obtained from Beckman Coulter (Villepinte, France). Anti–CD14-phycoerythrin was provided by Caltag Laboratories (Burlingame, CA) and anti–CD4-phycoerythrin by BD Pharmingen (San Jose, CA).

Flow Cytometry Analysis of Apoptotic Bodies

To check that our MP isolation procedure efficiently removes apoptotic bodies, we analyzed platelet-free plasma on a Guava easyCyte 8HT flow cytometer (Millipore, St Quentin-en-Yvelines, F). Regions corresponding to apoptotic bodies were identified in forward light scatter and side-angle light scatter intensity dot plot representation set at logarithmic gain (Supplementary Figure 1*A*). Apoptotic bodies gate was defined, using calibration beads (0.1-, 1-, 4-, and 10-μm beads; Invitrogen), as events with a 1- to 4-μm diameter[2] and then plotted on a fluorescence/fluorescence dot plot to determine apoptotic bodies counts positively labeled both by allophycocyanin-conjugated Annexin V (Becton Dickinson, Le Pont-de-Claix, F, as described previously) and by propidium iodide (1 μg/mL for 30 seconds).[3]

Organ Chamber Experiments

All experiments were performed in accordance with the guidelines formulated by the European Community for experimental animal use (L358-86/609EEC) and were approved by the French Ministry of Agriculture (agreement number A75-15-32).

Thoracic aortas were excised from Wistar rats (12- to 16-week-old male rats; Charles River Laboratories, Saint-Aubin-les-Elbeuf, France) killed under anesthesia using pentobarbital (CEVA santé, Libourne, France). Aortic rings were incubated (for 24 hours; 37°C in a 5% CO_2 incubator) in DMEM supplemented with antibiotics (100 IU/mL streptomycin, 100 IU/mL penicillin [Gibco, Invitrogen, Paisley, Scotland], and 10 μg/mL polymyxin B [Sigma, St Louis, MO]) either alone or in the presence of circulating MPs from patients or from controls at their circulating concentration or the corresponding volume of the 20,500*g* supernatant. In some experiments, rings were incubated with arachidonic acid (10^{-5} mol/L; Cayman Chemical, Ann Arbor, MI) or with its solvent, ethanol, for 2 hours because a longer incubation period had proven to cause nonspecific effects.[4] After this incubation period, the rings were mounted in organ chambers (Multi WireMyograph system, model 610 M; Danish Myo Technology, Aarhus, Denmark) filled with modified Krebs–Ringer solution supplemented with 10 μg/mL polymyxin B to study relaxation to acetylcholine chloride (10^{-9} to 10^{-4} mol/L) (Sigma) and contraction to L-phenylephrine hydrochloride (10^{-9} to 10^{-4} mol/L) (Sigma).[5] In some experiments, the effect of endothelium was tested by gently removing it mechanically. Concentration-response curves to phenylephrine were also performed after incubation of aortic rings with inhibitors of NOS (L-nitro arginine, 10^{-4} mol/L; Sigma), nonspecific inhibitor of COX (diclofenac, 10^{-6} mol/L; Research Biochemical International, Natik, MA), or inhibitors of COX-1 (SC-560, 10^{-6} mol/L; Cayman Chemical) or of COX-2 (NS-398, 10^{-6} mol/L; Cayman Chemical) incubated with aortic rings in organ chamber for 45 minutes before concentration-response curves; nonspecific inhibitor of protein synthesis (actinomycin D, 2 μg/mL; Calbiochem, Darmstadt, Germany) preincubated with aortic rings for 45 minutes before washing and exposure of aortic rings for 24 hours to MPs or supernatant; and nonspecific inhibitor of phopholipase A_2 (quinacrine dihydrochloride, 3×10^{-5} mol/L; Sigma) preincubated with aortic rings for 45 minutes and left for 24-hour incubation with MPs or supernatant. The implication of phosphatidylserine exposed at the surface of MPs was assessed by preincubating MPs with annexin V (4 μg/mL; Roche Diagnostics) for 30 minutes in appropriate buffer (140 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4) in the presence of $CaCl_2$ (5 mmol/L). Control experiments were performed with the same level of buffer and calcium in the absence of annexin V. The effect of diannexin (2 μg/mL for 1 hour

with MPs, a gift from Alavita), an annexin V homodimer, was also tested.[6]

Induction of Cirrhosis by Bile Duct Ligation

Cirrhosis was induced in male 12-week-old Wistar rats (Charles River Laboratories) by bile duct ligation, as described.[7] Briefly, under isoflurane anesthesia, a midline abdominal incision was made, and the common bile duct was occluded by double ligatures with a nonresorbable suture 5.0 silk. Then, the common bile duct was sectioned between the 2 ligatures. The abdominal incision was closed. Rats were killed 4 weeks after bile duct ligation under anesthesia using pentobarbital (CEVA santé). At that time, secondary biliary cirrhosis had developed and was confirmed by histologic examination of the liver. The thoracic aorta was collected and used for organ chamber studies.

Assessment of the Transfer of MPs to Endothelial Cells

To test the hypothesis of a transfer of MPs to endothelial cells, circulating MPs from patients with cirrhosis were incubated for 5 minutes with 5×10^{-6} mol/L fluorescent dye (CellTrace CFSE; Molecular Probes, Eugene, OR) at 37°C, washed twice by resuspending MPs in medium (DMEM supplemented with 25% fetal calf serum for the first centrifugation and phosphate-buffered saline for the second one), and centrifugation at 20,500*g* for 150 minutes at 4°C to pellet MPs. MPs (at their circulating concentration) or supernatant of the second centrifugation (used as negative control) were incubated with HUVECs. After 24 hours, HUVECs were washed with prewarmed phosphate-buffered saline and mechanically scraped. The percentage of fluorescent HUVECs was analyzed on a LSR II flow cytometer (BD Biosciences, San Jose, CA).

Western Blotting

Detection of COX-1, COX-2, NOS-2, NOS-3, and prostacyclin synthase in rat aortic rings and in HUVECs incubated for 24 hours with MPs from patients with cirrhosis at their circulating concentration, supernatant, or DMEM was performed as follows. HUVECs were washed twice with cold phosphate-buffered saline and scraped off in 50 μL of RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 2 mmol/L EDTA, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 2 mmol/L activated orthovanadate, complete protease inhibitor cocktail tablet, and complete phosphatase inhibitor cocktail tablet [Roche, Neuilly-sur-seine, France]).

Aortic rings were crushed and lysed in 50 μL of lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1% Igepal, 0.1% sodium dodecyl sulfate, 1 mmol/L activated orthovanadate, 1 mmol/L dithiothreitol, complete protease inhibitor cocktail tablet, and complete phosphatase inhibitor cocktail tablet [Roche]) for 1 hour at 4°C.

Lysates were then sonicated and centrifuged at 12,500*g* for 5 minutes, and protein content was quantified in the supernatant using the Lowry (Bio-Rad, Marnes-la-Coquette, France) protein assay. Lysates were mixed with the reducing sample buffer for electrophoresis and subsequently transferred onto nitrocellulose membranes (Bio-Rad). Equal loading (25 μg) was verified using Ponceau red solution. Membranes were incubated with rabbit anti–COX-1 (anti-human COX-1 for HUVECs, 1:1000; anti-murine COX-1 for aortic rings, 1:200; Cayman Chemical), rabbit anti–COX-2 (1:1000; Cayman Chemical), rabbit anti–NOS-2 (1:1000; Calbiochem, Darmstadt, Germany), rabbit anti–NOS-3 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-prostacyclin synthase (1:200; Santa Cruz Biotechnology) antibodies. After secondary antibody incubation (anti-rabbit 1:2000; Amersham, GE Healthcare, Courtaboeuf, France), immunodetection proceeded using an enhanced chemiluminescence kit (Immun-Star WesternC Kit; Bio-Rad, Hercules, CA) and bands were revealed using the Las-4000 Imaging System and Image Gauge software (Fujifilm, Tokyo, Japan). After initial immunodetection, membranes were stripped of antibodies and reprobed with goat anti-actine antibody (1:1000; Santa Cruz Biotechnology) and secondary antibody (anti-goat, 1:5000; Santa Cruz Biotechnology).

In Vivo Blood Pressure Measurement

Male C57Bl/6 mice (wild type; Charles River Laboratories) weighing 27 to 33 g and male BALB/C (Janvier Sas, Le Genest St Isle, France) nude mice weighing 30 to 36 g were injected retro-orbitally, under brief isoflurane anesthesia, with circulating MPs from patients with cirrhosis or the same volume of the corresponding 20,500*g* supernatant. The volume injected aimed at reaching in mice a concentration in cirrhotic patient MPs corresponding to 60% of the circulating concentration in patients (assuming that blood volume of a mouse is approximately 8% of its body weight). Two hours later, mice were anesthetized using intraperitoneal ketamine (100 mg/kg; Virbac, Carros, France) and xylazine (6.15 mg/kg Rompun 2%; Bayer HealthCare, Kiel, Germany). Body temperature was maintained at 37°C. In wild-type mice, after skin incision, a catheter was placed in the femoral vein. Response to L-phenylephrine hydrochloride (Sigma) 20 μg/kg body weight injected intravenously for 2 minutes was assessed. This dose was selected from a preliminary dose-response study (1 to 40 μg/kg body weight injected intravenously). Arterial blood pressure was measured every 30 seconds at mice tail using a CODA noninvasive blood pressure device (Kent Scientific Corp, Torrington, Connecticut, USA) before (mean value of 10 measures) and after phenylephrine injection (mean value of 4 measures). In BALB/C nude mice, only basal arterial blood pressure was measured.

Supplementary References

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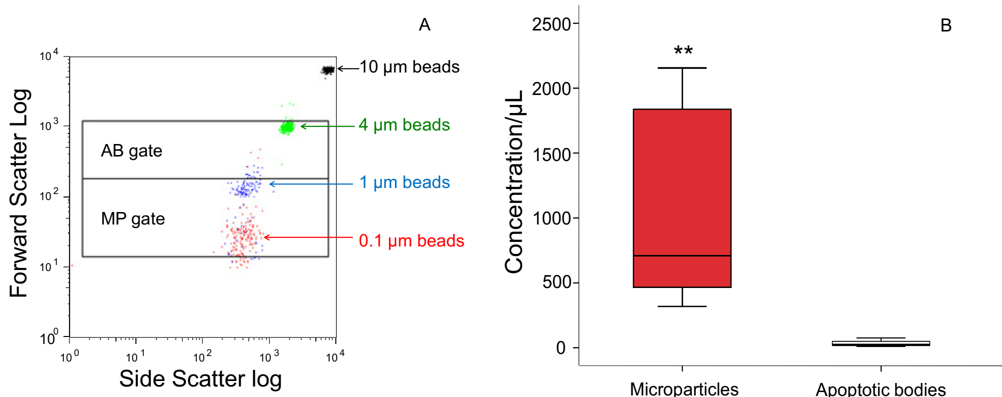


Supplementary Figure 1. (*A*) The MP gate and the apoptotic bodies (AB) gate were defined using calibration beads. These beads were identified by their fluorescence and retrogated on the forward scatter/side scatter graph to distinguish them from background noise, which is not shown in this figure for the sake of clarity. (*B*) The platelet-free plasma obtained after the 2 centrifugations (500*g* for 15 minutes and then 15,200*g* for 5 minutes) contains only rare apoptotic bodies (flow cytometry analysis; 6 patients with cirrhosis). Data are given as median (*horizontal bar*), 25th and 75th percentile (*boxes*), and extreme values, which are less than 3 box lengths from either end of the box (*error bar*). $^{**}P < .01$.

Supplementary Table 1. Baseline Characteristics

Characteristics	Patients with cirrhosis (pilot cohort) (n = 26)	Healthy controls (n = 30)	*P* value
Age (*y*)	55 (36–63)	52 (31–72)	.511
Male sex, n (*%*)	16 (61)	23 (77)	.219
Cardiovascular risk factors			
Diabetes, n (*%*)	8 (31)	10 (33)	.838
Hypertension, n (*%*)	6 (23)	10 (33)	.397
Smoking, n (*%*)	14 (54)	10 (33)	.122
Dyslipidemia, n (*%*)	2 (8)	10 (33)	.020
Body mass index (kg/m^2)	23.8 (18.0–36.5)	25.9 (19.0–36.6)	.038
Laboratory data			
Leukocytes ($\times 10^9/L$)	6.0 (1.1–15.6)	6.7 (4.2–13.1)	.495
Hemoglobin (*g/L*)	111 (75–165)	145 (119–162)	<.001
Platelet count ($\times 10^9/L$)	126 (44–356)	263 (99–357)	<.001

NOTE. Data are expressed as median (range) or frequency (%).

Supplementary Table 2. Cellular Origin of Circulating MPs in Patients With Cirrhosis and in Healthy Controls

Circulating MP concentration (*MP/µL*)	Patients with cirrhosis (pilot cohort) (n = 26)	Healthy controls (n = 30)	*P* value
Annexin V^+ MPs	825 (78–6394)	938 (80–18,577)	.869
Platelet MP ($CD41^+$)	493 (8–5634)	696 (12–17,182)	.490
Monocyte-macrophage MPs ($CD14^+$)	5 (0–143)	2 (0–24)	.053

NOTE. Data are expressed as median (range).

Supplementary Table 3. Univariate Analysis of Plasma MP Levels Predicting Survival

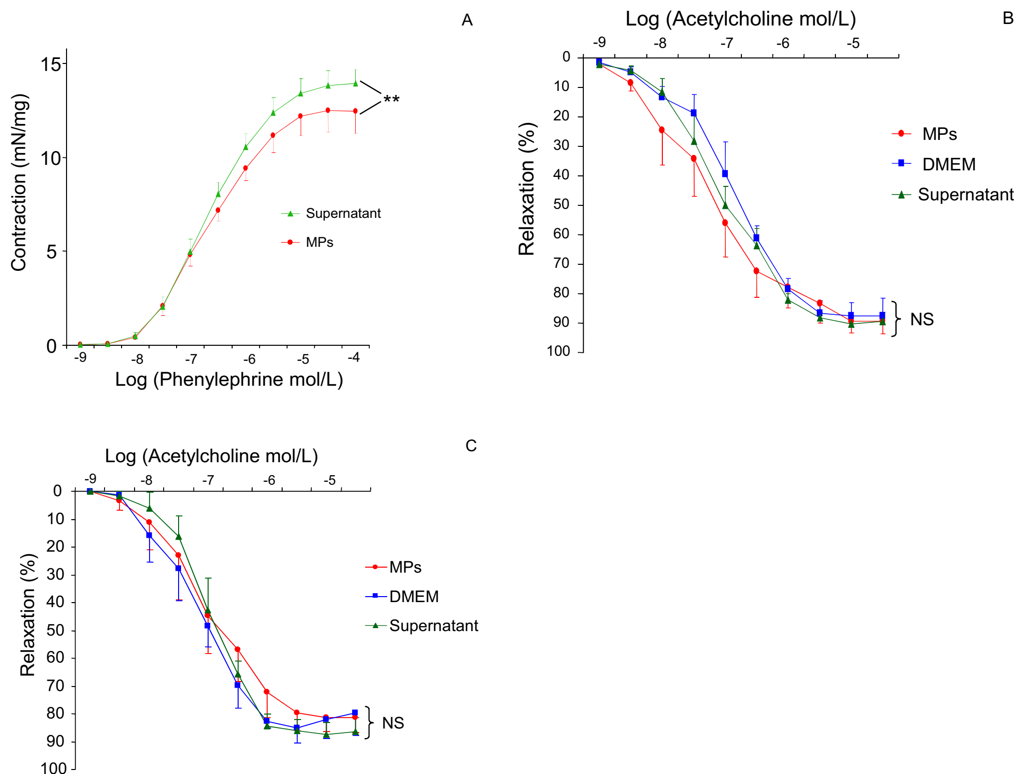
	Risk ratio	95% confidence interval	*P* value
MELD score	1.226	1.126–1.334	<.001
Child–Pugh score	1.432	1.096–1.870	.008
$10^2 \times$ CD31$^+$/41$^-$ MPs/μL	2.044	1.290–3.239	.002
$10^2 \times$ CD11a MPs/μL	1.111	1.010–1.222	.030
$10^2 \times$ CD4 MPs/μL	0.015	0.000–764.035	.448
$10^2 \times$ CD235 MPs/μL	1.011	0.818–1.250	.919
Cytokeratin-18$^+$ MPs (IU/L)	1.002	0.998–1.005	.359

NOTE. Except for MPs of hepatocyte origin, all data were available for 88 patients or more out of the 91 patients. Of these 91 patients, 31 underwent liver transplantation (counted as censored) and 16 died (counted as event). Of the 40 patients with available circulating levels of MPs of hepatocyte origin (cytokeratin-18$^+$), 15 underwent liver transplantation (counted as censored) and 6 died (counted as event).

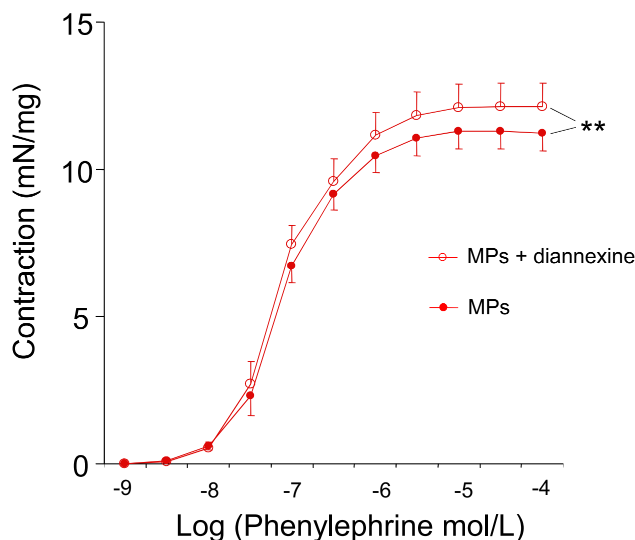
Supplementary Table 4. Results of Multivariate Cox Regression Analysis

Variable tested	Risk ratio	95% confidence interval	*P* value
$10^2 \times$ CD31$^+$/41$^-$ MPs/μL	1.268	0.763–2.108	.360
MELD score	1.213	1.110–1.325	<.001
$10^2 \times$ CD31$^+$/41$^-$ MPs/μL	1.630	1.005–2.642	.048
Child–Pugh score	1.356	1.044–1.763	.023
$10^2 \times$ CD11a MPs/μL	1.029	0.930–1.138	.578
MELD score	1.222	1.123–1.331	<.001
$10^2 \times$ CD11a MPs/μL	1.065	0.967–1.173	.201
Child–Pugh score	1.416	1.081–1.856	.012

NOTE. We fitted 4 Cox models including variables achieving a *P* value <.05 in Supplementary Table 3 (ie, $10^2 \times$ CD31$^+$/41$^-$ MPs/μL or $10^2 \times$ CD11a MPs/μL) with either Child–Pugh or MELD score, as previously reported.[8]



Supplementary Figure 2. (*A*) Concentration-response curves to phenylephrine of rat aortic rings previously incubated with plasma MPs from patients with Child–Pugh B or C cirrhosis at their circulating concentration or with supernatant. MPs impair response to phenylephrine even when aortas are isolated from cirrhotic rats (24-hour incubation, n = 6). (*B* and *C*) Concentration-response curves to acetylcholine of healthy rat aortic rings previously incubated for 24 hours with plasma MPs at their circulating concentration or with controls (ie, plasma supernatant or DMEM). Neither MPs from patients with Child–Pugh B or C cirrhosis (*B*; n = 6) nor MPs from healthy controls (*C*; n = 4) changed relaxation to acetylcholine. Data are expressed as mean ± SEM. NS, no significant difference; $^{**}P < .01$.



Supplementary Figure 3. Healthy rat aortic rings were incubated for 24 hours with plasma MPs from patients with Child–Pugh B or C cirrhosis at their circulating concentration. Then, response to phenylephrine was assessed. Preincubation for 30 minutes of MPs with diannexin (2 μg/mL) suppressed the cirrhotic MP effect. $^{**}P < 0.01$.