

# Red Blood Cell-Conditioned Media from Non-Alcoholic Fatty Liver Disease Patients Contain Increased MCP1 and Induce TNF- $\alpha$ Release

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## Abstract

**Background:** Non-alcoholic fatty liver disease (NAFLD) constitutes a global pandemic. An intricate network among cytokines and lipids possesses a central role in NAFLD pathogenesis. Red blood cells comprise an important source of both cytokines and signaling lipids and have an important role in molecular crosstalk during immunometabolic deregulation. However, their role in NAFLD has not been thoroughly investigated.

**Methods:** Conditioned media from erythrocytes derived from 10 NAFLD patients (4 men, 6 women, aged 57.875 $\pm$ 15.16) and 10 healthy controls (4 men, 6 women, aged 39.3 $\pm$ 15.55) was analyzed for the cytokines IFN- $\gamma$ , TNF- $\alpha$ , CCL2, CCL5, IL-8, IL-1 $\beta$ , IL-12p40, IL-17, MIP-1 $\beta$ , the signaling lipids sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), and cholesterol. Their effect on the cytokine profile released by RAW 264.7 macrophages was also studied.

**Results:** MCP1 levels were greater in conditioned growth medium from NAFLD patient erythrocytes than in that from healthy controls (37 $\pm$ 40 vs 6.51 $\pm$ 5.63 pg/ml). No statistically significant differences were found between patients and healthy controls with regard to S1P, LPA, cholesterol, or eight other cytokines. TNF- $\alpha$  release by RAW 264.7 cells was greater after incubation with patient-derived erythrocyte-conditioned medium than in medium without RAW 264.7 cells from either healthy or NAFLD subjects.

**Conclusions:** Erythrocytes may contribute to liver infiltration by monocytes, and macrophage activation, partially due to CCL2 release, in the context of NAFLD.

**Keywords:** Cytokines, Erythrocytes, Lipids, Non-alcoholic fatty liver disease, Signaling.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined as accumulation of fat in at least 5% of hepatocytes, after the exclusion of secondary causes, which include significant alcohol consumption, use of steatogenic medication, and hereditary, autoimmune, or viral hepatic disorders. NAFLD is histologically further categorized into nonalcoholic fatty liver (NAFL) (presence of hepatic steatosis with no evidence of hepatocellular injury in the form

of hepatocyte ballooning) and nonalcoholic steatohepatitis (NASH) (presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis) (1). NASH can evolve to fibrosis, cirrhosis, and hepatocellular carcinoma (2). However, the most frequent cause of mortality in NAFLD patients is cardiovascular complications (3).

NAFLD pathogenesis is orchestrated by an

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interplay of diverse molecular mechanisms. The recently proposed multiparallel hits hypothesis suggests that NASH results from numerous conditions acting in parallel; these conditions include genetic predisposition, abnormal lipid metabolism and trafficking, oxidative stress, lipotoxicity, mitochondrial dysfunction, altered cytokine and adipokine production, gut dysbiosis, and endoplasmic reticulum stress (4).

An intricate network among cytokines and lysophospholipids plays a central role in NAFLD pathogenesis. Deregulation of the synthesis and signaling of various cytokines and chemokines, including TNF- $\alpha$ , CCL2, CCL5, and IL-8, has been associated with the development and progression of NAFLD (5). In addition, sphingosine-1-phosphate (S1P) is increased in experimental NASH animal models (6), and its binding to its receptor, S1PR1, results in NFK $\beta$  activation and expression of pro-inflammatory cytokines, including tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and monocyte chemoattractant protein 1 (MCP1) (7). Furthermore, the role of S1P in steatohepatitis is highlighted by the fact that administration of S1P antagonists to experimental NASH animals eradicated the disease (8). Another signaling lipid, lysophosphatidic acid (LPA), also contributes to NAFLD pathogenesis: serum LPA levels were found to be associated with the degree of hepatic fibrosis and steatosis (8, 9). Finally, hepatic cholesterol accumulation contributes to mitochondrial dysfunction, endoplasmic reticulum stress, inflammation, and fibrosis (10).

Apart from their typical role as oxygen transporters, human erythrocytes retain a considerable immunomodulatory capacity (11, 12). Interestingly, erythrocytes constitute important pools of cytokines, chemokines, S1P, LPA, and cholesterol in the blood (13).

Despite the fact that erythrocyte dysfunction is implicated in the molecular mechanisms of NAFLD (14), erythrocyte involvement in NAFLD pathogenesis has not been studied in depth. It is known that during hepatic steatosis there is a remarkable erythrocyte accumulation in the liver both *ex vivo* and *in vivo* (15). These

erythrocytes externalize their phosphatidylserine (PSer) as a response to oxidative stress, which is then recognized by the Kupffer cell receptor, lactadherin, resulting in erythrophagocytosis. This interaction leads to an increase of hepatic oxidative stress and promotes inflammation (15). In addition, Unruh et al (16) have shown that a high-fat diet increases erythrocyte membrane cholesterol, externalized phosphatidylserine, bound MCP1, and reactive oxygen species (ROS). These erythrocytes also induce a proinflammatory phenotype in macrophages and monocyte adhesion to the endothelium in both control and high-fat diet-fed animals.

Taken together, these observations indicate that erythrocytes may mediate immunometabolic interactions in the context of metabolic disease. Hence, in this study, we sought to investigate whether erythrocytes participated in NAFLD pathogenesis by further, yet unexplored, immunomodulatory interactions with macrophages, through the release of cytokines, S1P, LPA, and cholesterol.

## Materials and Methods

### Materials

Dulbecco's phosphate buffered saline (PBS) w/o calcium w/o magnesium – 500 ml and RPMI 1640 were purchased from Biosera, France. MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel, NINE PLEX was purchased from Millipore, USA. VPC23019 and AM966 were purchased from Cayman Chemicals, USA. A human lysophosphatidic acid ELISA kit was purchased from Cubasio, USA, and a general sphingosine-1-phosphate ELISA kit was purchased from Fine Biotech, China. The cholesterol colorimetric assay was from Reagent Genie, Ireland.

### Patients

Ten patients (4 men, 6 women, age 57.86 $\pm$ 15.17) and 10 healthy controls (4 men, 6 women, age 39.3 $\pm$ 15.55) participated in our study. They were recruited by the 1st Pathology Clinic, Department of Medicine, Democritus University of Thrace,

Alexandroupolis, Greece. All patients presented hepatic steatosis according to ultrasonography. After exclusion of viral, alcoholic, drug, and other causes, patients were evaluated by NAFLD fibrosis score (FIB4), and AST/ALT ratio. Their

anthropometric characteristics are shown in Table 1. Our study was approved by the Scientific Council of the University Hospital of Alexandroupolis and the Ethics Committee after receiving informed consent from the participants.

**Table 1.** Basic characteristics of NAFLD patients and healthy subjects.

	NAFLD patients	Healthy Controls	P(Healthy< NAFLD) or P(Healthy> NAFLD)
Age (years)	57.86±15.17	39.3±15.55	98.2%
BMI	35.06±4.22	25.3±3.7	100.0%
AST (U/L)	49.29±22.19	21.7±8.1	98.3%
ALT (U/L)	82.43±74.45	21.9±6.55	95.1%
Triglycerides (mg/dL)	192.5±48.6	152±17.2	87.6%
Cholesterol (mg/dL)	201±45.34	154±13.5	91.2%
HDL-C (mg/dL)	43.41±3.91	65.1±13.29	99.3%*
LDL-C (mg/dL)	140.4±37.26	58.6±7.11	98.0%
Platelets	245.13±53.1	240±50.8	54.3%
FIB4	1.54±0.96	0.77±0.33	86.4%
AST/ALT	0.91±0.43	0.97±0.15	31%

\* Most probabilities are for P(Healthy< NAFLD). The probabilities marked by \* are P(Healthy> NAFLD).

P (Healthy< NAFLD) or P (Healthy> NAFLD) is the posterior probability that the difference between means of healthy subjects and NAFLD patients is less or greater than zero, respectively. (ALT: alanine aminotransferase; AST: aspartate aminotransferase; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol, FIB4: Non-Alcoholic Fatty Liver Disease Fibrosis score).

### ***Isolation of red blood cells***

Three ml of blood containing EDTA was centrifuged at 200 x g for 10 min at 4 °C. Plasma and buffy coat were removed. Then, the erythrocyte pellet was washed with cold saline solution and centrifuged at 200 x g for 10 min at 4 °C. The buffy coat was removed. This step was repeated three times. Next, 100 µl of erythrocytes from the bottom of the pellet were used for cell counting and production of conditioned media.

### ***Production of red blood cell-conditioned medium***

Erythrocytes were incubated in RPMI 1640, supplemented with 10% FBS and 1% streptomycin/penicillin, at 5% CO<sub>2</sub>, 37 °C, for 24 hours. Then, the red blood cell-derived

conditioned medium (RBC-CM), from both patients (P-RBC-CM) and healthy controls (H-RBC-CM) was collected and centrifuged at 200 x g for 10 min. Conditioned media were stored at -80 °C. As a control, growth medium was placed in 6-well plates in the same conditions following the same procedures as for RBC-CM. Spectrophotometric assay found no erythrocyte-released hemoglobin, ruling out hemolysis.

### ***Raw 264.7 macrophage culture***

RAW 264.7 macrophages were cultured in RPMI 1640, supplemented with 10% FBS and 1% streptomycin/penicillin. When passage 12 was reached, cells were seeded in 6-well plates at 2 x 10<sup>5</sup> cells/well. When confluency reached approximately 80%, RAW 264.7 macrophages

were exposed to red blood cell-conditioned medium, at 5% CO<sub>2</sub>, 37 °C, for 24 hours. VPC 23019, an antagonist for S1P receptors 1 and 3, and AM966, an antagonist for LPA receptor 1, (Cayman Chemicals, USA) were used at concentrations of 10 μM and 25 mM respectively, after diluting with DMSO. The culture media were collected after centrifugation, for cytokine measurement. DMSO, VPC23019, and AM966 had no effect on the cytokine profile released by RAW 264.7 macrophages.

#### ***Analysis of cytokines, S1P, and LPA***

IFN-γ, TNF-α, CCL2, CCL5, IL-8, IL-1β, IL-12p40, IL-17, and MIP-1β were studied by multiplex technology, according to manufacturer's instructions. S1P and LPA were analyzed by ELISA, following the manufacturers' instructions.

#### ***Cholesterol content determination***

Cholesterol was determined by a colorimetric assay, following the manufacturers' instructions.

#### ***Statistical analysis***

Results in the text are expressed as means±standard deviations, unless otherwise stated.

Since the sample size was small, we used a Bayesian approach for statistical analysis, which is suited to provide meaningful results for small datasets. Bayesian analysis does not assume large sample sizes, and small datasets can be analyzed while retaining statistical power and precision (17, 18).

Statistics were analyzed with the R programming language v. 3.6 (19). Differences between means were determined using the BEST package (20). The results are reported as the probability P of the difference between the means of healthy subjects and NAFLD patients being less than zero, P(Healthy < NAFLD), or greater than zero, P(Healthy > NAFLD). This probability is more intuitive than the more convoluted meaning of the frequently used p value, which is the probability of observing the data, assuming the

null hypothesis (no difference between means) is correct. Probabilities above 90% were considered statistically significant.

## **Results**

Patients included in our study presented statistically significant higher body mass indexes and levels of serum transaminases, triglycerides, low-density lipoprotein, and total cholesterol than healthy controls (Table 1). Meanwhile, healthy controls had higher high-density lipoprotein cholesterol. These results are indicative of the metabolic malfunction during NAFLD.

The boxplot in Figure 1 shows the levels of cytokines (Fig. 1A) and lipids (Fig. 1B) in the erythrocyte-conditioned medium of healthy and NAFLD subjects. The levels of the respective parameters in plain growth medium are shown as baseline reference. Growth medium from NAFLD patient erythrocytes had greater MCP1 concentration than that from healthy controls (37±40 vs 6.51±5.63 pg/ml) P(Healthy < NAFLD) = 99.4% (Fig. 1A). Also, IL-8 showed a marginal difference between means, with P(Healthy < NAFLD) = 94.6%. MIP1β, RANTES, and TNF-α levels did not significantly differ between the two groups, while IL-1β, IL-12p40, IFN-γ, and IL-17 levels were below the kit's detection limit.

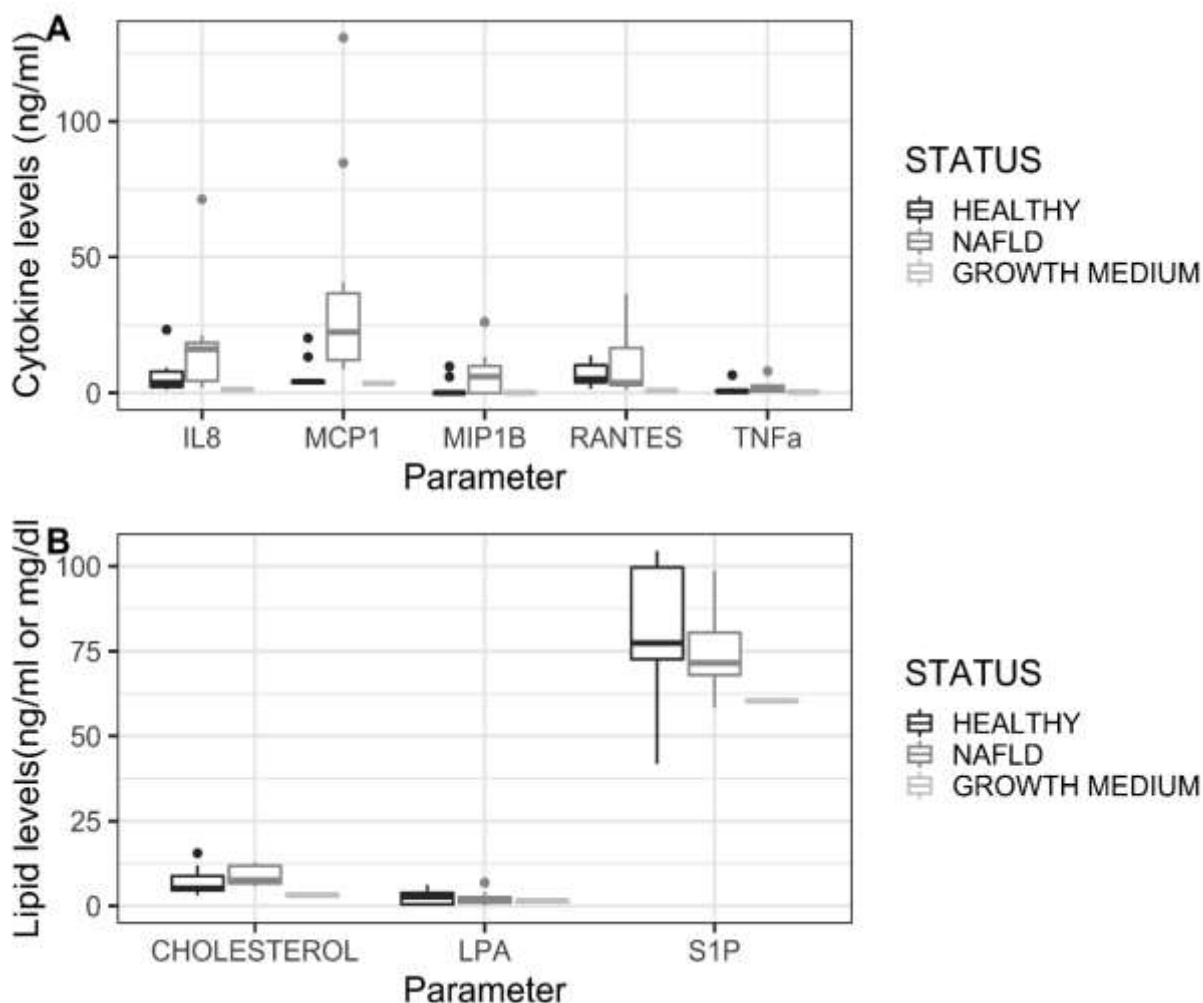
Since red blood cells secrete bioactive lipids, we examined S1P and LPA levels in RBC-CM. No statistically significant differences were found between patients and healthy controls (75.62±12.77 vs 81.18±19.72 for S1P, and 2.28±2.29 vs 2.59±2.15 pg/ml, for LPA) (Fig. 1B). Similarly, cholesterol levels did not differ significantly between the two groups (8.9±2.85 vs 4.51±5.23 mg/dl) (Fig. 1B).

Next, we examined the effect of conditioned medium both from patients and healthy controls (P-RBC-CM and H-RBC-CM respectively) on the cytokine profile released by RAW 264.7 macrophages (Fig. 2). The TNF-α concentration in P-RBC-CM-stimulated RAW cells was greater (36.84±25.21 pg/ml) than that in H-RBC-CM-stimulated RAW cells (1.67±1.23 pg/ml), P(H-RBC-CM-RAW < P-RBC-CM-RAW) =

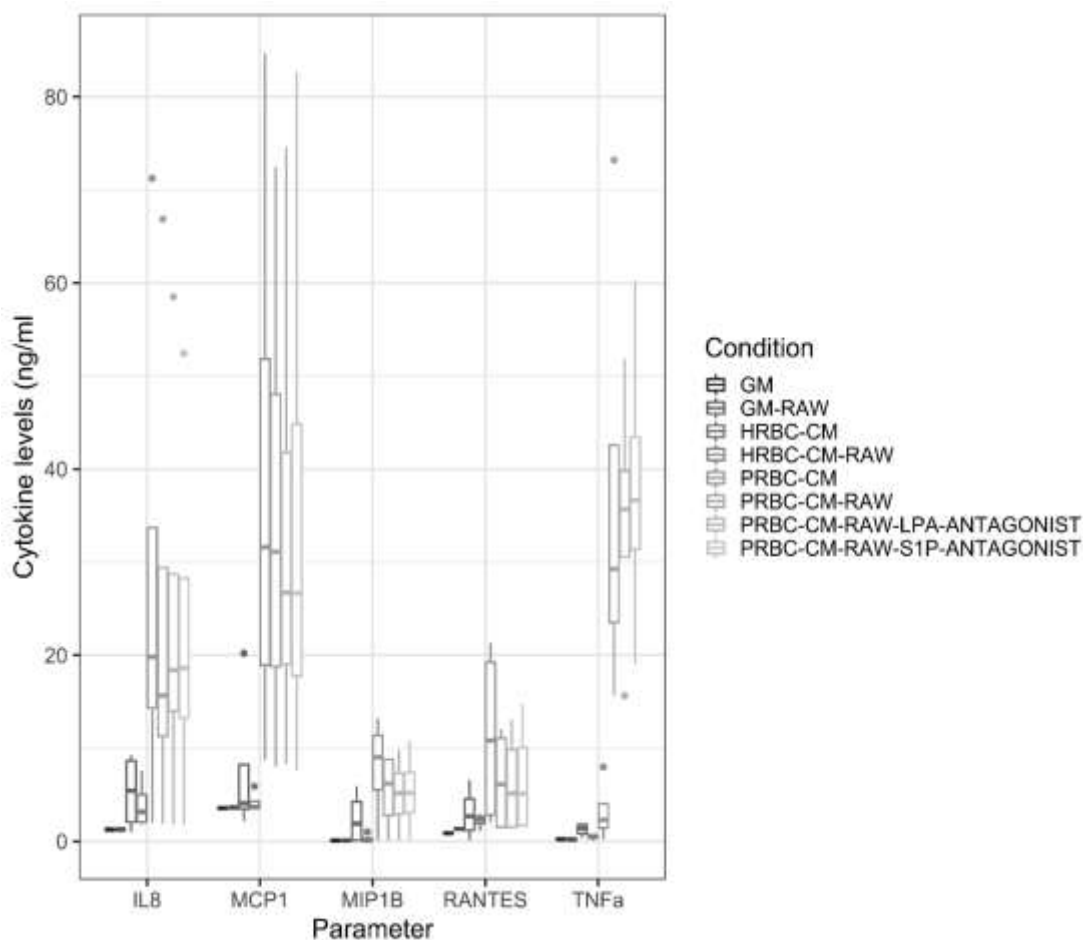
91.3%. This means that the patient erythrocyte-conditioned medium induced the release of TNF- $\alpha$  by RAW macrophages. Also, TNF- $\alpha$  levels of P-RBC-CM-RAW were greater than P-RBC-CM alone ( $3.19 \pm 3.65$  pg/ml),  $P(P-RBC-CM < P-RBC-CM-RAW) = 93.1\%$ . This observation demonstrates that the TNF- $\alpha$  increase is not inherent to the patient erythrocytes but is mainly released by the RAW macrophages.

Finally, we examined whether the effect of

erythrocytes from NAFLD patients was mediated through the action of S1P and/or LPA, using antagonists for S1P receptors 1 and 3, and LPA receptor 1 (Fig. 2). No statistically significant differences were found in the secreted cytokine profiles between cells treated with patient-derived conditioned medium in the presence or absence of VPC23019 or AM966. Therefore, our results indicate that S1PR1, S1PR3, and LPAR1 are not involved in the process.



**Fig. 1.** (A) Cytokine levels (ng/ml) in growth medium and red blood cell-conditioned medium from patients and healthy controls. (B): S1P and LPA (ng/ml) and cholesterol levels (mg/dl) in growth medium and red blood cell-conditioned medium from patients and healthy controls. LPA: lysophosphatidic acid; S1P: sphingosine-1-phosphate. The conditioned growth medium was prepared by incubating erythrocytes from patients or healthy controls in RPMI 1640, supplemented with 10% FBS and 1% streptomycin/penicillin, at 5% CO<sub>2</sub>, 37 °C, for 24 hours. The conditioned medium was then collected and centrifuged at 200 x g for 10 min to remove the erythrocytes. As a control, growth medium was placed in 6-well plates in the same conditions following the same procedures as for the erythrocyte-conditioned medium.



**Fig. 2.** Cytokine levels released by RAW 264.7 cells after incubation for 24 hours with growth medium and red blood cell-conditioned medium from patients and healthy controls. GM: growth medium; GM-RAW; RAW 264.7 conditioned medium; H-RBC-CM: healthy control-derived red blood cell conditioned medium, H-RBC-CM-RAW: healthy control-derived red blood cell conditioned medium incubated with macrophages RAW 264.7; P-RBC-CM: patient-derived red blood cell conditioned medium; P-RBC-CM-RAW: patient-derived red blood cell conditioned medium incubated with macrophages RAW 264.7; P-RBC-CM-RAW-LPA antagonist: patient-derived red blood cell conditioned medium incubated with macrophages RAW 264.7 pre-incubated with AM966, an antagonist for LPA receptor1 at concentration of 25 Mm; P-RBC-CM-RAW-S1P antagonist: patient-derived red blood cell conditioned medium incubated with macrophages RAW 264.7 pre-incubated with VPC 23019, an antagonist for S1P receptors 1 and 3, at a concentration of 10  $\mu$ M. RAW 264.7 macrophages were cultured in RPMI 1640, supplemented with 10% FBS and 1% streptomycin/penicillin. When passage 12 was reached, cells were seeded in 6-well plates at  $2 \times 10^5$  cells/well. When confluency reached approximately 80%, RAW 264.7 macrophages were exposed to red blood cell-conditioned medium, at 5% CO<sub>2</sub>, 37 °C, for 24 hours.

## Discussion

In this study, the role of red blood cells in the inflammatory response of macrophages in the context of NAFLD was investigated. We provide preliminary evidence that red blood cells release chemokines and induce TNF- $\alpha$  release from macrophages. This effect appeared to be independent of erythrocyte-derived S1P, LPA, and cholesterol.

Darbonne et al (21) were the first to show that erythrocytes bind chemokine CXCL8 (IL-8) and CCL2 (MCP1) through the Duffy

atypical receptor of chemokines. Apart from chemokine scavenging, red blood cells also possess the ability to release cytokines and growth factors. Wei et al (22), showed that IL-33 is released by erythrocytes through hemolysis. Next, Karsten et al (12) showed that erythrocytes from healthy volunteers can release more than 40 cytokines, chemokines, and growth factors. In addition, in a study by Unruh et al (16), erythrocyte-bound MCP1 was found to increase in high-fat diet-fed

experimental animals, and possibly, after release, participate in monocyte chemotaxis. Based on those observations, we explored a potential chemokine and cytokine releasing property of erythrocytes in the context of NAFLD, because these signaling mediators are implicated in a substantial part of the inflammatory cellular communication. Indeed, in our study, MCP1 was increased in NAFLD patient-derived red blood cell conditioned media. CXCL8 also displayed a small, but significant, increase.

Because MCP1 also induces pro-inflammatory cytokine expression in macrophages (23), we explored the effect of red blood cell-derived conditioned media on the cytokine profile secreted by RAW 264.7 macrophages. After examining the levels of nine cytokines by multiplex technology, we found that P-RBC-CM provoked a significant increase in TNF- $\alpha$  release. Since our protocol permitted the interaction with only erythrocyte-derived soluble factors, we hypothesized that the release of cytokines or signaling lipids could mediate the effects of red blood cells to activate monocytes or macrophages. In our study we excluded the role of both S1P and LPA, since their levels did not differentiate between patients and healthy controls, and receptor antagonism had no effect on RAW 264.7 macrophages. In addition, cholesterol levels did not differ between patients and healthy controls. However, erythrocyte-derived reactive oxygen species (ROS), free heme, hemoglobin, and microvesicles have all been implicated in the regulation of monocyte and macrophage function (24–26), and cannot be excluded from our study as potent mediators of the reported TNF- $\alpha$  increase. In fact, erythrocyte vesiculation is increased in both metabolic syndrome and hepatic cirrhosis (27, 28) and could explain MCP1 release (29) in our study.

Apart from microvesicle release, other mechanisms explaining the augmented chemokine release could be the differential binding capacity in the context of the disease. In particular, DARC sulfation levels could also lead to differential binding capacity, and thus, chemokine release (30). However, to what

extent this applies to all chemokines remains to be elucidated.

In our results, we observed a large variance regarding the degree of MCP1 and other chemokines released from patient erythrocytes. There are numerous reasons possibly explaining this fact. First, if chemokine release is mediated mainly through microvesicle formation, then a varied release is anticipated. Xiong *et al* (29) showed that microvesicles formed by red blood cells present altered binding ability and a large variance with regard to the dissociation constant of chemokines. Another reason could be erythrocyte variability between and within individuals. Especially, in NAFLD patients a high red-cell distribution width (RDW) is observed (31). Since RDW indicates the existence of various red blood cell subpopulations in the blood, these could have different physicochemical characteristics. Interestingly, in the context of NAFLD, angiotensin could be an important signaling molecule for the increased RDW and the subsequent erythrocyte malfunction (32–34).

We are aware that we analyzed samples from a limited number of patients with NAFLD who were not biopsy-proven. As a result, we are missing a possible relationship between NAFLD stage and cytokine profile released by erythrocytes and RAW 264.7 cells after interacting with RBC-CM. In view of the fact that the erythrocytes release such a plethora of signaling agents targeting a variety of cells implicated in NAFLD pathogenesis, further investigation is mandatory.

From these results, we conclude that erythrocytes may contribute to liver infiltration by monocytes and macrophage activation partially via MCP1 release, acting within the context of NAFLD pathogenesis. However, expansion of this study to a larger patient group and more experimental studies in animal models are needed.

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