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A novel CD14^{high} CD16^{high} subset of peritoneal macrophages from cirrhotic patients is associated to an increased response to LPS

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ABSTRACT

The aim of this study was to characterize monocyte-derived macrophages (M-DM) from blood and ascites of cirrhotic patients comparatively with those obtained from blood of healthy controls. The phenotypic profile based on CD14/CD16 expression was analyzed by flow cytometry. Cells were isolated and stimulated *in vitro* with LPS and heat killed *Candida albicans*. Phosphorylation of ERK, c-Jun, p38 MAPK, and PKB/Akt was analyzed by Western blotting. A novel CD14^{high}CD16^{high} M-DM subpopulation is present in ascites (~33%). The CD14^{++}CD16^{+} intermediate subset is increased in the blood of cirrhotic patients (~from 4% to 11%) and is predominant in ascites (49%), while the classical CD14^{++}CD16^{-} subpopulation is notably reduced in ascites (18%). Basal hyperactivation of ERK and JNK/c-Jun pathways observed in ascites M-DM correlates with CD14/CD16 high expressing subsets, while PI3K/PKB does it with the CD16 low expressing cells. *In vitro* LPS treatment highly increases ERK1/2, PKB/Akt and c-Jun phosphorylation, while that of p38 MAPK is decreased in M-DM from ascites compared to control blood M-DM. Stimulation of healthy blood M-DM with LPS and *C. albicans* induced higher phosphorylation levels of p38 than those from ascites. Regarding cytokines secretion, *in vitro* activated M-DM from ascites of cirrhotic patients produced significantly higher amounts of IL-6, IL-10 and TNF-α, and lower levels of IL-1β and IL-12 than control blood M-DM. In conclusion, a new subpopulation of CD14^{high}CD16^{high} peritoneal M-DM has been identified in ascites of cirrhotic patients, which is very sensitive to LPS stimulation.

1. Introduction

Hepatic cirrhosis is an end-stage disease characterized by a progressive replacement of the liver architecture by non-functional fibrotic tissue. The involvement of the innate immune system, especially monocytes and macrophages, in the pathogenesis of liver cirrhosis has been largely described (Ramachandran and Iredale, 2012; Zimmermann et al., 2012).

Macrophages constitute a functionally and phenotypically heterogeneous cell population that link innate and adaptive immunity and are critical in the development and maintenance of many inflammatory diseases. They are located in all tissues as resident cells in steady-state conditions, and also as immigrants recruited from peripheral blood monocytes in response to tissue injury, inflammation or pathogen invasion (Gordon and Taylor, 2005; Wynn et al., 2013). Studies on the role of the human immune system in inflammatory diseases largely rely on findings obtained from the blood compartment. However, the population of blood monocytes, which is the most extensively studied, does not constitute a homogeneous set of cells, but it is integrated by several not well characterized subpopulations with different phenotypes and functions (Ziegler-Heitbrock, 2007). According

Abbreviation: AF, ascites fluid; CCL2, chemokine (C—C motif) ligand 2; ERK, extracellular signal-regulated kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; M-DM, monocyte-derived macrophages; MAPK, mitogen-activated protein kinase; MIP, macrophage inflammatory protein; NFKB, nuclear factor kappa-light-chain-enhancer of activated B cells; PKB, protein kinase B; PMN, polymorphonuclear; SBP, spontaneous bacterial peritonitis; TGF-β, transforming growth factor beta; TLR-4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

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to the standard nomenclature approved by the Committee of the International Union of Immunological Societies to characterize subsets of human monocytes (Ziegler-Heitbrock et al., 2010), three subpopulations of peripheral blood monocytes can be distinguished in healthy people, the "classical" $CD14^{++}CD16^{-}$, the "intermediate" $CD14^{++}CD16^{+}$ and the "non-classical" $CD14^{+}CD16^{+}$ monocytes (Wong et al., 2011; Ziegler-Heitbrock and Hofer, 2013; Ziegler-Heitbrock et al., 2010). Several clinical studies have demonstrated that the blood $CD16^{+}$ subpopulations are expanded in acute (Mizuno et al., 2005) or chronic (Fingerle-Rowson et al., 1998; Horelt et al., 2002; Zimmermann et al., 2012, 2010) inflammatory conditions, so that they are referred to as pro-inflammatory monocytes. They produce *in vitro* the pro-inflammatory cytokines TNF-α, IL-6, MIP1α and MIP1β in response to LPS (Auffray et al., 2009; Ziegler-Heitbrock, 2007; Zimmermann et al., 2012), accumulate massively in fibrotic/cirrhotic livers, activate hepatic stellate cells and correlate with clinical progression (Zimmermann et al., 2010). They are probably expanded by undergoing a dynamic transition from classical to intermediate subset and from this to non-classical subpopulation. For instance, in a natural acute bacterial infection, the shift from classical to intermediate and from this to non-classical subpopulation is reverted along the course of the infection (Ziegler-Heitbrock and Hofer, 2013). The study of monocyte-derived macrophages (M-DM) from an inflammatory scenario can make valuable contributions to a better understanding of the pathophysiology of many human diseases (Liaskou et al., 2013; Wynn et al., 2013). Hence, human peritoneal macrophages are optimal for the study of the biological properties of this type of cells (Fieren, 2012). Furthermore, it has been described the existence of a significant heterogeneity in the studies carried out in macrophages, that seems to be the result of their specialization in each particular environment (Gordon and Taylor, 2005).

Since macrophages are involved in many of the pathophysiological events associated with liver cirrhosis, these cells are considered as current targets for anti-inflammatory therapy intended to avoid progression of liver damage (Heymann et al., 2009; Liaskou et al., 2013; Ramachandran and Iredale, 2012; Zimmermann et al., 2012).

In this work, we have studied the relationships among distribution of CD14/CD16 subsets in M-DM from the ascites and the blood of decompensated cirrhotic patients compared with the blood of healthy controls. Furthermore, the activation state of several intracellular signaling molecules in response to LPS or *Candida albicans* has been analyzed in peritoneal M-DM from the ascites of cirrhotic patients. A proper phenotypic and functional characterization of M-DM in ascitic fluid of cirrhotic patients will contribute to understand and perhaps, to find novel molecules or parameters that could be useful to predict the risk of suffering further episodes of SBP (Spontaneous Bacterial Peritonitis) that compromise the outcome in this clinical setting.

2. Materials and methods

2.1. Study subjects

Enrolled patients were admitted at the Liver Unit of Hospital General Universitario de Alicante or at the Liver Transplant Unit of Hospital Universitario Virgen de la Arrixaca, Murcia, Spain. Cirrhosis was diagnosed by histology or by clinical, laboratory, and/or ultrasonographic findings. Exclusion criteria included detectable levels of LPS in ascites, positive blood or ascites culture, an ascites polymorphonuclear (PMN) count equal or higher than 250/μl (Albillos et al., 1990), symptoms of systemic inflammatory response syndrome (Rangel-Frausto et al., 1995), upper gastrointestinal bleeding, hepatocellular carcinoma fulfilling Milan criteria (Mazzaferro et al., 1996) and/or portal thrombosis, previous liver transplantation, transjugular intrahepatic portosystemic shunt, norfloxacin treatment as prophylaxis of SBP, age older than 80 or younger than 18, etiology other than chronic alcohol intake or HCV chronic infection, and refusal to participate in the study.

Peripheral blood and ascites were collected from 48 patients with cirrhosis requiring a large-volume paracentesis at admission (Runyon, 1986). All patients received intravenous albumin after paracentesis (8 g/l of ascites) as routine protocol, if the volume of ascites evacuated was greater than 5 liters. Samples for routine biochemical study and PMN counts were obtained. Ascites was inoculated at bedside in aerobic and anaerobic blood culture bottles, 10 ml each (Runyon et al., 1988). Ascites samples were centrifuged at $500 \times g$; cells were collected and washed in PBS, and finally resuspended in DMEM (GIBCO Invitrogen, Paisley, UK). Peripheral blood of 27 healthy subjects was also obtained for flow cytometry analysis. Blood was diluted with sterile PBS and layered in tubes containing Ficoll (Axis-Shield PoC As Oslo, Norway). Lympho/monocytes fractions were collected and washed with DMEM (GIBCO Invitrogen, Paisley, UK).

The ethics committees (Comité Ético de Investigación Clínica Hospital General de Alicante, Hospital Universitario Virgen de la Arrixaca, and Comité de Bioética de la Universidad de Murcia) approved the study protocol according to the 1975 Declaration of Helsinki and all cirrhotic patients gave informed written consent to be included in this study.

2.2. Flow cytometry analysis

Cells from ascites or peripheral blood were stained with monoclonal antibodies and analyzed by flow cytometry. Antibodies used were mouse anti-human CD14-FITC (eBioscience, San Diego, CA), CD16-PE-Cy5, CD206-FITC, HLA-DR-FITC, CD3-FITC, CD33-PE and CD19-PE-Cy5 (BD-Pharmingen, San Diego, CA). The mouse IgG1-FITC, IgG1-PE and IgG1-PE-Cy5 antibodies used as isotype controls were from BD-Pharmingen. Fluorescence minus one (FMO) control is shown in Suppl. Fig. 1. In brief, 50 μl of peripheral blood or 0.2×10^6 white cells from AF were stained with 5 μl of the corresponding antibodies and incubated in the dark on ice, fixed with a fixing-lysing solution (Becton Dickinson, San José, CA) and then washed twice with PBS. Finally, fixed marked cells were resuspended in PBS and kept at 4 °C in the dark until data acquisition.

Flow cytometry analyses were performed on three-color fluorescence Epics XL (Beckman Coulter) using Cytomics RXP Analysis Software or version 2.5.1 of Flowing Software. 30,000–200,000 gated events were acquired and analyzed. Leukocytes were gated based on FCS *vs.* SSC (Forward *vs.* Side Scatter) on a lineal scale. Myeloid cells were gated on the base of $CD3^{-}CD33^{+}CD19^{-}$ cells and morphology. This gate was used to analyze the rest of markers studied. $CD14^{+}$ and $CD16^{+}$ cells were measured as a percentage of the total number of myeloid cells. Subpopulations were measured as a percentage of total number of $CD14^{+}$ cells and as a percentage of the total number of $CD16^{+}$ cells. Percentages of $CD206^{+}$ and $HLA\text{-}DR^{+}$ cells were analyzed in reference to their different levels of CD16 expression.

2.3. Isolation and stimulation of M-DM

Cells were seeded for panning at 0.2×10^6 M-DM/well in 96-well plates for ELISA, or $1–2 \times 10^6$ M-DM/well in 6-well plates for immunoblotting, according to the percentage of $CD14^{+}$ cells in the sample. After an overnight incubation at 37 °C in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin (complete culture medium, CCM), non-adhered cells were washed out with CCM. The purity of the M-DM in cell culture was >95%. Then M-DM were treated with 0.1 μg/ml LPS (*E. coli* serotype 0111.B4, Sigma Aldrich Co, Saint Luis, MO, USA) or heat-killed *C. albicans* SC5314