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Immuno-biological comparison of hepatic stellate cells in a reverted and activated state

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ABSTRACT

Human hepatic stellate cells (HSCs) demonstrated great immunological plasticity with important consequences for liver cell therapy. Activated HSCs (aHSCs) are *in vitro* reverted (rHSCs) to a quiescent-like phenotype with potential benefit to reduce liver fibrosis. The goal of this study is to establish and compare the immunological profile of activated and *in vitro* reverted HSCs and to investigate the impact of inflammatory priming on the immunobiology of both HSCs populations. The distribution of inflammatory primed activated and reverted HSCs across the different phases of the cell cycle is assessed by flow cytometry. In addition, Flow analysis was done to assess the expression level of neuronal, endothelial and stromal markers, cell adhesion molecules, human leucocyte antigens, co-stimulatory molecules, immunoregulatory molecules and natural killer ligands. Our results showed that the cell cycle distribution of both HSCs populations is significantly modulated by inflammation. Accordingly, activated HSC that were in G1 phase switch to S- and G2 phases when exposed to inflammation, while reverted HSCs mostly redistribute into sub-G0 phase. In a HSC state dependent manner, inflammatory priming modulated the expression of the stromal marker CD90, biological receptors (CD95 and CD200R), cell adhesion molecules (CD29, CD54, CD58, CD106 and CD166), human leucocyte antigen HLA-G, co-stimulatory molecules (CD40 and CD252), as well as the immunoregulatory molecules (CD200 and CD274). In conclusion, the immunologic profile of HSCs is significantly modulated by their activation state and inflammation and is important for the development of novel HSC liver cell-based therapy.

1. Introduction

The liver can adapt to injury more than any other solid organ since it is equipped with remarkable tissue repair mechanisms. Hepatic fibrosis is the wound-healing response of the liver to many causes of chronic injury of which viral infection, alcohol and non-alcoholic steatohepatitis (NASH) are the most common [1]. In general, fibrosis is preceded by inflammation, and elements of both the innate and adaptive immune systems are pivotal in regulating the fibrotic process [2]. Regardless of the underlying cause, iterative injury causes inflammatory damage, matrix deposition, parenchymal cell death and angiogenesis leading to progressive fibrosis. Ultimately, this can lead to cirrhosis and hepatocellular carcinoma (HCC), the primary malignancy of the liver [3]. The incidence of HCC is rising worldwide and is considered a major cause of liver-related death in patients with cirrhosis [4]. Today, the therapeutic challenge is to prevent the progression of liver fibrosis into more aggressive forms of chronic liver injury, and to promote its resolution by treating the underlying causative agent [5].

Abbreviations: aHSC, activated hepatic stellate cells; rHSC, reverted hepatic stellate cells; qHSC, quiescent hepatic stellate cells; MSCs, mesenchymal stem cells; IL-1β, interleukin-1β; IL-2, interleukin-2; IL-6, interleukin-6; IL-8, interleukin-8; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; INF-γ, interferon-γ; INF- α, interferon-α; CXCR4, C-X-C chemokine receptor type 4; CD, cluster of differentiation; CAM, cell adhesion molecule; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion protein-1; ALCAM, activated leukocyte cell adhesion molecule; LFA, lymphocyte function-associated antigen; HLA, human leukocyte antigen; MCP-1, monocyte chemoattractant protein-1; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; ECM, extracellular matrix; MMPs, matrix metalloproteases; DMEM, Dulbecco's Modified Eagle's medium; FGF-2, fibroblast growth factor-2

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Liver transplantation is a lifesaving intervention for patients with end-stage liver disease. Yet, this standard and efficient therapeutic approach faces a set of well-known challenges, including the shortage of living-donors liver transplant as well as complications of long-term immunosuppressive pharmacotherapy [6,7]. Cellular therapy, which includes bio-artificial liver support and hepatocyte transplantation, has emerged as an alternative and potential treatment for a variety of liver diseases [8]. Cell engraftment to replace damaged liver tissue, is considered a safer procedure than invasive liver transplantation [9]. Although clinical trials demonstrated long-term safety of liver cell transplantation, partial correction of metabolic disorders has been achieved and relatively poor long-term donor cell engraftment were a barrier against successful treatment of chronic diseases [9,10]. The use of hepatocytes as an alternative source for liver transplantation can overcome the shortage in liver donation [11,12]. Hepatic stellate cells (HSCs) have been shown to be good 'supportive' or 'accompanying' cells to enhance the engraftment and viability of infused hepatocytes and even extra-hepatic cells [13]. HSCs are derived from the non-parenchymal fraction of the liver and exhibit specific gene expression and secretion profiles allowing their discernment from other liver cell populations [14]. Low proliferative rate and hypofibrogenic features characterize quiescent HSCs (qHSCs). However, upon activation, HSCs differentiate into proliferative and extracellular matrix (ECM)-producing myofibroblasts (referred as activated HSCs; aHSCs), a hallmark of chronic liver injury and fibrosis [15,16]. The immunological behavior of HSCs occupies a central role in the pathophysiology of chronic liver injury [17]. In the injured site, long-term activation can in turn modulate the physiologic behavior of all adjacent liver cells including nearby qHSCs thus maintaining a permanent and irreversible activated phenotype of HSCs [18]. In normal conditions, the transient activated state of HSCs is terminated with inflammation resolution and expression of matrix metalloproteases (MMPs) that degrade accumulated matrix and thus resolution of fibrosis [19]. Recent studies suggested that reversion of aHSC phenotype into a quiescent-like phenotype could be a major cellular mechanism underlying fibrosis regression in the liver, thereby offering new therapeutic perspectives for the treatment of liver fibrosis. Thus, by combining different factors, we were able to revert *in vitro* the human primary aHSCs into a more quiescent-like phenotype (referred as reverted HSCs; rHSCs) [19]. As previously evoked, HSCs cell-based therapy for liver diseases has recently emerged as a promising alternative to liver transplantation and eligible cells should have an appropriate immunobiology state [20]. Indeed, qHSCs exhibit interesting immunomodulatory properties that may play a central role in successful liver transplantation [13,14,17]. *In vivo*, co-transplantation of hepatocytes with HSCs into a healthy liver recipient does not generate fibrosis, but significantly improves the engraftment of hepatocytes, probably by ameliorating cell homing [20]. The "immunological identity" acquired during inflammatory conditions by both quiescent and activated HSCs and responsible for the sustained activated state remains to be identified [21–23]. Recently, we reported that a regulated cytokine network balance is noted in HSCs under inflammatory priming [24]. In an attempt to determine pre-disposing factors of allograft fibrosis, we recently demonstrated that progressive fibrosis is driven by at least allo-immunity and inflammation in pediatric liver transplant recipients [25].

Considering that both rHSCs and aHSCs may have distinct immunobiology features and may differentially respond to an injury stimulus, it is then important to establish and compare their immunological profiles under normal and inflammatory contexts. Thus, their proliferative capacities were particularly contrasting as shown by their unequal distribution across the different cell cycle phases. In term of immunobiology, we observed several differences between activated and quiescent HSCs as demonstrated by a distinct inflammatory-modulated pattern of immunological markers expressed by both cell populations. This study provides for the first time a complete and detailed immuno-biological comparison of HSCs depending on their activation state. Therefore, successful liver therapy can be achieved with a well identified and tolerogenic HSCs immunological profile.

2. Materials and methods

2.1. Isolation and culture of human activated HSCs

HSCs were isolated and cultured as previously described [26]. Non-parenchymal liver cells were separated from parenchymal cells by sequential perfusion of human liver pieces with pre-warmed EGTA-containing EBSS medium (Lonza, Verviers, Belgium) and a collagenase P digestion solution (Roche) followed by low-speed centrifugation steps. HSCs were obtained by plating the low-density cell population obtained after subjecting the dissociated and washed non-parenchymal cells to an 8% Nycodenz (Myegaard, Oslo, Norway) gradient centrifugation step. Homogeneous populations of HSCs were obtained after three passages in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% Penicillin/Streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO2. HSCs were plated at a density of 1×104 cells/cm^2 and were passaged when reaching a confluence of 80–90%. Prior to each passage, cells were washed with phosphate buffered saline (PBS) and harvested by using 0.05% trypsin (Life Technologies). HSCs at passage three (P3) were thus used for the study. This is a brief, yet complete description of the isolation procedure. For the reader interested in more details, we maintain our referencing to two recent papers published by the co-authors of the present work [27,28]. These papers (El Taghdouini et al. [21]; Coll et al. [27]) are the first ones describing the isolation process in detail and provide an in depth characterization of the cells.

2.2. In vitro reversion of human activated HSCs

In vitro reversion was done as described in our previous work [28]. Briefly, for reversion of aHSCs to a more quiescent-like phenotype, cells were washed with PBS and incubated with DMEM supplemented with 1% FBS, 20 ng/mL epidermal growth factor (EGF) (Peprotech, London, UK), 10 ng/mL fibroblast growth factor 2 (FGF2) (Peprotech), 100 μM oleic acid (Sigma), 100 μM palmitic acid (Sigma) and 5 μM retinol (Sigma). The cells were incubated in these conditions for a total of 5 days and medium was refreshed every 2 days. The cells were harvested for further analysis at day 6.

2.3. Cell priming

The influence of inflammation was achieved according to our previous study [29]. Briefly, inflammation priming was performed by treating cells (overnight) with a pro-inflammatory cytokine cocktail containing IL-1β (25 ng/ml), TNF-α (50 ng/ml), IFN-α (10 ng/ml) and IFN-γ (50ng/ml) (all from Peprotech, Rocky Hill, NJ, USA). After the priming, the medium was removed and the cells were washed before analysis.

2.4. Cell phenotype

The cultured cells were harvested and analyzed by flow cytometry for the expression of several markers. Briefly, the cells were washed with phosphate buffered saline (PBS; GmbH, Bergisch, Germany) and incubated for 20 min with the following monoclonal antibodies listed in Table 1. After washing with the MACSQuant Running Buffer (Miltenyi Biotec), the cells were fixed with 4% formaldehyde solution.

2.5. Cell cycle analysis

The cell cycle distribution was analyzed by flow cytometry using the DNA Prep™ Reagent kit (Beckman Coulter) for iodure propidium staining, according to the manufacturer's instructions.

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