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Classical and alternative activation of rat hepatic sinusoidal endothelial cells by inflammatory stimuli

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ABSTRACT

The ability of rat hepatic sinusoidal endothelial cells (HSEC) to become activated in response to diverse inflammatory stimuli was analyzed. Whereas the classical macrophage activators, IFNγ and/or LPS upregulated expression of iNOS in HSEC, the alternative macrophage activators, IL-10 or IL-4 + IL-13 upregulated arginase-1 and mannose receptor. Similar upregulation of iNOS and arginase-1 was observed in classically and alternatively activated Kupffer cells, respectively. Removal of inducing stimuli from the cells had no effect on expression of these markers, demonstrating that activation is persistent. Washing and incubation of IFNγ treated cells with IL-4 + IL-13 resulted in decreased iNOS and increased arginase-1 expression, while washing and incubation of IL-4 + IL-13 treated cells with IFNγ resulted in decreased arginase-1 and increased iNOS, indicating that classical and alternative activation of the cells is reversible. HSEC were more sensitive to phenotypic switching than Kupffer cells, suggesting greater functional plasticity. Hepatocyte viability and expression of PCNA, β-catenin and MMP-9 increased in the presence of alternatively activated HSEC. In contrast, the viability of hepatocytes pretreated for 2 h with 5 mM acetaminophen decreased in the presence of classically activated HSEC. These data demonstrate that activated HSEC can modulate hepatocyte responses following injury. The ability of hepatocytes to activate HSEC was also investigated. Co-culture of HSEC with acetaminophen-injured hepatocytes, but not control hepatocytes, increased the sensitivity of HSEC to classical and alternative activating stimuli. The capacity of HSEC to respond to phenotypic activators may represent an important mechanism by which they participate in inflammatory responses associated with hepatotoxicity.

Introduction

Kupffer cells represent the largest population of macrophages in the body (Ishibashi et al., 2009). Like macrophages in other tissues, they play a key role in innate immune defense and in initiating inflammatory responses to injury and infection. Localized within the hepatic sinusoids, Kupffer cells are primed to rid the body of endotoxin and other foreign materials in the portal circulation *via* phagocytosis and the release of cytotoxic/proinflammatory mediators. Kupffer cells also contribute to the resolution of inflammatory responses and induction of tissue repair, and to the initiation of adaptive immunity (Ramadori et al., 2008; Tacke et al., 2009). Accumulating evidence suggests that the diverse activities of macrophages are mediated by distinct subpopulations that develop in response to inflammatory mediators in their microenvironment. Macrophages have been broadly classified into two major cell subpopulations: classically activated proinflammatory M1 macrophages induced by IFNγ, TLR-4 ligands and bacterial infection, and alternatively activated anti-inflammatory/wound repair M2 macrophages, which are further subdivided into M2a macrophages, induced by IL-4 and IL-13, M2b macrophages, induced by immune complexes in combination with IL-1β or LPS, and M2c macrophages, induced by IL-10, TGF-β or glucocorticoids (Martinez et al., 2008). It appears that Kupffer cells and infiltrating inflammatory macrophages undergo similar phenotypic activation *in vivo* during the pathogenic response to liver injury induced by hepatotoxicants such as acetaminophen (Laskin, 2009). Thus, while initially, macrophages responding to liver injury display a proinflammatory phenotype, later in the pathogenic process, they exhibit an anti-inflammatory/reparative phenotype. Findings that blocking M1 macrophages prevent acetaminophen-induced liver injury, while suppressing M2 macrophages exacerbate hepatotoxicity, provide evidence that both of these cell populations are important in the response to this liver toxicant (Blazka et al., 1995;

Abbreviations: HMGB1, high-mobility group protein B1; HSEC, hepatic sinusoidal endothelial cells; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NPC, nonparenchymal cell; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; TBS, tris-buffered saline; TLR, toll-like receptor; TNF, tumor necrosis factor; TGF, transforming growth factor.

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Dambach et al., 2002; Dragomir et al., 2012, in press; Gardner et al., 2012; Hogaboam et al., 2000; Holt et al., 2008; Ju et al., 2002; Laskin et al., 1995; Michael et al., 1999).

The walls of the hepatic sinusoids are composed of endothelial cells. These cells are distinct from vascular endothelial cells in that they are devoid of basement membrane (Enomoto et al., 2004); moreover, they possess pores or fenestrae, facilitating their ability to function as a selective barrier between the blood and the liver parenchyma. Hepatic endothelial cells also possess Fc receptors and scavenger receptors, and lysosome-like vacuoles, and are thought to play a role in the clearance of soluble macromolecules and small particulates (<0.23 μm) from the portal circulation (Elvevold et al., 2008; Kosugi et al., 1992; Lalor et al., 2006; Løvdal et al., 2000; Sano et al., 1990). Additionally, when Kupffer cell functioning is impaired, hepatic sinusoidal endothelial cell endocytosis is upregulated (Elvevold et al., 2008). In response to cytokines and bacterially-derived LPS, hepatic sinusoidal endothelial cells, like Kupffer cells, release inflammatory mediators including reactive oxygen and nitrogen species and eicosanoids, as well as chemokines, IL-1, IL-6, fibroblast growth factor, and IFN (reviewed in Gardner and Laskin, 2007). These findings suggest that endothelial cells play a role in hepatic inflammatory responses to tissue injury or infection. A question arises, however, as to whether the biological activity of endothelial cells, like macrophages, is mediated by phenotypically distinct subpopulations. To address this, we analyzed the response of hepatic sinusoidal endothelial cells to classical and alternative inducers of macrophage activation. Our findings that endothelial and Kupffer cells respond to inflammatory mediators in a generally similar manner developing into distinct pro- and anti-inflammatory/wound repair subpopulations provide support for the concept that both cell types contribute to innate immune responses in the liver.

Materials and methods

Reagents

Collagenase type IV, protease type XIV, DNase I, OptiPrep™, and *Escherichia coli* LPS (serotype 0128:B12) were purchased from Sigma Chemical Co. (St. Louis, MO). Leibovitz's L-15 medium and Liberase TM were from Roche Diagnostics Corporation (Indianapolis, IN). IL-4, IL-10 and IL-13 were from R&D Systems (Minneapolis, MN), and IFNγ from Invitrogen (Carlsbad, CA). Rat antibody to iNOS was from BD/Transduction Labs (San Jose, CA), rabbit antibodies to mannose receptor, arginase-1, MMP-9 and PCNA from Abcam (Cambridge, MA), and β-catenin from Santa Cruz (Santa Cruz, CA). Goat anti-rat and goat anti-rabbit HRP-conjugated secondary antibodies were from Santa Cruz.

Animals

Male Sprague–Dawley rats (100–150 g) were obtained from Harlan Laboratories (Indianapolis, IN). Rats were maintained on food and water *ad libitum* and housed in microisolation cages. All animals received humane care in compliance with the institution's guidelines, as outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

Liver cell isolation

Hepatocytes, endothelial cells and Kupffer cells were isolated from rat livers as previously described with some modifications (Ahmad et al., 1999; Gardner et al., 1998). For hepatocyte isolation, the liver was perfused *in situ* with Ca^{2+}/Mg^{2+}-free HBSS (pH 7.3) containing 0.5 mM EGTA and 25 mM HEPES, followed by Leibovitz's L-15 medium containing 25 mM HEPES and 0.27 μg/ml Liberase TM. After 15 min, the liver was excised, disaggregated and filtered through 220 μm nylon mesh. Hepatocytes were recovered by centrifugation at 50 ×*g* (5 min, 4 °C). For Kupffer cell and endothelial cell isolation, 0.05% protease type XIV was added to the perfusion buffer. After 15 min, the liver was excised, disaggregated and digested for an additional 45 min at 37 °C with 0.2% protease type XIV and 0.001% DNase I. The resulting cell suspension was filtered through 220 μm nylon mesh and hepatocytes separated from NPCs by four successive washes (50 ×*g*, 5 min, 4 °C) for 5 min. NPCs were recovered by centrifugation of the supernatant at 300 ×*g* for 5 min (4 °C). Macrophages and endothelial cells were purified on a Beckman J-6 elutriator (Beckman Instruments Inc., Fullerton, CA) equipped with a centrifugal elutriation rotor set to a pump speed of 12 ml/min and a rotor speed of 2500 rpm. Endothelial cells were collected between 12 and 18 ml/min and macrophages between 30 and 44 ml/min. Cells were enriched by differential centrifugation on a 40% OptiPrep™ gradient (400 ×*g*, 15 min, 4 °C). Macrophage purity was 80–85%, and endothelial cell purity >98%, as determined by differential staining and peroxidase staining, and by electron microscopy and flow cytometry (Ahmad et al., 1999; Chen et al., 2007; McCloskey et al., 1992).

Hepatocyte and NPC co-cultures

For experiments analyzing the effects of NPCs on hepatocytes, endothelial cells or macrophages were plated onto 6.5 mm transwell inserts in 24 well plates (2×10^5 cells/well). After overnight incubation, the cells were washed twice with warm William E medium containing 1% FBS and incubated for 48 h with PBS, IFNγ (10 ng/ml), IL-10 (10 ng/ml), or IL-4 (10 ng/ml) + IL-13 (10 ng/ml). The cells were then washed with serum free William's E medium, and co-incubated with hepatocytes cultured overnight on 24 well plates (3×10^5 cells/well). Hepatocyte lysates were collected in RIPA buffer consisting of ice cold PBS containing 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 1% protease inhibitor, and 1% phosphatase inhibitor cocktails (Sigma) 24 h later. For experiments analyzing the effects of parenchymal cells on endothelial cells, hepatocytes were plated onto 6.5 mm transwell inserts in 24 well plates (3×10^5 cells/well). After overnight incubation, the cells were washed and refed with 1% FBS William's E medium containing 5 mM acetaminophen or control. Two hours later, hepatocytes were washed twice with warm William's E medium containing 1% FBS and then co-incubated with endothelial cells, cultured for 24 h in 24 well dishes (2×10^5 cells/well), together with PBS, IFNγ (10 ng/ml) and/or LPS (100 ng/ml), IL-10 (10 ng/ml), or IL-4 (10 ng/ml) + IL-13 (10 ng/ml). Whole cell lysates were collected from endothelial cells in RIPA buffer 48 h later.

Measurement of hepatocyte viability

Hepatocytes were incubated with 0.5 mg/ml 3-(4,5-dimythylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) in serum-free/phenol red-free DMEM medium for 4 h. The medium was then carefully removed and the cells lysed in 0.5 ml/well 100% DMSO for 5 min at room temperature. Absorbance was measured at 550 nm on a Vmax microplate reader (Molecular Devices, Sunnyvale, CA).

Western blotting

Cells were rinsed with ice cold PBS (pH 7.2) and then lysed in RIPA buffer. After 30 min on ice, lysates were centrifuged at 14,000 *g* for 8 min at 4 °C and supernatants collected. Protein concentrations were assayed using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard. Samples were separated on 10–14.5% SDS-polyacrylamide gels and transferred overnight to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Nonspecific binding was blocked by incubation of the membranes for 1 h in 40 mM tris-buffered saline (TBS) (pH 7.5)