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

Yinglin Liu^a, Carol R. Gardner^a, Jeffrey D. Laskin^b, and Debra L. Laskin^{a,*}

^aDepartment of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, 160 Frelinghuysen Rd. Piscataway, NJ 08854

^bEnvironmental and Occupational Medicine, UMDNJ-Robert Wood Johnson Medical School, 170 Frelinghuysen Rd., Piscataway, NJ 08854

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, IFN γ 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 IFN γ 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 IFN γ 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.

Keywords

endothelial cells; Kupffer cells; acetaminophen; iNOS; mannose receptor; liver

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*Corresponding author: Fax: +1 732-445-2534, laskin@eohsi.rutgers.edu (D.L. Laskin).

Conflict of interest statement

The authors declare no conflict of interest.

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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 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 IFN γ , 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-1 β 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 prevents acetaminophen-induced liver injury, while suppressing M2 macrophages exacerbates hepatotoxicity provide evidence that both of these cell populations are important in the response to this liver toxicant (Blazka et al., 1995; Dambach et al., 2002; Dragomir et al., 2012a; Dragomir et al., 2012b; 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 comprised 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 μ m) 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 IFN γ 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 Ca²⁺/Mg²⁺-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 μ g/ml Liberase TM. After 15 min, the liver was excised, disaggregated and filtered through 220 μ m nylon mesh. Hepatocytes were recovered by centrifugation at 50 $\times$ 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 μ m nylon mesh and hepatocytes separated from NPCs by four successive washes (50 $\times$ g, 5 min, 4°C) for 5 min. NPCs were recovered by centrifugation of the supernatant at 300 $\times$ 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 12-18 ml/min and macrophages between 30-44 ml/min. Cells were enriched by differential centrifugation on a 40% OptiPrep™ gradient (400 $\times$ 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 $\times$ 10⁵ 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, IFN γ (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 $\times$ 10⁵ 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 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, IFN γ (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-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 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 14,000g 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 TBS (pH 7.5) containing 0.05% Tween-20 (TBS/Tween) and 5% nonfat dry milk. Membranes were incubated overnight (4°C) with primary antibody in 1% milk-TBS/Tween, washed for 1 h using TBS/Tween, and then incubated for 1 h with secondary antibody (1:5000) in TBS/Tween containing 2.5% nonfat dry milk. Antibody binding was visualized using an ECLTM Chemiluminescent Western Blotting Detection kit (Pierce). For each analysis, 5-7 mg protein/lane was analyzed on the gels. In all experiments, a housekeeping gene (actin) was run to ensure equal protein loading on the gels.

Real time-PCR

Cells were stored in RNA LATER solution (Ambion, Inc., Austin, TX) at -20°C until RNA isolation. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA). RNA purity and concentration were measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA (1 μ g) was converted into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Real time-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT thermocycler. PCR primer pairs were generated using Primer Express 3.0 (Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA). A minimum of three samples were analyzed for each experimental group and all samples were run in duplicate. Gene expression changes were normalized to GAPDH mRNA and expressed as fold change relative to control. Forward and reverse primer sequences used were: iNOS, TGGTGAAAGCGGTGTTCTTTG and ACGCGGAAGCCATGA; arginase-1, CTGCATATCTGCCAAGGACATC and GTTCCCCAGGGTCCACATC; mannose receptor, GCGCCATCTCCGTTTCTCAG and AGCGGAATTTCTGGGATTCA; and GAPDH, AAATGATACCCCACCGTGTGA and GCTGGCACTGCACAAGAAGAT.

Statistical analysis

All experiments were repeated at least three times. Data were analyzed in consultation with the Rutgers University Department of Statistics using two-way ANOVA or a paired t-test. A p-value of < 0.05 was considered statistically significant.

Results

In initial experiments, we determined if primary cultures of rat hepatic sinusoidal endothelial cells undergo phenotypic polarization in response to inflammatory mediators known to induce classical and alternative macrophage activation. For comparison purposes we also analyzed the response of rat Kupffer cells. Incubation of Kupffer cells with the classical macrophage activators IFN γ and LPS, alone or in combination, resulted in increased expression of iNOS protein, with no effect on arginase-1 and decreased expression of mannose receptor (Fig. 1). Similar results were observed in hepatic endothelial cells; however the responses to LPS and LPS + IFN γ were reduced, when compared to Kupffer cells. In endothelial cells, IFN γ alone was also less effective in inducing iNOS expression than LPS. To assess alternative activation, cells were treated with IL-10, or the combination of IL-4 + IL-13. In both endothelial cells and Kupffer cells, IL-10 treatment resulted in increased expression of mannose receptor; a small increase in arginase-1 was also observed in endothelial cells. The combination of IL-4 + IL-13 upregulated expression of arginase-1 in both cell types, while mannose receptor was only upregulated in endothelial cells. In general, endothelial cells were more sensitive to these cytokines than Kupffer cells. Neither IL-10 nor IL-4 + IL-13 altered iNOS protein expression.

We also analyzed mRNA expression for iNOS, arginase-1 and mannose receptor in endothelial cells and Kupffer cells treated with inducers of classical and alternative macrophage activation. Consistent with our findings on iNOS protein, iNOS mRNA expression increased in both liver macrophages and endothelial cells in response to IFN γ and/or LPS, with no major effects on arginase-1 or mannose receptor expression (Fig. 2 and not shown). Additionally, iNOS mRNA expression was not altered by incubation of the cells with IL-10 or the combination of IL-4 + IL-13. In endothelial cells, both IL-10 and IL-4 + IL-13 upregulated arginase-1 and mannose receptor mRNA; in Kupffer cells, IL-10 upregulated mRNA for mannose receptor, while IL-4 + IL-13 upregulated arginase mRNA.

We next determined if cytokine-induced activation of hepatic endothelial cells and macrophages persisted in the absence of inflammatory stimuli. In these experiments the cells were incubated with IFN γ , IL-10, or IL-4 + IL-13 for 48 h, washed, refed with fresh culture medium without cytokines, and incubated for an additional 24 h prior to analysis. We found that removal of IFN γ from the cultures had no effect on expression of iNOS protein by macrophages or endothelial cells (Fig. 3). Whereas in Kupffer cells, removal of IL-10 had no effect on mannose receptor expression, in endothelial cells, expression of this receptor increased. Similar increases in mannose receptor expression were noted in endothelial cells, but not Kupffer cells, after removal of IL-4 + IL-13.

In further experiments, we assessed whether cytokine-induced phenotypic polarization of hepatic endothelial cells and macrophages was reversible. As described above, treatment of both endothelial cells and macrophages with IFN γ for 48 h resulted in increased iNOS expression, while IL-4 + IL-13 treatment upregulated arginase-1 expression (Fig. 4). Subsequent washing and incubation of IFN γ treated cells with IL-4 + IL-13 resulted in increased expression of arginase-1 and decreased expression of iNOS. Conversely, washing and incubation of IL-4 + IL-13 treated cells with IFN γ resulted in increased iNOS expression and decreased arginase-1 expression. In general endothelial cells appeared to be more sensitive to phenotypic switching than Kupffer cells.

In our next series of studies, we assessed the consequences of classical and alternative activation of endothelial cells on hepatocytes. In these experiments, endothelial cells were stimulated with IFN γ and/or LPS, IL-10, or IL-4 + IL-13 for 48 h, washed, and then co-cultured in transwell dishes with hepatocytes. In some experiments hepatocytes were pretreated for 2 h with control or with 5 mM acetaminophen, prior to co-culturing with endothelial cells. Hepatocyte viability was assessed 24 h later. Whereas endothelial cells classically activated with LPS and IFN γ had no effect on control hepatocytes, a 30% decrease in viability was observed in hepatocytes pretreated with acetaminophen (Table 1). In contrast, the viability of control hepatocytes was increased in the presence of endothelial cells alternatively activated with IL-4 + IL-13. In acetaminophen pretreated hepatocytes, however, IL-4 + IL-13 activated endothelial cells caused a 20% decrease in hepatocyte viability. Additionally, IL-10 treated endothelial cells suppressed the viability of both control and acetaminophen-injured hepatocytes.

Since alternatively activated macrophages are known to promote tissue repair, we next analyzed markers of proliferation (PCNA and β -catenin) and extracellular matrix turnover (MMP-9) in hepatocytes. Consistent with this activity, we found that expression of PCNA, β -catenin and MMP-9 increased in hepatocytes co-cultured with IL-4 + IL-13 activated Kupffer cells (Fig. 5). IL-10 treated Kupffer cells also upregulated hepatocyte MMP-9 expression. Hepatocyte PCNA and β -catenin expression also increased in the presence of control Kupffer cells; IFN γ pretreated Kupffer cells upregulated hepatocyte PCNA. Endothelial cells alternatively activated with IL-4 + IL-13, as well as IL-10, were also found to upregulate markers of tissue repair in hepatocytes. Thus, hepatocyte expression of β -catenin and MMP-9 increased in the presence of endothelial cells activated with IL-4 + IL-13 or IL-10 (Fig. 5). IL-10 treated endothelial cells also upregulated PCNA expression in hepatocytes. In contrast to Kupffer cells, however, endothelial cells classically activated with IFN γ had no effect on hepatocyte expression of these markers. Additionally, while control endothelial cells upregulated hepatocyte MMP-9 expression, KC upregulated hepatocyte PCNA and beta-catenin.

In previous studies we demonstrated that injured hepatocytes release factors that induce classical activation of macrophages (Dragomir et al., 2011; Laskin et al., 1986). In further experiments, we determined if hepatocytes exert similar effects on sinusoidal endothelial cells. For these studies, endothelial cells were co-cultured in transwell dishes with hepatocytes pretreated for 2 h with control or 5 mM acetaminophen, in the absence or presence of classical and alternative macrophage activators. Endothelial cell expression of iNOS and mannose receptor was assessed 48 h later. Whereas the addition of LPS alone or in combination with IFN γ to co-cultures containing control hepatocytes resulted in increased expression of iNOS in endothelial cells, IL-10 or IL-4 + IL-13 caused an increase in mannose receptor expression (Fig. 6). The effects of these inflammatory stimuli on endothelial cells were markedly enhanced in co-cultures containing acetaminophen injured hepatocytes. Co-culture of endothelial cells and hepatocytes in the absence of inflammatory stimuli had no effect on endothelial cell expression of iNOS or mannose receptor.

Discussion

Hepatic sinusoidal endothelial cells have been implicated in tissue injury induced by chemical toxicants, ischemia-reperfusion, and in acute and chronic tissue rejection (DeLeve et al., 1997; Ikeda et al., 2009; McCuskey, 2008; Usui et al., 2009). Like Kupffer cells, hepatic endothelial cells respond to inflammatory stimuli and release cytotoxic and proinflammatory mediators, as well as growth factors and mediators important in extracellular matrix turnover (Chen et al., 2007; Feder and Laskin, 1994; Feder et al., 1993; Maher, 1993; McCloskey et al., 1992; Thiele et al., 2005; Wu et al., 2001). The present

studies suggest that these diverse activities of hepatic endothelial cells, like Kupffer cells, are mediated by distinct subpopulations. Thus, in response to IFN γ and/or LPS, hepatic sinusoidal endothelial cells expressed iNOS, a characteristic marker of classically activated macrophages, while IL-10 or IL-4 + IL-13 induced an alternatively activated phenotype, as measured by arginase-1 and/or mannose receptor expression. Moreover, while classically activated endothelial cells enhanced hepatocyte cytotoxicity, alternatively activated cells promoted hepatocyte viability, and upregulated hepatocyte proteins associated with tissue repair. These findings are novel and suggest that multiple sinusoidal cell populations contribute to hepatic inflammatory responses.

LPS and IFN γ are prototypical inducers of classical macrophage activation (Murray and Wynn, 2011). Thus, in response to these inflammatory stimuli, macrophages acquire a proinflammatory M1 phenotype characterized by expression of iNOS mRNA and protein and the release of reactive nitrogen and oxygen species (Sica and Mantovani, 2012). Similarly, we found that hepatic sinusoidal endothelial cells, like Kupffer cells, express iNOS mRNA and protein following stimulation with LPS and/or IFN γ . They also generate reactive nitrogen species and reactive oxygen species in response to these inflammatory stimuli (Feder and Laskin, 1994; McCloskey et al., 1992). For both iNOS expression and RNS production, macrophages were more sensitive to LPS than endothelial cells. This may be due to increased expression of LPS binding proteins and/or TLR4 on macrophages relative to endothelial cells (Aoyama et al., 2010; Uhrig et al., 2005).

Alternatively activated macrophages participate in down regulating inflammatory responses and in inducing tissue repair processes. These activities have been ascribed to M2a and M2c macrophages activated by IL-10 and IL-4 + IL-13, respectively (Martinez et al., 2008). While M2a macrophages express arginase, M2c macrophages express mannose receptor. Similarly, we found that IL-10 upregulated arginase expression in endothelial cells, while IL-4 + IL-13 upregulated mannose receptor expression. These data provide support for the concept that hepatic sinusoidal endothelial cells exhibit functional heterogeneity (Xie et al., 2010; Xie et al., 2012; Yee et al., 2007). Interestingly, low level constitutive mannose receptor expression was noted in both endothelial cells and Kupffer cells cultured for up to 48 h. The mannose receptor belongs to a class of scavenger receptors that are important in uptake of complex carbohydrates (Martinez-Pomares et al., 2006). Constitutive expression of mannose receptor is consistent with the ability of both of these NPC types to endocytize glycoproteins from the portal circulation. We also found that phenotypic activation of both hepatic endothelial cells and Kupffer cells persisted for at least 24 h in the absence of polarizing stimuli. Similar partial persistence of macrophage phenotype has been described in human monocytes differentiated with M-CSF (Porcheray et al., 2005). This may represent a mechanism for maintaining NPC functional activity at sites of inflammation, when activating signals are no longer present. Of note was our observation that mannose receptor expression continued to increase in hepatic endothelial cells in the absence of inducing stimuli. These data suggest that the effects of IL-10 and IL-4 + IL-13 on this protein are persistent.

Macrophages have been described as having a plastic gene expression phenotype that changes depending on the type, concentration, and longevity of exposure to stimulating agents (Murray and Wynn, 2011). In accord with this concept, we found that Kupffer cell phenotypic activation was reversible. Thus, classically activated liver macrophages could be reprogrammed into alternative activated cells, and alternatively activated cells into classically activated macrophages by incubation with IL-10 or IL-4 + IL-13, or LPS, respectively. However, it appears that this reprogramming was not complete, as evidenced by our findings that some iNOS expressing cells were detectable in cultures containing M2 phenotype inducing stimuli. These data are generally consistent with previous findings that

macrophages can be repolarized (Gratchev et al., 2006), and provide support for the idea that macrophage activation represents a continuum of cells at different stages of the activation process. Similar phenotypic repolarization was observed in hepatic sinusoidal endothelial cells demonstrating that these cells also display considerable plasticity with respect to their function that changes as inflammatory signals in their microenvironment change. Interestingly, it appeared that hepatic endothelial cells were more sensitive to phenotypic switching than were Kupffer cells. This may reflect the need for more prolonged macrophage responses, as their activity is key to maintaining inflammatory reactions. Our observation that the activation state of macrophages and endothelial cells can be reprogrammed suggests that both of these cell types participate in the induction and resolution of hepatic inflammatory responses.

Evidence suggests that activated macrophages play a role in the pathogenic response to drugs such as acetaminophen, a widely used analgesic known to induce centrilobular hepatic necrosis following intentional or accidental overdose (Cohen and Khairallah, 1997). Hence, following acetaminophen intoxication, macrophages rapidly accumulate in the liver and release mediators that contribute initially to tissue injury and inflammation, and subsequently to tissue repair (reviewed in Laskin, 2009). The present studies support the idea that these activities are mediated by macrophage subpopulations displaying a classically or alternatively activated phenotype. We further demonstrated that hepatic sinusoidal endothelial cells also have the capacity to develop into subpopulations exhibiting these different phenotypes. Moreover, while endothelial cells classically activated by LPS and IFN γ promote cytotoxicity in acetaminophen-injured hepatocytes, cells alternatively activated by IL-4 + IL-13 enhance the viability of uninjured hepatocytes. This latter activity would be expected to occur in remnant hepatocytes surrounding necrotic tissue, which is central to wound repair following acetaminophen-induced hepatotoxicity (Gieling et al., 2010; Sell, 2001). Surprisingly, alternatively activated endothelial cells were found to suppress the viability of acetaminophen-injured hepatocytes, although not as effectively as classically activated LPS and IFN γ treated cells. It remains to be determined if this reflects a unique contribution of alternatively activated endothelial cell subpopulations to tissue injury. We also found that alternatively activated endothelial cells induce proliferation and modulate extracellular matrix turnover in hepatocytes, as shown by increased expression of PCNA, β -catenin and MMP-9. IL-4, IL-13 and IL-10 have been identified in the liver following acetaminophen intoxication (Dambach et al., 2006; Gardner et al., 2003; Hogaboam et al., 2000). Moreover, transgenic mice lacking these cytokines exhibit increased susceptibility to the hepatotoxic effects of acetaminophen (Bourdi et al., 2007; Bourdi et al., 2002; Ryan et al., 2012; Yee et al., 2007). Our findings are consistent with the suggestion that NPCs play a role in tissue repair processes; however, their contribution appears to depend on their phenotype. IFN γ activated Kupffer cells, but not endothelial cells, also upregulated PCNA expression in hepatocytes. IFN γ has been reported to stimulate macrophage production of TNF α , a potent hepatocyte mitogen (Jia, 2011; Neta et al., 1992). Upregulation of PCNA in hepatocytes co-cultured with IFN γ activated Kupffer cells may be due to the release of TNF α by these cells. Unstimulated Kupffer cells also increased hepatocyte proliferative activity as measured by PCNA and β -catenin, while unstimulated endothelial cells upregulated MMP-9. These data are in accord with earlier studies showing that NPCs contribute to homeostatic maintenance of liver structure and function (Karlmark et al., 2010; McCuskey et al., 2005; Palmes et al., 2005; Ramadori et al., 2008; Tacke et al., 2009; Xie et al., 2012; Xu et al., 2011).

In previous studies we demonstrated that acetaminophen-injured hepatocytes release factors that induce classical activation of Kupffer cells (Dragomir et al., 2011; Laskin et al., 1986). The present studies demonstrate that endothelial cells are also activated by hepatocytes pretreated with acetaminophen, but not with control. Thus, endothelial cell expression of

iNOS in response to LPS or LPS and IFN γ was markedly increased when they were co-cultured with injured, but not control hepatocytes. Endothelial cell expression of mannose receptor in response to IL-10 and IL-4 + IL-13 was also upregulated. These findings demonstrate that endothelial cells are highly responsive to products released from hepatocytes following exposure to toxicants. The nature of the hepatocyte-derived factors that activate endothelial cells is unknown. Hepatocytes injured by acetaminophen have been reported to release damage associated molecular patterns such as HMGB1, which have been implicated in sterile inflammatory responses to tissue injury (Antoine et al., 2012; Antoine et al., 2009; Dragomir et al., 2011; Martin-Murphy et al., 2010). Endothelial cells possess receptors for HMGB1 including receptor for advanced glycation end products and TLR4 (Aoyama et al., 2010; Lohwasser et al., 2009; Uhrig et al., 2005). HMGB1 released by injured hepatocytes may contribute to increased iNOS expression in endothelial cells treated with LPS and IFN γ . The observation that mannose receptor is upregulated in endothelial cells by IL-10 and IL-4 + IL-13 suggests that additional factors are released by hepatocytes that promote an alternatively activated tissue repair phenotype. In this regard, previous studies have demonstrated that intoxication with acetaminophen induces an adaptive response characterized by the generation of proteins important in liver regeneration including TGF β (Chiu et al., 2003; Tygstrup et al., 1996).

The specific function of different endothelial cell subpopulations in nonspecific host defense in the liver is unknown. Data presented in this paper suggest a potential novel contribution of alternatively activated hepatic endothelial cells to down regulating inflammation and inducing wound repair. Further *in vivo* studies are required to elucidate the precise role of these cells, as well as classically activated endothelial cell subpopulations, in hepatic inflammatory responses to tissue injury.

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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

Highlights

- Hepatic endothelial cells can be classically and alternatively activated.
- Endothelial cell activation is persistent and reversible.
- Activated endothelial cells can modulate hepatocyte responses.

Fig. 1.

Classical and alternative activation of Kupffer cells and endothelial cells. Cells, incubated overnight in 12 well dishes (1×10^6 cells/well), were washed and then incubated with PBS, IFN γ (10 ng/ml), LPS (100 ng/ml), IFN γ + LPS, IL-10 (10 ng/ml), or IL-4 (10 ng/ml) + IL-13 (10 ng/ml). After 48 h, cell lysates were prepared and expression of iNOS, arginase-1 (Arg1), and mannose receptor (MR) analyzed by western blotting. One representative gel from 3 separate experiments is shown.

Fig. 2. Expression of mRNA for markers of classical and alternative activation in Kupffer cells and endothelial cells. Cells, incubated overnight in 6 well dishes (2×10^6 cells/well), were washed and then incubated with PBS, IFN γ , IL-10, or IL-4 + IL-13. After 24 h, RNA was extracted and analyzed for expression of iNOS, arginase-1 (Arg1), and mannose receptor (MR). Results are presented relative to GAPDH. Data are the mean $\pm$ S.E. from 3 separate experiments. *Significantly ($p < 0.05$) less than PBS control (Paired t test).

Fig. 3.

Persistence of a polarized phenotype in Kupffer cells and endothelial cells in the absence of inflammatory stimuli. Cells, incubated overnight in 12 well dishes (1×10^6 cells/well), were washed and incubated with PBS, IFN γ , IL-10, or IL-4 + IL-13 for 48 h (left panel). The cells were then washed and refed with fresh medium in the absence of stimuli (right panel). After an additional 24 h incubation, cell lysates were prepared and expression of iNOS and mannose receptor (MR) analyzed by western blotting. One representative gel from 3 separate experiments is shown.

Fig. 4. Reversible activation of Kupffer cells and endothelial cells. Cells, incubated overnight in 6 well dishes (2×10^6 cells/well), were washed and then incubated with PBS, IFN γ or IL-4 + IL-13. After 48 h, the cells were washed and IFN γ treated cells refed with medium containing IL-4 + IL-13, while IL-4 + IL-13 treated cells were refed with medium containing IFN γ . After an additional 24 h incubation, cell lysates were analyzed for iNOS and arginase 1 (Arg1) expression by western blotting. One representative gel from 3 separate experiments is shown.

Fig. 5. Effects of NPCs on hepatocyte expression of markers of proliferation and extracellular matrix turnover. Kupffer cells and endothelial cells, cultured overnight on transwell inserts in 24 well dishes, were washed and incubated with PBS, IFN γ , IL-10 or IL-4 + IL-13. After 48 h, the cells were washed and co-incubated with hepatocytes, cultured overnight on 24 well dishes. Hepatocyte lysates were prepared 24 h later and analyzed for expression of PCNA, β -catenin and MMP-9 by western blotting. One representative gel from 3 separate experiments is shown.

Fig. 6.

Regulation of endothelial cell activation by hepatocytes. Hepatocytes (HC), cultured overnight on transwell inserts in 24 well dishes, were washed and treated with medium control (Control HC) or medium containing 5 mM APAP (Injured HC). After 2 h, hepatocytes were washed and co-cultured with endothelial cells incubated for 24 h on 24 well dishes, together with PBS, IFN γ , LPS, IFN γ + LPS, IL-10, or IL-4 + IL-13. Endothelial cell expression of iNOS and mannose receptor (MR) were analyzed by western blotting 48 h later. One representative gel from 3 separate experiments is shown.

Table 1

Effects of classically and alternatively activated endothelial cells on hepatocyte viability

Endothelial cell treatment	Hepatocyte Viability (%)	
	Control HC	APAP-treated HC
Control	100 ± 1.2	95.8 ± 1.8
IFN γ	98.3 ± 0.1	94.0 ± 0.1
LPS	88.3 ± 0.6	93.3 ± 1.1 ^b
IFN γ + LPS	95.3 ± 0.3	70.9 ± 1.4 ^{a,b}
IL-10	84.9 ± 0.8 ^a	73.2 ± 0.3 ^{a,b}
IL-4 + IL-13	119.5 ± 1.2 ^a	82.7 ± 0.3 ^{a,b}

Endothelial cells were treated with IFN γ and/or LPS, IL-10, IL-4 + IL-13 or control for 48 h and then co-cultured in transwell dishes with control hepatocytes (HC) or HC pretreated for 2 h with 5 mM acetaminophen (APAP). Hepatocyte viability was assessed 24 h later using an MTT assay. Data are presented as % control; each value is the mean $\pm$ SE (n = 3-5).

^aSignificantly different (p < 0.05) from control endothelial cells;

^bSignificantly different (p < 0.05) from control HC (two-way ANOVA).