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LPS-mediated NFκB activation varies between activated human hepatic stellate cells from different donors

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Abstract

The activation of hepatic stellate cells (HSC) is recognized as the key event of hepatic fibrosis [Virchows Arch. 430 (1997) 195; Semin. Liver Dis. 21 (2001) 437; Front. Biosci. 7 (2002) d808]. NFκB has been associated with the development of the activated phenotype, the expression of proinflammatory genes, and with promoting survival of activated HSC. High levels of circulating endotoxin are observed in liver fibrosis and several lines of evidence indicate that LPS plays an important role in chronic liver disease. Here, we investigated the LPS-induced NFκB activation in activated HSC from different human donors. HSC were isolated from liver specimens obtained during surgical liver resection and were activated by culturing on plastic. LPS-induced NFκB activity and IL-8 expression revealed a significant correlation but differed significantly comparing HSC from individual donors. These variations seen in LPS mediated NFκB activation and chemokine secretion between HSC from different donors in vitro may contribute to differences seen in vivo between patients in the progression of fibrosis and the degree of inflammation during chronic liver disease.

Keywords: Heptic stellate cells; Endotoxin; LPS; NFκB; Chemokines; IL-8

Hepatic fibrosis represents the common end point of the majority of chronic liver injuries [2,3]. Well-characterized causal agents include chronic hepatitis C (HCV) infection and alcohol abuse. However, a broad spectrum of response to the same etiologic agent is seen in different individuals. For example, large-scale studies have allowed the identification of HCV-patients with rapid fibrosis progression and those with slow fibrosis progression per unit time [4].

Fibrosis can be viewed as a disease in which multiple genes and gene products interact with environmental factors [2]. Furthermore, genetic variability in factors influencing fibrogenesis and inflammation may be responsible for some of the variation in disease progression as seen in patients with chronic HCV infection [5,6] and alcoholic liver disease (ALD) [7].

More and more research is directed towards identifying and characterizing the interfaces of the cross-interactions between primary intrinsic factors and secondary risk factors to help understand individual predisposition to liver disease. Cell type-specific research helps to elucidate specific contributions not only of hepatocytes, but also of non-parenchymal cells to sensitizing and priming mechanisms.

There is a wealth of evidence indicating that hepatic stellate cells (HSC) represent the pivot in hepatic fibrosis. Following hepatic injury, HSC undergo an activation process and transform to an activated,

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myofibroblast-like phenotype. They start to proliferate and are responsible for the excessive hepatic matrix deposition during the fibrotic process. The hepatic matrix accumulation is the result of an overall increase in the number of HSC in addition to changes in the HSC gene expression [1–3].

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of gram-negative bacteria [8]. The gram-negative flora of the intestine is a substantial reservoir of endogenous LPS and serum LPS levels are significantly elevated in patients with chronic hepatitis and cirrhosis [9,10]. Several factors promote endotoxemia in this setting, including increased translocation of endotoxin from the gut lumen and a reduction in hepatic clearance capacity [11]. Both, clinical and experimental data, indicate that activation of Kupffer cells and HSC by gut derived endotoxin is an important mediator in liver injury [7,12].

Using a well-established model of HSC isolation and activation in culture and activated HSC from cirrhotic liver, Paik et al. [12] have shown that HSC activation is associated with an upregulation of TLR-4, CD-14, and MD-2 expression. It is known that TLR-4, in association with CD-14 and MD-2, is responsible for LPS-induced signal transduction leading to NFκB activation [8]. Consequently, they could demonstrate LPS-induced NFκB activation in activated HSC.

NFκB is a ubiquitous transcription factor that governs the expression of a wide array of proinflammatory and the immune system modulating genes [13]. The activation of NFκB has been associated with the development of the activated phenotype in the HSC and with promoting survival of activated HSC [14–19].

Complex interactions among primary mechanistic factors and between primary and secondary factors appear to be the basis for the heterogeneous response that patients exhibit for liver injury [7]. In this study, to elucidate parts of these complicated and interactive mechanisms in vivo, we investigated the LPS-induced NFκB activation in culture-activated HSC from different human donors in vitro. Interestingly, HSC from individual donors revealed notable differences in LPS-induced NFκB activity and IL-8 expression.

These findings may contribute to our understanding of the mechanism by which absorbed bacterial cell wall products can mediate hepatic inflammation and fibrosis, and may provide insights into differences seen in the clinical course of chronic liver disease between individuals.

Methods

Reagents. LPS from *Escherichia coli* (serotype 055:B5; phenol extracted and then chromatographically purified by gel filtration, protein concentration <1%) was purchased from Sigma (Steinheim, Germany).

Isolation and stimulation of activated hepatic stellate cells. Tissue samples from human liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the local Ethics Committee, University Hospital of Regensburg, and the charitable state-controlled foundation Human Tissue and Cell Research (HTCR), with informed patient's consent.

Only those liver tissues judged as non-cancerous by local pathologists were used for cell preparation. Further exclusion criteria were known liver disease or histological evidence for liver fibrosis or inflammation in surrounding non-tumorous liver tissue.

Cells were isolated using a modified two-step EGTA/collagenase perfusion procedure and sequential incubation of the cell suspension with pronase as described previously [6,20,21]. HSC were separated from other non-parenchymal liver cells by arabinogalactan gradient ultracentrifugation, yielding HSC that were more than 90% pure and viable, as determined by phase contrast- and UV-excited fluorescence microscopy, and Trypan blue exclusion, respectively. HSC were seeded on uncoated plastic culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μg/ml streptomycin in a 90% air–10% CO_2-humidified atmosphere. Growth medium was changed on a daily basis for the first 4 days in culture, then every second day thereafter.

Throughout the study, cell-culture activated HSC between week 4 and 6 of cell culture (reflecting approximately cell-passages 4 to 5) were used for the different in vitro experiments to ensure comparable conditions.

To study the effects of cell-isolation and cell-culture conditions on the parameters investigated, human HSC were isolated from two different pieces of liver tissue obtained during partial hepatectomy from the same patients. While isolating HSC from the first piece of tissue, the second piece of tissue was stored on ice. After approximately 1 h, HSC were also isolated from the second piece of liver tissue. Subsequently, also cell culture of HSC from both preparations was performed separately. After 4 weeks of cell culture, HSC of both preparations were seeded in parallel for subsequent experiments.

IL-8 ELISA. After 24 h stimulation, supernatants were collected, centrifuged to remove cellular debris and IL-8 concentration was analyzed by a sandwich enzyme-linked immunosorbent assay (ELISA) following the instructors manual (Biosource, Camarillo, USA).

Nuclear extracts. Activated HSC were stimulated with varying LPS concentrations (0.1–1000 ng/ml) for different time intervals (30 min to 8 h). Nuclear extracts were prepared as previously described [14,22]. Complete Mini tablets (Roche, Mannheim, Germany) were used as protease inhibitors.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts (5 μg) were incubated with a radiolabeled double-stranded oligonucleotide containing class I MHC κB binding site (GGCTGGGATT CCCCATCT), separated by electrophoresis, and analyzed by autoradiography as described previously [23]. For supershift analysis and competition assays, nuclear extracts were preincubated with antibodies to p50 and p65 (Santa Cruz) or 100-fold excess of unlabeled oligonucleotides.

Quantification of activated nuclear NFκB concentration. Activated NFκB was quantified in nuclear extracts with the ELISA based kit TransAm from Active Motif (Rixensart, Belgium) according to the manufacturer's instructions, as described previously [6,24]. ELISA-plates are coated with oligonucleotide (5′-GGGACTTTCC-3′) coding for an NFκB consensus site. Plates were preincubated with a binding buffer containing DTT and hering sperm DNA. Ten micrograms of nuclear extracts, solved in 20 μl lysis buffer containing DTT and protease inhibitors, was added per well and incubated at room temperature for 1 h. After a washing step an anti-p65 antibody was added and incubated for another hour at room temperature. After an additional washing step an HRP-conjugated secondary antibody was added, followed by 1 h further incubation at room temperature. After the last

washing step developing solution was added and the absorption was measured at 450 nm.

Statistical analysis. Results are expressed as means ± SD (range). Correlation between parameters was calculated with the Spearman test. A p value <0.05 was considered statistically significant. Computations were performed by using the SPSS-10 for Windows statistical computer package (SSPS, Chicago, IL).

Results

Time and dose dependence of LPS-induced NFκB activation in in vitro activated human HSC

Initially, we investigated the effect of different LPS doses and stimulation intervals on the NFκB activity of culture-activated human HSC. For this, the concentration of activated NFκB was analyzed in nuclear extracts using a new ELISA based technique [24]. We found a dose dependent increase of the nuclear concentration of activated NFκB in activated HSC after stimulation with LPS (Fig. 1A). Maximum of NFκB activity was seen at a LPS concentration of 100 ng/ml. These data were in accordance with a previous study investigating NFκB transcriptional activity in activated human HSC using NFκB-driven reporter assays [12].

Furthermore, we investigated the time-course of LPS-induced NFκB activation revealing a maximum of induction 1 h after stimulation (Fig. 1B). Again, these

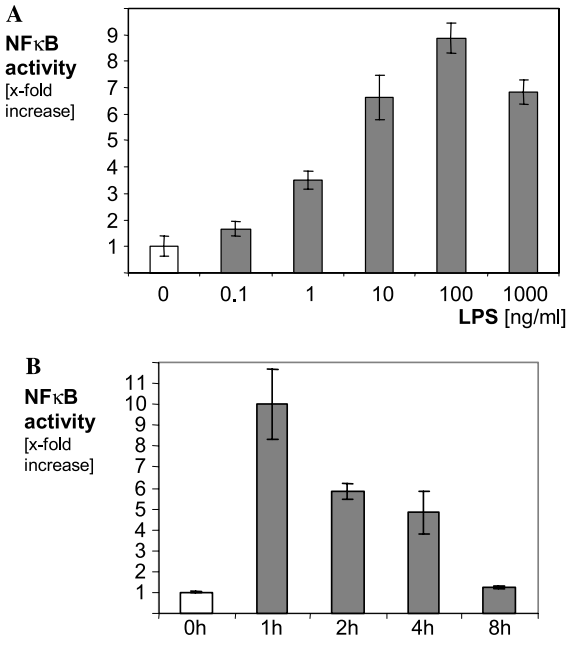

Fig. 1. Dose–response and time course of LPS-induced NFκB activation in culture-activated HSC. (A) HSC were stimulated with the indicated LPS concentrations for 1 h and (B) HSC were stimulated with 100 ng/ml LPS for different time intervals (0–8 h). Nuclear concentration of activated NFκB was analyzed by ELISA. Data represent means of three independent experiments and are expressed as fold increase over unstimulated cells.

data were in accordance with a previous study investigating p65 translocation in response to LPS stimulation in activated HSC [12].

Therefore, for the subsequent experiments a LPS dose of 100 ng/ml and an incubation period of 1 h were used.

Cell culture conditions do not influence LPS-induced NFκB activation in in vitro activated human HSC

Recently, Paik et al. reported de novo expression of components of the LPS receptor complex during the activation process of human HSC. Furthermore, the authors could demonstrate LPS-induced NFκB activation and chemokine expression in culture-activated human HSC, using HSC from cell-passages 3 to 9 [12]. Already previously it was shown that only fully activated HSC respond directly to LPS with the release of chemokines in the rat [25].

To investigate the effect of the duration of cell culture on the LPS-induced NFκB activation, we analyzed the concentration of activated NFκB in nuclear extracts of activated human HSC after 3, 5, and 7 weeks of cell culture, respectively. During that cell-culture interval (reflecting approximately cell-passages 3 to 6), basal as well as LPS-induced NFκB activity did not show significant differences between activated HSC from the same donor (Fig. 2A). Throughout this study, we used in vitro activated human HSC between week 4 and 6 of cell culture (reflecting approximately cell-passages 4 to 5).

Next, we wanted to analyze the influence of cell-isolation or cell-culture conditions on the LPS-induced NFκB activity in culture-activated human HSC. Therefore, we performed the cell isolation from two different pieces of liver tissue from the same donor separately. Furthermore, also cell culture was performed using separate incubators and separately prepared cell-culture media. Comparison of those separately isolated and cultured HSC from the same donor revealed only minimal differences in the basal as well as in the LPS-induced NFκB activity (Fig. 2B).

LPS-induced NFκB activation varies between in vitro activated human HSC from different donors

Based on these observations, we investigated the effects of LPS on NFκB activation in culture-activated HSC from nine different human donors (Fig. 3A). Notably, we found strong variation in the LPS-induced NFκB activation, ranging from no or only minimal effects (donor 2, 6, 7, and 9) to an up to 7-fold activation (donor 3).

To further confirm the interaction of NFκB with gene promoter elements, we analyzed nuclear extracts of activated HSC from six different donors with EMSA. As

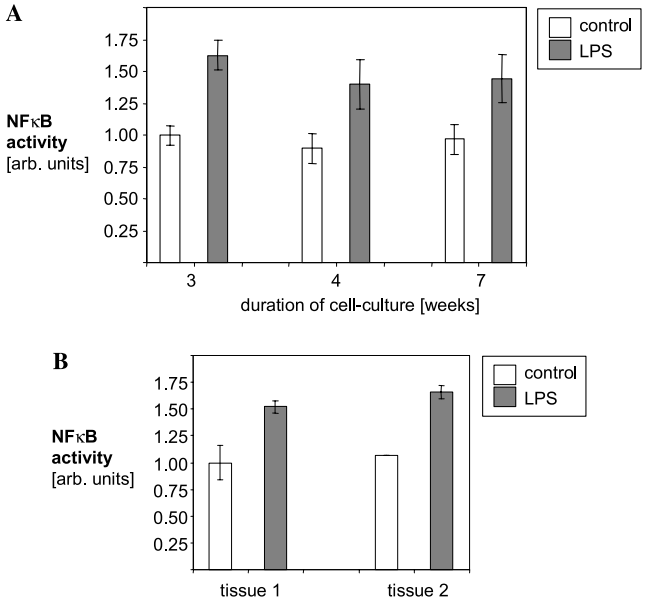

Fig. 2. Cell-culture conditions and cell-culture duration had only minimal effects on the basal and LPS-induced NFκB activity of culture-activated HSC. (A) After different duration of cell culture (3, 4, and 7 weeks, respectively) HSC were stimulated with LPS [100 ng/ml] for 1 h. (B) Human HSC were isolated subsequently from two different pieces of liver tissue of the same donor. Afterwards, HSC from the two pieces of tissue were cultured separately. After 4 weeks of cell culture, HSC were seeded in parallel and stimulated with LPS [100 ng/ml] for 1 h. Nuclear concentration of activated NFκB was analyzed by ELISA. Data represent means of three independent experiments and are expressed as fold increase over unstimulated HSC cultured for 3 weeks (A) or isolated from the first piece of liver tissue (B), respectively.

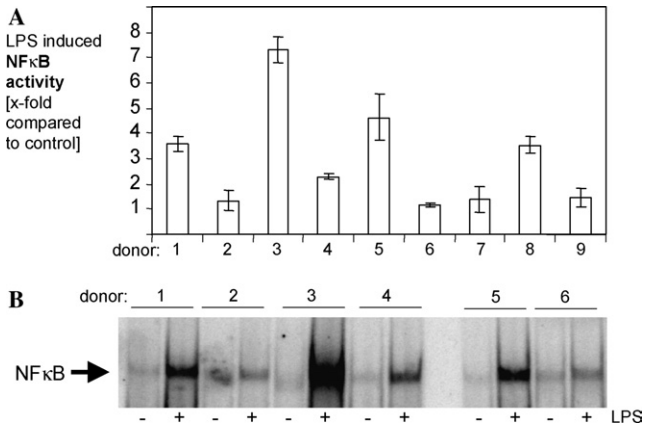

Fig. 3. LPS-induced NFκB activation varies between in vitro activated human HSC from different donors. (A) Culture-activated human HSC from nine different donors were stimulated with LPS [100 ng/ml] for 1 h. Nuclear concentration of activated NFκB was analyzed by ELISA. Data represent means of three independent experiments and are expressed as fold increase over unstimulated cells. (B) NFκB binding activity was determined by EMSA using six of the nine nuclear extracts also used for the NFκB-ELISA.

seen by determining the nuclear concentrations of activated NFκB, also NFκB binding activity showed notable differences between activated HSC from individual donors (Fig. 3B). In accordance, quantification of the EMSA binding activity using phosphoimager technology revealed a significant correlation between NFκB binding activity and the nuclear concentration of activated NFκB as determined by ELISA ($r = 0.95$; $p = 0.004$).

A complete supershift of the binding complex was obtained applying anti-p50 and anti-p65 antibodies, confirming a previous study that identified the p50/p65 heterodimer as the main NFκB binding complex in LPS stimulated activated human HSC (data not shown).

LPS-induced IL-8 expression varies between in vitro activated HSC from different donors

To investigate the functional relevance of the heterogeneous NFκB activation in response to LPS, we additionally analyzed IL-8 secretion of activated HSC from the nine different human donors.

IL-8 expression is regulated by NFκB and it has been shown previously that IL-8 expression is induced in activated HSC upon stimulation with cytokines or LPS [6,12,26].

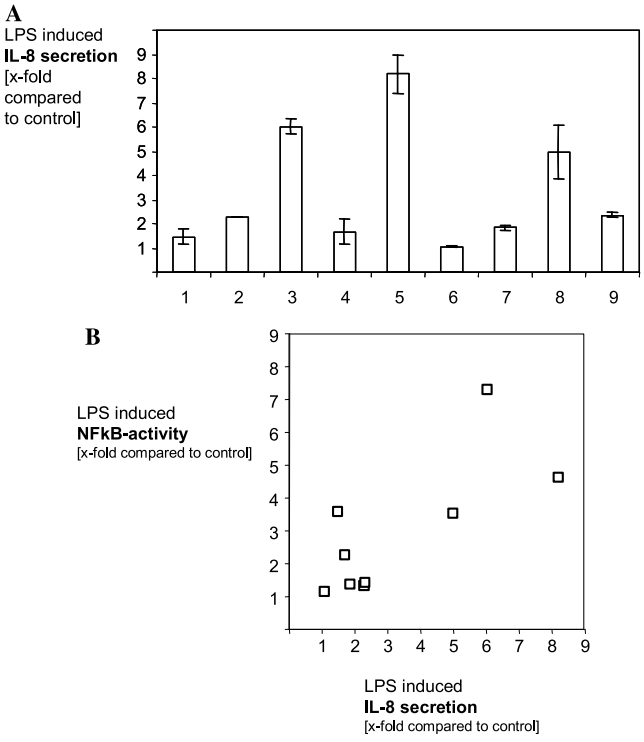

Fig. 4. LPS-induced IL-8 expression varies between in vitro activated HSC from different donors. (A) Culture-activated human HSC from nine different donors were stimulated with LPS [100 ng/ml] for 24 h. IL-8 secretion into the supernatant was analyzed by ELISA. Data represent means of three independent experiments and are expressed as fold increase over unstimulated cells. (B) LPS-induced NFκB activation and LPS-induced IL-8 secretion correlated significantly in culture-activated human HSC from nine different donors ($r = 0.75$; $p = 0.02$).

Notably, we found strong variation in the LPS-induced IL-8 secretion ranging from only minimal induction (donor 1 and 6) to an up to 8-fold induction (donor 5) (Fig. 4A).

Interestingly, we found a significant correlation between the LPS-induced NFκB activation and the LPS-induced IL-8 secretion in culture-activated human HSC from those nine different donors ($r = 0.75$; $p = 0.02$) (Fig. 4B).

Discussion

The aim of this study was to elucidate part of the molecular mechanisms responsible for the variations seen in the clinical course of chronic liver disease.

We focused on the role of LPS in the activation of the transcription factor NFκB in activated HSC from different human donors.

The rationale for this study was that activated HSC in addition to Kupffer cells were identified as a target for LPS induced liver injury, which provides a direct link between inflammatory and fibrotic liver injury. The activation of HSC is recognized as the key event of hepatic fibrosis [1–3] and several lines of evidence indicate that LPS plays an important role in chronic liver disease [7,9–12]. Moreover, a recent study revealed that LPS is capable to induce NFκB activity in activated HSC [12]. The transcription factor NFκB has been associated with the development of the activated phenotype in HSC, the de novo expression of several pathophysiologically relevant proinflammatory genes, and with promoting survival of activated HSC [14–19].

The study was based on the hypothesis that (1) the LPS effects on culture-activated HSC in vitro reflect the LPS effects in vivo on HSC activated in response to liver injury, and (2) the in vitro comparison of culture-activated human HSC from different donors allows the simulation of individual differences occurring in vivo.

The in vitro activation of activated HSC by cell culture on plastic is a well-defined and established cell culture model [1–3]. Moreover, de novo expression of compounds of the LPS receptor complex, LPS mediated NFκB activation, proinflammatory gene expression were reported in both, culture-activated human HSC and activated HSC isolated from diseased human livers [12]. This confirms that the in vitro model is suitable to simulate LPS mediated effects on activated HSC in vivo.

Several studies performed with skin and synovial fibroblasts from different donors reported variations such as in proliferation or chemotaxis in vitro that correlated with donor characteristics and also functionally with wound repair mechanisms in vivo [27–29]. Also cultured primary hepatocytes and liver slices from different human donors have been compared in in vitro experiments [30,31]. Mostly, these studies investigated drug metabolism and transport, and revealed significant differences between cells from different donors [30,31]. However, LPS mediated or other molecular mechanisms have not been compared so far in culture or in vivo activated human HSC from different donors.

Importantly, as a requirement to identify donor related differences in culture-activated HSC cell-isolation and cell-culture were performed under strictly standardized conditions. Furthermore, experiments were performed with activated HSC at relatively early culture-periods (or passages, respectively) compared to most other studies reported. Under this condition, LPS-induced NFκB activation in activated HSC from the same donors was found to be similar over a time range of several weeks of cell culture, excluding the possibility that the relatively minimal differences in cell-culture age caused the notable, phenotypical differences seen between the HSC from individual donors. Moreover, no differences were found in LPS-induced NFκB activation between separately isolated and cultured HSC from the same donor. These findings additionally confirmed that cell-isolation and cell-culture conditions were standardized and reproducible, and were not responsible for the variations seen in LPS-induced NFκB activity between culture-activated HSC from different donors.

Based on the dose–response curve (Fig. 1) and the previous study by Paik et al. [12], an LPS dose of 100 ng/ml was used for the stimulation experiments. It cannot be excluded that activated HSC from individual donors with only minimal NFκB activation under this experimental conditions might have revealed a stronger response applying higher endotoxin concentrations. Due to the limited number of cells we did not address this question systematically but repeated the dose–response curve only three times revealing similar results (data not shown). However, even if higher LPS doses might have induced NFκB activation in certain cases, differences between HSC from individual donors in the susceptibility to LPS would have remained.

The phenotypic differences in LPS mediated NFκB activation correlated functionally with the LPS-induced IL-8 secretion of activated human HSC. It was shown previously that the LPS mediated IL-8 expression is regulated via NFκB [32]. Activated HSC promote hepatic inflammation by production of potent neutrophil chemoattractants such as cytokine-induced neutrophil chemoattractant, the rat homolog to human IL-8 [6,12,26].

IL-8 expression is of particular interest in the context of alcohol-induced hepatitis, which is characterized by a marked neutrophil infiltration and is associated with a poor prognosis and a rapid progression to cirrhosis [33]. Furthermore, serum and hepatic levels of IL-8 correlate with the severity of viral hepatitis and cirrhosis [34].

These findings highlight a potential mechanism whereby high levels of circulating endotoxin observed in cirrhosis might result in perpetuation of an inflammatory response mediated via HSC chemokine secretion. The observed phenotypic variations might contribute to differences seen in the clinical course of liver disease.

In addition, the heterogeneous LPS effects on NFκB activation in activated HSC may have further implications for the progression of chronic liver disease as well as for the resolution of hepatic fibrosis. Several studies have recently shown that apoptosis of activated HSC is a major mechanism mediating recovery from fibrosis [19]. In parallel, it is becoming increasingly recognized that NFκB activation is associated with enhanced survival and resistance to the effects of survival factor withdrawal in HSC [19,35]. The enhanced activation of NFκB occurring as a result of endotoxin stimulation might, therefore, promote HSC survival even after withdrawal of the initial stimulus and potentially perpetuate the hepatic fibrotic response. Variations seen between HSC from different donors in vitro might indicate one potential mechanism responsible for the heterogeneous response that patients exhibit for hepatic injuries as well as during the resolution of hepatic fibrosis after withdrawal of the causing agent or during antifibrotic therapies [36].

It is intriguing to speculate that phenotypical differences seen in vitro between HSC from different human donors might reflect part of the in vivo situation that would occur in those individuals in response to chronic liver injury. Ultimately, this hypothesis could only be proven by comparison of in vitro studies with human HSC and clinical or histological data of the clinical course of liver injuries of the individual cell donors. However, in our study HSC donors neither had nor developed chronic liver disease, a situation probably similar as in most other centers.

As an alternative approach, further studies may address the question which genetic and other host factors are responsible for the differences between HSC from individual donors observed in vitro. Subsequently, those factors have to be evaluated in large cohorts of patients with chronic liver disease to verify the data obtained in the simplified in vitro systems.

In summary, our data further support the hypothesis that HSC are potential mediators of LPS-induced liver injury. Enhanced endotoxin levels found in chronic liver injury may increase and perpetuate the fibrotic process by promoting the survival of HSC. Among the multifactorial nature and complex interactions between primary mechanistic factors and secondary factors that appear to be the basis for the heterogeneous response that patients exhibit in response to liver injury, phenotypic differences between HSC in LPS-induced NFκB activation may contribute to variations seen in the clinical course of chronic liver disease.

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