Loss of NF-κB activation in Kupffer cell-depleted mice impairs liver regeneration after partial hepatectomy

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Short title: liver regeneration and Kupffer cells

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Abstract

Kupffer cells (KCs) are located in the liver sinusoids adjacent to hepatocytes and are capable of producing important growth-regulating mediators which exert both stimulatory and inhibitory influences on hepatocyte proliferation by paracrine mechanisms. To elucidate the overall effect of KC-depletion on liver regeneration, mice were selectively and long-standing depleted of KCs by liposome-encapsulated dichloromethylene diphosphonate. Using in vivo fluorescence microscopy, immunohistochemistry, Western blot analysis, NF-κB transcription factor DNA binding activity and cytokine assays, livers of KC-depleted and KC-competent mice were analyzed at day 3, 5 and 8 after partial, i.e. 68% hepatectomy (PH). Selective KC elimination delayed cell proliferation, as given by significantly reduced PCNA and cyclin B1 protein expression in liver tissue at day 3 post PH. This was associated with a lower liver weight at day 8 upon PH. Resection-associated activation of NF-κB with translocation into parenchymal and nonparenchymal cell nuclei was diminished in livers of KC-depleted mice, primarily at day 3 after PH. KC-depleted mice further lacked the resection-induced rise in TNF-α and IL-6 serum concentrations. These findings imply that KCs play a stimulatory role in liver regeneration, mainly by activating NF-κB with influence on the cell cycle, and by enhancing expression of the proliferative cytokines TNF-α and IL-6.

Keywords: in vivo fluorescence microscopy, hepatocytes, clodronate, tumor necrosis factor alpha, interleukin-6, proliferating cell nuclear antigen
Introduction

Liver regeneration is a complex and evolutionarily conserved process that involves paracrine interactions of numerous hepatic cell types. KCs are the liver macrophages predominantly located in the lumen of hepatic sinusoids, where they mainly clear particulate and foreign materials from the portal circulation (28). Because KCs are known to produce a variety of growth- and immuno-modulating mediators which have stimulatory and inhibitory effects on liver regeneration, they have been postulated to play a key role in liver regeneration (35). However, their function in liver regeneration after PH and the underlying mechanisms are not fully understood (12).

Macrophage depletion constitutes a widely accepted and useful approach to elucidate their functional aspects in vivo. Liver tissue macrophages can selectively be depleted by intravenous administration of liposome-encapsulated dichlormethylene diphosphonate (Cl2MDP) without activation or damage to other liver cells (36, 37). So far, there are only a limited number of studies available using this KC-depleting approach to clarify their role in liver regeneration (7, 26, 35). While Boulton et al. noted an augmentation of the early phase of liver regeneration following KC-depletion (7), it has also been reported that KC-depletion exerts an inhibitory effect on liver regeneration by alteration of hepatic cytokine expression (26, 35). Most recently, KCs have been shown to express NF-κB (30, 42) which in turn is advocated to be a critical effector of the cascade initiating the regenerative process (23). Experiments in hepatectomized animals have shown that inhibition of NF-κB results in massive hepatocyte apoptosis and decreased mitosis with impairment of liver function (19).

The so far contradictory data on KCs in liver regeneration prompted us to precisely study the process of liver regeneration in KC-depleted animals with the emphasis on NF-κB activation and its downstream signaling.

Materials and Methods

Liposome preparation and administration. Cl2MDP was kindly provided as gift from Roche Diagnostics GmbH (Mannheim, Germany). Cl2MDP encapsulated in multilamellar liposomes (400nm) was prepared using the method described by Van Rooijen & Sanders (36) with minor modifications. Inactive PBS-containing liposomes were prepared under identical conditions at the same time. All liposome preparations were stored under nitrogen gas and used within 14 days.

Experimental groups and protocol. For depletion of KCs, mice were applied Cl2MDP-liposomes (0.1ml/10g body weight (bw)) via the tail vein under light ether anaesthesia at 24h prior
to PH and at every 72h after PH. Control animals received equivalent volumes of PBS-liposomes (0.1ml/10g bw). In KC-depleted and KC-competent mice (controls), livers were analyzed at day 3, 5 and 8 after PH (n=6 animals per group and time point) as well as in non-resected animals (day 0; n=6 animals per group and time point).

**Liver regeneration model.** Upon approval by the local government, all experiments were performed in accordance with the German legislation on protection of animals and the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animal Resources, National Research Council; NIH publication 86-23 revised 1985). Female C57BL/6J mice (10-12 week old, Charles River Laboratories, Sulzfeld, Germany) were anesthetized by breathing isoflurane (1.5 vol%) and subjected to a 68% PH (13, 17). The animals were allowed to recover from anesthesia and surgery under a red warming lamp and were held in single cages until the subsequent experiments followed at postoperative days 3, 5 and 8 (n=6 animals per time point). Sham-operated animals without hepatic resection served as group day 0.

**In vivo fluorescence microscopy for analysis of KC phagocytic activity.** Under ketamine/xylazine anesthesia (90/25mg/kg bw ip) sham-treated controls and hepatectomized animals were subjected to intravital fluorescence microscopy, as previously described in detail by our group (1). Animals were placed in supine position on a heating pad and underwent insertion of a polyethylene catheter (PE 50, ID 0.58 mm, Portex, Hythe, UK) in the right carotid artery. After laparotomy and exteriorization of the left liver lobe, KC phagocytic activity was assessed by intra-arterial injection of plain fluorescent latex particles (3x10^6 beads/10g bw in isotonic saline; diameter 1.1 μm; Polysciences Inc., Warrington, PA) (38). For assessment of kinetics of particle adherence, 20-25 selected observation areas per experiment were videotaped successively within 10 min after injection using blue light epi-illumination (excitation/emission wavelength: 450-490nm/>520nm) and a water immersion objective (x20/0.50W, Zeiss). The kinetics of adherence was quantified off-line by counting the number of particles moving in sinusoids as percentage of all particles visible in the area during observation for 10 s (38). After the microscopy, liver tissue was sampled for subsequent histochemical and molecular biology analysis (n=6 animals per time point and group).

**Regeneration study.** Following in vivo microscopy animals were sacrificed and the remnant livers were harvested, weighed and processed for subsequent analysis. The weight of regenerated liver was used to calculate the growth of residual liver lobes according to weight of regenerated liver/preoperative liver weight × 100 (%). Preoperative liver weight was assumed as 4.3% of body weight.
Being aware that liver weight is influenced by various extrinsic and intrinsic factors that are often unrelated to hepatic regeneration, we additionally used 5-bromo-2-deoxyuridine (BrdU) incorporation to study DNA synthesis upon liver resection by immunohistochemistry. For this purpose, additionally pretreated mice were given BrdU (50mg/kg bw ip) 1h prior to harvest of liver tissue (n=4 animals per group and time point) (4, 5, 13).

**Western blot analysis of liver tissue.** For Western blot analysis of PCNA, cyclin B1 and NF-κB, liver tissue was homogenized in lysis buffer (1M Tris pH 7.5, 5M NaCl, 250mM EDTA, 10% Triton-X 100, 4% NaN₃, and 100mM PMSF), incubated for 30 min on ice, and centrifuged for 15 min at 10,000xg. Prior to use, all buffers received a protease inhibitor cocktail (1:100 vol/vol; Sigma). Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard. Equal amounts of whole protein extracts (PCNA: 20μg; cyclin B1 and NF-κB65: 60μg) were separated discontinuously on sodium dodecyl sulphate polyacrylamide gels (12% SDS-PAGE), and transferred to a polyvinylidifluoride membrane (Immobilon-P transfer membrane; Millipore, Billerica, MA, USA). After blockade of non-specific binding sites, membranes were incubated for 2h at room temperature with monoclonal rabbit anti-PCNA (1:2,000; Santa Cruz Biotechnology, CA, USA), polyclonal rabbit anti-cyclin B1 (1:1,000; Santa Cruz) and polyclonal goat anti-NF-κB (1:500, Santa Cruz) followed by peroxidase-conjugated goat anti-rabbit IgG antibody (PCNA: 1:8,000, cyclin B1: 1:10,000; Cell Signaling Technology, Frankfurt, Germany) or rabbit anti-goat IgG antibody (NF-κB65: 1:40,000; Santa Cruz) as secondary antibodies. Protein expression was visualized by means of luminol enhanced chemiluminescence (ECL plus; Amershamp Pharmacia Biotech, Freiburg, Germany) and exposure of the membrane to a blue light sensitive autoradiography film (Kodak BioMax Light Film, Kodak-Industrie, Chalon-sur-Saone, France). Signals were densitometrically assessed (Quantity One, Gel Doc XR, Bio-Rad Laboratories GmbH, Munich, Germany) and normalized to the β-actin signals (monoclonal mouse anti-β-actin antibody, 1:20,000; Sigma Aldrich, Saint Louis, USA) or β-tubulin signals (polyclonal rabbit anti-β-tubulin antibody, 1:500; Santa Cruz).

**Immunohistochemistry.** Liver tissue of animals at day 0, 3, 5 and 8 after resection was excised, fixed in 4% phosphate buffered formalin for 2-3 days and then embedded in paraffin. From the paraffin-embedded tissue block, 5μm sections were cut.

The degree of KC-depletion was analyzed immunohistochemically by the F4/80 antigen (1:10, Serotec, Oxford, UK), a marker for resident tissue macrophages (35). Over night incubation (4°C) with the first antibody (polyclonal rat anti-F4/80) was followed by alkaline-phosphatase (AP)
conjugated mouse anti-rat immunoglobulin (1:200, Santa Cruz). The sites of AP-binding were detected by fuchsia (Dako Cytomation, Hamburg, Germany).

For the demonstration of DNA-incorporated BrdU in liver cells, sections collected on poly-L-lysine-coated glass slides were incubated with monoclonal mouse anti-BrdU antibody (1:50; Dako) over night at 4°C followed by horseradish-peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (LSAB kit plus; Dako). The sections were counterstained with hemalaun. The sites of peroxidase-binding were detected by 3,3'-diaminobenzidine. BrdU-positive hepatocellular nuclei were counted within 50 consecutive high power fields (HPF) (x40 objective, numerical aperture 0.65) and are given as cells/HPF (13). In analogy, BrdU-expressing non-parenchymal cells were assessed and also given as cells/HPF.

The detection of active NF-κB was performed using a polyclonal goat antibody directed against the epitope mapping within the amino terminal domain of NF-κBp65 (1:100, Santa Cruz) over night at 4°C, followed by HRP-conjugated rabbit anti-goat immunoglobulin (LSAB kit plus; Dako). Cell-specific expression of NF-κBp65 was analyzed by counting NF-κBp65-positive hepatocytes and non-parenchymal cells within 50 HPF with discrimination of the intracellular distribution serving as an indicator of NF-κB nuclear translocation. The nuclear-to-cytoplasmic ratio of parenchymal and non-parenchymal cells is calculated by dividing the number of cells with nuclear NF-κBp65-positivity by the number of cytoplasmic NF-κBp65 positive cells.

**Bioplex protein array system.** A panel of serum cytokines was measured in duplicate using the Bioplex Protein Array System (BioRad), according to the instructions of the manufacturer. This novel multiplexed, particle-based, flow cytometric assay which utilizes anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes enables quantification of several mediators in a sample volume as small as 12.5μl. With the Bioplex cytokine assay kit in combination with the Bioplex Manager software serum TNF-α, IL-6 and IL-10 were assessed. For each cytokine, standards ranged from 1.5 to 32,000 pg/ml serum. Values of the respective serum cytokine levels of mice at day 0 were set as 1 and all other data were given as x-fold increase.

**NF-κB transcription factor assay.** DNA-binding activity of hepatic NF-κBp65 was measured in duplicate using a non-radioactive transcription factor assay (Chemicon International, Inc., Temecula, CA, USA) according to the instructions of the manufacturer. This method combines the principle of the electrophoretic mobility shift assay (EMSA) with the 96-well based enzyme-linked immunosorbent assay (ELISA). During the assay a double stranded biotinylated oligonucleotide containing the flanked DNA binding consensus sequence for NF-κB (5'-
GGGACTTTCC-3`) is mixed with nuclear extract of liver tissue (50 µg/well) and transferred to the streptavidin-coated plate well. The bound NF-κB transcription factor subunit p65 is detected with a specific primary antibody, followed by a highly sensitive HRP-conjugated secondary antibody, which colorimetric signals were detected in a spectrophotometric plate reader (Magellan, Tecan Sunrise, Tecan Trading AG, Männedorf, Switzerland).

**Statistical analysis.** All data are expressed as means ± SEM. After testing for normality and equal variance across groups, differences between the groups were assessed by one-way ANOVA, followed by the appropriate post-hoc comparison test, including Bonferroni probabilities to compensate for multiple comparisons. Statistical significance was set at $P<0.05$. Statistics were performed using the software package Sigma-Stat (Jandel Corporation, San Rafael, CA, USA).

**Results**

**Evidence of KC-depletion.** In all experiments we verified the completeness of KC-depletion by immunostaining for the F4/80 antigen. Treatment with Cl$_2$MDP-liposomes at -24h and subsequently every 72h guaranteed absence of KCs at the time point of resection as well as during the whole 8-d period of regeneration (Fig. 1, A and B). There was no histological evidence of damage to hepatocytes or other cell subpopulations (Fig. 1, A and B).

Uptake of fluorescent latex particles was used as functional parameter of KC phagocytosis. Following Cl$_2$MDP-liposome treatment there was a distinct attenuation in clearance of latex particles from blood, however without complete blockade of particle adherence (Fig. 1C).

**Effect of KC-depletion on liver regeneration.** There was a constant increase of liver weight upon PH with return to almost pre-operative values at day 8 after resection in KC-competent mice, while KC-depleted animals showed only a limited restoration up to 79% of the initial weight (Table 1).

**Effect of KC-depletion on proliferation markers in liver regeneration.** The cumulative hepatocyte DNA-synthesis, as histochemically determined in liver sections by BrdU incorporation, was slightly decreased at day 3 after PH in KC-depleted mice when compared to control mice with a displacement of the proliferation maximum of parenchymal cells from day 3 in KC-competent mice to day 5 in KC-depleted mice (Fig. 2, A and B). In contrast, DNA synthesis phase of non-parenchymal cells was not found altered in KC-depleted animals, however a second proliferation peak was observed at day 8 after resection (Fig. 2, C and D).

To exclude that the proliferating NPCs are KCs we performed double immunostaining for BrdU and F4/80. The immunohistochemical images of KC-depleted animals did not show F4/80-
BrdU-double positive cells, confirming that hepectomy of these animals did not induce KC repopulation and proliferation (data not shown). This indicates that BrdU-positive NPCs in KC-depleted livers can most probably be attributed to proliferation of sinusoidal endothelial cells and stellate cells.

Densitometric analysis of PCNA protein expression served as parameter for proliferation and showed a significant reduction in KC-depleted mice. In particular, at day 3 and 5 after resection hepatic PCNA expression in KC-depleted mice was less than 50% of that found in KC-competent control mice (Fig. 3, A and B).

Densitometric analysis of cyclin B1 protein expression, a M-phase-specific protein of the cell cycle (40, 43), served to distinguish the mitotic rate in KC-competent and KC-depleted livers. Cyclin B1 revealed an expression pattern comparable to that of PCNA, characterized by significantly reduced values in KC-depleted mice at day 3 after PH (Fig. 3, C and D).

**Effect of KC-depletion on transcriptional factor NF-κB during liver regeneration.** Due to the lack of KC-specific NF-κBp65 expression densitometric analysis of total NF-κBp65 levels in whole liver protein extracts showed a marked decrease at day 3 after resection in KC-depleted mice (Fig. 4, A and B). Furthermore, DNA binding activity of NF-κBp65 in hepatic nuclear extracts was found significantly reduced to 25% at this early time point after resection when compared to extracts of KC-competent mice (Fig. 4C).

Using immunohistochemistry with subsequent quantitative analysis of the intracellular distribution of NF-κBp65, sham-treated non-resected animals showed little NF-κBp65 staining in the cytoplasmic but in particular the nuclear compartment of liver cells (Fig. 5, B and D). On the contrary, NF-κBp65 expression was found significantly enhanced upon resection with a preferential location in nuclei of hepatocytes at day 3 and of nonparenchymal cells, namely KCs, at day 3 and 5 (Fig. 5, A-D). Cl₂MDP-liposome application strikingly attenuated this increase of NF-κBp65 expression in hepatocytes and nonparenchymal cells (Fig. 5, A-D). In these animals, NF-κBp65 staining revealed a perinuclear pattern and was found rarely present in the nuclei of parenchymal and nonparenchymal cells (Fig. 5, B, C and D).

**Effect of KC-depletion on cytokine expression pattern in liver regeneration.** To examine the role of KCs in PH-evoked cytokine production, serum concentrations of several cytokines were measured using a multiplex-cytokine assay. As indicated in Fig. 6, serum TNF-α and IL-6 concentrations increased up to day 5 in KC-competent mice, whereas in KC-depleted mice cytokine expression failed to rise or was even found repressed at day 3 and 5 after resection (Fig. 6, A-D).
6, A and B). Serum IL-10 concentrations of KC-competent and KC-depleted mice revealed a similar pattern such as TNF-α (data not shown).

Discussion

In recent years, a number of in vivo and in vitro methods have been used to study the role and function of KCs under physiological and pathological conditions. These studies have led to controversial findings without any conclusive evidence on whether the net influence of KCs in liver regeneration is to stimulate or to diminish hepatocyte proliferation. This study provides now direct in vivo evidence for the stimulatory role of KCs in parenchymal cell proliferation upon hepatectomy.

Methodological considerations. Various models have been employed to clarify the functional role of KCs. However, many agents, such as gadolinium chloride (GdCl₃), might be inappropriate for this purpose, as they activate macrophages to secrete biologically active substances (35). The rare earth metal GdCl₃ itself activates intrahepatic cytokines, is retained in hepatocytes and is indeed highly toxic (31). Using this substance to deplete KCs in vivo, Rai et al. reported that GdCl₃ enhances the expression of hepatic TNF-α mRNA and promotes liver regeneration after subsequent PH (31). As TNF-α has been shown to promote hepatocyte mitosis upon PH (21), increased expression of hepatic TNF-α mRNA in GdCl₃ treated animals might account for the observation of Rai et al. (31). IL-10, a cytokine that down-regulates TNF-α, was found to be abolished in GdCl₃-treated mice. Thus, GdCl₃-treatment might enhance liver regeneration due to the absence of IL-10 in limiting the TNF-α surge (31). Apart from this, the use of GdCl₃ as an agent to induce KC inhibition provides further substantial difficulties in interpretation. For example, there is an ongoing controversy as to whether GdCl₃ eliminates KCs or just alters macrophage function, phenotype (25) and acinar distribution (24).

In contrast, application of liposome encapsulated Cl₂MDP seems to be a more appropriate approach for investigating the effect of KC-depletion on liver regeneration. Encapsulated Cl₂MDP is non-toxic and upon intracellular release of the substance KCs are selectively eliminated without activation (29, 36, 37). Therefore we have chosen this approach for KC-depletion in the present study. Our data show that KCs were eliminated within 24h and during the whole observation period of Cl₂MDP-liposome treatment, as demonstrated by the absence of F4/80 expressing cells in the present study as well as in studies of other groups (28, 35). In addition, the marked attenuation of phagocytosis of latex particles underlines the effectiveness of KC-depletion in animals receiving Cl₂MDP-liposomes, as the remaining latex particle adherence can be attributed to sinusoidal endothelial cells (7, 39).
Effect of KC-depletion on liver regeneration. The present study shows unequivocally that KC-depletion by \( \text{Cl}_2\text{MDP}-\text{liposomes} \) results in reduced liver weights at day 5 and 8 after PH. With respect to the fact that KC-depleted mice revealed an increase of cumulative BrdU-incorporation not only in hepatocytes, but also in non-parenchymal cells, limited liver regeneration might be attributed to a delay rather than to a harm in proliferation (26). In line with this view, Takeishi et al. (35) applied \( \text{Cl}_2\text{MDP}-\text{liposomes} \) first at day 3 after resection and, consequently, observed a reduction in the rate of hepatocyte proliferation at later stages of liver regeneration. However, the proliferation marker PCNA which arises during the entire cell cycle (11) has been found to be markedly decreased over the whole observation period in KC-depleted mice. Additional analysis of the M-phase specific protein cyclin B1 (40, 43) revealed an expression pattern comparable to that of PCNA, characterized by a significantly decreased protein expression at day 3 after PH in KC-depleted mice. Thus, it might be speculated that KC-depletion interferes with the cell cycle at the transition from the S- to the G\(_2\)/M-phases because BrdU incorporation only reflects the synthesis phase of the cell cycle.

KCs are known to produce several kinds of biologically active mediators and seem to be the most important source of TNF-\( \alpha \) (10). In contrast to KC-competent animals revealing a rise of TNF-\( \alpha \) and IL-6 serum concentrations upon liver resection, KC-depleted mice failed to mount an equivalent cytokine response. Consistent with the ability of TNF-\( \alpha \) to induce IL-6 (14, 27), the rise of TNF-\( \alpha \) in KC-competent mice was paralleled by an increase of serum IL-6, while this autocrine effect was missing in KC-depleted mice. Though TNF-\( \alpha \)-deficient mice showed normal liver regeneration (16, 18), TNF-\( \alpha \) has been regarded as an important promoter of hepatocyte proliferation (22). In support of this view, studies using antibodies against TNF-\( \alpha \) demonstrated a reduction of hepatocyte proliferation (2, 33). In addition, livers of MyD88-deficient mice exhibited an impaired regeneration also accompanied by blunted TNF-\( \alpha \) and IL-6 expression (32). Using TNFR-1-deficient mice, it has been shown that impaired liver regeneration resulted from the failure of NF-\( \kappa \)B activation and subsequent suppression of IL-6 production in the liver of these mice after PH (41). Furthermore, using a murine model of bone marrow transplantation Aldeguer et al. showed clearly an importance of KC-derived IL-6 for adequate liver regeneration (3).

PH induces an early cellular response involving the transcription factor molecule NF-\( \kappa \)B (15) which has been attributed a key role in the initiation of hepatocellular proliferation (23). Upon nuclear translocation, NF-\( \kappa \)B induces the transcription of a large set of immediate early genes (20, 34) which are particularly involved in the cell cycle regulation (6). Moreover, NF-\( \kappa \)B is responsible for the expression of numerous pro-inflammatory mediators including TNF-\( \alpha \) (6, 20). In KC-
competent mice we observed highest NF-κBp65 nuclear staining in hepatocytes at day 3 after resection, whereas in non-parenchymal cells NF-κBp65 nuclear staining occurred over day 5. In line with this, it has most recently been shown that after PH NF-κB expression mainly occurs in KCs (42). Now, we demonstrate that in KC-depleted mice both hepatocytes and non-parenchymal cells almost completely lacked the initial nuclear shuttle of NF-κBp65. In parallel, DNA binding activity of nuclear NF-κBp65 was found significantly reduced. This indicates that KC-depleted liver tissue desiderates appropriate NF-κB activation and subsequent TNF-α release. In support of this view, it has been shown that inhibition of NF-κB activation by overexpression of the inhibitor IκB reduces hepatocyte proliferation after PH by downregulation of IL-6 as a down-stream event (9, 19, 42). Most notably, however, specific inhibition of hepatocellular NF-κB by using a transgenic mouse model did not impair DNA-synthesis (8), indicating that KC-confined NF-κB expression in liver regeneration is more important than hepatocellular NF-κB activation. These data are consistent with the results of our study that decreased NF-κB activation is due to the absence of KCs which in turn results in retardation of liver regeneration. Therefore, we finally suggest that hepatocyte proliferation during liver regeneration can be triggered by a signal transduction pathway that involves KC-specific NF-κB, TNF-α, and IL-6.

At first sight increased BrdU staining in the present study contrasts the finding of reduced PCNA and cyclin B1 protein expression. However, it has been reported that inhibition of NF-κB results in a significant decrease in the number of mitotic figures in the regenerating liver without impairment of DNA-synthesis (6). Because of this finding and the present results of decreased cyclin B1 expression in KC-depleted mice we suppose a NF-κB-dependent alteration of the cell cycle at the S-to-G2/M-phase transition. This might explain the observation that KC-depleted mice present with reduced liver regeneration despite of an increased DNA-synthesis. However, further investigations of KC-depleted livers are necessary to specify the impact of KC-specific NF-κB on the cell-cycle.

Taken together, KCs turn out to be essential for intact liver regeneration by their NF-κB activation and effector downstream signals, such as TNF-α and IL-6 release. Thus, KCs play a crucial role in liver regeneration by supporting cellular proliferation.

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References


Figure legends

**Figure 1:** Representative images of hepatic tissue for F4/80 antigen immunostaining at day 5 after PH of a control (A) and a KC-depleted animal (B). While KCs are easily identified in control sections, KCs are missing upon Cl₂MDP-liposome treatment. Scale bars represent 50μm. Latex particle adherence (C), as assessed by the fraction of non-adherent particles using intravital fluorescence microscopy, at day 5 after PH in KC-competent (filled circles) and KC-depleted animals (open circles). Values are means ± SEM of six independent experiments per group. Note the marked delay and reduction of latex particle adherence in the KC-depleted animal, confirming the absence of KC-confined phagocytic activity.

**Figure 2:** Representative immunohistochemical images (A and C; original magnification x400) and quantitative analysis (B and D) of BrdU stained parenchymal (PCs) and non-parenchymal cells (NPCs) in tissue sections at multiple time points upon PH in controls (black bars) and KC-depleted mice (grey bars). Values are means ± SEM of four independent experiments per group and time point. ANOVA and post-hoc comparison; * p<0.05 vs PBS.

**Figure 3:** Representative Western Blot analysis (A and C) and densitometric analysis (B and D) of PCNA (A and B) and cyclin B1 (C and D) expression in liver tissue at multiple time points upon PH in controls (black bars) and KC-depleted mice (grey bars). Western blot signals were corrected with that of β-actin and β-tubulin serving as internal control. Values are means ± SEM of six independent experiments per group and time point. ANOVA and post-hoc comparison; * p<0.05 vs PBS.

**Figure 4:** Representative Western Blot analysis (A) and densitometric analysis (B) of NF-κB p65 levels as well as NF-κBp65 transcription factor DNA-binding activity in hepatic nuclear extracts (C) at multiple time points after resection in controls (black bars) and KC-depleted mice (grey bars). Western blot signals were corrected with that of β-actin serving as internal control. Values are means ± SEM of six independent experiments per group and time point. * p<0.05 vs PBS.

**Figure 5:** Representative images of NF-κBp65 immunohistochemistry of liver tissue at day 3 upon PH in KC-competent (A) and KC-depleted mice (C). Note the increase and the nuclear location of NF-κBp65 immunostaining at day 3 upon PH in KC-competent mice (A), while there was a marked
reduction of NF-κBp65 positive cells in the KC-depleted mice (C). In addition, in KC-depleted animals NF-κBp65 immunostaining was found almost exclusively restricted to the cytoplasmic compartment of hepatocytes. Original magnification x400.

Quantitative analysis of NF-κBp65 immunohistochemistry with cellular and subcellular distribution, as given by the nuclear-to-cytoplasmic ratio of NF-κBp65 positivity in parenchymal (B) and nonparenchymal cells (D) at day 0 (non-resected), 3, 5 and 8 after PH in KC-competent (black bars) and KC-depleted mice (grey bars). Note the predominant nuclear location of NF-κBp65 at day 3 after PH in hepatocytes (B) and at day 3 and 5 after PH in nonparenchymal cells in KC-competent animals (D), while in KC-depleted animals the nuclear-to-cytoplasmic ratio of NF-κBp65 positivity in both hepatocytes and non-parenchymal cells hardly exceeds 1. Values are given in means ± SEM of six independent experiments per group and time point. ANOVA and post-hoc comparison; * p<0.05 vs PBS.

**Figure 6:** Serum concentrations of TNF-α and IL-6 at day 0, 3, 5 and 8 after PH in KC-competent (black bars) and KC-depleted mice (grey bars). Values are given as means ± SEM of six independent experiments per group and time point. ANOVA and post-hoc comparison; * p<0.05 vs PBS.
**Table 1:** Liver weight at day 0, 3, 5 and 8 after PH in mice with treatment of either PBS-liposomes or Cl₂MDP-liposomes.

<table>
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<th>liver weight (%)</th>
<th>PBS</th>
<th>day 0</th>
<th>day 3</th>
<th>day 5</th>
<th>day 8</th>
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<td></td>
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<td>79.2±3.8</td>
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</table>

Values of six animals per group and time point are given as means ± SEM; ANOVA and post-hoc comparison; * p<0.05 vs PBS.
Fig. 1

A

B

C

non-adherent latex particles (%)

0 200 400 600 800
time (s)

Fig. 1

A

B

C

non-adherent latex particles (%)

0 200 400 600 800
time (s)

5d PBS

5d Cl₂MDP
Fig. 3

Panel A: Western blots showing PCNA and β-actin expression over time after resection.

Panel B: Bar graph depicting the relative density of PCNA/β-actin over days after resection.

Panel C: Western blots showing cyclin B1 and β-tubulin expression over time after resection.

Panel D: Bar graph depicting the relative density of cyclin B1/β-tubulin over days after resection.
Fig. 4

A

B

C

Days after resection

Relative density (NF-κBp65/β-actin)

NF-κBp65 binding activity (OD)

PBS     CLMPD
0d  3d  5d  8d

0  0.5  1.0  1.5

0  0.4  0.8  1.2  1.6

0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

*
Fig. 5

A

B

C

D

NF-κB p65 expression of PCs nuclear-to-cytoplasmic ratio

NF-κB p65 expression of NPCs nuclear-to-cytoplasmic ratio

days after resection

days after resection

3d PBS

3d Cl₂MDP

0 3 5 8

0 3 5 8

*
Fig. 6

A

B

Days after resection

serum TNF-α (x-fold increase)

Days after resection

serum IL-6 (x-fold increase)