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Direktor: Prof. Dr. J. Werner

Modulating CD4+ T-Cell Migration in the Postischemic Liver: Hepatic Stellate Cells as New Therapeutic Target?

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Jörg Andreas Reifart

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Berichterstatter:	Priv. Doz. Dr. med. Andrej Khandoga
Mitberichterstatter:	Prof. Dr. Alexander L. Gerbes Priv. Doz. Dr. Elisabeth Deindl Prof. Dr. Christian Schulz Prof. Dr. Daniel Wolff
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
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1. INTRODUCTION

1.1. Clinical relevance of ischemia reperfusion damage

In patients that require liver surgery organ damage caused by ischemia (loss of organ perfusion) and subsequent reperfusion (I/R) play an important role regarding the outcome of the patient. I/R damage can occur after liver transplantation (LT), liver resection, liver trauma as well as after hemorrhagic or septic shock during which blood flow to the liver is also reduced. Liver transplants are the most common cause for hepatic I/R. In 2013, Eurotransplant registered 1491 liver transplants from deceased donors that were performed in Europe - most of them in Germany. The number of livers needed was higher by 522 [Eurotransplant, 2013].

Additionally, the shortages in livers for transplantations has led to increased use of alternatives to "classic" cadaveric LT (liver transplants) such as split (one part of the liver is given to an adult, a smaller part to a child), domino (from a patient with amyloidosis), or living related LTs. These alternative methods now account for 15% of all procedures [Adam et al., 2012]. Furthermore, there has also been increased use of livers from extended criteria donors [Durand et al., 2008].[1] In these scenarios post procedural liver damage might be even more detrimental.

During classic liver transplantation, the organ´s perfusion is completely disconnected from the patient's circulation system (starting ischemia) and, after some time during which the organ is conserved in a cold solution, reconnected to the circulation (starting

[1] Extended criteria donors normally mean that the patients suffered from steatosis, were no older 80 years, had serum sodium levels higher than 165mmol/L or had other criteria that resulted in a higher risk in comparison with a classic donor [Bruzzone, P., Giannarelli, D., Nunziale, A., Manna, E., Coiro, S., De Lucia, F., Frattaroli, F.M., Pappalardo, G.
Extended criteria liver donation and transplant recipient consent: the European experience.
Transplantation proceedings. 43 (2011) 971-973.]

reperfusion). Early organ failures after LTs is one of the worst complications possible and ten percent of early organ failures are attributed to ischemia reperfusion injury (I/R injury) [Fondevila et al., 2003].

Post-operative non-functioning of the organ caused by damage from I/R can lead to need for re-transplantation or even the patient's death.

Of 100 liver transplanted patients 61 live after 10 years. In 7% of the whole patient collective re-transplantation is needed [Adam et al., 2012].

Failure of a transplanted liver is not only an extreme hardship on the patient, it also dramatically increases the associated costs. *The Annals of transplantation* estimated the mean cost of a liver transplantation in Germany at 52.570€. Any complications post transplantation increase the cost by at least 62%. Graft failure, which can be the result of I/R injury increases the cost by up to 227% [Lock et al., 2010].

The points discussed above are some of the various reasons why a better understanding of hepatic I/R is something to strive for. Additionally, I/R specific pathophysiological events do not only take place in the context of liver surgery but also to a similar extend in other organs such as the heart, kidneys and lungs so a better understanding of hepatic I/R might reveal management strategies for other organs as well.

1.1.1. Hepatic ischemia

Looking more closely at the pathomechanism of ischemia we see that there are several processes that lead to tissue damage when the blood supply to an organ is compromised. Initially the oxygen which is predominantly used for aerobic glycolysis is depleted. In aerobic glycolysis metabolization of one mol of glucose results in 36 mole ATP (Adenosine tri phosphate)[Lehninger AL, 1993]. Without oxygen, anaerobic glycolysis is the main source of ATP which only offers 2 moles per every 1 mole of glycose. Because the ATP synthesis is now anaerobic, lactate is created, which leads to the acidification of the cell environment which is detrimental to most enzyme dependent processes. There is no alternate source of molecules for metabolism available at that point, so the concentration of energy rich phosphates continues to decline while the concentration of acidic metabolites increases.

This in itself can make the cell environment inhospitable and even result in cell death. Longer ischemia times lead to greater hepatic damage [Gujral et al., 2001].

Before the cells become necrotic they swell up due to the inability to keep up transmembrane electrolyte gradient, a process that requires a lot of energy (in form of ATP). This swelling of cells can then restrict capillary blood flow upon reperfusion which is known as “no reflow” phenomenon [Kloner et al., 1974]. Although there are more complex mechanisms to consider the vast amount of damage during ischemia can be attributed to hypoxia.

1.1.2. Hepatic reperfusion

Restoring the blood flow initiates reperfusion which brings new nutrients and oxygen to the distressed tissue. Paradoxically reperfusion leads to additional cell death. This is known as the "reflow paradox" [Menger et al., 1992].

Several intra- and extracellular mechanisms are known to be involved in this process. One of the mechanism is caused by intracellular reduction of ATP to AMP during ischemia. This leads to accumulation of adenosine. To re-phosphorylate adenosine to adenosine-tri-phosphate (ATP), oxygen is required so the excess of adenosine is partially metabolized, which leads to accumulation of hypoxanthine (under anaerobic conditions). Without oxygen hypoxanthine, cannot be turned to xanthine. When the tissue is finally reperfused and reoxygenated hypoxanthine is quickly metabolized to xanthine, a process in which reactive oxygen species are created. In part the "extra" damage is caused by the reactive oxygen species which can damage the DNA and other cellular structures but are also known to initiate other cascades that lead to cell death. These processes are in part driven by immunologic cells. Through activation of redox-sensitive transcription factors a complex immunological response in the hepatic microvascular system involving pro-inflammatory cytokines and heightened expression of adhesion proteins is set in motion. In contrast to hypoxia driven organ damage during ischemia, during reperfusion the damage is predominantly inflammation-mediated.

1.1.3. Leucocytes in I/R injury

The protagonists of inflammation dependent damage of hepatic I/R are leukocytes. There are several leukocyte subgroups that are involved in I/R: Neutrophil granulocytes, Kupffer-cells (hepatic macrophages) and as we will explore later, lymphocytes. Kupffer cells as well as activated neutrophils are especially active in the initial phase of reperfusion, as they create reactive oxygen species and other pro-inflammatory chemokines that promote I/R damage [Jaeschke et al., 1991, Wanner et al., 1996]. This spirals into a self-promoting process during inflammation: Damaged endothelial cells activating leukocytes that reciprocally cause more damage to the endothelial cell. Destruction of the endothelium increases tissue edema and negatively affects perfusion. Post-ischemia leukocytes are found in the hepatic sinusoids and also post-sinusoidal venules.

It is unclear whether the intravascular or the already extravasated leukocytes are primarily responsible for immunogenic I/R damage [Jaeschke, 2006]. The leukocyte extravasation has several steps to it. Leukocytes interact with specific adhesion molecules expressed by the hepatic microvasculature endothelium. In stress situations, these adhesion molecules, such as ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular adhesion molecule 1) from the immunoglobulin superfamily are highly expressed leading to increased interaction. This mechanism is not specific to I/R injury and is seen in almost all inflammatory environments.

"*Homing*" starts when leucocytes are captured by the endothelium. Endothelial cells (EC) express multiple selectins (like CD62P, CD62E) and adhesion molecules (see above) that interact in a characteristic on-off way with L-selectins and integrins on the leukocyte. This interaction causes the leukocytes to be slowed down. They are not

flowing freely in the vessel anymore but "roll" along the endothelium getting increasingly activated by cytokines, which were initially excreted by the post-ischemic tissue, now being presented in the glycocalyx of the endothelial cells. The activation of the leukocyte is G-protein mediated and causes a stronger bond between the endothelial cell and the leukocyte by means of LFA-1 (lymphocyte function associated antigen 1), β1/β2-integrins as well as the endothelial adhesion molecules of the Ig-family. Next, processes called intraluminal *crawling* and *transmigration* take place. The leucocyte crawls along the endothelial barrier to find a site of transmigration. The proteins LFA-1 (see above) and MAC-1 (Macrophage antigen-1) play an important role here [Phillipson et al., 2006].

The actual transendothelial and interstitial migration is not as well explored. In the subsequent phase of ischemia derived hepatic damage, leukocytes follow a gradient of chemokines (like IL-8, MIP-2, KC and CXC [Colletti et al., 1996, Li et al., 2004, Simonet et al., 1994]) that drive their migration. After the leukocytes passed the endothelial layer they migrate toward the inflammatory epicenter excreting enzymes like matrix metalloproteinases (also called gelatinases), which are suspected to play a vital role during hepatic I/R. Supposedly they are essential to facilitate leukocytes crossing the basal membrane. Studies where MMP-9 was inhibited showed ameliorated hepatic damage after I/R [Hamada et al., 2008].

But not just leukocytes contribute to hepatic I/R damage. Thrombocytes also seem to be involved in this process [Khandoga et al., 2002a]. Activated thrombocytes release oxygen- and nitrogen-radicals as well as other proinflammatory and procoagulatory mediators [Khandoga et al., 2003].

1.2. CD4 T-cells in I/R

More recent studies showed that CD4+ T-lymphocytes, besides other leukocytes mentioned above, might also play a critical role in the development of hepatic I/R-injury [Khandoga et al., 2006, Kuboki et al., 2009, Mende et al., 2014, Shen et al., 2009, Zhang et al., 2013].

Analogous to other leukocytes, CD4+ T-cells (of which there are several identified subsets[2]) migrate through the hepatic parenchyma to the afflicted sites during the post-ischemic inflammatory reaction after diapedesis [Khandoga et al., 2006, Schrage et al., 2008]. There they can promote leukocyte recruitment via IL-17 as well as suppress it [Caldwell et al., 2005]. This in itself shows their ability to impact hepatic I/R.

Historically, first signs of lymphocyte involvement in hepatic I/R were seen in experiments in a model of isolatedly perfused rat livers [Clavien et al., 1991]. A defined number of lymphocytes were added to the solution the liver was perfused with. What stood out was that the number of lymphocytes in the solution decreased over time, more so in liver tissues with longer ischemia times. Post-experimental immunofluorescence studies also showed T-cell infiltration into hepatic tissue.

Later T-cell deficiency ([nu/nu] mice) was observed to lead to a reduction in hepatic I/R damage [Zwacka et al., 1997]. The hepatoprotective effect of lymphocyte absence was then reversed through adoptive transfer of CD4 and CD8 positive T-cells, showing these

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[2] **Type 1 T helper (Th1) cells** which are responsible for cell-mediated immunity and phagocyte-dependent protective responses by activating macrophages and type **2 T helper (Th2) cells** which are responsible for strong antibody production, eosinophil activation, and inhibitio n of several macrophage functions are the main classes of CD4 T-cells. Additionally regulatory T-cells, NK cells and certain cytotoxic T-cells can also express CD4. [Romagnani, S. Th1/Th2 cells. Inflamm Bowel Dis. 5 (1999) 285-294]

cells as the culprit. In subsequent studies with "signal transducer and activator of transcription" (STAT)-deficient mice the role of Th1-lymphocytes in the induction of I/R damage could be demonstrated [Shen et al., 2003]. STAT represents a family of transcription factors that are activated by cytokine receptor stimulation through phosphorylation and then proceed to activate further cytokine-induced transcription factors upon translocation into the cell nucleus [Hoey et al., 1999, Wurster et al., 2000]. While the transcription factors STAT 1 through 6 have many different functions in angiogenesis, tumor suppression and immunosuppression, only STAT-4 deficiency leads to attenuated hepatic I/R injury [Shen et al., 2003]. Th1-lymphocyte differentiation and proliferation is greatly depended on STAT-4 [Nishikomori et al., 2002].

Research targeted at CD4+ T-cells has found a protective effect of immunosuppressive drugs (like CsA, FK506 und FTY720) on the allo-antigen-independent post-ischemic liver injury [Anselmo et al., 2002, Kawano et al., 1995] as well as reduction of hepatic post-ischemic damage in mice with reduced CD4 T-cells [Le Moine et al., 2000, Martin et al., 2010].

The findings mentioned above underline the role of CD4+ T-cells in hepatic I/R but do not clarify the processes involved. CD4 T-cells marked with immunofluorescent dye have been shown to accumulate in hepatic sinusoids and ,to a lesser extent, in post-sinusoidal venules [Khandoga et al., 2006]. Their transendothelial emigration into the tissue has been shown to be dependent on the adhesion molecules ICAM-1 and VAP-1 [Lalor et al., 2002] but also on several endothelial cytokines [Cinamon et al., 2001].

Overall CD4 T-cells are not known to be an aggressive cell type: They do not produce

ROS or excrete proteolytic enzymes. They can, however, modulate neutrophil granulocyte-activity through IL-17 excretion [Caldwell et al., 2005] but also interact with these and other cell types: Interactions between CD4 T-cells and platelets[3] as well as the sinusoidal endothelium[Khandoga et al., 2006] and interactions between CD4 and Kupffer cells [Hanschen et al., 2008] have been documented.

Additionally, hepatic endothelial cells selectively express adhesion molecules like CD54 (ICAM-1) und CD106 (VCAM-1) as well as MHC- I and II, and the costimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD40 (76) that potentially play an important part in endothelium-T-cell interaction [Lohse et al., 1996]. The endothelial cells are able to present previously endocyted antigens on MHC I and MHC II molecules – therefore acting as antigen presenting cells (APC) to CD4 and CD8 lymphocytes [Bertolino et al., 2002]. Both the endothelial cells as well as the T-cells get activated by I/R related mediators (TNF-α und IL-6) and ROS [Hanschen et al., 2008].

Recently the interaction between CD4 + T-cells and another APC, the hepatic stellate cells (HSC) was observed *in vitro* [Langhans et al., 2014, Wu et al., 2012]. This supports the idea of their interaction *in vivo*, which was also alluded to in studies of viral hepatitis where NK-cells as well as CD8 and CD4 cells were found in proximity to HSC in fibrotic liver samples [Muhanna et al., 2008]. We assume that an interaction between T-cells (CD4+, NK cells, regulatory T-cells) and hepatic stellate cells (HSCs), is critical for inflammatory response, regeneration and fibrosis formation [Glassner et al., 2013, Muhanna et al., 2008, Muhanna et al., 2007] [Knolle et al., 2014].

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[3] 30% of intravascularly adherent T-cells colocalize with thrombocytes, which suggests interaction.

1.3. Hepatic stellate cells

HSCs are pericytes found in the space of Disse. They are also called hepatic non-parenchymal cells (HNPCs) (Figure 1) or Ito-cells and they are the major cell type involved in liver fibrosis in response to liver injury [Bissell, 2010]. The name stellate comes from their dendritc ultrascructure. The cell's dendrites stretch in the subendothelial space and serve to receive chemotactic signals.

HSC exhibit two states in which they behave very differently: Deactivated/quiescent or activated. In the quiescent cell status, these stellate cells have many vitamin A-rich droplets in their cytoplasm. A characteristic which helped distinguish them from other cells. Upon activation during injury, however, HSC lose the storage droplets, develop a comparatively larger rER with Golgi-apparatus [Friedman et al., 1985], produce microfilaments that appear beneath the cell membrane and turn into myofibroblast like cells [Enzan et al., 1999, Friedman, 2008].

Hepatic stellate cells express a multitude of receptors such as neurotrophin receptors [Cassiman et al., 2001], toll-like receptors (TLR) [Paik et al., 2003], proteinase-activated receptors [Gaca et al., 2002] and also CB receptors [Teixeira-Clerc et al., 2010, Teixeira-Clerc et al., 2006] as well as a multitude of cytokines and chemokines such as IL-17 and CCL2 [Weiskirchen et al., 2014].

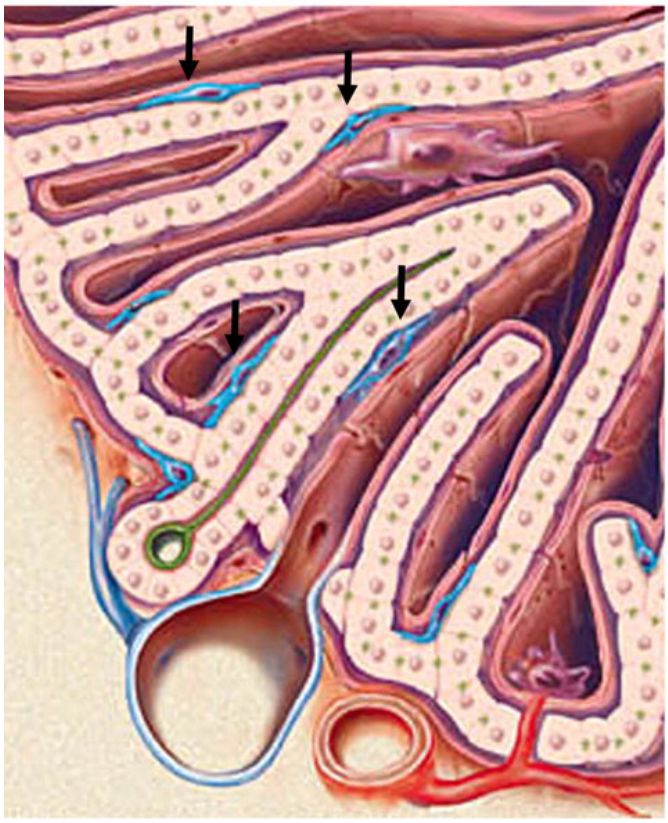
Especially activated HSCs have immune regulatory functions and are thought to exert an immunomodulatory effect on lymphocyte reactions *in vitro* [Langhans et al., 2014, Wu et al., 2012] [Kobayashi et al., 2003]and *in vivo* [Friedman, 2008, Yin et al., 2013]. APC features and even phagocytic properties have been observed [Jiang et al., 2009].

In fibrotic murine livers lymphocytes were seen in proximity to HSCs, mainly in the periportal area and along fibrotic septa, which suggests a direct interaction. Interestingly,

hypoxia alters the sensitivity of HSCs to certain molecules such as chemotaxins through activation of HIF-1α and also regulates the expression of genes that are important for angiogenesis and collagen synthesis [Copple et al., 2011].

According to recent data in literature, HSC-T-cell interactions have varying immunomodulatory effects which strongly depend on the character/location of the inflammatory reaction. Indeed, HSCs are potent antigen-presenting cells (APC) and can activate NKT cells as well as conventional T lymphocytes [Winau et al., 2007]. Such activation would enhance the immune response after liver transplantation, accelerate the T-cell-induced I/R injury, and even increase the graft rejection rate. On the other hand, HSCs were also reported to prevent activation of naïve T-cells by dendritic cells or artificial antigen presenting cells in a cell contact-dependent mechanism [Schildberg et al., 2011]. The pathophysiological relevance of HSC-CD4+ T-cell interaction during hepatic I/R has not been investigated, so whether CD4+ T-cells interact with HSCs during I/R-induced inflammation remains unclear. We propose an interaction of these two cell types after hepatic I/R and believe a modulation of HSC activity can influence their interaction.

A



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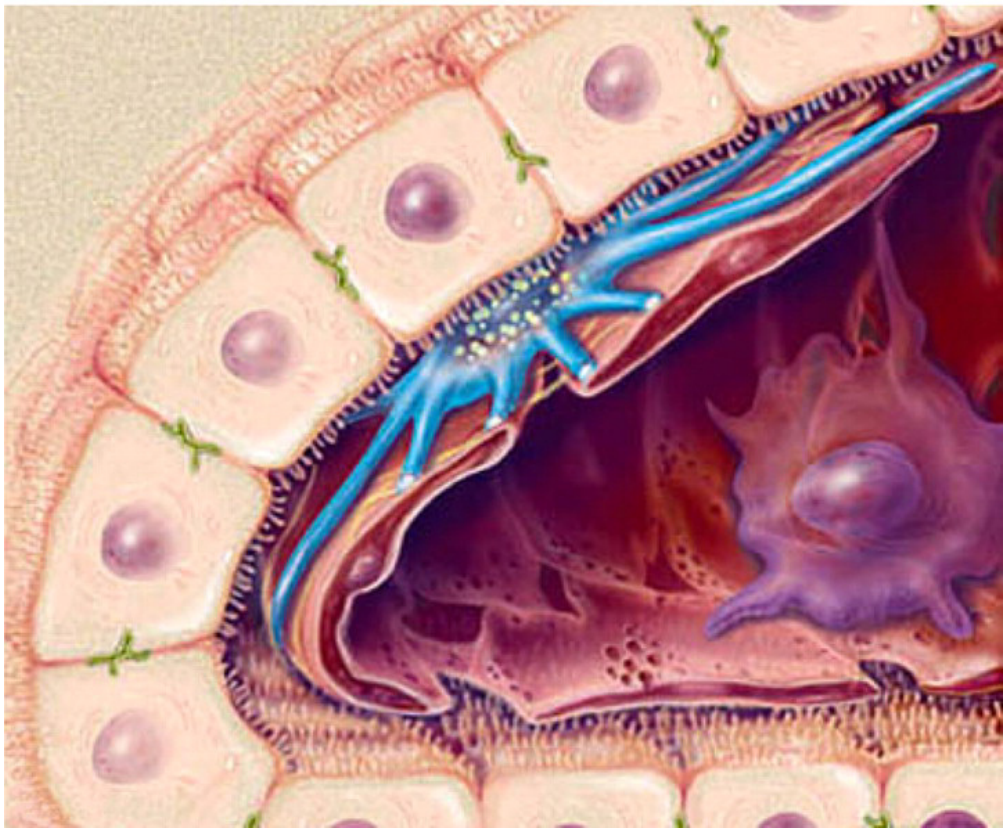

Figure 1 Morphology of hepatic stellate cells in normal liver. A: A hepatic sinusoid with stellate cells (in blue, indicated with arrows) within the sinusoidal architecture. B: higher resolution drawing of stellate cells in the perivascular space. From Friedman SL, ***Arthur MJ.*** *Reversing hepatic fibrosis. Science Medicine 8: 194–205, 2002*

2. HYPOTHESIS

The hypothesis of the following experimental study was that:

1. Hepatic stellate cells (HSCs) interact with CD4+ T-cells during I/R of the liver and
2. that modulation of HSC activity affects intrahepatic T-cell migration and T-cell dependent I/R injury.

3. MATERIAL AND METHODS

3.1. Preliminary remark

The following experiments were performed from November 2008 to July 2012 at the Walter Brendel Zentrum and contributed to a published article [Reifart et al., 2014]. The grant to work with animals was approved by the government of Oberbayern, filed under: 55.2.1.54-2532-8-13. All experiments were carried out according to the German legislation on animal protection.

3.2. Experimental model

The majority of the experimental modalities used in our study are established procedures that have proven to be reliable interventions to assess the investigated effects. Only the western blot to show hepatic stellate cell activity and the pharmacological treatment of stellate cells are methods that were scarcely described in research literature. More detailed information on the different techniques are described below.

3.3. Animals

The animals used in these experiments were female 5- to 7-week-old wild type Charles river mice (Charles River, Sultzfeld, Deutschland), as well as GFP (green fluorescent protein) positive Cx3CR1(gfp/gfp) obtained from the European Mouse Mutant Archive

(Monterotondo, Italy). The mice weight ranged from 20 for 25 g and the dosages were adjusted accordingly. Within the facility the animals had regular 12-hour day-night cycles and were housed in groups of 3 to 5 mice per makrolon-cage. Water and regular food (Ssniff Spezialdiäten, Soest Deutschland) were always available and unrestricted.

3.3.1. Anesthesia

Narcosis was initiated with isoflurane inhalation anesthesia (Forene®,

Abbott GmbH, Wiesbaden, Deutschland) at 5% of total gas volume, laughing gas (N2O, 1,5l/min) and an inspiratory oxygen level of 35%. After approximately 20 to 30 seconds the animals were fully anesthetized. The subsequent surgery was performed with isoflurane levels turned down to 1 - 2% and 3 l/min flow (50% O_2, 50% N_2O) was kept steady. 0.1mg/kg buprenorphine was injected subcutaneously for additional analgesia.

After the anesthesia took effect, the animals were fixed to a heated metal plate which served as surgical field. Throughout the experiments the body temperature was controlled intra-abdominally as reference to keep the temperature steady between 36 and 37 degrees Celsius. This procedure was adhered to for all animals in this study.

3.3.2. Surgical procedure

3.3.2.1. Intra-peritoneal drug injection

To ensure intraperitoneal and atraumatic intraperitoneal injection of the different substrates (vehicle, vehicle plus JWH-133, vehicle plus ACPA) the mice were put under isoflurane anesthesia as described above. We made a very small incision in the median

abdominal line to give way to a clear view of the linea alba. The abdominal wall was then pulled up with forceps to be able see the injection of the substances into the abdominal cavity without damaging the intestine. After injection, the small cut was sutured with one knot using Ethibond 2-0.

3.3.2.2. Intra-arterial catheter placement

The surgery always started with a small incision (1cm) on the mouse`s throat giving way to access the left carotid artery after bluntly dissecting the fat tissue and thyroid gland.

The carotid artery was then marked and ligated cranially to improve accessibility. This operation was performed at a micro-surgical work station using an operation microscope (Leitz, Wetzlar, Deutschland) that allowed magnification of the operation field by 5 to 42 times. Then the artery was clipped caudally. A small incision was made to allow a polypropylene catheter (inner diameter 0,28mm, Portex, Lythe, Great Britain) to enter the vessel lumen. The catheter was then fixed with two ligatures. Afterward the clip was removed allowing real-time measurement of mean arterial pressure, as well as a way for continuous intravenous administration of 0,2ml/g (body weight) /h saline solution (0,9%) to compensate for volume loss due to bleeding and perspiration. The mean arterial pressure was then continuously registered by a transducer (Statham Transducer Typ P 23 ID, Statham Instruments Inc., Oxnard, USA) and an electric manometer (Press. Ampl. 863E154E, Siemens Medizinische Technik, München, Deutschland), which allowed measurements in mmHg. These measurements, as well as temperature measurements were displayed on a workstation computer und saved for

eventual analysis.

The intra-arterial catheter was also used to inject, CD4 positive, T-cells and fluorescent dye. Upon completion of the experiment blood samples were taken from the site where the carotid catheter had been removed. The mean amount of blood was about 1mL.

3.3.2.3. Reversible partial liver ischemia

After a median laparotomy, reversible warm liver ischemia was induced by clipping the left liver lobe with a 11mm straight Yarsagil Aneurysm Clip (Medicon®), clamping the supplying nerve vessel bundle[Biberthaler et al., 2001b]. The laparotomy was then closed with 5-0 polyester sutures to prevent the liver and bowels from drying out and heat loss. The mean time from the start of the operation till ischemia was 20 minutes. 90-minute warm ischemia was monitored by mean arterial pressure and intraperitoneal temperature sensors.

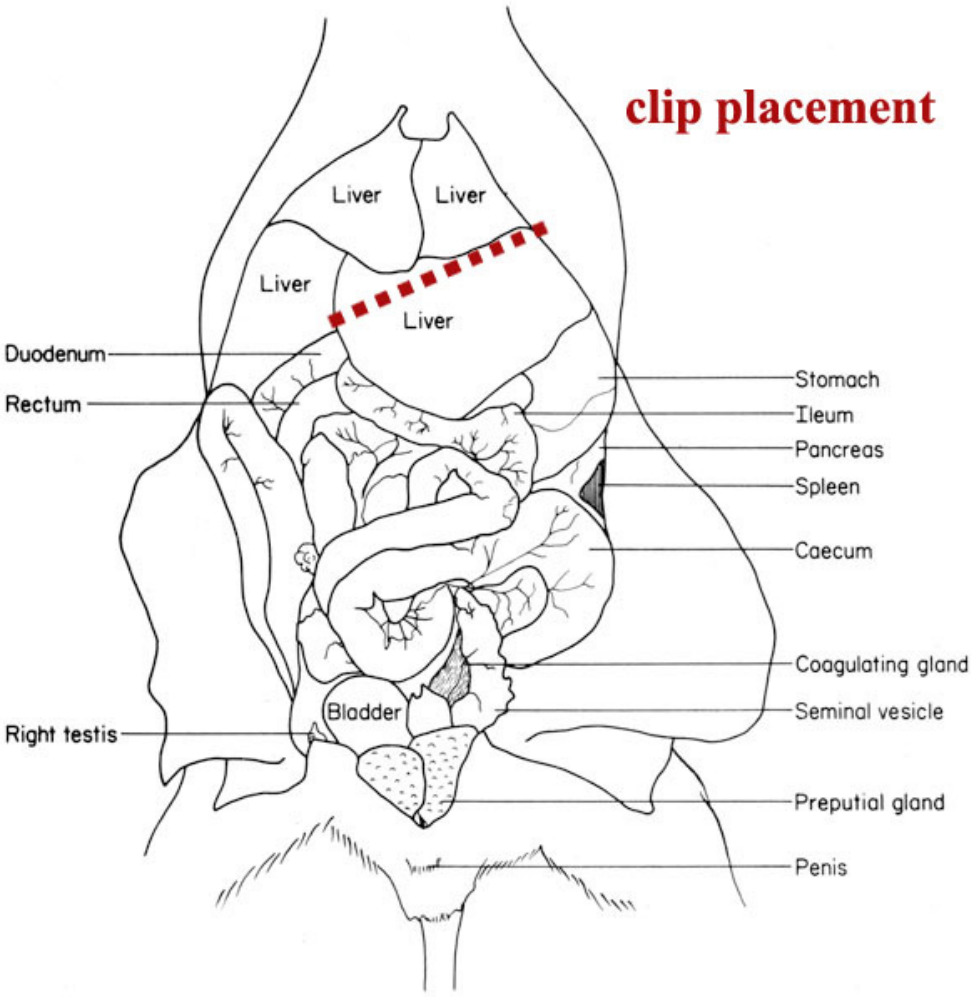

Figure 2 Murine anatomy. The mark of the clip placement shows the dominant left liver lobe. Note that only female animals were used in the experiments. Modified from: Margaret J. Cook, The Anatomy of the Laboratory Mouse, 54. Abdominal viscera. [http://www.informatics.jax.org/cookbook/figures/figure54.shtml]

3.3.2.4. Reperfusion

After a 90-minute period the abdomen was reopened and the clip removed, restoring blood-flow to the left liver-lobe. The total reperfusion time was 120 minutes. Sham-operated animals underwent the same procedures with the difference of a short (3 sec) clamping of the left liver lobe instead of full ischemia. All other procedures were the same. They were monitored under anesthesia for a total time of 230 minutes (20minutes +90minutes +120minutes).

3.3.3. Isolation and labeling of CD4+ T-cells

For the intravital microscopic studies, CD4+ T-cells were isolated from spleens of syngeneic mice (C57/BL6). In order to do this the mice were anesthetized in the manner mentioned above. A median laparotomy gave way to access the spleen which was then carefully extracted to minimize the amount of peri-splenic fat and vessel tissue extracted. The spleen was then homogenized with a S24 glass pestle homogenizer adding 3ml of a buffer solution (DPBS, no Mg, no Ca^{2+}, 2mM EDTA, 2,5ml albumin 5% solution in 500ml). In the next step the T lymphocytes were isolated with the MACS® Separation System (Miltenyi Biotec, Bergisch-Gladbach, Germany) using anti-mouse CD-4^{+} antibody labelled magnetic beads following the manufacturer's instructions [Miltenyi et al., 1990]. Here positive separation was used.

This process uses anti bodies that are linked to superparamagnetic biotinylated-micro particles (about 100 nm diameter), so called Microbeads. After homogenization, the mixture ran through a 30-micrometre filter to avoid congestion of the columns that were

7used later. Then the homogenate was centrifuged for the first time at 4 degrees Celsius for 10 minutes at 1500 rounds per minute. At the end of this cycle the fluid was emptied and the bottom pellet re-suspended in 2ml of the buffer solution. The cell suspension was then incubated with the CD4 Microbead-Antibody solution for 15 minutes at 4 degrees Celsius. After the incubation, the mixture was replenished with buffer to reach 10ml again and a second cycle of centrifuging allowed to get rid of superfluous antibodies. The pellet was suspended in 2ml buffer again. Then the solution was passed through the Miltenyi column (containing steel wool) that was mounted on a magnetic manifold. This column had been rinsed with rinsed with PBS puffer. Cells that were labeled with the antibody stick to the column due to the magnetic force. Unlabeled cells passed through the column.

In the second part of the isolation process the column with the target cells was removed from the magnetic manifold. The Microbeads no longer had extra support in the column and could be flushed out with PBS using a specially designed plunger. The result was a solution rich in CD4 T-cells. The concentration of Isolated CD4+ T-cells was assessed with a Coulter-Counters (Coulter® AC-T Series; Coulter Corporation, Miami, USA) and then labeled with the fluorescent dye CFSE (carboxyfluorescein diacetate succinimidyl ester, 5 μM, Molecular Probes, Eugene, OR, 10 min incubation). A total of $1x10^7$ CFSE-labeled CD4+ T-cells was infused intra-arterially after 120 min of reperfusion and then visualized using intravital microscopy.

The isolation procedure did not lead to T-cell activation as measured by expression of the markers CD62L, CD44, and CD69 and the purity of the CD4+ T-cells subsets was routinely >95% as determined by fluorescence activated cell sorting analysis. The viability of CD4+ cells following the isolation procedure was approximately 94% as

determined in our own previous studies (see [Hanschen et al., 2008, Khandoga et al., 2006]).

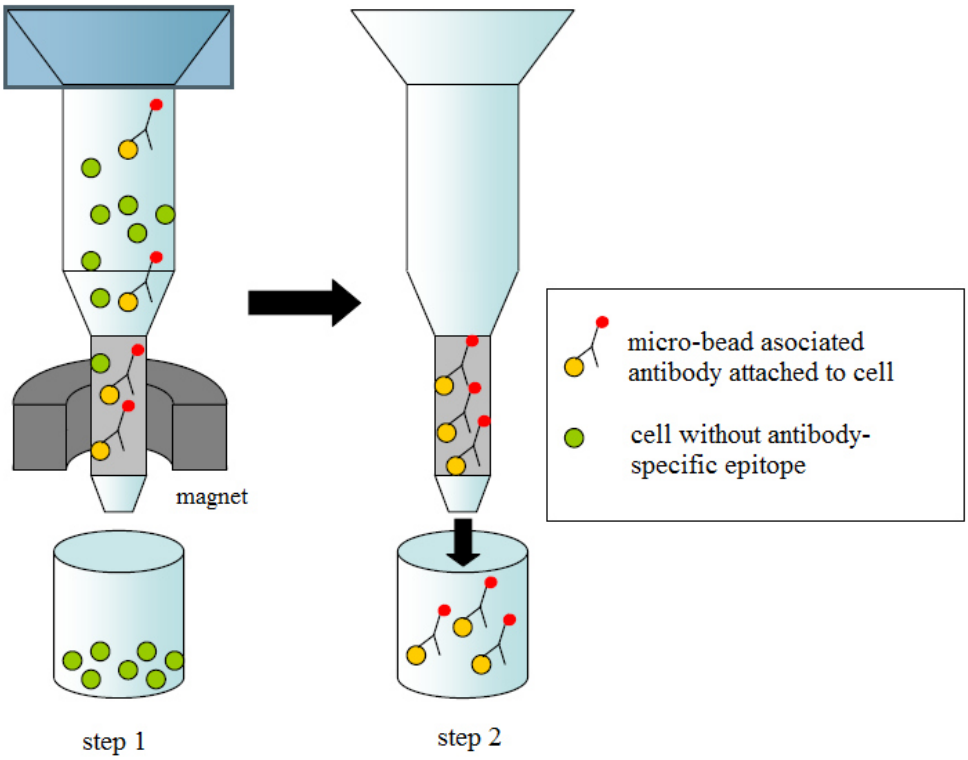

Figure 3 Positive selection of CD4 T-cells with magnetic mircrobead antibodies

3.3.4. Intravital fluorescence microscopy

3.3.4.1. Experimental set-up

Intravital fluorescence microscopy was performed using a modified Leitz-Orthoplan microscope with a 100 Watt HBO mercury lamp attached to a Ploemopak illuminator (Leitz, Wetzlar, Germany). This served to achieve epi-illumination. To get adequate microscopic pictures the left liver lobe was propped up on a small plate holding a spoon-like structure made of synthetic modeling clay. Then a thin cover glass was laid on top of this contraption, not touching the liver tissue. Saline solution was used to have the liver tissue be suctioned to the cover glass. This allowed us to correct for natural convexity of the liver, maximizing the number of acini that could be seen in focus. During the intravital microscopy a special saline drip contraption continuously replenished the fluid the liver was floating in. This also prevented the liver tissue from drying out. Intravital microscopic images at a magnification level of 500 – a water immersion objective (W 25/0.6; Leitz) was used - were recorded by a charge-coupled device camera (FK 6990, Cohu; Prospective Measurements, San Diego, CA) and tape-recorded for off-line evaluation (S-VHS Panasonic AG 7330; Matsushita Electric, Tokyo, Japan). Videotaped images were quantitatively analyzed offline in blinded fashion using an image analysis program (CAPIMAGE®, Dr. Zeintl, Heidelberg, Germany).

Initially the CFSE labeled CD4 T-lymphocytes were injected into the carotid catheter and acini were filmed.

Thereafter, Fluorescein isothiocyanate (FITC)-labeled dextran (MW 150000; 0.1ml,

5%, Sigma-Aldrich) was infused. This showed sinusoidal perfusion. Erythrocytes were seen in black contrasting the then fluorescent blood plasma. Intravital microscopy lasted approximately 20 min before the experiments were concluded.

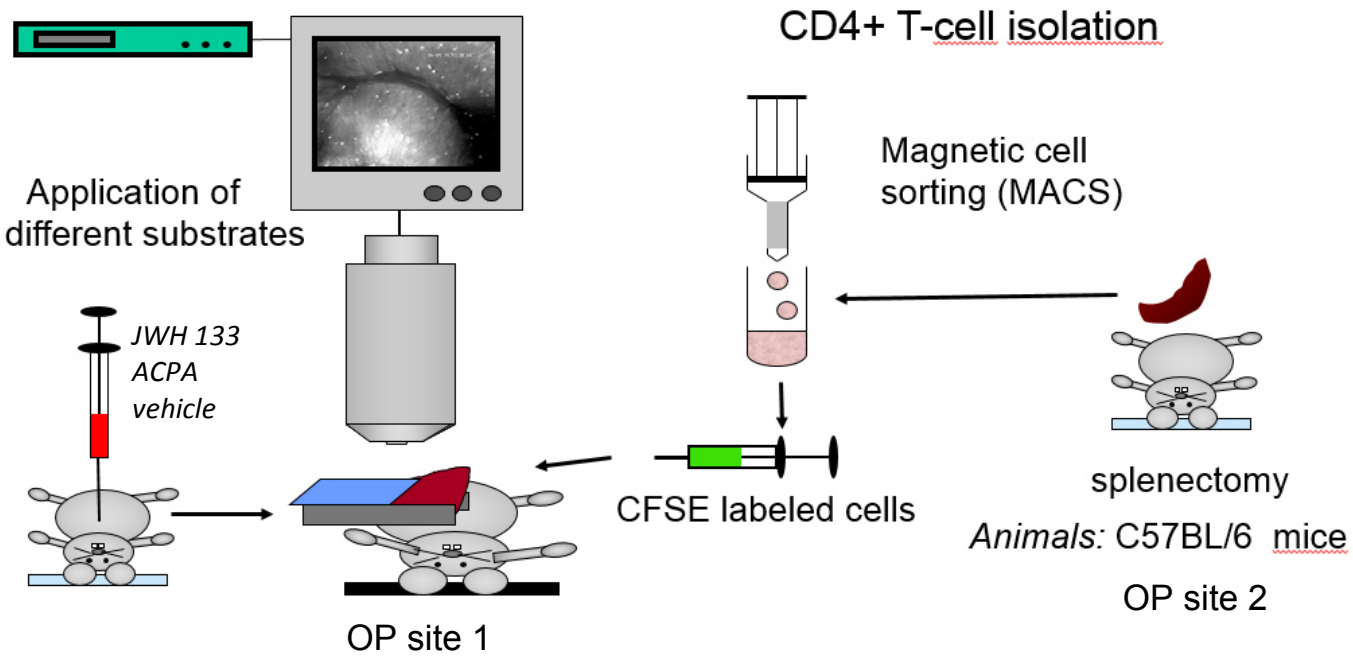

Figure 4 The experiments were conducted in two different operation sites. The main operation site (1) featured the mouse undergoing hepatic ischemia. The second operation site (2) was needed to simultaneously explant the spleen for further processing and CD4 T-cell isolation as described above. Prior to the experiment the animals were treated with the different endocannabinoid agonists (ACPA, JWH 133) or the vehicle solution (tocrissolve) as pictured on the left of the figure.

3.3.4.2. CD4 T-cell visualization

CD4+ T-cells were visualized in seven to 10 randomly chosen acini using an I2/3 filter block (excitation: 450-490 nm, emission >515 nm; Leitz). Each acinus was put into focus and then recorded on VHS for approximately 13 seconds. In a second step the recordings were evaluated with CAPIMAGE (a video analysis software) and the number of T-cells per acinus were counted. The results are stated in number of CD4 T-cells per acinus.

3.3.4.3. Sinusoidal perfusion

To assess the sinusoidal perfusion the plasma was contrasted with FITC-labeled dextran. Then Seven to ten acini were recorded to show the hepatic perfusion. Non-perfused sinusoids per acinus were counted. The results of this measurements are stated in % of occluded sinusoids of all visible sinusoids in the acinus

3.3.5. Liver enzyme measurements

To quantify the hepatocellular damage aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured in the blood samples that had been taken from the carotid artery at the end of the experiment. The volume of the blood samples averaged around 1ml. These samples were drawn with a small plastic syringe that had been exposed to a 20.000 IE/ml heparin solution. The sample was then put in a small plastic vial and immediately centrifuged at 2000 x g for 10 min.

The plasma was then separated from the cellular components and stored at -80°C until all collected samples were brought to the laboratory for activity measurements. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined at 37°C with an automated analyzer (Hitachi 917, Roche-Boehringer, Mannheim, Germany) using standardized spectrometric test systems (HiCo GOT and HiCo GPT, Roche-Boehringer). The values are presented in IU/L as recommended by the German society for clinical chemistry.

3.3.6. Histology

After the end of the experiments the left liver lobe was extracted and partitioned into six equally sized pieces. Four samples were immediately frozen at -80 degrees Celsius to later be used for western blots. Two samples were put in a solution of 4% paraformaldehyde (pH 7,4) to be conserved. These samples were embedded into paraffin at a later point in time.

3.3.6.1. Staining for α-smooth muscle actin (α-SMA)

Four micrometer thick microtome cut Paraffin-fixed, liver sections were incubated with Animal Research Kit Peroxydase (Dako). We used anti-α-SMA as primary monoclonal antibody (dilution 1:200, Abcam, Cambridge, UK). Next the slices were incubated with horseradish peroxidase-conjugated Streptavidin and diaminobenzidine, and then counter-stained with haemalaun. As negative controls, primary antibody was replaced with non-immune immunoglobulin at the same concentration. No staining was observed in the negative controls. The staining was performed on the liver tissue samples from

the set of experiments including the pharmacological interventions. Therefore, we could study the effects of the substrates on α-SMA expression in response to the I/R damage.

3.3.6.2. Ki67 staining

The Ki67 is a nuclear protein that became a universally used marker of cell proliferation [Gerdes et al., 1983], yet its functional significance has not been fully understood. It is only present within the nucleus during interphase but can then be seen on the surface of chromosomes during mitosis [Scholzen et al., 2000]. Therefore, we used it to assess the hepatocellular proliferation. Ki67 staining of paraffin sections was performed with a commercially available kit (Dako, Hamburg, Germany). The number of Ki67-positive hepatocytes was counted in 10 high-power fields (microscope magnification x 400). All cell counts were performed in a blinded fashion.

3.3.7. Western Blot

Protein expression of CCR5, CCL21, and RANTES was assessed with Western blot in all experimental groups. Western blot was initially carried out in shock-frozen tissue samples. Since we detected little expression in the frozen tissues, we performed a new set of experiments with n=2 in each group to assess the expression from freshly isolated protein. To prep the frozen samples for western blotting a freshly prepared lysis buffer solution was used. The buffer solution consisted of 2ml fresh red blood cell lysis buffer with added DTT (1mM), PMSF (1mM) and fresh serine protease inhibitor at a

concentration of 1:100. Initially the tissue samples were homogenized with the buffer solution (20 µl lysis buffer per mg liver tissue) in a S24 glass pestle homogenizer and then incubated for 15 minutes and swiveled after 10 minutes to mix up the solution. The samples were chilled on ice throughout the preparation. After incubation, the samples were centrifuged at 4 degrees Celsius at 13000 rounds per minute for 15 minutes. The centrifuged sample was then pipetted to a new chilled Eppendorf container without touching the bottom pellet. In this new container, the samples were then mixed up. 50 µl of it was pipetted into a different Eppendorf container for total protein concentration measurements. Both samples frozen at -80 degrees Celsius and later used for western blot. Western blot protocols are widely described in scientific literature and will not be reiterated here.

3.4. Experimental protocols

In reference to the previously stated goals of this study, there were two different study groups with slightly differing protocols:

1. Co-localization protocol of hepatic stellate cells and CD4 positive T-lymphocytes. Because of better spatial resolution and improved ability to use two different dyes, this set up required confocal microscopy.
2. HSC depletion / activation-protocol studying the effect on CD4+ T-cell recruitment and I/R injury.

3.4.1. Co-localization between HSC and CD4+ T-cells after I/R

CD4 T-cell behavior in relationship to hepatic stellate cells was studied in GFP positive mice under identical conditions as the main experimental groups.

3.4.1.1. Experimental groups

As mentioned in the introduction we assumed an interaction between hepatic stellate cells and CD4 T-lymphocytes during post-ischemic reperfusion. To assess this interaction in the hepatic microcirculation after I/R (I: 90 min, R: 120min) we studied an intervention group of n=2 and a control/sham-group of n=3. A greater number of animals was not deemed to be necessary because we expected the used two-photon microscopy to have high specificity and sensitivity to show co-localizations.

There were no drug intervention groups.

3.4.1.1.1. Green fluorescent protein (GFP) positive mice

CX(3)CR1 is a seven-transmembrane receptor which is specific for the chemokine fractalkine. Stimulation of the receptor mediates leukocyte capture in perfused blood vessels [Fong et al., 1998]. This receptor is expressed on activated endothelial cells [Bazan et al., 1997, Pan et al., 1997], neurons [Harrison et al., 1998, Nishiyori et al., 1998] and dendritic cells (DC) [Kanazawa et al., 1999, Papadopoulos et al., 1999] such as the investigated hepatic stellate cells. The mice mentioned here express eGFP at the locus of the Cx3cr1 gene. They can otherwise be considered genetically identical to C57-BL6 mice.

3.4.1.2. Two-photon microscopy

For in vivo two-photon microscopy, we used an upright microscope with a water immersion objective (20 x 0.95 NA; Olympus, Tokyo, Japan) which was connected to a TriM Scope II two-photon microscope (LaVision Biotech, Gottingen, Germany). The two-photon microscope unit that was equipped with a Mai Tai laser tuned at 870nm. The fluorescence signal emitted from the tissue (filters LP495, BP525/50, LP560, LP665) was detected by four photomultiplier tubes. The software system ImSpector (LaVision Biotech) was used for image acquisition and processing. The three-dimensional scans per time point were flattened and visualized in two dimensions as projections onto the X-Y axes over time. In this experimental set, isolated CD4+ T-cells were labeled with eFluor 660 (eBioscience).

3.4.1.3. Experiment procedure

The utilized heterozygote Cx3CR1(gfp/gfp) mice exhibiting green fluorescent protein in HSC [Lee et al., 2010] were used as the experiment animals receiving the standard 90-minute ischemia procedure. CFDA-SE-labeled CD4+ T-cells, isolated and stained with the aforementioned protocol were injected into Cx3CR1 mice after 120 minutes of reperfusion. Interactions between both cell types were analyzed using *in vivo* two-photon microscopy, recorded and later analyzed. CD4+ T-cell-HSC interactions were assessed in 5-7 acini per experiment. In each analyzed acinus, we counted CD4+ T-cells firmly adherent in sinusoids as well as the number of adherent CD4+ T-cells which were colocalized with HSCs within the same acinus. The percentage of colocalized T-cells was calculated as follows: T-cells colocalized with HSCs/adherent CD4 T-cells per acinus x 100%.

3.4.2. HSC activity modulation

3.4.2.1. Experimental groups

Animals	Group	Number
WT C75/BL6 +CD4 T-cells	**Sham no intervention**	**N=7**
WT C75/BL6 +CD4 T-cells	**I/R + JWH 133**	**N=7**
WT C75/BL6 +CD4 T-cells	**I/R + ACPA**	**N=7**
WT C75/BL6 +CD4 T-cells	**I/R + Vehicle (Tocrisolve)**	**N=7**

Table 1 Experimental groups for pharmacological intervention of CD4 T-cell - HSC interaktion

In an attempt to analyze the effect of HSC targeting (depletion vs. activation) a sham-operated group and four I/R groups (I: 90 min, R: 120 min) were analyzed (n=7 each).

3.4.2.2. Experiment procedure

The experimental procedure required to work simultaneously. The different substrates, which were injected intra-peritoneally 24 hours prior to the experiment were:

- Tocrisolve TM 100 solution (200µl, i.p., Tocris Bioscience, Bristol, UK) as vehicle.
- CB-2 agonist JWH-133 [Batkai et al., 2007, Teixeira-Clerc et al., 2010] (i.p., 0.2 mg/kg body weight in 200µl Tocrisolve, Tocris Bioscience) to reach HSC

depletion.

- CB-1 agonist ACPA [Feizi et al., 2008] (i.p., 1 mg/kg body weight in 200µl Toscisolve, Tocris Bioscience) for HSC activation.

Intravital microscopy was started after 120 min of reperfusion and took approximately 20 min. Tissue and blood samples were taken at the end of the experiment.

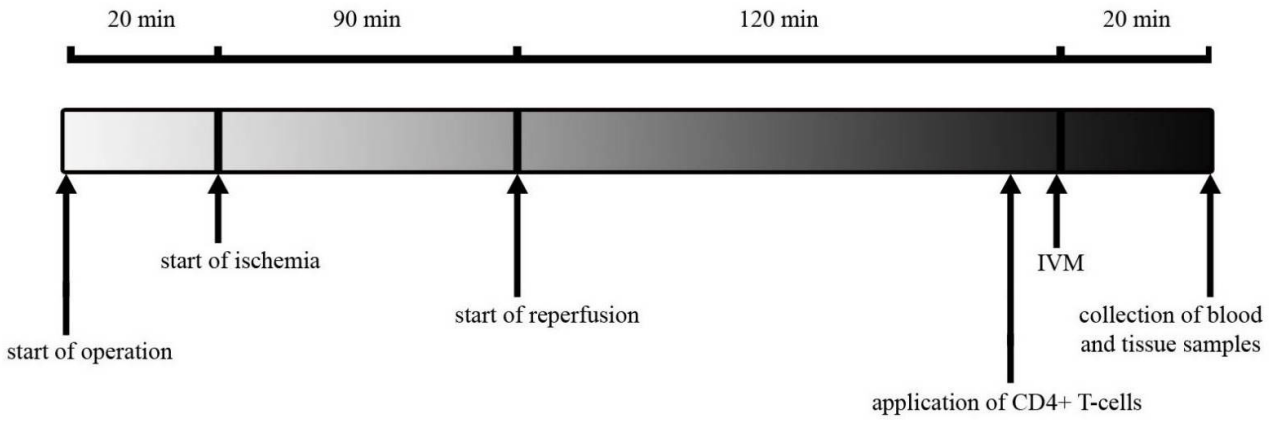

Figure 5 Approximately 250 min of total procedural time. There were of course slight variations in the time prior to ischemia because of anatomic differences between the animals.

3.4.2.2.1. Hepatic stellate cell depletion/activation

Both CB agonists are suspended in Tocrisolve, a water-soluble emulsion composed of a 1:4 ratio of soya oil/water that is emulsified with the block copolymer Pluronic F68.

The CB-2 agonist JWH-133 (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo [b,d]pyran; MW 312, Cat. No 1343) is very potent and selective (Ki = 3.4 nM) to CB-2 receptors. Its affinity towards the CB-2 receptor is approximately 200-fold selective over CB-1 receptors.

ACPA (Arachidonylcyclopropylamide, MW 344, Cat No.1781) is a potent and selective CB1 agonist (Ki = 2.2 nM) that displays 325-fold selectivity over CB-2 receptors [Hillard et al., 1999]. The effects of these agonist were verified with the expression of reelin as well as, more importantly, the anti-α-SMA staining mentioned above.

There were further attempts to portray the effects on the hepatic stellate cells more precisely by assessing the expression of CCR5, CCL21, and RANTES in all experimental groups. Western blot was carried out in shock-frozen tissue samples from all experimental groups as described above. Since we did not detect expression in the frozen tissues, we performed a new set of experiments with n=2 in all groups to assess the expression from freshly isolated protein. As shown by the blots, the expression of the mediators was too low to be detected by the used approach, even if assessed in freshly isolated proteins. Apparently, more sensitive techniques, more strong inflammatory stimulation or longer reperfusion time are required.

The applied concentrations of JWH133 and ACPA were established in separate dose-

finding experiments (n=3 in each substrate group) and are in line with published studies in mice [Feizi et al., 2008, Hillard et al., 1999].

3.5.Statistics

ANOVA on ranks followed by Student-Newman-Keuls method were used for the estimation of stochastic probability in intergroup comparison (SigmaPlot 12, Jandel Scientific, Erkrath, Germany). Mean values ±SEM are given. P values less than 0.05 were considered significant.

4. RESULTS

4.1. Co-localization between HSC and CD4+ T-cells

In our first experiments, we tackled the question of whether CD4+ T-cells are colocalized with HSC in the post-ischemic liver. Freshly isolated and fluorescence-labeled CD4+ T-cells were infused into heterozygote Cx3CR1 (gfp/gfp) mice (mice exhibiting green fluorescent protein-labeled HSCs), and the interaction between CD4+ T-cells and HSCs was visualized in the hepatic microcirculation using intravital microscopy. In sham-operated controls, no CD4+ T-cell-HSC colocalizations were observed. After I/R, 26%±3% of all accumulated CD4+ T-cells were colocalized with HSCs in sinusoids (Figure 6**Error! Reference source not found.** A). This suggests a direct interaction between both cell types. There were more colocalizations (31%±5%) after a prolonged reperfusion time (140 min vs. 120 min, Fig. 1B). The CD4+ T-cell-HSC colocalizations were also shown using the intravital two-photon microscopy. This technique is a superior alternative to intravital fluorescence microscopy and confocal microscopy because of its deeper tissue penetration, efficient light detection, and reduced photo toxicity. Figure 6 (C) to (E) demonstrates proximity and attachment of both cell types in the liver microcirculation.

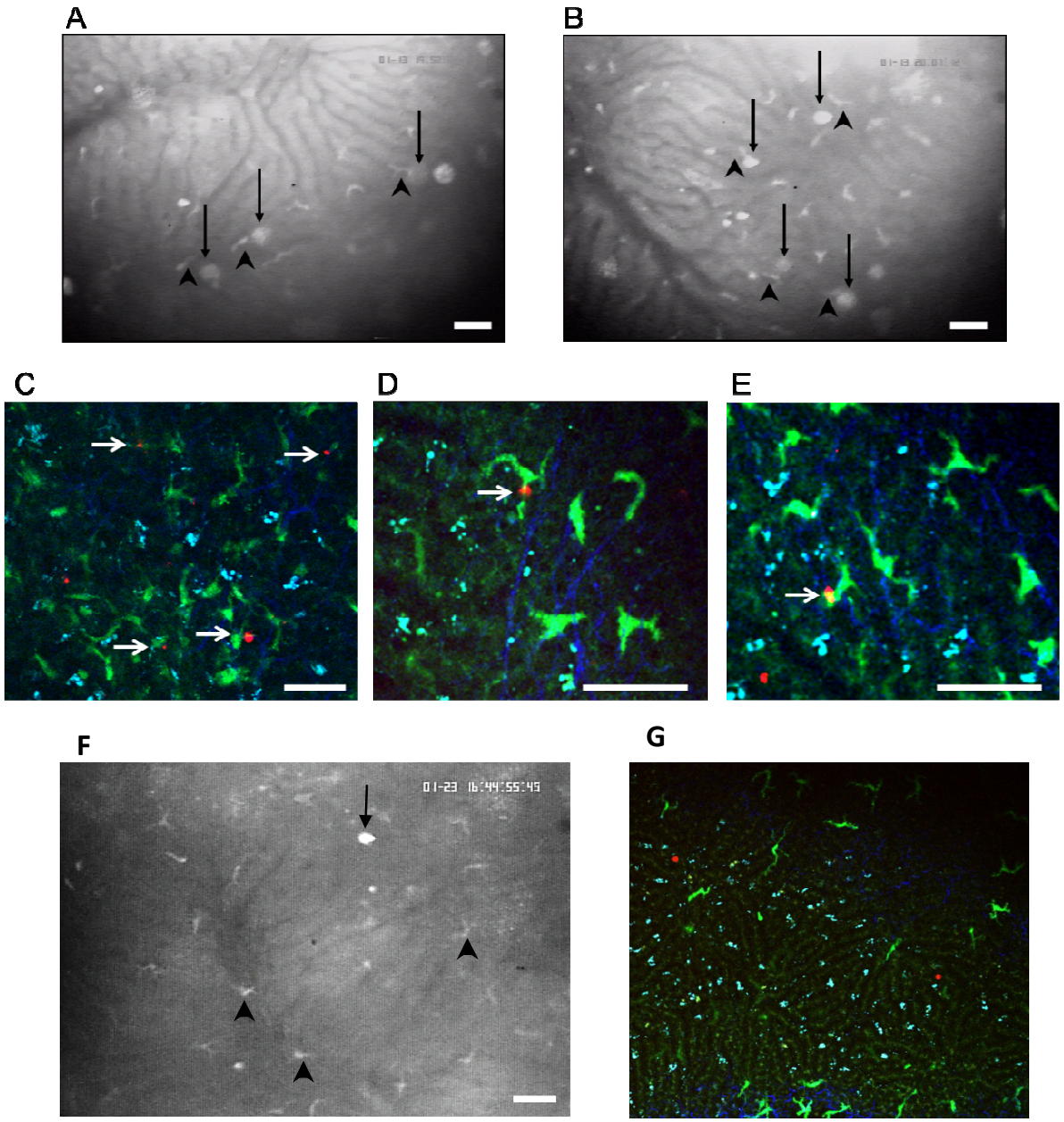

Figure 6 Interactions between HSCs and CD4+ T-cells in vivo. CD4+ T-cell-HSC interactions were analyzed in vivo after infusion of fluorescence-labeled CD4+ T-cells into Cx3CR1 (gfp/gfp) mice (mice exhibiting GFP-labeled HSCs). The intravital microscopic images (upper panel) demonstrate co-localization of CFDA-SE-labeled CD4+ cells (arrows) with GPF-expressing HSCs (arrow heads) in hepatic sinusoids of a mouse after 90 min of ischemia following 120 min (A) or 140 min (B) of reperfusion. Representative images from at least three independent experiments. The bottom panel (C-E) presents imaging of the hepatic microcirculations with multiphoton microscopy in vivo. CD4+ T-cells are labeled ex vivo with eFluor ®660 (red), HSCs are GPF-positive (green). Image C (arrows) shows co-localizations between T-cells and HSCs. We observed proximity between both cell types (D) as well as their attachment (E). The yellow-colored area in (E) is a mixed result of the green and red, reflecting the overlay and attachment of CD4+ T-cells and HSCs to each other. Size bar 50 µm in all images. No co-localization was observed in sham-operated animals (F: intravital microscopy; G: two-photon microscopy). The findings are representative of at least three different experiments per group. [Reifart et al., 2014]

4.2. Immunostaining for α-SMA as a marker of HCS activation

In the next set of experiments, we modulated the HSC activity by stimulating CB-1 and CB-2 receptors with specific agonists. A stimulation of CB-2 leads to HSC apoptosis, whereas CB-1 agonists activate HSCs. Expression of smooth muscle actin (SMA) is a recognized marker of HSC activation in the liver tissue. Using immunostaining, we showed that α –SMA expression was enhanced in the untreated group after I/R as compared to the sham operated controls (Figure 7). In contrast, α -SMA expression was almost absent in the I/R group pretreated with CB-2 agonist JWH-133. In the post-ischemic group, pretreated with the CB-1 agonist arachidonylcyclopropylamide (ACPA), the α -SMA expression was strongly enhanced. Taken together, the HSC activity was negatively influenced by the CB-2 agonist and stimulated by the CB-1 agonist in our model of hepatic I/R.

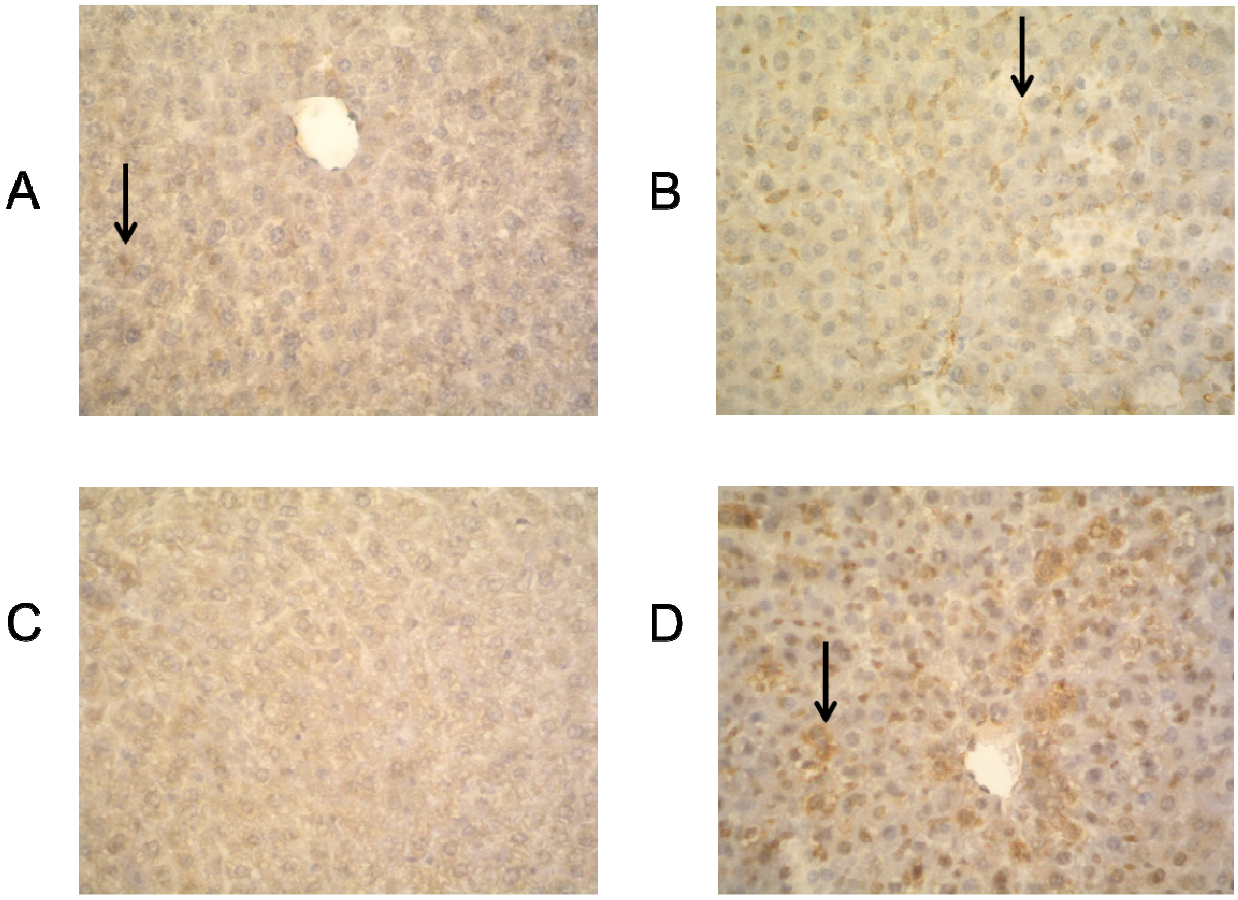

Figure 7 Expression of α-SMA. Microphotographs show immunostaining for α-SMA (arrows) as a marker of HSC activation in the liver tissue of a sham-operated mouse (A), a mouse after I/R (90min/120min) pre-treated with Tocrisolve as vehicle (B), a mouse after I/R pre-treated with CB-2 agonist JWH-133 (C), and a mouse after I/R pre-treated with CB-1 agonist ACPA (D). Microscope magnification ×400. Representative images from seven experiments per group.

4.3. CCR5, CCL21, and RANTES Western blot results

Unfortunately, there was no experimental group in which the expression of CCR5, CCL21, and RANTES could be detected by the used approach. Even when assessed in freshly isolated proteins there was no signal.

4.4. CD4+ T-Cell recruitment

To answer the question of whether the targeting of HSCs through the CB receptors can affect T-cell migration, the recruitment of CD4+ T-cells in the hepatic microvasculature was analyzed using intravital microscopy. As shown in Figure 8, only few CD4+ T-cells were found accumulated in sinusoids of sham-operated mice (2.8±0.2/acinus). In contrast, CD4+ T-cell recruitment was significantly enhanced in the vehicle-treated group after I/R (8.4±0.4/acinus). In mice undergoing HSC depletion with JWH-133, the post-ischemic CD4+ T-cell accumulation was reduced by about 60% ($p<0.05$). The activation of HSCs using ACPA did not significantly influence the post-ischemic T-cell recruitment as compared with the I/R group treated with the vehicle solution. As observed in an additional set of experiments using Cx3CR1(gfp/gfp) mice, the percentage of CD4+ T-cell colocalized with HSCs remained almost unchanged after pretreatment with ACPA (28%±5% and 30%±3% after 120 min and 140 min of reperfusion, respectively) as compared to the vehicle-treated I/R group. Thus, HCS depletion attenuates the I/R-induced CD4+ T-cell migration, whereas HSC activation does not affect it.

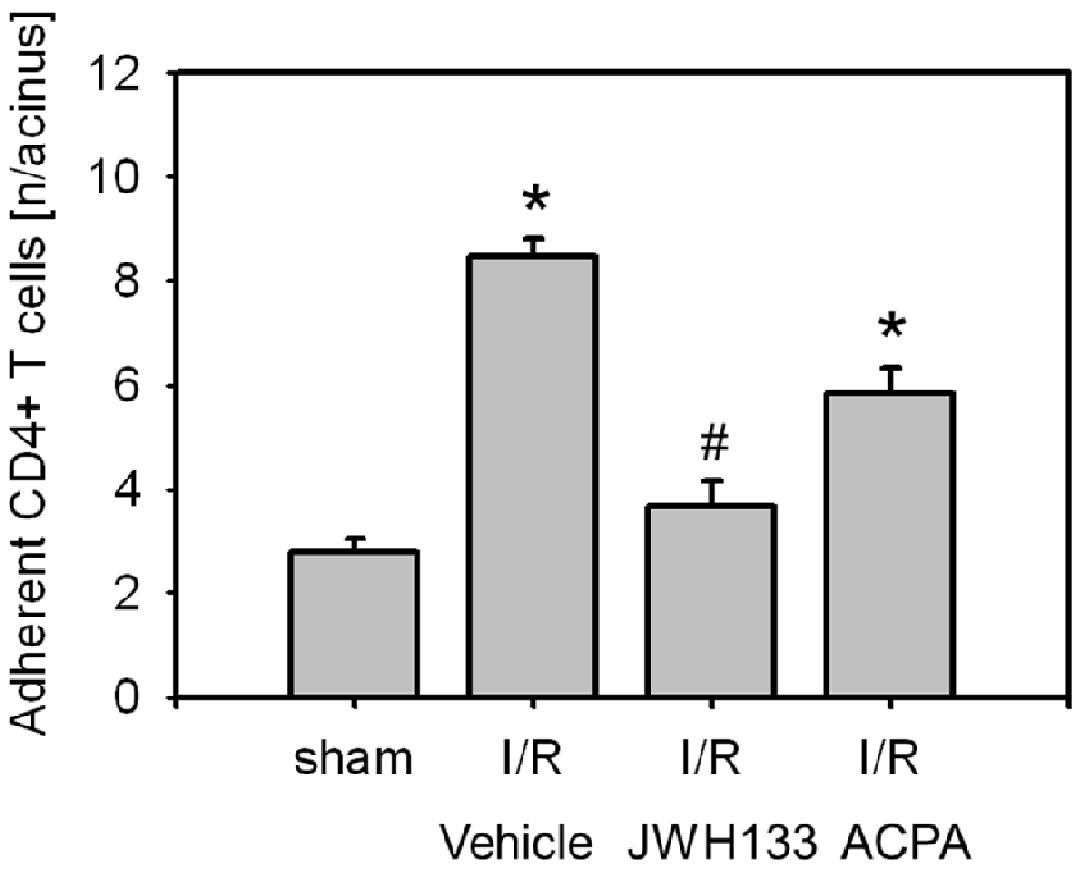

*Figure 8 CD4+ T-cell recruitment. Accumulation of CD4+ T-cells was quantitatively analyzed using intravital microscopy in sham-operated mice (A), mice after I/R (90min/120min) pre-treated with Tocrisolve as vehicle (B), mice after I/R pre-treated with CB-2 agonist JWH-133 (C), and mice after I/R pre-treated with CB-1 agonist ACPA (D). N=7 animals per group, mean ± SEM, *p<0.05 vs. sham-operated group, #p<0.05 vs. I/R + vehicle.*

4.5. Microvascular and hepatocellular I/R injury

Sinusoidal perfusion failure was quantified using intravital microscopy after plasma labeling with FITC-dextran. The data are presented as a percentage of non-perfused sinusoids to all sinusoids visible per acinus. In the sham-operated group, only 7%±1% of all sinusoids were not perfused. In contrast, sinusoidal perfusion failure was 27%±3% in the vehicle-treated I/R group. The pretreatment with JWH-133 significantly improved the post ischemic perfusion, whereas the perfusion failure was even higher (53%±2%) in the ACPA pretreated group as compared to the vehicle-treated I/R group (Figure 9). The liver enzyme activities were determined in serum as markers of hepatocellular necrotic injury. In line with the data on sinusoidal perfusion, we observed a dramatic increase of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the vehicle-treated I/R group. The HSC depletion with JWH-133 significantly reduced the liver enzyme activity (AST by ~2.5-fold, ALT by ~3-fold). The I/R group which underwent HSC stimulation with ACPA, showed comparable AST-ALT activities as the I/R vehicle group (Figure 10).

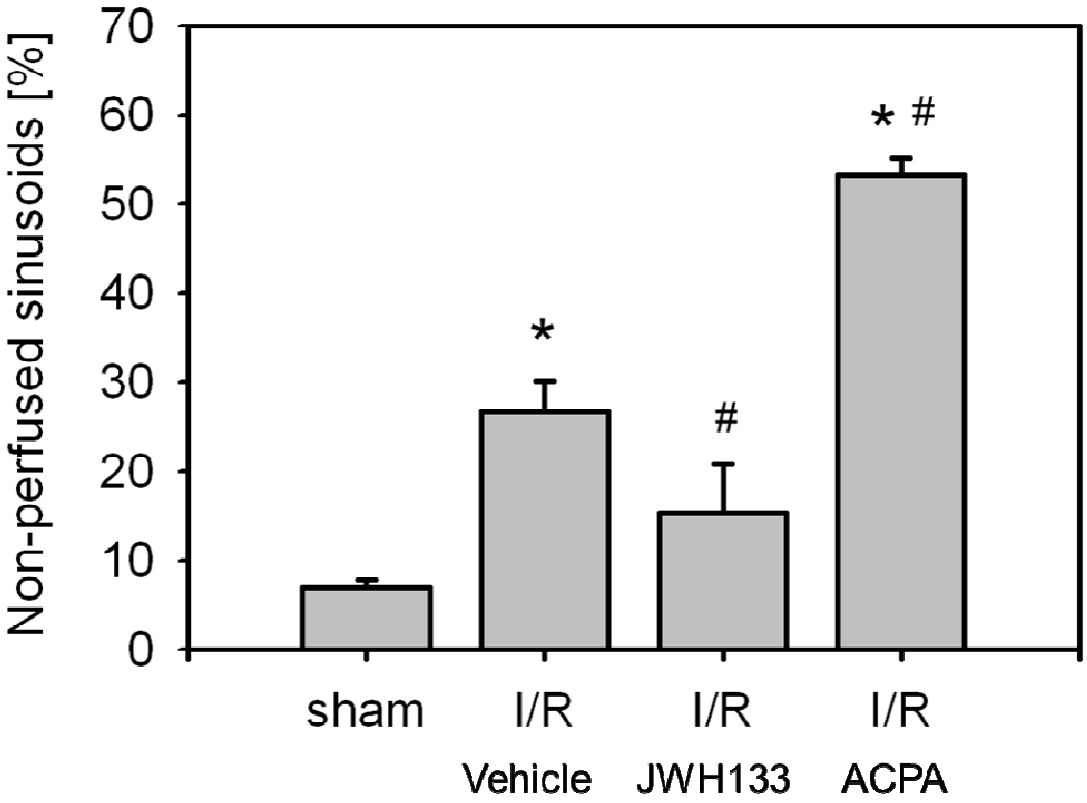

*Figure 9 Sinusoidal perfusion failure. Sinusoidal perfusion failure (=percentage of non-perfused sinusoids) was measured using intravital microscopy as a parameter of microvascular hepatic injury in sham-operated mice (A), mice after I/R (90min/120min) pre-treated with Tocrisolve as vehicle (B), mice after I/R pre-treated with CB-2 agonist JWH-133 (C), and mice after I/R pre-treated with CB-1 agonist ACPA (D). N=7 animals per group, mean ± SEM, *p<0.05 vs. sham-operated group, #p<0.05 vs. I/R+ vehicle.*

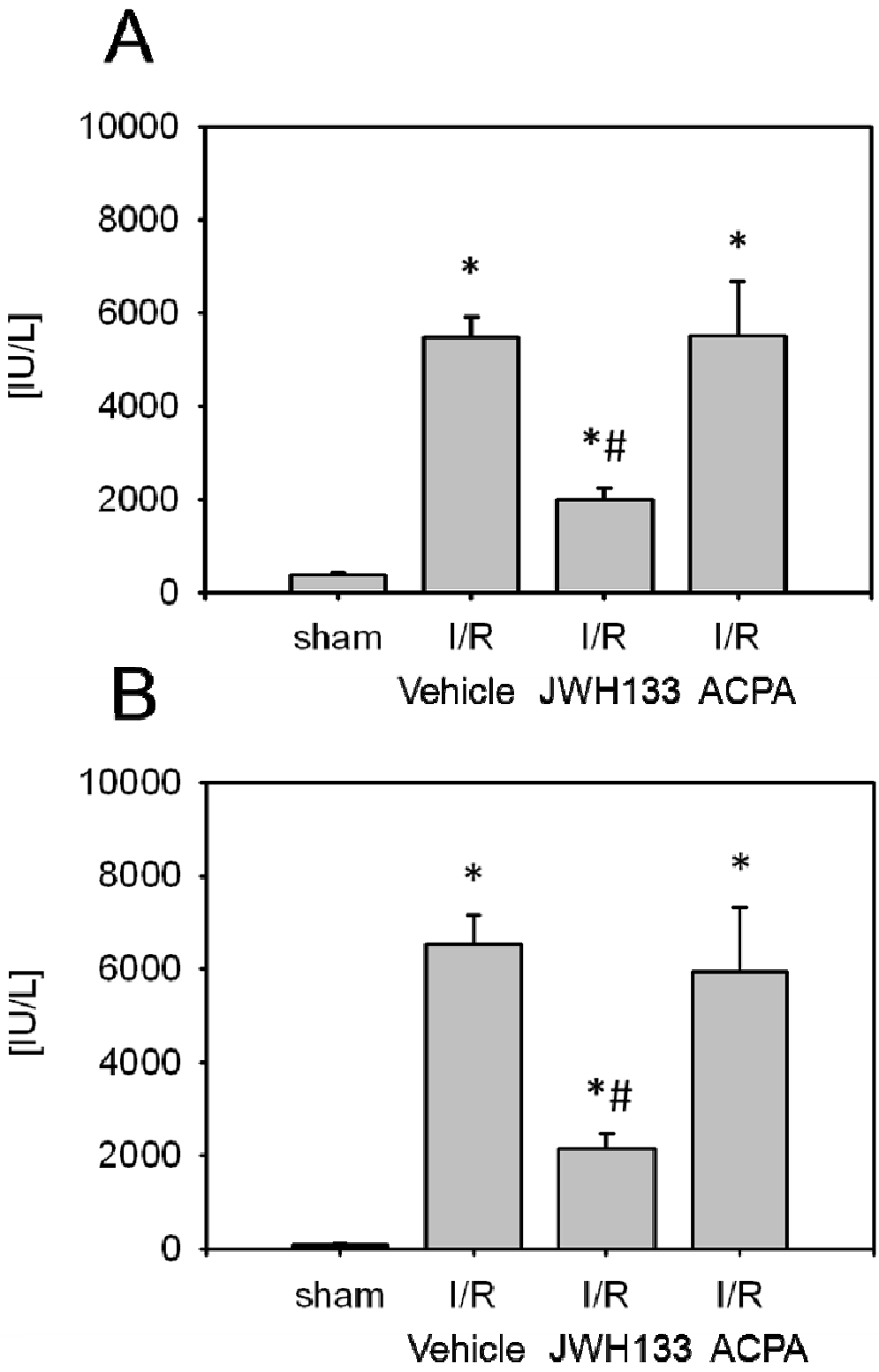

*Figure 10 Liver enzyme activity. Serum activity of the liver enzymes AST and ALT was determined as a marker of hepatocellular necrotic injury in sham-operated mice (A), mice after I/R (90min/120min) pre-treated with Tocrisolve as vehicle (B), mice after I/R pre-treated with CB-2 agonist JWH-133 (C), and mice after I/R pre-treated with CB-1 agonist ACPA (D). N=7 animals per group, mean ± SEM, *p<0.05 vs. sham-operated group, #p<0.05 vs. I/R+vehicle.*

5. DISCUSSION

5.1. Synopsis

This experimental study was designed to observe the modulation of hepatic stellate cells with cannabinoid receptor agonists and the effects of these pharmacological interventions on CD4 T-cell behavior during hepatic I/R in regards to HSCs and total I/R damage. Our main finding is that depleting or deactivating HSCs, with a CB2 receptor agonist, attenuates the I/R-induced CD4+ T-cell recruitment and reduces tissue injury. In contrast, hyper-activation of HSCs, with a CB1 agonist, does not affect CD4+ T-cell migration and even enhances I/R injury, in particular, microvascular perfusion failure. Furthermore, we showed that CD4 + T-cell co-localize with hepatic stellate cells in the perisinusoidal space.

We will initially discuss the methods used and then address points related to our results and what conclusions we draw from our findings.

5.2. Model

5.2.1. Animals

In this study, we strived to show interactions of CD4 T-lymphocytes and hepatic stellate cells in the post-ischemic liver. The pathophysiology of post-ischemic hepatic injury is characterized by the complex interactions of different systems within the healthy macro-organism. Since not all components and influencing factors surrounding the process of I/R are known there is no accurate *in vitro* model. I/R injury is best studied in animals. Two strains of mice, were used. The vast majority of animals used were C57 Black 6 mice. Some experiments required use of heterozygote CxCR1 mice.

To visualize the cells behavior *in vivo,* we needed to use an animal model that allows intravital-microscopic analysis of hepatic microcirculation. Since this is only established in small animals such as mice, hamsters and rats, the possible species for this model were narrowed down. Of these three species, mice are the most immunologically studied. There is a vast array of antibodies available to reliably target specific cell types. The decision to use C57 Black6 mice was fortified by the fact that these animals are especially favored in studies regarding liver microcirculation because of their liver architecture. The sites of relevant pathophysiological effects such as the presinusoidal arterioles, the sinusoids and the post-sinusoidal venules are situated very close to the liver surface and can therefore be studied microscopically [Menger et al., 1999]. The model of reversible liver lobe ischemia in mice is well established [Biberthaler et al., 2001a, Brown et al., 1997, Horie et al., 1997, Khandoga et al., 2002a,

Khandoga et al., 2002b]. Moreover, CD4 T-cells can reliably be isolated from murine spleens. Lastly the availability of GFP positive mice, that allowed us to have hepatic stellate cells be fluorescent was a point in favor this species.

The decision to use heterozygous Cx3CR1(gfp/gfp) mice exhibiting green fluorescent protein in HSC [Lee et al., 2010] was based on the fact that these mice express eGFP at the locus of the Cx3cr1 gene. This causes hepatic stellate cells, as well as other cell types to be fluorescent upon excitation with 395 nm and also, to a lesser extend at 475 nm [Bazan et al., 1997, Harrison et al., 1998, Nishiyori et al., 1998, Pan et al., 1997]. The emission peak of GFP is at 509 nm, which can be seen as green light. Cx3CR1 is a seven-transmembrane receptor for the chemokine fractalkine. Stimulation of the receptor aids leukocyte capture and adhesion in the blood vessel [Fong et al., 1998]. In heterozygous Cx3CR1 (gfp/gfp), fractalkine receptor function is not inhibited which is why these mice are considered phenotypically identical to C57-BL6 mice [Jung et al., 2000, Lee et al., 2010].

5.2.2. Anesthesia and surgical technique

The main anesthetic effect during the surgical intervention can be attributed to isoflurane, which is known to have a low rate of hepatic metabolization in lab animals [Fiserova-Bergerova, 1973, Stevens et al., 1975]. In previous studies by this group the surgical technique proved to give good results in regards to hemodynamic stability [Khandoga et al., 2002b]. The surgery was reproducible at a high standard of quality after a short learning period. As reported before, there were no significant effects on murine mean arterial pressure and temperature comparing sham and ischemia

interventions [Hanschen et al., 2008, Khandoga et al., 2002a, Khandoga et al., 2004, Khandoga et al., 2002b]. It seems possible that the median laparotomy as well as the placement of the micro clip on the left liver lobe and the subsequent manipulation of the liver to allow intravital microscopy could hemodynamically affect the other abdominal organs but this would lead to an equal effect in all intervention groups. Regarding the inhalation anesthesia with persistent spontaneous breathing: Any negative influence of these effects on the studied parameters were deemed to be negligible [Andreen et al., 1981, Gelman, 1976]. By closing the median laparotomy with a suture during all periods that did not require hepatic manipulation or inspection we also designed to keep a more physiologic intra-abdominal environment with as little temperature and fluid loss as possible.

The intraperitoneal injection of the CB agonists prior to the experiments could be seen as potential cause of inflammation. Especially if the intestinal wall is penetrated. However, the surgical technique – injecting the solution under vision - makes this an unlikely factor.

5.2.3. T-cell isolation and staining

To track CD4+ T-cells movement *in vivo* during the intravital microscopic studies T-lymphocytes were isolated from spleens of syngeneic mice (C57/BL6) and then dyed with CFSE (carboxyfluorescein diacetate succinimidyl ester). CFSE is well established to dye and track lymphocytes *in vitro* and *in vivo* [Parish, 1999]. Since CFSE can be toxic for cells [Quah et al., 2007] we chose a concentration well within the non-toxic concentrations. Since the cells only had to be tracked for a short time as opposed to

studies that investigate lymphocyte proliferation. Lower concentrations of dye did not affect cell tracking quality. FITC-labeled dextran was used for sinusoidal perfusion assessment.

The T-lymphocytes were isolated with the MACS® Separation System (Miltenyi Biotec, Bergisch-Gladbach, Germany) using anti-mouse CD 4+ antibody labelled magnetic beads following the manufacturer's instructions [Miltenyi et al., 1990].

The result is a solution rich in CD4 T-cells. The concentration of isolated CD4+ T-cells was assessed with a Coulter-Counters (Coulter® AC-T Series; Coulter Corporation, Miami, USA). In investigations establishing this method the purity of the CD4+ T-cells subsets was routinely >95% as determined by fluorescence activated cell sorting analysis [Hanschen et al., 2008]. A total of $1x10^7$ CFSE-labeled CD4+ T-cells was infused intra-arterially after 120 min of reperfusion and then visualized using intravital microscopy. Because prior research in a similar model showed that injection of activated T-cells can lead to near exclusive accumulation in the liver [Klugewitz et al., 2002] it was important to verify that the isolation process did not activate the T-cells. This was tested by measuring the expression of CD62L, CD44, and CD69, which are known parameters of cellular activity [Goodison et al., 1999, Lopez-Cabrera et al., 1993, Ryan et al., 1992]. CD4+ cell viability following the isolation procedure was tested using propidium iodide. T-cell vitality depends on the integrity of the cell membrane. Propidium iodide will only be enriched in cells with a compromised membrane, which are therefore not seen as vital [Hanschen et al., 2008, Jin et al., 2007]. Viability was approximately 94% as determined when this model was established [Hanschen et al., 2008, Khandoga et al., 2006]. It was therefore save to conclude that

the injected T-lymphocyte solutions were rich in viable, non-activated CD4 positive T-cells. Splenic CD4 T-cells are described to be slightly different to hepatic CD4 T-cells in regards to their cytokine profile [Katz et al., 2005]. It is possible that splenic CD4 T-cells therefore have varying response patterns as compared to peripheral or hepatic T-lymphocytes.

5.2.4. Intravital fluorescent microscopy

In vivo intravital microscopy is a well-established method to observe physiological, as well as pathophysiological processes in different organs. Applying this method to observe the microcirculation [Messmer et al., 1998] is also very common. Some examples are the study of microcirculation in the arteries [Massberg et al., 2003], the lung [St Croix et al., 2006], the skin [Lindenblatt et al., 2007], the muscle [Rotter et al., 2012] or the pancreas [Cardini et al., 2014, Preissler et al., 2006], to mention a few. Different wave-lengths of light and a variation of available dyes allows selective staining of cells and compartments. Phototoxic effects [Saetzler et al., 1997] of exposure to light and any of the dyes can be disregarded since this effect is not reported for any of the dyes used. Nonetheless we tried to keep the time of intra-vital microscopy short (approximately 20 minutes) and use the smallest amount of light intensity without compromising the quality of the results in any way [Steinbauer et al., 2000].

5.2.4.1. Measuring adherent T-cells

Quantitative analysis of leucocyte accumulation and emigration in post-ischemic liver tissue has previously been studied with intravital microscopy [Vollmar et al., 1995, Vollmar et al., 1994b, Vollmar et al., 1996]. Newer work of our study group demonstrated that CD4 T-cells were behaving similarly [Hanschen et al., 2008,

Khandoga et al., 2006], although involvement of this cell type in I/R pathophysiology had already been seen *ex vivo* in immunohistology [Zwacka et al., 1997].

Counting the labeled lymphocytes per acinus in up to 10 acini per animal allowed us to calculate mean adherent T-cell level that could be compared between the different groups.

5.2.4.2. Sinusoidal perfusion

Sinusoidal perfusion is crucial to maintain regular organ function and serves as an important functional parameter of hepatic microcirculation [Horie et al., 1998]. Measuring the sinusoidal perfusion deficit is an established method that correlates with the severity of hepatic I/R tissue damage *in vivo* [Biberthaler et al., 2001b, Kondo et al., 1998, Menger et al., 1999] but in itself is also causing a prolongation of focal hypoxia /anoxia as well as loss of endothelial integrity, which lead to edema formation and oncotic necrosis as alluded to in the introduction[Mende et al., 2014].

In our experiments, several acini were filmed for approximately 20 seconds. The captured images were then evaluated off-line.

5.2.5. Ischemia and reperfusion times

Hepatic ischemia times from 30 to 90 minutes' lead to clinically relevant damage [Sawaya et al., 1999, Yadav et al., 1998]. Past 90 minutes of warm ischemia the damage to the murine liver is thought to be irreversible and the modulation of cell interactions does not seem to have a relevant effect anymore [Yadav et al., 1999]. Because of this

we decided to have 90 minutes of ischemia.

A reperfusion time of 120 minutes was chosen to allow enough time for reperfusion damage and the resulting inflammatory cascades to take effect as well as leaving sufficient time to be able to maintain a high quality of T-cell isolation and staining, which had to be done simultaneously.

5.2.6. Pharmacological intervention with CB-1 and CB-2 agonists.

We were investigating the interaction of T-cells with HSC and the effect of pharmacological depletion/deactivation or activation of HSC. There are different ways to deplete HSC. Using Ganciclovir in transgenic mice expressing herpes simplex virus thymidine kinase (TK) under the glial fibrillary acidic protein (*GFAP*) promoter is a new and successful method [Puche et al., 2013, Stewart et al., 2014]. Also, the use of gliotoxin [Orr et al., 2004] or gliotoxin-coupled antibodies [Ebrahimkhani et al., 2011] showed promise. However, gliotoxin also has broad effects in vivo and in culture, targeting not only HSC, but also immune and endothelial cells and hepatocytes [Hagens et al., 2006]. We decided to use substrates that act through CB-1 and CB-2 cannabinoid receptors because this gave us the opportunity to make the desired interventions on the same receptor family while using the same vehicle medium (Tocrissolve). Both cannabinoid receptors are highly expressed on HSCs and their activation leads to opposite effects on HSC activity [Siegmund et al., 2008]. JWH-133 that acts through CB-2 stimulation was used to deplete HSCs [Siegmund et al., 2007, Siegmund et al., 2008] *in vivo.* The hepatoprotective effect of CB-2 stimulation was demonstrated before [Teixeira-Clerc et al., 2010]. Increased activation of HSC as opposed to depletion was the result of pretreatment with ACPA, a CB-1 agonist. The dosage of 0.2 mg/kg of

JWH-133 was chosen because of successful ischemia/reperfusion damage experiments with this drug in murine kidneys [Feizi et al., 2008].

CB-1 agonist ACPA was used at a dosage of 1 mg/kg for HSC activation. This dosage was used after own dosage finding experiments as well as existing literature [Feizi et al., 2008, Hillard et al., 1999].

CB receptor are present on immune cells at different concentrations [Bouaboula et al., 1993, Galiegue et al., 1995] - any modification of the injected T-lymphocytes can however be dismissed since they were not exposed to the cannabinoid agonists. Still effects of the CB agonists on other cell types within the mouse that is being investigated are hard to control for and may for example affect the amount of adherent T-cells because of reduced interaction with endocannabinoid-affected endothelial cells [Rajesh et al., 2007].

Direct toxic effects of the agonists on the liver can be disregarded, there are no known reports of hepatotoxicity for these two substances in the scientific literature.

5.2.7. Liver enzymes as markers of hepatocellular damage

Serum activity levels of AST and ALT correlate with hepatocellular damage [Balazs et al., 1961, Korsrud et al., 1972, Korsrud et al., 1973] - they are commonly referred to as transaminases. Alanine transaminase (ALT, also ALAT or GPT) is routinely found in the plasma and other tissues but is most strongly associated with liver damage [Ghys et al., 1975]. Aspartate transaminase (AST, also ASAT or GOT) is also somewhat specific to the liver but is also found in the heart [Wu, 1999], the skeletal muscle [Nathwani et al., 2005], the small intestine [Yamamoto et al., 2001] and the kidneys [Bhargava et al., 1968]. Since a combination of both of these enzymes is more specific to hepatic

damage than just one, we used them as an additional marker to compare the results of our experimental groups.

5.2.8. Immunohistochemistry

To gain more detailed data about HSC activity levels in response to I/R and our pharmacological intervention we prepared immunohistochemical staining of paraffin slices. Immunohistochemistry is well established since the middle of the last century [Coons, 1951]. We settled on α-SMA and reelin staining, although recent findings show, that cytoglobin an ubiquitously expressed type of hemoglobin also serves as marker of HSC fibrosis induction/activation [Motoyama et al., 2014].

5.2.8.1. Staining for alpha-SMA

Expression of α-SMA is a recognized parameter of HSC activation routinely used in numerous studies investigating effects of HSC depletion or activation *in vivo* [Bansal et al., 2014, Fan et al., 2013, Puche et al., 2013, Taimr et al., 2003, Yang et al., 2014].

Some authors even define the induction of α-SMA as the single most reliable marker of stellate cell activation because it is absent from other resident liver cells in either normal or injured liver except the smooth muscle cells surrounding large vessels [Friedman, 2008]. Hepatic stellate cells reproducibly express alpha-SMA in inflammatory settings preceding liver fibrosis [Carpino et al., 2005].

5.2.8.2. Staining for CCR5, CCL21, and RANTES

The Chemokines CCL21 [Bonacchi et al., 2003], RANTES, and CCR5 [Schwabe et al.,

2003] are produced by activated stellate cells. But even though methodically the established staining resulted in positive controls. The samples of hepatic tissue showed no results for these markers. In all likelihood, these markers are not produced in quantities that allow for significant staining.

5.2.9. CCR5, CCL21, and RANTES Western blot results

Analogous to the immunohistochemistry there were no usable results of our Western blots investigating HSC activity. Presumably, more sensitive techniques, more strong inflammatory stimulation or longer reperfusion time are required.

However, the expression of these chemokines seems to be more interesting in context of mechanisms potentially responsible for the cross-talk between HSCs and T-cells [Bonacchi et al., 2003, Seki et al., 2009] rather than their value as markers of HSC activation. In fact, these chemokines are also expressed in other cells (monocytes, T-cell subpopulations, platelets) and none of them is very specific for HSCs. In an ongoing follow-up study, we analyze the role of chemokines for HSC-CD4 T-cell interactions during hepatic I/R and are establishing in situ hybridization to assess the expression of chemokines and their receptors in our model.

5.3. Discussion of results

Research on hepatic I/R injury is clinically relevant for liver-transplantation as well as hepatic trauma or shock. The main site in which I/R injury occurs is the hepatic microvasculature [Bilzer et al., 2000, Clemens et al., 1997, Jaeschke, 1998, Menger et al., 1999]. There is increasing evidence how critically involved CD4+ T-cells are in the induction of I/R injury of the liver [Anselmo et al., 2002, Caldwell et al., 2005, Hanschen et al., 2008, Khandoga et al., 2006, Kuboki et al., 2009]. This study, just as studies showing the protective effects of immune-suppressant drugs like Tacrolimus on antigen independent ischemia-reperfusion damage [Kawano et al., 1995] and studies involving T-cell deficient mice [Shen et al., 2003] demonstrate this clearly.

5.3.1. T-lymphocyte migration

The exact mechanisms that control post-sinusoidal CD4+ T-cell migration and cell-cell interaction during alloantigen-independent post-ischemic inflammation remain unclear and it will take many more experimental studies to illuminate this topic. Undoubtedly there is a multistep cascade that controls T-cell migration into inflamed tissue. This process includes intravascular adhesion, transendothelial migration and finally, interstitial migration to the site of injury within the parenchyma, similar to what has been described for neutrophils [Ley et al., 2007].

In our previous work, we described the recruitment of T-cells in the post-ischemic hepatic microcirculation *in vivo* and analyzed the mechanisms of their activation and *intravascular* adhesion during alloantigen-independent hepatic inflammation

[Khandoga et al., 2006].

This could also be seen in this study. We previously also described interactions between CD4+ T-cells and platelets in post-ischemic hepatic sinusoids and suggested a reciprocal activation between CD4+ T-cells and endothelial cells via CD40-CD40L and CD28-B7 co-stimulation, which was independent from MHC II-TCR binding. In yet another study Kupffer cells triggered CD4+ T-cell activation in the post-ischemic liver by releasing reactive oxygen species, IL-6, and TNF-α [Hanschen et al., 2008]. Depletion of Kupffer cells with GdCl3 led to decreased numbers of CD4 T-cells in post-ischemic liver tissue. T-cell activation by Kupffer cells is MHC II independent as well.

Even though hepatic immune-dependent damage is formally alloantigen-independent there seems to be some evidence that CD4 activation in I/R is possible through both allogen-independent –chemokine driven- and antigen-dependent - MHC II driven-pathways [Kuboki et al., 2009].

After their intravascular adherence CD4+ T-cells migrate through the sinusoidal layer to the site of injury. Matrix metalloproteinases play a role in this process. MMP-9 hat been shown to be activated in the post-ischemic liver. MMP-9 seems to be crucial for the recruitment of not only CD4+ T cells but also neutrophils [Leppert et al., 1995, Weeks et al., 1993]. A pharmacological MMP-9 inhibition led to moderate attenuation of early microvascular, necrotic, and apoptotic hepatocellular I/R damage as well as postoperative survival.

There are multiple ways to attenuate hepatic I/R damage. Targeting T-cells is a newer therapeutic focus although multiple strategies such as application of augmenter of liver

regeneration (ALR) and blocking protease-activated receptor 4 have been seen to reduce I/R damage as well as CD4 T-cell infiltration [Khandoga et al., 2014, Mende et al., 2014].

The exact pathophysiological role of emigrated T-cells remains unclear, but in this study, we were able to add hepatic stellate cells to the list of cells T-lymphocytes potentially interact with following I/R.

5.3.2. Hepatic stellate cells

HSCs are located in the subendothelial space, between the basolateral surface of hepatocytes and the anti-luminal side of sinusoidal endothelial cells. They comprise approximately one-third of the non-parenchymal cell population and approximately 15% of the total number of resident cells in the liver. HSCs are normally in a quiescent state and change into their activated state upon injury such as viral infections or hepatic toxins. Once activated, HSCs receive signals from damaged hepatocytes and immune cells through secreted molecules, causing them to differentiate into activated myofibroblast-like cells (reviewed in [Friedman, 2008]). As the primary extracellular matrix–producing cells in the liver, activated stellate cells generate a temporary scar at the site of injury to protect the liver from further damage. They also secrete an array of cytokines and growth factors which promote the regeneration of hepatic epithelial cells [Yin et al., 2013].

Hepatic stellate cells are known to express cannabinoid receptors (CB1 and CB2) and their cell status can be modified by these receptors. Seemingly the role of Cannabinoid receptors and their stimulation in the setting of I/R has not been focused on. CB1 is said to have a profibrotic effect, whereas CB2 is said to have an antifibrotic effect.

Cannabinoid receptor recently emerged as a potential target for antifibrotic therapies [Mallat et al., 2007].

We documented the effects of CB1 and CB2 cannabinoid receptor agonists on hepatic I/R damage as well as on the recruitment of CD4 positive T-cells because it has been established that HSC could be deactivated/depleted using endocannabinoids [Wojtalla et al., 2012]. Deactivating HSC could therefore possibly protect from HSC-mediated injury and hepatic fibrosis - a feared long term complication of liver transplants [Giannone et al., 2012].

The modulation of HSC activity has been a difficult task. In particular, because HSCs activated in culture do not fully reproduce the changes in gene expression observed *in vivo*, making it difficult to correlate *in vitro* results with HSC behaviors *in vivo* [De Minicis et al., 2007]. As discussed above several models have been established to deplete HSCs *in vivo* so far. In our study, we modulated HSC activity by using CB-1 and -2 agonists. Both cannabinoid receptors are highly expressed on HSCs and their activation leads to opposite effects on HSC activity [Siegmund et al., 2008]. We observed in our study that CB-2 stimulation resulted in HSC depletion *in vivo*, since only a very low expression of α-SMA was detectable in the post-ischemic liver tissue. In contrast, pretreatment with a CB-1 agonist massively affected α-SMA expression. Our data suggests that CB-1 stimulation induces a (hyper-)activation of HSCs as compared to the sham-operated animals without ischemia and even to the vehicle-treated mice undergoing I/R.

5.3.3. HSC – T-cell interaction

Central findings of our study are two photon microscopy visualizations of co-localized adherent CD4+ T-cells and HSCs during hepatic I/R *in vivo*. Such proximity suggests a direct interaction between these cell types. A direct interaction like such requires a binding between an adhesion receptor and a corresponding counter-receptor or a ligand on the cell surface.

Interaction of CD4 T-cells with APC has been documented numerous times. Dendritic cells (DC) are activated by a Toll-like receptor-mediated pathway and respond with increased expression of MHC class II during hepatic I/R injury, which leads to antigen-dependent activation of CD4+ T cells [Loi et al., 2004, Tsung et al., 2007].

The integrin protein LFA-1 on the T-cell and Intercellular Adhesion Molecule-1 (ICAM-1) on the APC are the primary molecules of adhesion in this cell-cell interaction [Dustin et al., 1989]. In fact, ICAM-1, a member of the immunoglobulin superfamily as well as MHC II, is expressed and up-regulated on activated HSCs and therefore may be responsible for the observed HSC-CD4+ T-cell interactions [Yin et al., 2007].

The HSC-T-cell proximity was more frequently observed after prolongation of reperfusion time. This is not surprising, since we also see more adherent CD4+ T-cells in the hepatic microvasculature after longer reperfusion times. Moreover, HSCs are able to move towards certain stimuli [Yang et al., 2003]. Such chemotaxis of HSCs might also increase the frequency of T-cell-HSC interactions.

As shown by our immunostaining results, hepatic I/R leads to HSC activation during early reperfusion measured by α-SMA expression in the liver tissue. We believe these more active HSCs are more likely to interact with T-cells leading increased immune-

derived post-ischemic damage. Therefore, it is of interest to modulate HSC activity to then reduce the subsequent damage.

Depletion of HSCs with a CB-2 agonist not only attenuated CD4+ T-cell migration but also reduced the microvascular and hepatocellular injury as shown in our study by measurement of the liver enzyme activity and by analysis of the sinusoidal perfusion *in vivo*. Whether the effect of CB-2 stimulation is gradual and dose dependent or if it is a binary effect that is seen at a certain concentration of JWH-133 cannot be concluded from our data and needs to be further investigated.

The significant role of CD4+ T-cells during alloantigen-independent post-ischemic hepatic inflammation has been demonstrated previously and interventions targeted at CD4+ T-cell activation or migration have had a clear therapeutic impact [Khandoga et al., 2006, Shen et al., 2009, Zhang et al., 2013]. The effects of the CB-2 agonists could possibly protect the liver via pathways other than the here postulated CB-2-HSC-T-cell axis. Activation of CB-2 receptors by specific agonists, such as JWH133 and HU-308, protected against I/R damage by decreasing neutrophil infiltration, tissue and serum TNF-α, chemokines macrophage-inflammatory protein-1α and macrophage-inflammatory protein-2 levels, caspase 3 activity, tissue lipid peroxidation, and expression of adhesion molecule intercellular adhesion molecule-1 [Batkai et al., 2007, Mukhopadhyay et al., 2011]. These agonists also decreased the TNF-α-induced expressions of endothelial adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 in human liver sinusoidal endothelial cells *in vitro.* This effect was prominent when the CB-2 agonist was applied shortly before the damage [Feizi et al., 2008] therefore we believe this effect to be a more immanent response to CB-2

stimulation and hence not completely applicable to our experimental setting. We believe this because any G-receptor mediated effects might not be as prominent after 24 hours as compared to irreversible effects such as HSC apoptosis.

5.3.4. Hepatocellular damage and perfusion failure

Hyper-activating HSCs with the CB-1 agonist ACPA does not additionally enhance hepatocellular injury (determined by the liver enzyme activity) compared with the I/R-induced activation. It did however, markedly increase sinusoidal perfusion failure. The post-ischemic shutdown of the hepatic microcirculation is triggered by sinusoidal narrowing caused by endothelial cell edema [Vollmar et al., 1994a], by activated Kupffer cells and by HSC-mediated vasoconstriction [Bauer et al., 1994, Pannen et al., 1998]. Therefore, it seems likely that HSC hyper-activation via CB-1 receptors increases sinusoidal resistance through stellate cell contractility, which further deteriorates post-ischemic tissue perfusion. There was a non-significant difference in numbers of adherent T-cells and almost equal transaminase levels between the CB-1 and the regular I/R group. It is not clear if a greater number of experiments would have shown a significant difference in T-cell adherence between these two groups. The very similar transaminase levels surprisingly did not really reflect the increased perfusion damage. One could speculate that the effect of the CB-1 agonist on the liver led to decreased immune mediated damage (which could be demonstrated by lower leukocyte adherence) but increased perfusion mediated damage. It seems the increased activation of HSCs as measured through alpha-SMA did not lead to immanently increased expression of the factors involved in T-cell attraction/interaction.

5.3.5. Markers of proliferation

Although activated HSCs promote liver regeneration after liver injury [Khan et al., 2007], we did not observe any changes in the number of Ki67-positive, proliferating cells in all experimental groups. We assume, the reperfusion time of two hours to be too short to detect a proliferative response in this model.

There is significant evidence that HSCs can modulate the hepatic immune response. They are known to have various immune functions, which range from immunogenic antigen presentation over mechanism for inhibiting T-cell-mediated cytotoxicity to even the induction of T-cell apoptosis [Jiang et al., 2013, Xia et al., 2013, Zhao et al., 2012] . Indeed, activated HSCs can stimulate T lymphocytes and cause lymphocyte proliferation due to their function as professional APC [Unanue, 2007, Vinas et al., 2003, Winau et al., 2007]. Furthermore, activated HSCs produce chemokines such as monocyte chemotactic peptide, CCL21, RANTES, and CCR5, which could play a role for T-cell activation during inflammation or I/R but direct detection of these chemokines was not possible [Schwabe et al., 2003].

Activated HSCs do express the co-stimulatory molecule B7-H1, which can bind to counter-receptor programmed death ligand-1 (PD1) on T-cells. PD1 is expressed on a range of immune cells including CD4+ T-cells and, at very low levels, PD1 activation is sufficient to inhibit the earliest stages of T-cell activation [Friedman, 2008, Yu et al., 2004]. This shows that hepatic stellate cells can exert a protective effect on hepatic tissue. An immunotolerizing role is also suggested by experimental models in mice in which transplanted stellate cells protect islet allografts from rejection [Chen et al., 2006] and enhance engraftment of transplanted hepatocytes [Benten et al., 2005]. Therefore,

there might be more negative effects to HSC depletion that could not be observed in this acute study. Yet the overall results lead to an improved understanding of HSC in hepatic I/R.

5.4. Conclusion

In summary, our *in vivo* data suggest that

I. CD4+ T-cells co-localize and interact with HSCs upon their migration into the hepatic parenchyma;

II. a selective depletion/deactivation of HSCs via CB-2 activation reduces CD4+ T-cell-dependent I/R injury,

III. HSC hyperactivation via CB-1 receptors is not protective and even enhances perfusion failure.

5.5. Prospects

In light of our findings we can assume that HSCs might represent a potential target for future therapeutic strategies against T-cell-mediated I/R injury during liver transplantation. The results do not allow us to draw the conclusion that HSC depletion only attenuates injury mediated by T-lymphocytes. In all likelihood, more cells are involved and responsible for I/R injury in this context. There is a multitude of aspects that must be clarified to advance on this topic and a potential use of CB agonists in the context of liver transplantation cannot be expected any time soon. The next step might be to investigate other cell types that interact with hepatic stellate cells, as well as trying to grasp possible negative effects of hepatic stellate depletion, as well as boarder effects of CB-2 receptor stimulation in a chronic mouse model. Still vast amount of research in this field shows that there are many possible to approaches to tackle the problems associated with hepatic I/R. Hopefully some of these creative solutions will transition into the clinical work and someday help to improve patient's prognosis.

6. ABSTRACT

Background. CD4+ T-cells play a critical role during hepatic ischemia-reperfusion (I/R) injury although the mechanisms of their migration in the post-ischemic liver remain unclear. Recent studies suggest that emigrated T-cells are colocalized with hepatic stellate cells (HSCs) during viral hepatitis. We answered the questions of whether hepatic stellate cells (HSCs) interact with CD4+ T-cells during I/R of the liver and whether modulation of HSC activity affects T-cell-dependent I/R injury.

Methods. In mice, migration of CD4+ T-cells was analyzed in vivo using conventional intravital microscopy and two-photon microscopy in sham-operated mice and in mice after I/R (90 min/120min). CD4+ T-cell-HSC interactions were visualized after infusion of fluorescence-labeled CD4+ T-cells into Cx3CR1 mice (mice exhibiting GFP-labeled HSCs) after I/R. Because the activation of HSC is controlled by endocannabinoid receptors, CB-1 and CB-2, the mice received treatment before I/R with the CB-2 agonist JWH-133 to reach HSC depletion or the CB-1 agonist arachidonylcyclopropylamide to activate HSCs. Sinusoidal perfusion and liver transaminases were used as markers of I/R injury.

Results. Hepatic I/R induced CD4+ T-cell recruitment in sinusoids. More than 25% of adherent CD4+ T-cells were colocalized with HSCs during reperfusion after ischemia, but not in the sham-operated mice. This is suggesting a direct cell-cell interaction. The HSC deactivation with JWH- 133 significantly attenuated the CD4+ T-cell recruitment

in the post-ischemic liver and reduced I/R injury as compared to the vehicle-treated group. The HSC hyperactivation by CB-1, however, did not affect T-cell migration and even increased perfusion failure.

Conclusion. Our in vivo data suggest i) that CD4+ cells interact with HSC upon their migration thought the endothelial layer; ii) a selective depletion/deactivation of HSC reduces T-cell-dependent I/R injury, whereas a HSC hyperactivation even accelerates the injury. Taken together, HSC might represent a potential target for future therapeutic strategies against T-cell-mediated I/R injury.

7. ZUSAMMENFASSUNG

CD4+T Zellen spielen eine zentrale Rolle während des Ischämie-Reperfusions Schadens. Dennoch sind die genauen Mechanismen der Zell Migration in der post-ischämischen Leber noch nicht verstanden sind.

In unserer Studie versuchten wir zu beantworten ob **a)** die CD4 T-Zellen während Ischämie-Reperfusion mit hepatischen Sternzellen interagieren und ob **b)** die pharmakologische Beeinflussung der Sternzellaktivität einen protektiven Effekt auf den T-Zell vermittelten I/R Schaden hat.

Die Migration von frisch isolierten und mit Immunfluoreszenzfarbstoff markierten CD4 positiven T-Zellen wurde mittels Intravitalmikroskopie in einer Sham- und der I/R Gruppen von Mäusen untersucht.

Sinusoidale Leberperfusion und Leberenzyme wurden als Marker zur Abschätzung des Leberzellschadens verwendet.

Die in Punk **a)** postulierte Interaktion zwischen T-Zellen und hepatischen Sternzellen wurde durch Injektion von fluoreszenzmarkierten T-Zellen in post-I/R-Mäuse mit GFP-exprimierenden Sternzellen (Cx3CR1) mittels Two-photon Mikroskopie untersucht.

Nachdem in Studien gezeigt wurde, dass hepatische Sternzellen in ihrer Aktivität durch Stimulierung von Endocannabinoidrezeptoren (CB1 /CB2) beeinflusst werden können, erhielten die Tiere in unseren Versuchen entweder den CB-2 Agonist JWH-133 zur Depletion von Sternzellen oder den CB-1 Agonist ACPA zur Aktivierung dieser. Hepatische I/R führte in allen Gruppen zu T-Zell Rekrutierung in den Sinusoiden. In der JWH-133 Gruppe führte die Deaktivierung der Sternzellen zu einer deutlich verringerten Ansammlung von T-Lymphozyten in die Sinusoide sowie signifikant

geringeren hepatischen I/R Schaden verglichen mit den anderen Gruppen. HSC über-Aktivierung durch CB-1 Stimulation hatte keinen Einfluss auf T-Zell Rekrutierung, ging jedoch mit höherem hepatischem Perfusionsversagen einher.

Demnach suggerieren unserer Daten, dass **a)** in der Tat eine Interaction zwischen HSC und T-Zellen während der Postischämie stattfindet und **b)** selektive Depletion/Deaktivierung von HSC durch CB-2 Agonisten zu geringerem I/R Schaden führt.

Somit könnten HSC in Zukunft als ein potentielles Ziel für therapeutische medikamentöse Intervention im Rahmen der T-Zell vermittelten I/R Schaden gesehen werden.

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List of abbreviations:

ALT	Alanine aminotransferase
α-SMA	α-smooth muscle actin
AST	Aspartate aminotransferase
ACPA	Arachidonylcyclopropylamide
CB-1 and CB-2	Cannabinoid Receptor type 1 and 2
CFSE	Carboxyfluorescein diacetate succinimidyl ester
FITC	Fluorescein isothiocyanate
GFP	Green fluorescence protein
HGF	Hepatocyte growth factor
HSC	Hepatic stellate Cell
ICAM-1	Intercellular adhesion molecule-1
I/R	Ischemia-reperfusion
LFA-1	leukocyte function- associated antigen-1
NK cells	Natural killer cells
PD-1	Programmed death ligand-1

Eidesstattliche Versicherung

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