Enhancement of liver regeneration and liver surgery
Olthof, P. B.

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CHAPTER 1

Warm ischemia time-dependent variation in liver damage, inflammation, and function in hepatic ischemia/reperfusion injury

PB Olthof, RF van Golen, B Meijer, AA van Beek, RJ Bennink, J Verheij, TM van Gulik, M Heger

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Chapter 1

ABSTRACT

Background: Hepatic ischemia/reperfusion (I/R) injury is characterized by hepatocellular damage, sterile inflammation, and compromised postoperative liver function. Generally used mouse I/R models are too severe and poorly reflect the clinical injury profile. The aim was to establish a mouse I/R model with better translatability using hepatocellular injury, liver function, and innate immune parameters as endpoints.

Methods: Mice (C57Bl/6J) were subjected to sham surgery, 30 min, or 60 min of partial hepatic ischemia. Liver function was measured after 24 h using intravital microscopy and spectroscopy. Innate immune activity was assessed at 6 and 24 h of reperfusion using mRNA and cytokine arrays. Liver inflammation and function were last profiled in two patient cohorts subjected to I/R during liver resection to validate the preclinical results.

Results: In mice, plasma ALT levels and the degree of hepatic necrosis were strongly correlated. Liver function was bound by a narrow damage threshold and was severely impaired following ≥ 60 min of ischemia. Severe ischemia (≥ 60 min) evoked a neutrophil-dominant immune response, whereas mild ischemia (≤30 min) triggered a monocyte-driven response. Clinical liver I/R did not compromise liver function and displayed a cytokine profile similar to the mild I/R injury model.

Conclusions: Mouse models using ≤30 min of ischemia capture the clinical liver I/R injury profile in terms of liver function dynamics and type of immune response.

General significance: This short duration of ischemia therefore has most translational value and should be used to increase the prospects of developing effective interventions for hepatic I/R.
INTRODUCTION

Hepatic ischemia and reperfusion (I/R) injury is characterized by hepatocellular damage, sterile inflammation, and compromised postoperative liver function after surgical procedures such as liver resection or transplantation. The extent of postoperative liver injury is measured by several methods, which include biochemical markers such as plasma alanine aminotransferase (ALT) and dynamic liver function tests such as hepatobiliary scintigraphy (HBS). Of these, ALT is the most universally measured parameter in both the clinical and experimental setting. However, a recent study in patients revealed that postoperative peak transaminase levels following I/R-inducing major liver surgery do not correlate with postoperative outcome or the duration of liver ischemia. Consequently, alternative markers should be used to more accurately determine post-operative liver injury so as to better manage the patients following liver surgery.

After resection or transplantation, the single most important clinical outcome parameter is residual liver function. The remnant liver or transplanted liver must be able to support the patient across the entire liver functional spectrum, which includes basolateral uptake-, hepatocellular biotransformation-, and canalicular excretion of compounds, protein synthesis, as well as regulation of immunological and metabolic homeostasis. The manifestation of considerable defects in any of these functions may have deleterious consequences on liver surgery patients. Accordingly, liver quantitative function tests should be considered important tools in the clinical management of these patients.

In light of the clinical findings, it is currently not known whether the lack of correlation between ALT and post-ischemic liver damage and function also applies to standardized hepatic I/R mouse models. These models are abundantly employed to investigate I/R-mediated processes and interventions, whereby ALT is used as a hallmark outcome parameter for liver damage. Investigating the relationship between liver damage, the putative parenchymal damage marker (ALT), and liver function in the most commonly used animal model is therefore imperative for validating past research results and properly conducting future studies.

Here we employed a standardized mouse I/R model to determine (1) the prognostic power of plasma ALT levels and histological necrosis score for post-ischemia liver function, (2) the correlation between plasma ALT levels and liver function, and (3) the representability of mouse I/R models in terms of the clinical situation, in line with and . The main findings were that (1) plasma ALT levels and the degree of hepatic necrosis are strongly correlated, (2) immune signaling differs with the degree of hepatocellular damage, (3) liver function is
bound by a liver damage threshold in mice, and (4) mouse models of I/R might not all be
representative of the clinical situation in terms of inflammation and function.

MATERIALS AND METHODS

References to supplemental information are indicated with a prefix ‘S’.

Animals
Male specific pathogen-free C57Bl/6J mice weighing 25-30 g were obtained from Charles
River Laboratories (JAX stock number #000664, Saint-Germain-sur-l’Arbresle, France). The
animals were housed in groups of 6 in standard Makrolon type II cages placed in a humidity-
and temperature-controlled cabinet (21 °C) with 12-h dark-light cycle and ad libitum access
to CRM pellet food (Special Diet Services, Essex, UK) and water. Animals were acclimated for
7 days before inclusion in the experiments. All experimental procedures were approved by
the animal ethics committee of the Academic Medical Center (BEX 119) and animals were
treated according to the Guide for the Care and Use of Laboratory Animals (8th edition,
National Institutes of Health, Bethesda, MD) and institutional guidelines.

Mouse model of I/R
All experimental and surgical procedures were performed as described before. In
brief, mice received 0.06 mg/kg of buprenorphine (Temgesic, Schering-Plough, Kenilworth,
NJ) subcutaneously for preoperative analgesia. A mixture of oxygen:air (0.5:0.5, 1 L/min)
with 3% isoflurane (Forene, Abbott Laboratories, Queensborough, UK) was used to induce
and maintain anesthesia. Core body temperature was maintained at 37 ± 0.3 °C during the
procedure with a heating pad and heating lamp. Following a midline laparotomy, the liver
was exteriorized and a 4 × 1.0-mm microvessel clip (MEHDOMDORN, Aesculaep, Center Valley,
PA) was used to clamp off blood flow to the left lateral and median lobes and induce ~70%
liver ischemia (Figure S1). After clamping, the liver was placed back into the abdominal cavity
and ischemia was maintained for 30 or 60 min, confirmed by blanching of the liver at the
end of the ischemia period (Figure S1). Animals with livers that did not exhibit blanching
were excluded from the experiment. In the sham group, the same surgical procedure was
performed without clamping of the hepatic vessels. Slightly before 24-h reperfusion, the
mice were anesthetized as described above, and the protocol was divided into 2 test arms.

In the first test arm, intravital fluorescence microscopy was performed on the livers as
described in section ‘Intravital fluorescence microscopy and spectroscopy’. In both test
arms, blood (1 mL) was collected from the heart by cardiac puncture and directly transferred
Ischemia time-dependent variation in hepatic I/R

into heparin-containing tubes for analysis by clinical chemistry (section ‘Clinical chemistry’). Next, the liver was excised and sections from the left lateral lobe were fixed in 10% buffered formalin for histological processing (section ‘Histology’). In the second test arm, biopsies ~30 mg from the excised lobe were transferred to RNAlater (Qiagen, Venlo, the Netherlands), incubated for 24 h at 4 °C, and stored at -20 °C for transcriptomic analysis (section ‘RNA extraction from mouse liver tissue and analysis by PCR’).

Intravital fluorescence microscopy and spectroscopy

In the first test arm, the liver was mobilized following re-laparotomy and prepared for intravital fluorescence microscopy as described before. The microscopy setup consisted of a custom-modified stereo fluorescence microscope (model M165 FC, Leica Microsystems, Wetzlar, Germany) equipped with a Peltier-cooled DFC420C camera, a Planapo 1.0× objective lens, a 0.5× video objective (C-mount), a time-based acquisition module, filter sets for brightfield (420 nm cut on filter) and fluorescence (λex = 650 ± 30 nm, λem = 695 nm long pass) microscopy, and a Leica EL6000 light source. The C-mount adapter was modified at the Department of Medical Innovation and Development to house a spectroscopy component. The C-mount adapter was cut directly above the lens such that a light separation module was placed between the microscope aperture and the camera. The module contained an interference filter that was positioned at an angle relative to the incident light, causing ~1% of light to be diverged orthogonally into an optical fiber connected to a spectrometer (model QE65000, Ocean Optics, Dunedin, FL) while ~99% of light was transmitted onto the camera’s CCD chip. This allowed concomitant microscopy and spectroscopy.

DY-635 (NHS-ester, product number 635-01, Dyomics, Jena, Germany) was dissolved in DMSO to a stock concentration of 3 µmol/mL. DY-635 was dissolved to a 25 nmol/mL working solution using 0.9% NaCl. Absorption and emission maxima of DY-635 in ethanol are 647 nm and 671 nm respectively.

The mice were fixed on a custom-designed temperature-controlled stage to which the anesthesia tubing was secured. The liver lobes that had been subjected to I/R were positioned onto a custom-built transabdominal stage and covered with saran wrap to prevent desiccation during imaging (Figure S1). The stage was placed under the microscope, with the field of view encompassing most of the left lateral lobe and part of the median liver lobe. The baseline fluorescence was recorded at t = 0 min and t = 1 min, after which DY-635 was administered systemically through the penile vein (167 pmol/g body weight, 200 µL working solution per 30 g body weight). Imaging and spectroscopy were performed at a frequency of 1 min⁻¹ for 60 min. For microscopy, the exposure time was 10 s using automated shutter control of the excitation light source, with 10× gain and 0.73× optical
zoom. For spectroscopy, the signal was integrated over a 5-s time frame with dark current correction.

**Analysis of hepatic DY-635 kinetics**

For analysis of DY-635 uptake and excretion, the area under the curve of the spectral data in the range of 671-761 nm was calculated and normalized to baseline ($t = 0$ min). To eliminate DY-635 dose-dependent variation in signal intensity and to analyze uptake and excretion kinetics, data were normalized to the maximal value per individual animal. The mean data were fitted using the kinetics of first-order uptake and elimination formula:

$$F = \frac{1}{(k_\text{e} / k_\text{u}) - 1} e^{-k_\text{et}} (1) / (1 - e^{-k_\text{et}})$$

where $t$ is time (min), and $k_\text{u}$ and $k_\text{e}$ are the uptake and elimination rate constants ($\text{min}^{-1}$).

To assess liver uptake and excretory function, several pertinent pharmacokinetic parameters were calculated from the data sets. The time to maximum concentration ($\text{Tmax}$, in min) is the time until the highest measured value was achieved in each individual animal. The maximum concentration ($\text{Cmax}$, in arbitrary units (a.u.)) represents the highest background-corrected fluorescence emission intensity obtained throughout measurement in each individual animal. Best-fit first order uptake and elimination rate constant for the formula above were determined for each individual animal in GraphPad Prism (GraphPad Software, La Jolla, CA).

**Clinical chemistry**

Heparin anti-coagulated blood samples were centrifuged at 10,000 × g for 5 min at 4 °C. Plasma was aspirated and assayed for serum alanine aminotransferase (ALT) at the Department of Clinical Chemistry using a Cobas 8000 modular analyzer (Roche Applied Sciences, Basel, Switzerland). ALT values were expressed as units/liter (U/L).

**Histology**

Following fixation in 10% (vol/vol) formalin solution, the histological specimens were dehydrated in graded steps of ethanol, embedded in paraffin, cut to 3-μm thick sections, and stained with haematoxylin and eosin (H&E). One slide per animal was scored by an experienced hepatopathologist (J.V.) blinded to the experimental groups for the percentage of confluent necrosis in the total slide.10
**RNA extraction from mouse liver tissue and analysis by PCR**

RNA was extracted from liver biopsies using the RNAeasy Mini Kit (Qiagen) and Mouse Th17 For Autoimmunity & Inflammation PCR arrays (catalogue number PAMM-073A, 96-wells configuration, SABiosciences, Venlo, the Netherlands) were performed using 1 μg of input RNA on a LightCycler 480 (Roche) as described.\(^{12, 15}\) Fluorescence data were processed according to Ruijter et al.\(^{16}\) and all samples were normalized to the housekeeping gene that showed the most stable expression over all arrays (i.e., Hprt). Data were expressed as fold difference compared to the sham group. Heat maps of the expression profiles were generated using GENE-E.\(^{17}\) Primers of genes not included in the PCR array (i.e., Slc01b2, Mdr2, Mrp2, Bsep) were designed using NCBI Primer Blast to span an exon-exon junction and ordered from Life Technologies (Eugene, OR). The primer sequences were: Slco1b2, forward 5′-GCCACACTTTGTAGAAGGCC-3′, reverse 5′-TGACCCTACCCTATGCC-3′; Mdr2, forward 5′-ATCCTATGACTGCCCTTCTTGGT-3′, reverse 5′-GAAAGCATCAATAACAGGGGCAG-3′; Mrp2, forward 5′-ACTGGACAAGCCACATTCC-3′, reverse 5′-CTGCAGGAGTTGCTCTGATCA-3′; Bsep, forward 5′-ACATCTGTAGGGTGTTTAGTGA-3′, reverse 5′-GGGCACAACACTTCCCATAA-3′; β-actin, forward 5′-GCCCTCTCTTTGCTGATGG-3′, reverse 5′-CAGCTCAGTACACGTCGCC-3′; and Hprt, forward 5′-GAGACCGTTGGGCTTCACCT-3′, reverse 5′-ATCGCTAATACGACGCTTG-3′. Primer validation and qRT-PCR were performed as described\(^{12}\) and gene expression levels were normalized to the housekeeping gene Hprt.

**Determination of cytokines in mouse plasma**

Mouse plasma samples were assayed for mouse granulocyte macrophage colony-stimulating factor (Gm-csf), Interferon gamma (Ifnγ), interleukin (Il) 1β, II-2, II-4, II-5, II-6, II12p70, II-13, II-18, and tumor necrosis factor alpha (Tnfa) using a ProcartaPlex Mouse Th1/Th2 extended 11-plex kit and mouse B-cell activating factor (Baff), II-10, II-22, Rantes/Ccl5, thymic stromal lymphopoietin (Tslp) and vascular endothelial growth factor (Vegf) using ProcartaPlex simplex kits on the LumineX platform (Affymetrix, Santa Clara, CA), according to the manufacturer’s instructions. In brief, samples were thawed on ice. The antibody-coated beads were mixed and washed, and subsequently incubated overnight at 4 °C with 1:1 diluted standards or samples. After washing, the beads were incubated with detection antibody mix for 30 min at room temperature. The beads were washed and incubated for 30 min at room temperature with streptavidin-PE. After another washing step, the beads were measured with a LumineX instrument (Bio-Plex 200, Bio-Rad Laboratories, Hercules, CA) which was calibrated using Bio-Rad calibration beads. Standard curves were calculated using 5 parameter logistic regression in Bioplex 5.0 software (Bio-Rad Laboratories).
Clinical validation of post-I/R liver function and cytokine expression

To evaluate the effects of hepatic I/R on postoperative liver function, a subgroup of patients was selected from a historical cohort. In this cohort, patients scheduled for liver resection were subjected to preoperative assessment of future remnant liver function by HBS using technetium-99m (99mTc)-mebrofenin and analysis was repeated at postoperative day one. Detailed information on the future remnant liver volume and function analysis are available in the original report. Patients who had undergone major liver resection (≥ 3 liver segments) and who had been subjected to at least 40 min of intermittent or continuous vascular inflow occlusion (VIO, i.e., ischemia) were selected from the cohort. Preoperative 99mTc-mebrofenin uptake was compared to uptake at postoperative day one to evaluate the effects of VIO on liver function. The study protocol was approved by the medical ethics committee of the Academic Medical Center and every participant gave written informed consent before inclusion in the trial.

Patients from an ongoing randomized clinical trial (clinicaltrials.gov identifier: NCT01499979) were included in this study to IL-1β levels in VIO-subjected patients. Patients who had undergone a right hepatectomy were included. The presence of hepatic co-morbidity was determined as described elsewhere and patients with severe steatosis, fibrosis, cholestasis, or hepatitis were excluded to exclude an effect of pre-existent parenchymal liver disease on post-operative outcomes. Patients were operated using intermittent VIO using cycles of 20 min hepatic ischemia followed by 10 min of reperfusion. Blood samples were collected at baseline and at 1 and 6 h of reperfusion. Plasma was assayed for human IL-1β using ProcartaPlex simplex kits on the Luminex platform (Affymetrix) according to the manufacturer’s instructions and as described in section ‘Determination of cytokines in mouse plasma’. The study protocol was approved by the medical ethics committee of the Academic Medical Center and every participant gave written informed consent before inclusion in the trial.

Statistical analysis

All statistical analyses were performed with GraphPad Prism. A P-value of < 0.05 was considered statistically significant. All variables that followed a Gaussian distribution according to a D’Agostino-Pearson omnibus test were analyzed using a student’s t-test or one-way ANOVA with Dunnett’s post-test. Non-Gaussian data were analyzed with Mann-Whitney U-test or a Kruskal-Wallis tests with a Dunn’s post-test. Spearman rank correlation was used to test correlations between variables. A paired t-test was used to compare paired, normally distributed data.
RESULTS

The extent of hepatocellular damage is proportional to the duration of ischemia
The first aim was to investigate the relation between the duration of liver ischemia and hepatocellular injury in mice. All mice tolerated both 30 and 60 min of partial hepatic ischemia and there was no mortality. At 24 h after ischemia, plasma ALT levels (Figure 1A) and the degree of confluent necrosis (Figure 1B) were directly proportional to the duration of ischemia, supported by the strong correlation between ALT and necrosis (Figure 1C). The manifestation of hepatocellular damage (Figure 1D) was visible during intravital microscopy, where severely afflicted livers in the 60-min ischemia group were entirely pale and exhibited disorganized liver morphology (Figure 1D).

Differential inflammatory response after 30 and 60 min hepatic ischemia
In previous work we observed that the degree to which the innate immune system is activated after I/R depends strongly on the extent of hepatocyte damage. Moreover, the released cytosolic components and cell fragments from damaged hepatocytes triggered the production of tumor necrosis factor α (Tnfα), interleukin 1β (Il-1β), and Il-6, which are key pro-inflammatory mediators of hepatic I/R injury. Given the differential histological damage profiles in the 30- and 60-min ischemia groups (Figure 1D), we next determined whether the extent of inflammation was proportional to the degree of damage and therefore could lie at the basis of the damage. Accordingly, inflammation-related PCR arrays comprising 57 inflammation-related genes (Table S1) and cytokine arrays comprising 17 proteins (Table S3) were performed at 6 h and 24 h after sham operation, 30 min, or 60 min of liver ischemia. The function(s) of the genes (denoted in italics) and proteins (denoted in regular font) are described in Table S1 and Table S3. Heat maps of the differential transcriptomic and proteomic expression profiles at 6 h and 24 h reperfusion are presented in Figure 2. The actual values of the heat map data are provided in Table S2 (genes) and Figure S2 (proteins). Only statistically significant data were considered.
Figure 1: A: Plasma alanine transaminase (ALT, y-axis) levels 24 h after 30 or 60 min of partial hepatic ischemia or sham procedure. Levels are displayed as mean (SEM) for N = 3-6 per group. B: Extent of confluent necrosis as quantified by a hepatopathologist on the basis of H&E-stained liver histology. Data represent mean ± SEM with N = 3-6 per group. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$, compared to the sham group. # indicates $P < 0.05$ and ## indicates $P < 0.01$ between the groups indicated by the solid line. C: Correlation plot of plasma alanine transaminase with the extent of confluent necrosis. Correlations were tested using Spearman's rank correlation coefficient. D: Representative hematoxylin and eosin-stained liver sections, where necrotic regions are characterized by high neutrophil infiltration (top row, 50× magnification), and intravital microscopy images of the in situ liver (bottom row, 0.73× magnification, brightfield mode). Sections and intravital images were acquired 24 h after the start of reperfusion.
Figure 2: Differential inflammatory response following 30 and 60 min partial hepatic ischemia in mice. The left heat map displays the differential expression of genes after 30 or 60 min of ischemia and 6 or 24 h of reperfusion. Data are displayed as log2 fold expression compared to the sham group, whereby downregulated genes are shown in green and upregulated genes are shown in red. Missing data are shown in grey. Genes also included in the cytokine (protein) array were excluded from the transcriptome heat map. The right heat map displays plasma cytokine levels after 6 and 24 h of reperfusion, shown as fold expression compared to sham-operated animals. Interleukin-2 was excluded since the levels were below the detection range in most samples. Readers are referred to the supplemental material for quantitative transcript (Table S2) and protein levels (Figure S2). ‘30-6’ and ‘60-6’ refer to 6 h reperfusion after 30 min and 60 min ischemia, respectively. ‘30-24’ and ‘60-24’ refer to 24 h reperfusion following 30 min and 60 min ischemia, respectively. In the heat maps, a dot indicates $P < 0.05$ compared to the sham group. $^1$ indicates $P < 0.05$ between the 30-6 and 60-6 group. $^2$ indicates $P < 0.05$ between the 30-24 and 60-24 group. The cartoon summarizes the differential immune response in the 30 min and 60 min ischemia model based on the observed gene and protein expression.
I/R-induced molecular regulation of immunity is temporally heterogeneous

The first major finding was that the post-I/R immune response exhibited an expressional and temporal heterogeneity at the transcriptomic and proteomic level that varied with the severity of liver injury. In terms of expressional heterogeneity, 15 genes were differentially regulated following 30-min ischemia, associated with moderate injury (Figure 1A and B), compared to 9 genes following 60-min ischemia, which is associated with severe injury (Figure 1A and B). Of the 15 dysregulated genes in the 30-min ischemia group, 11 genes (73%) were upregulated and 4 genes (27%) were downregulated. Of the 9 dysregulated genes in the 60-min ischemia group, 5 genes (56%) were upregulated and 4 genes (44%) were downregulated. Moreover, only 3 genes overlapped in both ischemia groups (Icos, Stat6, Il6ra), underscoring the expressional heterogeneity.

With respect to temporal heterogeneity, the most profound mRNA dysregulation occurred 24 h after ischemia in the 30-min ischemia group (14 of the 15 genes). In contrast, the most extensive mRNA dysregulation in the 60-min ischemia group occurred 6 h after ischemia (6 of the 9 genes). These data reflect time-based differences in I/R-induced transcriptional dysregulation. An opposite effect was observed in the protein profiles. The most profound changes were observed in the 60-min ischemia group (16 proteins versus 10 proteins in the 30-min ischemia group). All of the 16 proteins in the 60-min ischemia group were upregulated, while 9 out of 10 proteins were upregulated in the 30-min ischemia group. Most dysregulation occurred at 6 h post-ischemia, particularly in the 60-min ischemia group in terms of cytokine signaling.

Post-I/R innate immune signaling proceeds via different pathways depending on the duration of ischemia

The second major finding was that innate immune cell signaling might proceed via different pathways in an ischemia duration-dependent manner. For example, neutrophil recruitment in the 60-min ischemia group most likely proceeded via Il-1β and Il-6, in accordance with previous reports.2, 20 Both transcript and protein levels of Il-1β/Il-1β and Il-6/Il-6 were markedly elevated after 60 min but not after 30 min of ischemia. Molecules involved in monocyte and neutrophil production (Csf3, Gm-csf) and chemotaxis (Cxcl2, Ccl5) were elevated in the 60-min ischemia group but not in the 30-min ischemia group. The high levels of Ccl5 following 60 min ischemia could indicate platelet activation.21 In the 30-min ischemia group, neutrophil dynamics were most likely regulated through Cxcl1 (chemotaxis) and Mmp9 (migration). Moreover, the monocyte-activating chemokine Ccl7 as well as Ccl2, which are known to mediate monocyte migration and infiltration, were upregulated following 30 min but not 60 min of ischemia.
Post-I/R adaptive immune signaling proceeds via different pathways depending on the duration of ischemia

The third major finding was that the adaptive immune response was activated in both groups, but that the underlying signaling was reliant on the duration of ischemia. In case of the 30-min ischemia group, the adaptive immune response was regulated through Il-4, Tsip, Baff, Il-15, Nfatc, and Tgfβ1 (upregulated) and Il-5, Icos, and Stat6 (downregulated). In the 60-min ischemia group, adaptive immunity was regulated by Il-1β, Il-6, Il-10, Il-12, Il-18, Tnfa, Il-27, Stat6, SykB (upregulated) and Cxcl12, Icos, and Rorc (downregulated). The increased Il-12 levels following 60 min but not 30 min ischemia are in line with the elevated Tnfa and Ifny concentrations, the production of which by T cells is stimulated by Il-12.22 The upregulation of Il-1β, Il-6, and Il-12 in the 60-min ischemia group but not the 30-min ischemia group suggests a response towards neutrophil-mediated tissue injury in the 60 min ischemia group,20 which is reflected by the higher degree of necrosis in this group.

Additional observations spawned by the post-I/R molecular signatures

The data yielded several noteworthy observations. Firstly, Stat6 not only regulates T helper 2 cell differentiation23 but also regulates Il-4-mediated cell survival through the overexpression of anti-apoptotic proteins Bcl2l1/Bcl-x(l).24 Given the profound injury in the 60-min ischemia group and the overexpression of Il-4 and Stat6 in this group, afflicted hepatocytes may have engaged cell survival pathways to offset extensive cell death and curtail liver injury.

Secondly, Foxp3 was more strongly upregulated in the 30-min ischemia group compared to the 60-min ischemia group (Figure 2). Foxp3 is the master regulator of regulatory T cells, which are induced by cytokines including Tgfβ (Figure 2),25 inhibit the development of pro-inflammatory effector T cells,26 and promote tolerance to self-antigens.26 The elevated Foxp3 and Tgfβ expression following 30 min but not 60 min of ischemia implies development of regulatory T cells in response to moderate liver injury. The significance of this signaling axis is currently elusive, but it may serve to attune the extent of (injurious) immune signaling to the degree of liver damage.

Thirdly, increased Il-10 expression was found in the 60-min but not 30-min ischemia model. Il-10 inhibits the activation of T cells, monocytes, and macrophages.27 To date, conventional dendritic cells (cDCs) have been implicated as the source of Il-10 in response to the uptake of apoptotic cells.28 Inasmuch as necrosis was the predominant form of cell death after 60 min of ischemia (Figure 1), the source and trigger of Il-10 are unknown in our model, as is the role of this cytokine in severe I/R injury.
Liver uptake and excretion function is more sensitive to ischemia/reperfusion than hepatocellular damage

In light of the increasing damage profile and differential immune signaling with longer I/R times, the effect of ischemia time on liver uptake and excretion was determined next. For these purposes the intravenously administered fluorescent dye DY-635 was employed in combination with the I/R model and intravital fluorescence spectroscopy. DY-635 is specifically taken up and excreted by hepatocytes. Its rapid clearance from the circulation and favorable spectral properties (i.e., deep tissue penetration of excitation light) make DY-635 ideal for intravital assessment of liver function. Our stereo fluorescence microscopy setup allowed measurement in a large tissue volume, spanning almost the entire left lateral lobe (Figure 1C), thereby providing representative information on regional liver function. We hypothesized that liver function would deteriorate proportionally to ischemia time and that there would be a negative correlation between liver injury (ALT and necrosis) and liver function.

The integrated emission of DY-635 (671-761 nm) at 24 h reperfusion is plotted as a function of time following intravenous administration in sham-operated mice and in mice that had been subjected to 30 min or 60 min of ischemia (Figure 3A). A kinetics curve in healthy livers is comprised of 3 phases: uptake of the probe (upward slope), intrahepatic saturation of the probe (plateau at maximum emission intensity), and excretion of the probe (downward slope). This pattern is exemplary in the sham-operated animals, where the maximum intrahepatic DY-635 concentration was reached within 7 min and the probe was largely excreted during the next 50 min. Livers that had been subjected to 30 min of ischemia exhibited stalled DY-635 uptake and excretion, while uptake and excretion were profoundly hampered in severely damaged livers (60 min ischemia). The findings indicate that liver function was inversely proportional to liver damage and therefore support our hypothesis.

The pharmacokinetic parameters of DY-635 corroborate that uptake and excretion were impaired in an I/R injury-dependent manner (Figure 3B-E). The Tmax, which is essentially a measure of basolateral uptake efficiency, was lowest in the sham group and highest in the 60-min ischemia group (Figure 3B). Healthy livers were able to effectively extract DY-635 from the blood and reach hepatocellular saturation (Tmax) 7 min after probe administration. Ischemically compromised livers exhibited a greater Tmax and were therefore less effective in basolateral transport of the probe. This is also evident from the uptake constants (Figure 3C), which reflect the slope coefficient of the curve in the uptake phase. Accordingly, the Tmax and hepatocellular damage (ALT) exhibited a strong positive correlation (Figure 3F).
Figure 3: A: Liver function as assessed by DY-635 uptake and excretion kinetics by intravital spectroscopy in sham-operated animals (black circles) and mice that had been subjected to 30 min (red squares) or 60 min of ischemia (blue triangles). Measurements were performed at 24 h of reperfusion. The emission intensities were background-corrected to \( t = 0 \) min (before DY-635 administration), normalized to the maximum emission intensity, and integrated over the 671 to 761 nm range. Data were expressed as mean ± SEM with \( N = 3-6 \) per group and fitted using first-order uptake and elimination kinetics. B: \( T_{\text{max}} \), differences in time to maximum DY-635 fluorescence intensity (i.e. basolateral uptake efficiency). C: Uptake constant (i.e., slope of the curve in the uptake phase). D: Cmax, differences in maximum fluorescence emission intensity (i.e., hepatic probe loading) E: Elimination constant (i.e., slope of the curve in the excretion phase). * indicates \( P < 0.05 \), ** indicated \( P < 0.01 \), and *** indicates \( P < 0.001 \) compared to the sham-operated group. F-J correlation plots of liver uptake and excretory function parameters with plasma ALT levels. Linear regression lines were shown in panel F-H and one-phase decay curves in panel I-J. Correlations were tested using Spearman’s ranked correlation coefficient.
The Cmax equals the amplitude of the kinetic curve at maximum fluorescence intensity obtained during the 60-min spectral acquisition, and therefore corresponds to the highest intracellular DY-635 concentration achieved during the experiment. Livers that rapidly excrete the probe have a low Cmax, while livers with impaired canalicular excretion will exhibit a high Cmax due to hepatocellular DY-635 loading. As shown in Figure 3D, the greater the ischemia time (i.e., ischemic injury), the larger the Cmax. This finding also confirms that the probe was adequately delivered to the liver and not impeded by I/R-induced microvascular defects. Consequently, a strong positive correlation was found between ALT and Cmax (Figure 3G). A strong positive correlation was also found between ALT and the area under the curve (AUC, Figure 3H), which reflects the general dynamics of uptake and excretion. Principally, rapid uptake and poor excretion yields a high AUC, while rapid uptake and rapid excretion yields a low AUC. Clearly, the transport machinery was debilitated in more damaged livers, as further evidenced by the substantially lower elimination constants in the 30-min and 60-min ischemia groups compared to sham-operated animals (Figure 3E).

Our study confirmed the linear relationship between the ischemia time and liver damage (Figure 1A and B) and the strong positive correlation between ALT and necrosis (Figure 1C) in mice. The data indicate that liver damage occurred in direct proportion to ischemia time, up to a certain period of ischemia. Contrary to expectations, the most striking observation was the relationship between hepatocellular damage (ALT) and the uptake and elimination constants, which were not linear but exponential (Figure 3I and J). Accordingly, liver uptake and excretion function seem to have a narrow and rather low-threshold tolerance for ischemic damage, at least in mice. The ischemic damage threshold occurred at an ALT of 1000-1500 U/L (Figure 3I and J), which corresponds to less than 30 min ischemia (Figure 1A).

Organic anion transporting polypeptide (Oatp)1b2 (Slco1b2) is the basolateral transporter mainly responsible for the uptake of DY-635 by hepatocytes. Previous studies in rats demonstrated post-ischemic downregulation of this transporter. Slco1b2 mRNA expression was therefore assayed to ascertain the possible cause of the impaired uptake function. As shown in Figure S3A, Slco1b2 was downregulated in proportion to the extent of ischemia, which may therefore have (partly) accounted for the reduced DY-635 uptake. Multidrug resistance protein 2 (MDR2), multidrug resistance-associated protein 2 (MRP2), and bile salt export pump (BSEP) are the most prominent transporters in the canalicular membrane of hepatocytes and facilitate the export of various compounds into the biliary system, which may include DY-635. The exact export mechanisms of DY-635 are currently elusive but likely embody one or more of these canalicular transporters. In line with the elimination constant data (Figure 3J), the transcript levels of these exporters were also reduced in an ischemia-
dependent manner (Figure S3B-D), possibly accounting for the impaired hepatocellular excretion of DY-635.

**Hepatic ischemia in patients mildly impairs liver function and does not induce IL-1β production**

To determine the translational value of the in vivo findings, liver uptake and excretory function were determined in a well-defined historic cohort of patients who had undergone resection of at least 3 liver segments with at least 40 min of VIO. Patient characteristics are provided in Table S4. Liver function was measured before and 24 h after liver resection with 99mTc-mebrofenin HBS in order to determine the functional recovery of the liver following surgery. Mebrofenin is basolaterally taken up by OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3) and canalically excreted by MRP2 (ABCC3). Of all liver function probes, mebrofenin is most analogous to the DY-635 probe used to determine liver function in mice. The change in liver function was calculated. Liver damage was assessed by routine clinical chemistry (peak plasma ALT). Correlation analysis was performed between the duration of ischemia and ALT, and between the duration of ischemia and liver function.

As shown in Figure 4A, postoperative peak ALT levels did not correlate with the postoperative change in liver function on postoperative day (POD) 1 (Spearman’s $\rho = -0.200$, $p = 0.92$). In these four patients, preoperative liver function did not differ from postoperative liver function (4.9 %/min/m$^2$ (range = 4.5-5.7) vs. 4.7 %/min/m$^2$ (3.8-5.2), $p = 0.08$) (Figure 4B). However, there was a trend towards decreasing liver function in all patients with a median (range) drop in mebrofenin uptake of 9% (6-27) (Figure 4B). These data support previous clinical findings that ALT does not reflect postoperative liver function. In terms of animal model translatability, the absent to mild impairment of liver uptake and excretory function on POD 1 in these patients suggests that the 30-min liver ischemia mouse model better emulates the clinical scenario than the 60 min model, in which the impairment of liver uptake and excretory function was (too) severe (Figure 3A).

Furthermore, it was shown in Figure 2 that moderate (30 min) and severe (60 min) hepatic I/R injury in mice markedly differ with respect to the mode and severity of innate immune activity, which was exemplified by the lack of IL-1β induction in case of moderate liver injury. Since the contribution of IL-1β to post-I/R sterile immune responses has been widely investigated and shown to affect most cell types involved, IL-1β levels were determined in liver ischemia-subjected patients. To assess which of these two scenarios best mimics the clinical situation, blood samples were collected from 9 patients who underwent a right hemihepatectomy with intermittent VIO (Table S4). In these patients, postoperative peak ALT levels did not correlate to cumulative VIO duration (Figure 4C, Spearman’s $\rho = -0.460$, $p = -0.200$, $p = 0.92$).
Chapter 1

p = 0.21), as was also observed in the previous cohort (Figure 4A). Furthermore, ALT levels were not related to plasma Il-1β levels at 6 h of reperfusion (Figure 4C, Spearman’s ρ = 0.091, p = 0.81). No increase in Il-1β was measured after 1 and 6 h of reperfusion compared to baseline (Figure 4D). The absence of Il-1β release in clinical liver I/R again suggests that the 30-min ischemia model in mice is more representative compared to the 60-min ischemia model, where hepatic I/R does induce an increase in plasma Il-1β (Figure 2).

**Figure 4:**

A: Correlation between hepatocellular damage (ALT) and VIO duration (black squares, left y-axis) and the change in liver function on postoperative day (POD) 1 (grey triangles, right y-axis) in 4 patients subjected to major liver resection with at least 40 min of VIO. B: Future remnant liver (FRL) liver function before and 24 h after liver resection with at least 40 min VIO. Data represent measurements in each individual patient. C: Correlation between hepatocellular damage (ALT) and VIO duration (black squares, left y-axis) and plasma IL-1β levels 6 h after reperfusion (grey squares, right y-axis) in nine patients subjected to a right hemihepatectomy using intermittent VIO. D: Plasma IL-1β levels in patients subjected to major right liver resection using intermittent VIO. Data represent mean ± SEM for N = 9.

**DISCUSSION**

We investigated the relation between duration of hepatic ischemia (30 and 60 min) and the extent of hepatocellular injury, immune signaling, and liver function in liver I/R in mice. The
data were juxtaposed to similar parameters derived from 2 patient cohorts to assess the level of translatability of the animal models. The main findings in mice were that (1) plasma ALT levels and the degree of hepatic necrosis were strongly correlated, (2) immune signaling differs with the degree of hepatocellular damage, and (3) liver function is bound by a liver damage threshold and is most likely a more relevant outcome parameter than plasma ALT levels. In patients it was shown that (1) ischemia time does not correlate with ALT levels, (2) I/R does not significantly impair liver function, and that (3) I/R does not induce IL-1β production. Accordingly, commonly employed mouse models of severe I/R injury (≥ 60 min) lack translational value, at least in terms of the gold standard parameters tested in this study. Less severe I/R injury models (≤ 30 min) that better approximate the clinical injury profile should therefore be used in translational liver I/R research.

With respect to ischemia time and damage profiles, a recent study revealed that 67 out of 87 reports (77%) on mouse models of hepatic I/R used at least 60 min of liver ischemia and only 7 (8%) studies used less than 40 min. The reported percentage of confluent necrosis in all reports was median (range) 60% (38 - 92) at 24 h of reperfusion. Besides the extent of necrosis, the employed animal models also varied in almost all other aspects (i.e., used anesthetics, ischemic durations, and reported ALT levels). The translational bottleneck is that necrosis of > 50% and ALT levels of > 2500 U/L are usually not encountered in patients subjected to hepatic ischemia during liver resection. Accordingly, the clinical situation is ill-represented in a large portion of translational liver I/R research. This incongruence may also in part account for the persistent difficulty of implementing I/R-targeted interventions validated in murine models into clinical practice.

The ischemia time-dependent differential immune response is also an important observation in terms of I/R injury because sterile inflammation dictates the extent of post-ischemic liver injury, at least in rodents. The pro-inflammatory cytokines IL-1β and IL-6 are often used as main endpoints in experimental studies of hepatic I/R. As opposed to the 60-min ischemic injury model, plasma IL-1β and IL-6 were not elevated following 30-min ischemia. In mice, IL-1β contributes to hepatic I/R injury following activation of the NALP3 inflammasome. However, these studies employed 60 to 90 min partial hepatic ischemia and clinical validation is currently lacking. Furthermore, our work revealed a primarily monocyte-driven immune response via chemokines Ccl2 and Ccl7 following 30 min ischemia, whereas the immune response following 60 min of ischemia seemed to be mainly neutrophil-driven via Gm-csf and CCL5. Monocytes may fulfill a regenerative role in sublethally afflicted livers given their pleiotropic plasticity, whereas neutrophils chiefly act as ‘cellular vultures’ that scavenge damaged liver tissue that is beyond repair. In support of this hypothesis, monocytes are involved in heart tissue remodeling and repair of cardiac I/R...
injury. In analogy, monocytes may be involved in liver repair or regeneration after hepatic I/R, where neutrophils seem to ubiquitously induce injury. It should be noted that monocytes have also been found to cause post-ischemic hepatocellular injury. However, these results were obtained in a severe injury model using 60 min of ischemia, and the monocyte subset was not characterized. The monocytes responsible for injury may differ from the monocyte subset in our 30-min ischemia model. Also, regulatory T cells are protective in hepatic I/R and, on the basis of the Foxp3 and Tgfβ data, may have been involved following 30 min ischemia but not 60 min ischemia. Taken together, these results suggest a controlled immune response under mild injury conditions that is geared towards liver regeneration and repair, while the profound necrosis-driven immune response after 60 min ischemia likely overwhelms this regenerative response and is orchestrated to remove irreparably damaged tissue.

To assess the mouse-to-human translatability of the immunological findings, IL-1β levels were determined in patients that had been subjected to liver resection with intermittent liver ischemia. No increase in plasma IL-1β was found at 6 h of reperfusion. The median 41 min of intermittent ischemia applied in this cohort is likely to have induced an I/R response with some hepatocellular damage. The complete absence of any elevation of plasma IL-1β in any of the nine included patients throughout 6 h of reperfusion suggests the clinical I/R response differs from the response seen after severe injury models in mice, in which IL-1β is usually abundantly present. Although this is just one of many cytokines, it confirms the discrepancies in I/R-induced inflammation between mice and humans. Future clinical I/R studies should further elaborate on the clinical inflammatory syndrome and compare results to patients subjected to liver resection without ischemia to exclude confounders such as inflammation induced by surgical stress.

In light of the damage-function relationship, liver function was assessed in mice exposed to various durations of ischemia. Functional impairment in murine livers was governed by a liver damage threshold. ALT values above 1000-1200 U/L at 24 h of reperfusion were associated with a steep decline in DY-635 uptake and excretion. Beyond this damage threshold the liver is no longer able to cope with the I/R injury, resulting in an acute and severe deterioration of liver function. As liver function encompasses basolateral uptake, hepatic detoxification, and subsequent canalicul export of compounds, liver failure theoretically can emanate from any of these three compartments. The current data indicate that I/R injury primarily affects hepatocyte canalicul export when it exceeds the damage threshold. A similar collapse of canalicul traffic is seen in experimental models of cholestasis and sepsis, in clinical settings of drug-induced liver injury, and in critically ill patients. As most canalicul exporters are ATP dependent and hepatic I/R is characterized by ATP depletion, the lack
of ATP could impair the clearance (i.e., export) of toxic metabolites such as bile acids from hepatocytes. This could in turn trigger cellular stress and hepatocyte necrosis, as apoptosis requires sufficient residual ATP to be completed. The possible link between ATP depletion and decay of liver function beyond a damage threshold is supported by earlier findings that hepatocyte necrosis is inevitable when ATP reserves fall below ~10% of baseline values. The downregulation of basolateral import might serve to compensate for the impaired hepatocyte export capacity, as slowing down hepatocyte uptake of toxicants should attenuate hepatocyte injury. Toxic injury due to impaired canalicular export in a liver already challenged by post-ischemic hepatocellular death further deteriorates postoperative liver function and could ultimately result in post-hepatectomy liver failure.

In the clinical setting, dynamic liver function tests are a robust tool to assess liver injury. To select the most clinically relevant model, the liver function data were compared to postoperative liver damage and function in I/R-exposed patients. In patients subjected to major liver resection and at least 40 min of ischemia, liver function was only mildly impaired post-I/R compared to preoperative measurements. A similar sustenance of adequate liver function was reported on day 3 after surgery in a larger cohort of patients who were exposed to intermittent or continuous hepatic ischemia during major liver resection. It therefore seems that hepatic I/R encountered in patients does not reach the damage threshold seen in mice and only transiently compromises liver function, which fully recovers within 3 days. These findings moreover indicate that the current criteria used to select patients that are eligible for major liver surgery effectively prevent post-hepatectomy liver failure. It also confirms that the moderate (i.e., 30-min ischemia) injury model has more translational value than more severe models, as post-operative liver failure is rarely seen and patients at-risk for severe post-operative morbidity are most likely considered unfit for surgery in preoperative patient work-up.

Although the current clinical data suggest a correlation between transaminase levels and ischemic duration, two recent larger studies demonstrated that postoperative transaminase levels depend on the duration of surgery and not on the duration of hepatic ischemia. Therefore, transaminase levels are likely not indicative of the degree of liver injury. Liver function is likely to better represent actual liver injury and most likely dictates clinical outcome. Future studies should address the exact interplay between biomarkers of hepatic injury, liver function test outcomes, and clinical outcomes to confirm the results found in mice.

In conclusion, mouse models of hepatic I/R that result in > 75% necrosis and severely impaired liver function do not represent the clinical situation of hepatic I/R in the context of
liver surgery. Furthermore, hepatic I/R injury in mice is associated with marked differences in the immune response that are governed by the severity of liver damage. The innate and possibly adaptive immune activation pathways observed in mild mouse liver I/R injury models best approach the clinical setting. Mouse models of hepatic I/R should therefore not exceed 30 min of hepatic ischemia and all studies should aim to validate translational data in a clinical cohort. The proposed approach is expected to yield more reliable and translatable research and increase the prospects of implementing effective interventions for hepatic I/R.
REFERENCES


Ischemia time-dependent variation in hepatic I/R


SUPPORTIVE INFORMATION

Intravital fluorescence microscopy and spectroscopy

Figure S1: Top left panel: Placement of the clamp over the liver hilum to induce 70% hepatic ischemia. The arrow indicates the afferent hepatic vessels. Top right panel: Ischemic left lateral and median liver lobes, evidenced by hepatic ‘blanching’, directly prior to removal of the clamp. Bottom panel: In vivo microscopy setup. The mouse is fixed on a custom-designed temperature-controlled stage. The liver lobes that had been subjected to 60 min I/R were positioned onto a custom-built transabdominal stage and covered with saran wrap to prevent desiccation during imaging.
Differential inflammatory response after 30 and 60 min hepatic ischemia

Figure S2: Plasma cytokine levels after 6 and 24 h of reperfusion. 30-6 and 60-6 indicate the 6 h reperfusion time for the 30 min and 60 min ischemia group, respectively. 30-24 and 60-24 indicate the 24 h reperfusion time points for the 30 min and 60 min ischemia group, respectively. Data are presented as mean ± SEM for N = 5-6 per group. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared to the sham group. # indicates $P < 0.05$ comparing the 30 and 60 min groups at the same reperfusion time point. Il-2 results are not displayed as levels were below the range of detection in most samples.
<table>
<thead>
<tr>
<th>Gene abbreviation</th>
<th>Gene Function of the gene product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2, monocyte chemotactic protein 1.</td>
<td>Induces migration and infiltration of monocytes/macrophages</td>
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<td>Ccl22</td>
<td>Chemokine (C-C motif) ligand 22</td>
<td>Chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells</td>
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<tr>
<td>Ccl7</td>
<td>Chemokine (C-C motif) ligand 7, monocyte-specific chemokine 3</td>
<td>Activates monocytes, dendritic cells, lymphocytes, natural killer cells, eosinophils, basophils, and neutrophils</td>
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<td>Cd2</td>
<td>Cluster of differentiation 2, T-cell surface antigen T11/Leu-5, LFA-2, LFA-3 receptor, erythrocyte receptor and rosette receptor</td>
<td>T cell-specific adhesion molecule</td>
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<td>Cd28</td>
<td>Cluster of differentiation 28</td>
<td>Costimulator, leads to T cell proliferation and cytokine secretion</td>
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<td>Cd34</td>
<td>Cluster of differentiation 34</td>
<td>Hematopoietic progenitor cell antigen</td>
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<td>Cd4</td>
<td>Cluster of differentiation 4</td>
<td>T-helper cell marker</td>
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<tr>
<td>Cd40lg</td>
<td>Cluster of differentiation 40 ligand</td>
<td>Activates CD40 to induce expression of pro-inflammatory mediators</td>
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<tr>
<td>Cd8a</td>
<td>Cluster of differentiation 8a</td>
<td>Cytotoxic T cell marker</td>
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<tr>
<td>Cebpb</td>
<td>CCAAT/enhancer-binding protein β</td>
<td>Transcription factor regulating macrophage function</td>
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<td>Csf3</td>
<td>Colony-stimulating factor 3</td>
<td>Stimulates the bone marrow to produce and release granulocytes</td>
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<td>Cx3cl1</td>
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<td>Chemoattracts lymphocytes</td>
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<td>Cxcl2</td>
<td>Chemokine (C-X-C motif) ligand 2</td>
<td>Chemoattracts leukocytes and hematopoietic stem cells</td>
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<td>Cxcl5</td>
<td>Chemokine (C-X-C motif) ligand 5, ENA-78</td>
<td>Chemoattracts neutrophils</td>
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<td>Foxp3</td>
<td>Forkhead box P3</td>
<td>Master regulator of regulatory T cells</td>
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<td>Gata3</td>
<td>GATA binding protein 3</td>
<td>Master regulator of T helper 2 cells</td>
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<tr>
<td>Icam1</td>
<td>Intercellular adhesion molecule 1</td>
<td>Potentiates neutrophil migration</td>
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<tr>
<td>Icos</td>
<td>Inducible T cell costimulator</td>
<td>Costimulator of T cells</td>
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<tr>
<td>Ifng</td>
<td>Interferon γ</td>
<td>Activates macrophages and promotes T helper 1 cell differentiation</td>
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<td>Il-10</td>
<td>Interleukin 10</td>
<td>Inhibits activation of T cells, monocytes, and macrophages</td>
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<td>Il-12rb1</td>
<td>Interleukin 12 receptor, β1</td>
<td>Receptor for Il-12</td>
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<td>Interleukin 15</td>
<td>Regulates T cell and natural killer cell activation and proliferation</td>
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<td>Il-17c</td>
<td>Interleukin 17c</td>
<td>Production of antimicrobial peptides</td>
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<td>Il-17d</td>
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<td>Stimulates Ccl2 production</td>
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<tr>
<td>Il-17rb</td>
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<td>Mediates NF-kB activation after Il-17E or Il-25 stimulation</td>
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<td>Il-17 receptor subtype</td>
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<td><strong>Il-18</strong></td>
<td>Interleukin 18, Induces IFNγ</td>
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<td><strong>Il-1b</strong></td>
<td>Interleukin 1β, Induces Cox-2 and Icam1 expression and IL-6 and chemokine production</td>
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<td><strong>Il-23α</strong></td>
<td>Interleukin 23α, Stimulates Ifn-γ production and T cell proliferation</td>
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<td><strong>Il-23r</strong></td>
<td>Interleukin 23 receptor, Receptor for IL-23</td>
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<td><strong>Il-27</strong></td>
<td>Interleukin 27, Regulates T cell function</td>
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<td><strong>Il-4</strong></td>
<td>Interleukin 4, Induces T helper 2 cell differentiation</td>
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<td><strong>Il-5</strong></td>
<td>Interleukin 5, Stimulates proliferation and activation of eosinophils and basophils</td>
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<td><strong>Il-6</strong></td>
<td>Interleukin 6, Regulates neutrophil recruitment through cytokine and chemokine signaling</td>
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<td><strong>Il-6ra</strong></td>
<td>Interleukin 6 receptor α, Receptor for IL-6</td>
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<td>Interleukin 7 receptor, Receptor for IL-7</td>
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<td><strong>Isg20</strong></td>
<td>Interferon-stimulated gene 20 kDa protein, Involved in Ifn-γ anti-viral defenses</td>
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<tr>
<td><strong>Jak1</strong></td>
<td>Janus kinase 1, Kinase essential for cytokine signaling, such as IL-2, IL-4, IL-6, IL-12, IL-10 and IFN signaling.</td>
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<td><strong>Mmp3</strong></td>
<td>Matrix metalloproteinase 3, Involved in extracellular matrix degradation</td>
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<td><strong>Mmp9</strong></td>
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<td><strong>Nfatc2</strong></td>
<td>Nuclear factor of activated T-cells, cytoplasmic 2, Transcription factor that regulates inflammatory gene expression upon T cell activation</td>
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<td><strong>Nfkβ1</strong></td>
<td>Nuclear factor NF-kappa-B p105 subunit, Transcription factor that induces inflammatory gene expression</td>
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<td><strong>Rorc</strong></td>
<td>RAR-related orphan receptor γ, Transcription factor characteristic of T helper 17 cells</td>
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<td>Sphingosine-1-phosphate receptor 1, Regulates regulatory and T helper 17 cell differentiation</td>
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<td><strong>Socs1</strong></td>
<td>Suppressor of cytokine signaling 1, Suppressor of cytokine signaling</td>
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<td><strong>Stat3</strong></td>
<td>Signal transducer and activator of transcription 3, Induction of the acute-phase response in the liver</td>
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<td>Signal transducer and activator of transcription 5A, Regulates lymphocyte and NK cell proliferation and is involved in hematopoiesis of lymphocytes</td>
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<td><strong>Sykb</strong></td>
<td>Spleen tyrosine kinase, Signaling kinase downstream of the T cell receptor</td>
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<td><strong>Tbx21</strong></td>
<td>T-box transcription factor 21, Regulates T helper 1 cell cytokine expression</td>
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<td><strong>Tgfβ1</strong></td>
<td>Transforming growth factor β1, Induces regulatory T cell development; can stimulate or inhibit macrophages</td>
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<td><strong>Tlr4</strong></td>
<td>Toll-like receptor 4, Initiates immune response induces by pathogen- or damage associated molecular patterns</td>
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<td><strong>Tnf</strong></td>
<td>Tumor necrosis factor α,Induces macrophage activation, neutrophil recruitment, and apoptosis</td>
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<td><strong>Traf6</strong></td>
<td>Tumor necrosis factor receptor-associated factor 6, Signaling factor downstream of Tnf, Il-1, and Toll-like receptors</td>
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Table S2: Fold expression of Th17 For Autoimmunity & Inflammation PCR Array genes compared to the sham group after hepatic I/R. Results are expressed as mean ± SEM for N = 3-5 per group.

<table>
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<tr>
<th>Gene abbreviation</th>
<th>Sham 30 min ischemia</th>
<th>Sham 6 h reperfusion</th>
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<td>Cebpb</td>
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<td>0.9 ± 0.0</td>
<td>0.5 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.8 ± 0.0</td>
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<tr>
<td>Csf3</td>
<td>1.0 ± 0.2</td>
<td>4.5 ± 0.6</td>
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<td>32.5 ± 0.2</td>
<td>9.4 ± 5.0</td>
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<tr>
<td>Cx3cl1</td>
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<td>0.3 ± 0.1</td>
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<td>0.6 ± 0.0</td>
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<td>Cxcl1</td>
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<td>2.5 ± 0.3</td>
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<td>Cxcl12</td>
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<td>0.8 ± 0.1</td>
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<td>0.2 ± 0.1</td>
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<tr>
<td>Cxcl2</td>
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<td>4.0 ± 1.2</td>
<td>18.1 ± 9.6</td>
<td>51.5 ± 13.9</td>
<td>19.6 ± 4.7</td>
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<tr>
<td>Cxcl15</td>
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<td>26.5 ± 10.3</td>
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<td>20.2 ± 4.4</td>
<td>16.0 ± 6.4</td>
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<td>Foxp3</td>
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<td>1.3 ± 0.2</td>
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<td>0.6 ± 0.1</td>
<td>0.7 ± 0.4</td>
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<td>Il10</td>
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<tr>
<td>Il-12rb1</td>
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<td>0.1 ± 0.0</td>
<td>0.6 ± 0.3</td>
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<tr>
<td>Il-15</td>
<td>1.0 ± 0.3</td>
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<td>2.3 ± 0.6</td>
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<tr>
<td>Il-17c</td>
<td>1.0 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>15 ± 0.4</td>
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<td>Il-17d</td>
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<td>Il-1b</td>
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<td>Il-23a</td>
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<tr>
<td>Il-27</td>
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<td>Il-4</td>
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<tr>
<td>Il-5</td>
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<td>6.1 ± 2.8</td>
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<td>Il-6</td>
<td>1.0 ± 0.2</td>
<td>3.7 ± 1.8</td>
<td>9.6 ± 7.3</td>
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<td>Il-7r</td>
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<td>0.8 ± 0.4</td>
<td>1.5 ± 0.4</td>
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<td>Nfkbia</td>
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<td>1.1 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>0.8 ± 0.3</td>
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<td>Rorc</td>
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<td>0.7 ± 0.1</td>
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<td>0.2 ± 0.0</td>
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<td>0.6 ± 0.1</td>
<td>1.2 ± 0.3</td>
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<td>1.4 ± 0.2</td>
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<td>1.6 ± 0.6</td>
<td>0.9 ± 0.2</td>
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Table S3: Function of proteins included in the cytokine array.

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<tr>
<th>Protein abbreviation</th>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Tnfa</td>
<td>Tumor necrosis factor α</td>
<td>Induces macrophage activation, neutrophil recruitment and apoptosis</td>
<td>56</td>
</tr>
<tr>
<td>Il-1β</td>
<td>Interleukin 1β</td>
<td>Induces Cox-2 and Icam-1 expression and IL-6 and chemokine production and affects cells such as macrophages and endothelial cells.</td>
<td>30</td>
</tr>
<tr>
<td>Il-4</td>
<td>Interleukin 4</td>
<td>Induces T helper 2 cell differentiation</td>
<td>34</td>
</tr>
<tr>
<td>Il-5</td>
<td>Interleukin 5</td>
<td>Stimulates proliferation and activation of eosinophils and basophils</td>
<td>35</td>
</tr>
<tr>
<td>Il-6</td>
<td>Interleukin 6</td>
<td>Regulates neutrophil recruitment through cytokine and chemokine signaling</td>
<td>36</td>
</tr>
<tr>
<td>Il-10</td>
<td>Interleukin 10</td>
<td>Inhibits activation of T cells, monocytes, and macrophages</td>
<td>22</td>
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<tr>
<td>Il-12</td>
<td>Interleukin 12</td>
<td>Activates T cells and natural killer cells, enhances proliferation of hematopoietic progenitor cells, stimulates production of IFN-γ and TNF-α in T cells and natural killer cells</td>
<td>58, 59</td>
</tr>
<tr>
<td>Il-13</td>
<td>Interleukin 13</td>
<td>Inhibits cytokine production by monocytes and regulates IFN-γ production by lymphocytes</td>
<td>60</td>
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<tr>
<td>Il-18</td>
<td>Interleukin 18</td>
<td>Induces IFN-γ</td>
<td>29</td>
</tr>
<tr>
<td>Il-22</td>
<td>Interleukin 22</td>
<td>Promotes cell proliferation and survival</td>
<td>61</td>
</tr>
<tr>
<td>Ccl5</td>
<td>Chemokine (C-C motif) ligand 5</td>
<td>Induces chemotaxis of leukocytes and lymphocytes</td>
<td>62</td>
</tr>
<tr>
<td>Gm-csf</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
<td>Induces production differentiation, proliferation, and activation of granulocytes and monocytes</td>
<td>63</td>
</tr>
<tr>
<td>Ifny</td>
<td>Interferon γ</td>
<td>Activates macrophages and promotes T helper 1 cell differentiation</td>
<td>21</td>
</tr>
<tr>
<td>Baff</td>
<td>B cell activating factor</td>
<td>Stimulates B cell proliferation and function</td>
<td>64</td>
</tr>
<tr>
<td>Tslp</td>
<td>Thymic stromal lymphopoietin</td>
<td>Induces T cell signaling and enhances maturation of dendritic cells</td>
<td>65</td>
</tr>
<tr>
<td>Vegf</td>
<td>Vascular endothelial growth factor</td>
<td>Is mitogenic and angiogenic; mediates vascular permeability</td>
<td>66</td>
</tr>
</tbody>
</table>
Liver uptake and excretion function is more sensitive to ischemia/reperfusion than hepatocellular damage

Figure S3: A-D. Solute carrier organic anion transporter family member 1B2 (SLCO1b2), multidrug resistance protein 2 (MDR2), multidrug resistance-associated protein 2 (MRP2), and bile salt export pump (BSEP) mRNA expression at 24 h of reperfusion after sham procedures, 30 min, or 60 min of hepatic ischemia in mice. Data represent mean ±SEM for N = 3-6 per group. ** indicates P < 0.01, and *** indicates P < 0.001 compared to the sham group.

Hepatic ischemia in patients mildly impairs liver function and does not induce IL-1β production

Table S4. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Liver function cohort</th>
<th>IL-1β cohort</th>
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<tbody>
<tr>
<td></td>
<td>N = 4</td>
<td>N = 9</td>
</tr>
<tr>
<td><strong>Age, years, median (IQR)</strong></td>
<td>66 (63-75)</td>
<td>67 (51-70)</td>
</tr>
<tr>
<td><strong>Gender, male, n (%)</strong></td>
<td>1 (25)</td>
<td>5 (56)</td>
</tr>
<tr>
<td><strong>Diagnosis, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Colorectal liver metastasis</td>
<td>2 (50)</td>
<td>4 (44)</td>
</tr>
<tr>
<td>- Hepatocellular carcinoma</td>
<td>1 (25)</td>
<td>2 (22)</td>
</tr>
<tr>
<td>- Cholangiocarcinoma</td>
<td>1 (25)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>- Benign lesion</td>
<td></td>
<td>2 (22)</td>
</tr>
<tr>
<td><strong>ASA classification, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- I</td>
<td></td>
<td>2 (22)</td>
</tr>
<tr>
<td>- II</td>
<td>3 (75)</td>
<td>3 (33)</td>
</tr>
<tr>
<td>- III</td>
<td>1 (25)</td>
<td>4 (44)</td>
</tr>
<tr>
<td><strong>Procedure, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Right hepatectomy</td>
<td>2 (50)</td>
<td>6 (67)</td>
</tr>
<tr>
<td>- Extended right hepatectomy</td>
<td>1 (25)</td>
<td>3 (33)</td>
</tr>
<tr>
<td>- Left hepatectomy</td>
<td>1 (25)</td>
<td>-</td>
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<tr>
<td><strong>FRL volume, mL, median (IQR)</strong></td>
<td>810 (753-1364)</td>
<td>591 (336-646)</td>
</tr>
<tr>
<td><strong>FRL function, %/min/m², median (IQR)</strong></td>
<td>4.9 (4.5-5.7)</td>
<td>3.2 (2.7-4.5)</td>
</tr>
<tr>
<td><strong>VIO duration, min, median (IQR)</strong></td>
<td>45 (41-56)</td>
<td>42 (33-50)</td>
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<tr>
<td><strong>Morbidity, Clavien-Dindo grade ≥ IIIa, n (%)</strong></td>
<td>1 (25)</td>
<td>1 (11)</td>
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<tr>
<td><strong>90-day mortality, n</strong></td>
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</tbody>
</table>

Abbreviations: IQR, interquartile range; ASA, American Society of Anesthesiologists; FRL, future remnant liver.
REFERENCES


Chapter 1


