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Role of Nucleotide Phosphohydrolysis and Nucleoside Signaling in Ischemic Preconditioning of the Liver

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I. ABBREVIATIONS

APCP	Alpha-Beta-Methylene-Adenosine Diphosphate
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
APCP	Alpha-Beta-Methylene-Adenosine Diphosphate
ARDS	Acute Respiratory Distress Syndrome
ATP	Adenosine Triphosphate
CD73	Ecto-5'-nucleotidase
CD39	Ecto-apyrase
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
E-NTPDase	Ecto-nucleoside Triphosphate Diphosphohydolase
IR	Ischemia Reperfusion
IP	Ischemic Preconditioning
IL	Interleukin
KO	Knock-out
MPO	Myeloperoxidase
mRNA	Messanger Ribonucleic Acid
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear Leukocyte (Neutrophil)
RNA	Ribonuclein Acid
ROS	Reactive Oxygen Species
RT-PCR	Realtime Polymerase Chain Reaction
RT	Room Temperature
TNF-α	Tumor Necrosis Factor-alpha
WT	Wildtype

II. TABLE OF CONTENTS

III. INTRODUCTION

Comparison of the human and the murine liver

The human liver is divided into a pars hepatis dextra consisting of the right liver lobe and the right half of the caudate lobe and a pars hepatis sinistra consisting of the left liver lobe, the quadrate lobe and the left half of the lobus caudatus. This functional anatomy reflects the blood supply and bile drainage from the right and left branch of the portal triad which includes the portal vein, hepatic artery, and bile duct. As shown in Figure 1, the widely accepted Couinaud classification of liver anatomy divides the liver further into eight functionally indepedent segments (I-VIII) based on a transverse plane through the bifurcation of the main portal vein [1]. The numbering of the segments is in a clockwise manner. Segment IV (quadrate lobe) is sometimes divided into segment IVa and IVb according to Bismuth [2]. Segment I (caudate lobe) is located posteriorly. It is not visible on a frontal view. Each segment has its own vascular inflow, outflow and biliary drainage. In the center of each segment there is a branch of the portal vein, hepatic artery and bile duct. In the periphery of each segment there is vascular outflow through the hepatic veins. The gall bladder lies in the Fossa vesicae biliaris, between the quadrate (IV) and right liver lobe.

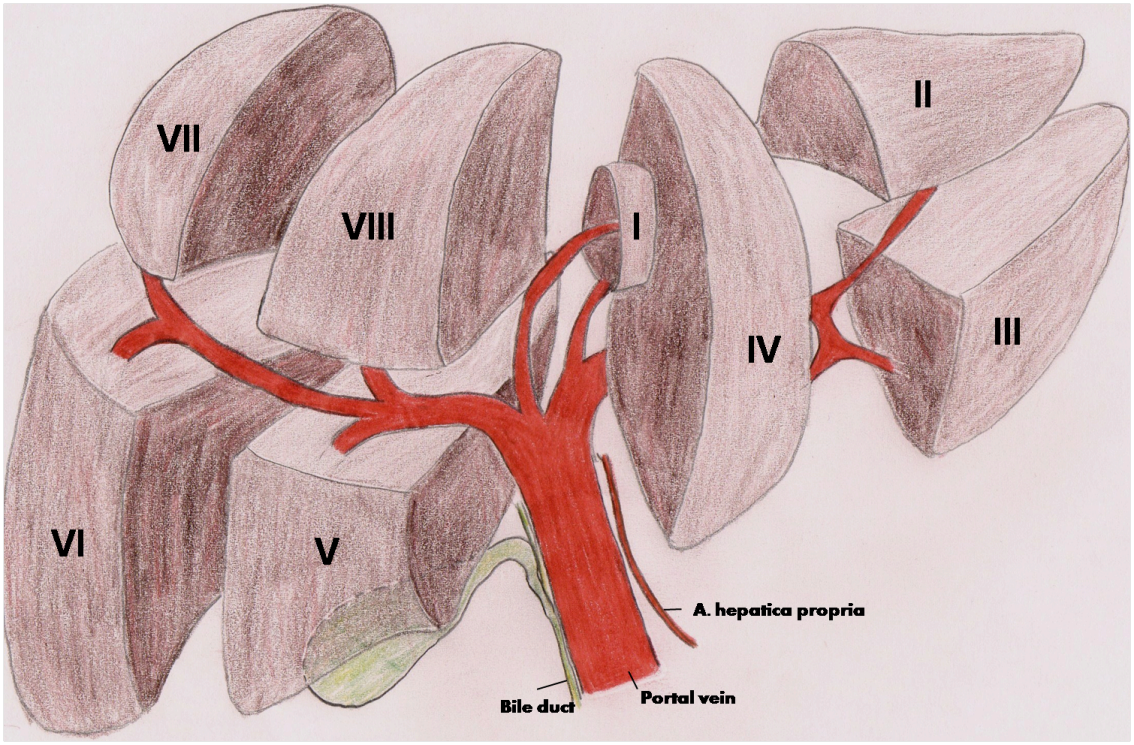

Fig. 1: Couinaud system. Eight functional liver lobes based on a transverse plane through the bifurcation of the portal vein. The bile duct and hepatic artery run and branch together with the portal vein known as the portal triad. The liver veins are not shown on this drawing.

As shown in Figure 2, the mouse liver is divided into four main lobes: caudate lobe, right lobe, median lobe and left lobe. The right liver lobe is subdivided into the superior right lobe and inferior right lobe. The median lobe is also divided into two parts: the right median lobe and the left median lobe. Similar to the human liver, the mouse liver is divided into lobes according to the branches of the portal triad, which in the mouse liver also consists of the portal vein, hepatic artery and bile duct. A study from Kongure and colleagues utilized liver corrosion casts to characterize rat liver lobes and compared them with the human liver segmentation defined by Couinaud. The lobe anatomy of the rat

liver is similar to the mouse liver. According to their study, the rat caudate lobe, left lobe, left median lobe, right median lobe, inferior and superior right lobe represent in humans segments: (I and IX); II; (III and IV); (V and VIII); VI; and VII respectively [3]. The gall bladder in the mouse is embedded between the right and left median lobes. In the mouse the inferior vena cava runs intrahepatic [4].

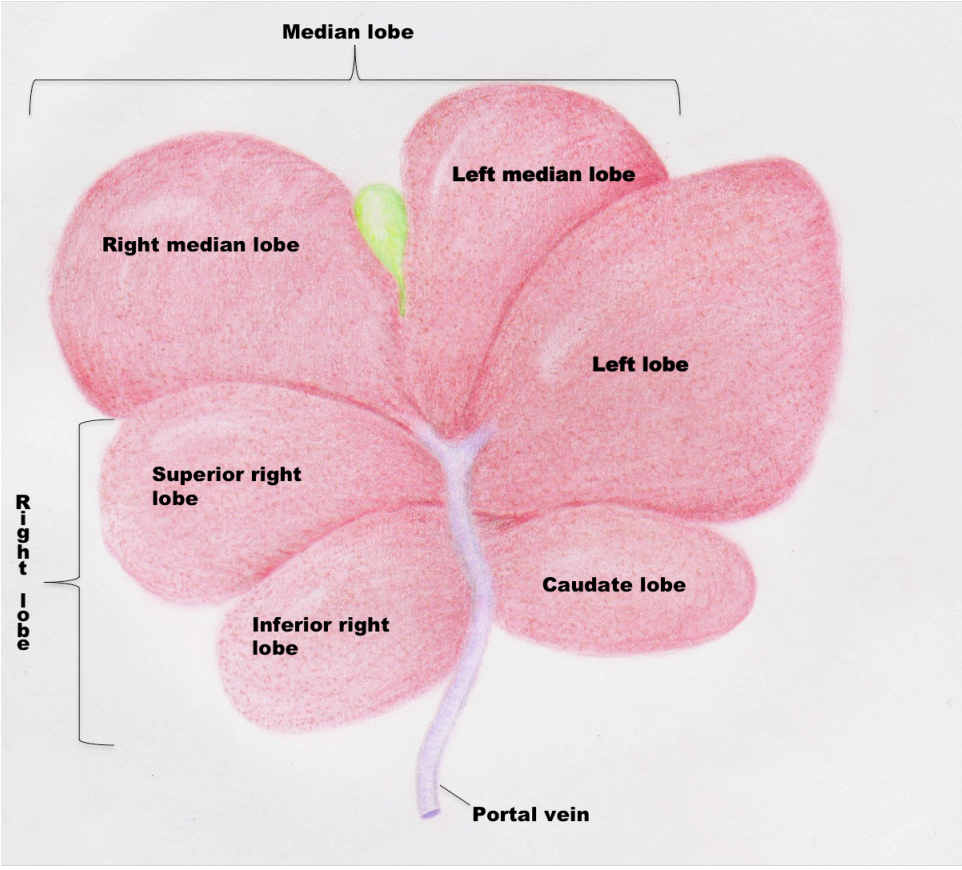

Fig. 2: Mouse liver lobe anatomy. The mouse liver is divided into four main lobes: caudate lobe, right lobe, median lobe and left lobe. Portal vein, hepatic artery and bile duct run and branch together and are known as the portal triad, similar to the human liver.

Definition of liver ischemia reperfusion injury and epidemiology

Ischemia/reperfusion (IR) injury is a phenomenon whereby cellular damage in an ischemic organ is elevated after the reestablishment of oxygen flow. Although restoration of blood flow to the ischemic organ is essential to prevent irreversible tissue injury, reperfusion also augments tissue injury in excess of that produced by ischemia alone by causing destruction of vascular integrity, tissue edema and disturbances in cellular energy balance.

Liver dysfunction or failure remains to be a significant clinical problem following transplantation surgery, tissue resections and hemorrhagic shock [5]. It is well known that the liver tolerates prolonged ischemia poorly [6] and IR injury is the main cause of hepatic damage and contributes to morbidity and mortality during these events [5, 6]. Furthermore, the shortage of organs available for transplantation increase the use of steatotic or cadaveric livers, which have a even lower tolerance to hypoxia and thus are more susceptible to reoxygenation damage, thereby increasing the risks of IR injury [5, 6]. Severe hepatic IR injury causes not only liver failure but may also result in multiple organ failure and systemic inflammatory response syndrome [7, 8]. Inflammatory events associated with hepatic reperfusion include disruption of the vascular endothelium and sinusoids, activation of immune cells, chemokine/cytokine secretion, and complement activation [9-11].

Pathophysiology of hepatic ischemia reperfusion

Ischemia and reperfusion injury to the liver occurs during liver resections performed under temporary inflow occlusion (Pringle manoeuvre) or inflow and outflow occlusion commonly used to reduce intraoperative blood loss, and during storage and implantation of livers for transplantation. Ischemia-induced decreases in cellular oxidative phosphorylation result in a failure to resynthesize energy-rich phosphates including ATP [12]. Thus, membrane ATP-dependent ionic pump function is altered, supporting the entry of calcium, sodium and water into the cell [12]. Ischemia also promotes expression of certain proinflammatory gene products (e.g. leukocyte adhesion molecules, cytokines) and bioactive agents (e.g. endothelin, thromboxane A2) within the endothelium, while repressing other “protective” gene products (e.g. constitutive nitric oxide (NO) synthase, thrombomodulin) and bioactive agents (e.g. prostacyclin, NO) [13, 14]. The regulation of these metabolites cause cellular damage and as a result lead to progressive cellular alterations, culminating in cell death by either necrosis or apoptosis [15, 16]. The hepatic production of TNF-α also propagates the inflammatory response to other organs, particularly to the lung, causing pulmonary insufficiency [17]. Ischemia activates Kupffer cells, which are the main source of vasclular reactive oxygen formation during the initial reperfusion period [18, 19]. Furthermore, with increasing length of the ischemic episode, intracellular generation of reactive oxygen species (ROS) may also contribute to liver dysfunction and cell injury during reperfusion. During ischaemia adenine nucleotide catabolism results in intracellular accumulation of hypoxanthine,

which is subsequently converted into toxic ROS when molecular oxygen is reintroduced (i.e., reperfusion) [20]. Finally, ROS increase leukocyte adhesion molecules and a proinflammatory state that increases tissue vulnerability to further injury on reperfusion. Consequently, ROS can cause tissue injury through several mechanisms including direct damage of cellular membranes through lipid peroxidation [21, 22] and via formation of arachidonic acid, an important precursor of eicosanoid synthesis (e.g. thromboxane A2 and leukotriene B4) which stimulates leukocyte activation and chemotaxis of polymorphonuclear cells (PMN or neutrophils) [22]. Furthermore these substances can cause vasoconstriction, vasodilatation, increased vascular permeability and stimulate platelet cytokine gene expression [22].

Adenosine signaling

It is well known that inflammatory tissue damage is accompanied by accumulation of extracellular adenosine, a naturally occurring anti-inflammatory agent [23-26]. Local tissue hypoxia or ischemia in inflamed areas represents one of the most important conditions leading to adenosine release and accumulation [23, 24, 26-30]. Recent in vitro and in vivo studies clearly confirm the beneficial role of adenosine as an immune modulator [27]. Furthermore, adenosine receptors (ARs) have been shown to modulate inflammation [31] [32].

Additionally, several murine models of inflammation provide evidence for adenosine receptor signaling as a mechanism for regulating inflammatory responses in vivo [24, 31, 33-56].

Four subtypes of G protein-coupled adenosine receptors exist, A2, A2a, A2b and A3. Originally, these receptors were classified based on their affinities for adenosine analogues and methylxanthine antagonists [57]. However, presently they are classified according to utilization of pertussis toxin–sensitive pathways (A1 and A3) or adenylate cyclase activation pathways (A2A and A2B) [58]. The A1AR and A3AR are coupled to inhibitory G (G_i) proteins, through which signaling results in decreased cAMP levels. In contrast, the A2ARs (A2A, A2B) are coupled to stimulatory G (G_s) proteins, which results in increased cAMP concentrations, an intracellular “off” signal which inhibits signaling pathways, leading to the interruption of proinflammatory processes in immune cells in a delayed negative feedback manner [29, 59]. Thus adenosine has been shown to control function of virtually every organ and tissue [59]. However, the exact source of adenosine during hypoxic or ischemic events is not well defined, but likely results from a combination of increased intracellular metabolism and amplified extracellular phosphohydrolysis of adenine nucleotides via surface ecto-nucleotidases.

Nucleotide metabolism and the role of ectonucleotidases

During ischemia, extracellular nucleotides (ATP/ADP) liberated at inflammatory or hypoxic tissue sites from various cells, including neutrophils, platelets, mast cells and endothelial cells [23, 24] are metabolized to adenosine via surface expressed ecto-nucleotidases (CD39 and CD73) [60]. As shown in Figure 3, ectoapyrase (CD39) converts ATP/ADP to AMP and ecto-5'-nucleotidase (CD73) subsequently converts AMP to adenosine [61]. Thus CD73 represents the major extracellular pathway for adenosine generation, especially during conditions of limited oxygen availability as may occur during hepatic ischemia.

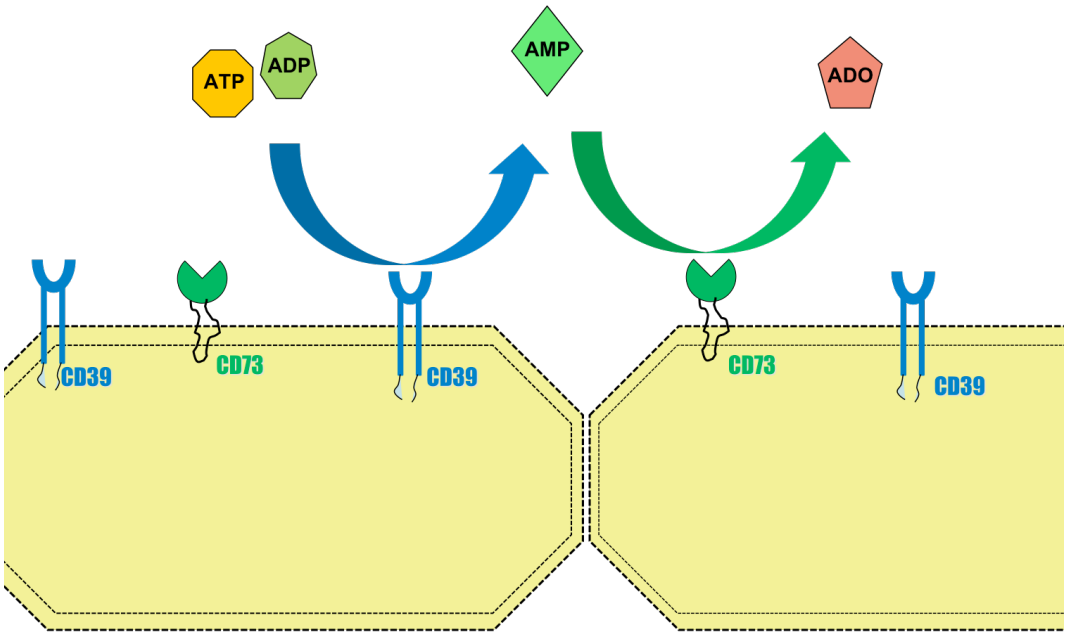

Fig. 3: Nucleotide Metabolism. CD39 converts ATP/ADP to AMP and CD73 subsequently converts AMP to adenosine.

Ecto-apyrase (CD39)

CD39 is an ecto-nucleotidase or ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and is expressed by the endothelium, dendritic cells, B cells and activated T cells [62]. The main property of this enzyme is to hydrolyse nucleoside tri- and diphosphates (i.e., ATP/ADP) to generate monophosphates (AMP) of both purine and pyrimidine nucleosides that are converted by the ubiquitous CD73 to the respective nucleosides.

Previous studies revealed an increase in CD39 in hypoxic endothelial and epithelial cells [34, 35]. Using $CD39^{-/-}$ animals it was shown that extracellular adenosine, produced through adenine nucleotide metabolism during hypoxia, is a potent anti-inflammatory signal for neutrophils *in vitro* and *in vivo* [34]. In addition CD39 has been considered to have high thromboregulatory potential [63] and to play a functional role in promoting endothelial permeability during hypoxia [34]. Thus, CD39 is a critical regulatory element in the control of inflammatory response by providing increased adenosine concentrations.

Ecto-5'nucleotidase (CD73)

Ecto-5'-nucleotidase is a 70-kDa glycosylphosphatidylinositol (GPI)-linked, membrane-bound glycoprotein which functions to hydrolyze extracellular nucleotides into bioactive nucleoside intermediates [64, 65]. Surface bound CD73 produces adenosine via enzymatic conversion of AMP. Adenosine then

activates one of four types of ARs or can be internalized through dipyridamole-sensitive carriers [23]. Adenosine generated by CD73 expressed on barrier cell types (e.g. endothelia, epithelia) has been shown to result in such diverse endpoints as regulation of endothelial permeability [66], attenuation of neutrophil adhesion [31] and stimulation of epithelial electrogenic chloride secretion [67]. Endothelial cells of many origins express CD73 constitutively. The primary function attributed to endothelial CD73 is catabolism of extracellular nucleotides (i.e., AMP to adenosine). Using $CD73^{-/-}$ mice, studies show that extracellular adenosine produced through adenine nucleotide metabolism during hypoxia is a potent anti-inflammatory signal for PMNs *in vitro* and *in vivo* [31, 33, 35-37]. These findings identify CD73 as a critical control point for endogenous adenosine accumulation and implicate this pathway as an innate mechanism to attenuate excessive tissue PMN accumulation. In addition to its role in limiting excessive neutrophil tissue accumulation, CD73 is also important for maintaining vascular permeability in multiple organs [34-36] and preventing intestinal barrier dysfunction during hypoxia [35].

Ischemic preconditioning

Ischemic preconditioning (IP) is a technique whereby an organ is rendered resistant to the damaging effects of IR by prior exposure to brief periods of vascular occlusion followed by short intervals of reperfusion. The current understanding of the underlying biologic principle is that cells primed by various

kinds of subinjurious stress trigger defense mechanisms against subsequent lethal injury [5, 68, 69].

IP was first described by Murry et al. in 1986 using a canine model in which they demonstrated that multiple brief ischemic episodes protected the heart from a subsequent sustained ischemic insult [70]. Since then myocardial IP has been shown to occur in many animal species [71] and in humans [72] and subsequently IP has been observed in other organ systems including skeletal muscle [73], brain [74], spinal cord [75], kidney [76], intestine [77] and liver [78].

Role of adenosine in hepatic IP

IP represents one of the strongest forms of *in vivo* protection of the liver [79]. Furthermore, its benefits have been suggested clinically by Clavien et al. who showed in a study with 100 patients undergoing major liver resection (> bisegmentectomy) under inflow occlusion for at least 30 minutes that postoperative serum transaminase levels were significantly lower in preconditioned patients versus control patients [80]. Thus IP is a promising strategy for improving the outcome of hepatic surgery.

Although the benefits of IP in the liver have been suggestged clinically [80-82], knowledge of the molecular mechanisms remains vague [6, 79, 83]. Previous studies suggest that adenosine generation plays a protective role in liver

protection from ischemia [53, 84]. For example Peralta et al. showed that the administration of adenosine mimics the effect of preconditioning in ischemic livers, and that the metabolization of endogenous adenosine by adenosine deaminase abolished the protective effect of preconditioning [85]. These observations were extended by Nakayama et al. who showed that rat pretreatment with the adenosine A2 receptor agoinist CGSS21680, but not with the adenosine A1 receptor agoinst N-pheyl-isoproyladenosine, before total hepatic ischemia enhanced tolerance against hypoxia/reperfusion damage [86].

During liver ischemia extracellular levels of ATP are dramatically increased [87]. ATP/ADP at inflammatory hypoxic tissue sites procduced from neutrophils, platelets, mast cells and endothelalial cells are metabolizied to adenosine via surface expressed ecto-nucleotidases (CD39 and CD73) [23, 60]. This extracellular adenosine released in large quantities during ischemia is believed to play a role in the protective effect of IP during reperfusion of ischemic tissue. IR injury is associated with neutrophil and leukocyte activation and primary microvascular failure. Adenosine inhibits leukocyte adhesion, decreases expression of adhesion molecules and inhibits neutrophil and platelet function [88, 89]. Adenosine also inhibits free radical production [26, 90, 91], which are important mediators of cellular damage in the early phase of IR injury, and is a potent vasodilator [92]. The above would suggest adenosine may be protective against ischemia reperfusion injury and the effects of adenosine in IP are likely to be multifactorial.

Despite the fact that liver cells express both A1- and A2A-type adenosine receptors, it is believed that liver preconditioning specifically relies on the activation of A2A -type receptors [45]. These receptors have been shown to be anti-inflammatory and to reduce IR injury in the liver [45, 47, 93]. Linden et al. also showed that the pharmacological activation of A2AAR procduced a profound protection from liver IR injury that was absent in congenic animals lacking the A2AAR gene.

Objective and experimental setting

Ischemic preconditioning represents a powerful experimental strategy to identify novel molecular targets to attenuate hepatic injury during ischemia. As a result, murine studies of hepatic IP have become an important field of research. However, murine IP is technically challenging and experimental details can alter the results. Therefore, we systematically tested a novel model of hepatic IP by using a hanging-weight system for portal triad occlusion. This system has the benefit of applying intermittent hepatic ischemia and reperfusion without manipulation of a surgical clamp or suture, thus minimizing surgical trauma. Previous studies have demonstrated that liver IP can be performed in mice [83]. However, these studies differ in their experimental approach, particularly with regard to details of intermittent occlusion of blood flow to the liver. Furthermore, occlusion is achieved by use of a surgical clamp which can be technically

challenging. In particular, placement of the clamp without injury to the hepatic lobes is technically difficult and this technique may be associated with further tissue trauma during IP by removing and replacing the clamp. Repeated removal and replacement of the surgical clamp also makes it difficult to produce exact reproducible durations of IP intervals since the lobes of the liver frequently shift and thus disrupt the view of the portal triad. Moreover, if the clamp is not strong enough or is misplaced due to slight movements, inadvertent reperfusion due to imperfect occlusion may affect the results. Based on these potential problems associated with portal triad occlusion by a clamp, we developed a model of IP using a hanging-weight system for intermittent occlusion of the portal triad. We hypothesized that this model would yield results with highly reproducible injury markers and hepatic protection by IP, as it combines the advantage of immediate and reliable occlusion/reperfusion with avoidance of tissue trauma due to manipulation of the hepatic lobes by reapplication of a clamp.

With a reliable experimental setting, we decided to further investigate the role of CD73 for hepatic IP. To date, the role that CD73, the “pacemaker” enzyme of extracellular adenosine production, plays in hepatic IP is not clear. Therefore we sought to determine weather CD73 can be implicated as a key mediatior in protection against hepatic IR induced injury by IP and identify novel pharmacological targets for liver protection against ischemia. We performed murine pharmacologic and genetic studies using mice gene targeted for *cd73*.

IV. MATERIAL AND METHODS

Mice

All animal experiments were in accordance with the German guidelines for use of live animals approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen. C57BL/6 and SV129 mice (8 to 12 week old) were purchased from Charles River Laboratory. Mice deficient in cd73 ($cd73^{-/-}$) [94] were compared to $cd73^{+/+}$ (WT) mice. All mice maintained on a 12:12-h light/dark cycle at an ambient temperature of 24°C and 60% humidity. Food and water were provided ad libitum. In some experiments, mice were treated with 5'-[αβ-methylene] diphosphate (APCP, Sigma, 40 mg/kg/h, i.p.), soluble 5'-nucleotidase (5'-NT) (Sigma, 2 U i.v., followed by 10 U/kg/h, i.p.), adenosine (Sigma, 1 mg/kg/h, i.a.) or dipyridamole (GensiaSicor, Irvine, CA, 0.5 mg/kg, i.p.) prior to ischemia.

Anesthesia and surgery

Mice were anesthetized with pentobarbital sodium (70 mg/kg body weight, i.p.) and placed on a temperature-controlled heated table with a rectal thermometer probe attached to a thermal feedback controller to maintain body temperature at 37°C. Mice were then secured in a supine position, with the upper and lower extremities attached to the table with removable tape.

Technique of portal triad occlusion

Operations were performed under an upright dissecting microscope (Olympus, SZX7, Hamburg, Germany). After a midline laparotomy and incision of the linea alba, the peritoneal cavity was exposed. The stomach and duodenum were caudally displaced using a wet cotton tip swab to expose the portal triad and caudate lobe. The caudate lobe was gently separated from the left lobe and the right lobe was then slightly shifted to clearly view the portal triad above the bifurcation of right, median, and left lobes. We used a partial hepatic ischemia model that avoids mesenteric congestion by allowing blood flow through the right lobe. Once visually identified, the needle, followed by suture (7/0 nylon suture, Ethicon, Norderstedt, Germany) was placed under the portal triad, including the hepatic artery, hepatic vein and common bile duct (Fig. 4A, B). The left end of the suture was then placed over the right pole, while the right end of the suture was placed over the left pole and a weight of 2 grams was attached to each end (Fig. 4A, C). While the weights were placed over the poles, the triad was immediately occluded, causing blood supply to the left, median and caudate lobes of the liver to be interrupted (Fig. 4D). Successful occlusion was confirmed by visual inspection of pale blanching in the ischemic lobes (i.e., a change in color from red to a pale color). In contrast, the change of color immediately disappeared when the hanging weights were removed from the poles and the liver was reperfused. In an additional experiment, successful occlusion to the left, median and caudate lobes was confirmed by injection of Evan's Blue into the portal vein caudal to the liver (Fig. 4E). During surgery, the

liver was kept wet and warm with a wet swab soaked with saline at 37°C. The surgical wound was closed using continuous sutures of the muscle wall and skin. Mice underwent 30 or 75 min ischemia, followed by 3 h reperfusion under a heating lamp or IP (4 cycles of 3 min ischemia/3 min reperfusion) prior to IR. Sham operated mice served as the control and underwent anesthesia, laparotomy, and exposure of the portal triad without IR or IP. All animals survived the surgical procedure and no complications were observed with portal triad occlusion using the hanging-weight system or in control mice.

Changes in liver perfusion

Changes in liver perfusion were determined by injecting a solution of fluorescein isothiocyanate (FITC)-labeled dextran (0.06g/ml; FITC-dextran; mol wt 4000; Sigma, St. Louis, MO) into the carotid artery (200 µl dissolved in saline), followed by 200 µl of saline. FITC-dextran was allowed to perfuse through the entire mouse for approximately 2 min to ensure maximal perfusion of the liver. The right followed by median lobe was quickly excised from the mouse prior to ischemia, at 30 min ischemia or following 30 min ischemia and 15 min reperfusion and placed into formamide (100 mg tissue/ml; Sigma, St. Louis, MO) for 2 h at 55°C. The fluorescence of FITC-dextran was determined using a fluorescence spectrophotometer with 485-nm excitation and 535-nm emission.

Preconditioning Protocols

First, the influence of different ischemia times (10, 20, 30, 40 and 50 min) on hepatic function was investigated. Then hepatic protection by IP was assessed in this model comparing different IP cycles consisting of 3 or 5 min ischemia and 3 or 5 min reperfusion, followed by 30 min ischemia and 3 h reperfusion. For CD73 studies, mice underwent 4 cycles of IP (3 min ischemia/3 min reperfusion), followed by 30 min ischemia and 3 h reperfusion.

Alanine (ALT) and aspartate aminotransferase (AST*)* serum activity

Serum ALT and serum AST levels were measured using a microtiter plate adaptation of a commercially available kit (Teco Diagnostics, Anaheim, CA, USA) [95]. Serum samples were added to ALT or AST Substrate Solution in a 96-well microtiter plate. After incubation (at 37°C) the corresponding Colour-Reagent was added. The reaction was stopped with ALT-Colour-Developer for ALT measurement and 2 N HCl for AST measurement. The optical density was measured at 510 nm using a photometer.

Lactate dehydrogenase activiy

Lactate dehydrogenase (LDH) activity was measured using a kit purchased from Randox (Crumlin, United Kingdom). Briefly, Serum samples were added to a 96 well Elisa Microplate. When NAD-Reagent was added to the serum the

samples incubated for 30 seconds. Then, LDH activity was measured every 30 seconds over 3 minutes at 355nm using a photometer.

Interleukin-6 (IL-6) serum activity

Cytokine activity was measured in serum using a commercially available ELISA kit (R+D Systems, Minneapolis, MN, USA). Briefly, serum samples were added to a 96-well microtiter plate, which was coated with rat anti-mouse antibody to IL-6. The unbound protein was removed by washing and a biotinylated goat anti-mouse antibody to the appropriate cytokine was added. After washing and adding streptavidin-HRP to each well, substrate solution was added after 20 min incubation and repeated washes and the plate was again incubated. The optical density of each well was measured photometrically at excitation of 450 nm and emission of 540 nm.

TTC staining for determining percent infarct

After mice underwent 30 min ischemia, followed by 3 h reperfusion (with or without prior IP), the ischemic area (area at risk, AAR, Fig. 4E) and the size of the infarct itself were determined using an adaptation of the previously described triphenyltetrazolium chloride (TTC) staining technique [96]. In brief, the left and median lobes of the liver were carefully removed following reperfusion, washed in ice-cold 0.9% saline, placed on parafilm, frozen at -20˚C

for 30 minutes and cut into 1mm slices. The slices were then incubated with 1% TTC at 37°C for 30 min and fixed in 10% formaldehyde to allow differences between viable and necrotic tissue to become apparent. Thus, TTC stains all cells red except those that are depleted in NADPH and therefore allows one to visualize the viable tissue which appears red versus the infarcted tissue which appears pale. Using computer-assisted planimetry, the boundaries of the whole lobe versus the infarcted areas were defined and calculated using NIH software Image 1.0. The infarct size and therefore degree of damage was calculated as the percentage of infarcted AAR compared to the whole lobe (=100%).

Myeloperoxidase activity

Myeloperoxidase (MPO) analysis was performed using the Fluorescent Myeloperoxidase Detection Kit (Cell Technology, Mountain View, CA, USA). In brief, 0.5g of the median and left liver lobes were homogenized in 500 µl 1X Assay Buffer which was provided with the kit. Diamide (10mM, Sigma, St. Louis, MO), a glutathione inhibitor, was added to each sample and incubated for 30 min at room temperature (RT). Homogenates were centrifuged and 0.5 ml Solubilization Buffer (provided in the kit) was added to the pellet. Samples were homogenized for an additional 30 sec followed by 30 sec of sonication and 2 cycles of freezing/thawing. After centrifugation, 20mM 3-Amino-1,2,4-triazole (Sigma, St. Louis, MO), a catalase inhibitor, was added to the supernatant and incubated at RT for 1 h. A standard curve was prepared by using the standard

provided in the kit and the value for each sample was read from this curve. Fluorescence was measured at excitation of 550 nm and emission at 590 nm using a fluorescent plate reader.

Histological assessment of damage

The median and left liver lobes were harvested and placed in cryomolds containing OCT Tissue-Tek embedding medium (Sakura Finetek Europe, Zoeterwoude, NL). Samples were immediately frozen in 2 methylbutane (Sigma, St Louis, MO, USA), pre-cooled in liquid nitrogen, and stored at -80ºC until further processing. Frozen tissues were subsequently sectioned on a cryostat, 10 µm thick, collected onto (+) charge slides and stained with hematoxylin and eosin. Examination and scoring of each lobe was carried out by a pathologist who was blinded to the experimental group. A semi-quantitative grading scale of 0-4, as outlined by Suzuki et al. [97] was used for the histopathological assessment of liver necrosis where 0 = no liver necrosis, 1 = single cell necrosis, 2 = up to 30% lobular necrosis, 3 = up to 60% lobular necrosis, and 4 = more than 60% lobular necrosis.

For C3 despositon frozen tissue sections were air dried, fixed with 100% methanol at -20°C and washed with PBS. After blocking, slides were washed and C3 deposition was determined using rabbit polyclonal IgG anti-C3 (1:100;

Abcam, Cambridge, MA) and Cy5-coupled donkey anti-rabbit IgG (1:200; Dianova, Hamburg, Germany). Nuclei were counterstained with 4',6-diamidino-2-phenylindole.

RT-PCR

We performed four cycles of IP (3 min ischemia, 3 min reperfusion) and excised the median and left lobes after different reperfusion times, followed by isolation of RNA, reverse transcription, and quantification with ENT-, complement- or CD73-specific primers by real-time RT-PCR (iCycler; Bio-Rad Laboratories, Munich, Germany). In short, total RNA was isolated from liver tissue using the total RNA isolation NucleoSpin RNA II Kit according to the manufacturer's instructions (Macherey and Nagel, Dueren, Germany). For this purpose, tissue from the liver was homogenized in the presence of RA1 lysis buffer (Micra D8 homogenizer, ARTLabortechnik, Muellheim, Germany), and after filtration lysates were loaded on NucleoSpin RNA II columns, followed by desalting and DNase I digestion (Macherey and Nagel, Dueren, Germany). RNA was washed, and the concentration was quantified. cDNA synthesis was performed by using reverse transcription according to the manufacturer's instructions (i-script Kit, Bio-Rad Laboratories, Munich, Germany). The primer sets for the PCR reaction contained 10 pM sense and 10 pM antisense with SYBR Green I (Molecular Probes, Leiden, Netherlands). The following murine primer sequences were used (sense and anti-sense, respectively): ENT1 (5'-

CTTGGGATTCAGGGTCAGAA-3', 5'-TCAGGTCACACGACACCAA-3'); ENT2 (5'-CATGGAAACTGAGGGGAAGA-3', 5'-GTTCCAAAGGCCTCACAGAG-3'); ENT3 (5'AACCTGGGCTACAGGAGACA-3', 5'-TAGAACAGGGAGCCCTGAGA-3'), ENT4 (5'-AGGGGGCGTTTATTCA-GTCT-3', 5'-AGAACGGAGTTGGGGAC-TTT-3'), MBL-A (5'-CCAAAGGGG-AGAAGGGAGAAC-3', 5'-GCCTCGTCCG-TGATGCCTAG-3'); MBL-C (5'-GACGTGACGGTGCCAAGGG-3', 5'-CTTTCTGGATGGCCGAGTTTTC-3'); C3 (5'-CACCGCCAAGAATCGCTAC-3', 5'-GATCAGGTGTTTCAGCCGC-3'); C5 (5'-CAAAGGATCCAGAAAAGAAGCCTGTAAACC-3', 5'-CCTTAAGCTTCGTGCA-GCAGAACTTTTCATTC-3'); C9 (5'-CCACCGAAGTACCTGAAAAG-3', 5'-AGGAAAGTTGACCTCAGCAC-3'); CD73 (5'-CAAATCCCACACAACCACTG-3' and 5'-TGCTCACTTGGTCACAGGAC-3'). The primer set was amplified by using increasing numbers of cycles of 94°C for 1 min, 58°C for 0.5 min, and 72°C for 1 min. Murine β-actin (sense primer, 5'-ACATTGGCATGGCTTTGTTT-3' and antisense primer, 5'-GTTTGCTCC-AACCAACTGCT-3') in identical reactions were used to control for the starting template.

Western Blot

Western blotting technique was used to examine total CD73 protein level. Briefly, protein extracts from the median lobe were solubilized in reducing Laemmli sample buffer and heated to 70°C for 10 min. Samples were resolved

on a 10% polyacrylamide gel and transferred to PVDF membranes (BioRad Laboratories, Munich, Germany). The membranes were blocked at 4°C overnight in 5% nonfat dry milk (Applichem, Cheshire, USA) in Tris-buffered saline with Tween-20 (TBS-T). The membranes were incubated in 4 µl/ml CD73 rabbit polyclonal antibody raised against the C-terminus (ABGENT, San Diego, USA) for 2 h at room temperature (RT), followed by 5 min washes in TBS-T. The membranes were incubated 1:2000 in goat anti-rabbit HRP (Santa cruz, Danvers, USA). The wash was repeated and proteins were detected by enhanced chemiluminescence. To ensure equal loading, membranes were detected for β-Actin. Blots were stripped for 15 min at RT in Stripping Buffer (PIERCE, Rockford, USA) and washed and blocked as mentioned above. Membranes were incubated with β actin rabbit monoclonal antibody at dilution of 1:1000 (Cell Signaling, Danvers, USA) for 2 h at RT followed by 5 min washes in TBS-T. The membranes were then incubated with 1:2000 goat anti-rabbit HRP (Santa Cruz, Danvers, USA). The wash was repeated and proteins were detected by enhanced chemiluminescence.

Adenosine measurements

The left and median liver lobes were removed and immediately snap frozen with clamps pre-cooled to the temperature of liquid nitrogen within a time lag of 3-5 sec. The frozen tissue was pulverized under liquid nitrogen, protein was precipitated with ice-cold 0.6 N perchloric acid and tissue adenosine levels

were determined [98].

Statistical analysis

Hepatic injury score data are given as median (range) and analyzed with a Kruskal-Wallis rank test. All other data are presented as mean±SEM and analyzed using one-way analysis of variance.

V. RESULTS

Technique of portal triad occlusion

Based on potential problems associated with portal triad occlusion by a clamp, we developed a model of hepatic IP using a hanging-weight system for intermittent occlusion of the portal triad (Fig. 4A-D). Once the portal triad was visually identified, the needle with suture was placed under the portal triad as indicated (Fig. 4A, B). The sutures, with weights attached to each end, were then placed over opposite poles (Fig. 4A, C). Once the weights were placed over the poles, the triad was immediately occluded, causing blood supply to the left, median and caudate lobes of the liver to be interrupted, with blood flow continuing through the right lobe (Fig. 4D). Successful occlusion was confirmed by visual inspection of pale blanching in the ischemic lobes (i.e., a change in color from red to a pale color). In contrast, the change of color immediately disappeared when the hanging weights were removed from the poles and the liver was reperfused.

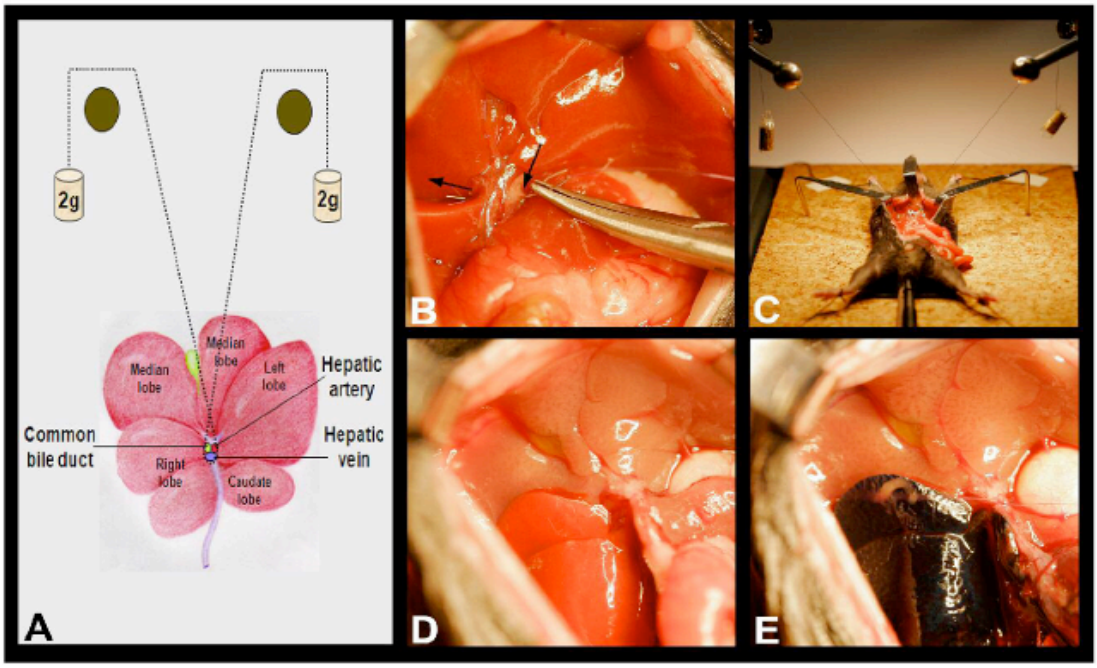

Fig. 4: Model of liver ischemic preconditioning (IP) using a hanging-weight system for portal triad occlusion. After a midline laparotomy the stomach and duodenum were caudally displaced to expose the portal triad. *(A)* The caudate lobe was gently separated from the left lobe and the right lobe was then slightly shifted to clearly view the portal triad above the bifurcation of right, median, and left lobes. *(B-C)* Once visually identified, a 7/0 needle and nylon suture was placed under the portal triad, including the hepatic artery, hepatic vein and common bile duct. The left end of the suture was then placed over the right pole, while the right end of the suture was placed over the left pole and a weight of 2 grams was attached to each end. *(D)* While the weights were suspended by the poles, the triad was immediately occluded, causing blood supply to the left, median and caudate lobes of the liver to be interrupted. Successful occlusion was confirmed by a change of color from red to a pale color. In contrast, the change of color immediately disappeared when the hanging weights were removed from the poles and the liver was reperfused. *(E)* In an additional experiment, successful occlusion to the left, median and caudate lobes was confirmed by injection of Evan's Blue into the portal vein caudal to the liver. The ischemic (pale colored) area represents the area at risk (AAR).

In an additional experiment, the portal triad was occluded and ischemia was confirmed via injection of Evan's Blue into the portal vein caudal to the liver (Fig. 4E). Evan's blue was unable to enter the left, median and caudate ischemic lobes and therefore these lobes appeared pale colored, while the non-ischemic

right lobe was intensely stained with Evan's blue. To quantify the observed changes in hepatic perfusion, mice were given FITC-dextran via a carotid artery catheter prior to ischemia (sham), at 30 min ischemia or following 30 min ischemia and 15 min reperfusion and the perfused:nonperfused FITC-dextran ratio of the liver lobes was calculated. As shown in Figure 5A, during ischemia (30 min I) perfusion to the ischemic lobe was significantly decreased ($P<0.001$) compared to the sham control or following 30 min ischemia and 15 min reperfusion (30 min I/15 min R).

Total hepatic ischemia has been shown to produce several pathologic events, including splanchnic congestion, severe intestinal ischemia, mesenteric congestion and systemic shock, thereby resulting in a high mortality rate [99-102]. Furthermore, occlusion to all lobes of the liver causes venous congestion in the mesenteric bed and compromises the intestinal mucosa, resulting in bacterial translocation and onset of systemic inflammatory response syndrome (SIRS) [103, 104]. To confirm these results we compared intestinal congestion following partial versus total hepatic ischemia using the hanging-weight system. As shown in Fig. 5, in sharp contrast to mice that underwent total hepatic ischemia, mice that underwent partial hepatic ischemia revealed little to no intestinal/mesenteric congestion. Therefore we used a partial hepatic ischemia model that avoids congestion to the intestine by allowing blood flow through the right lobe.

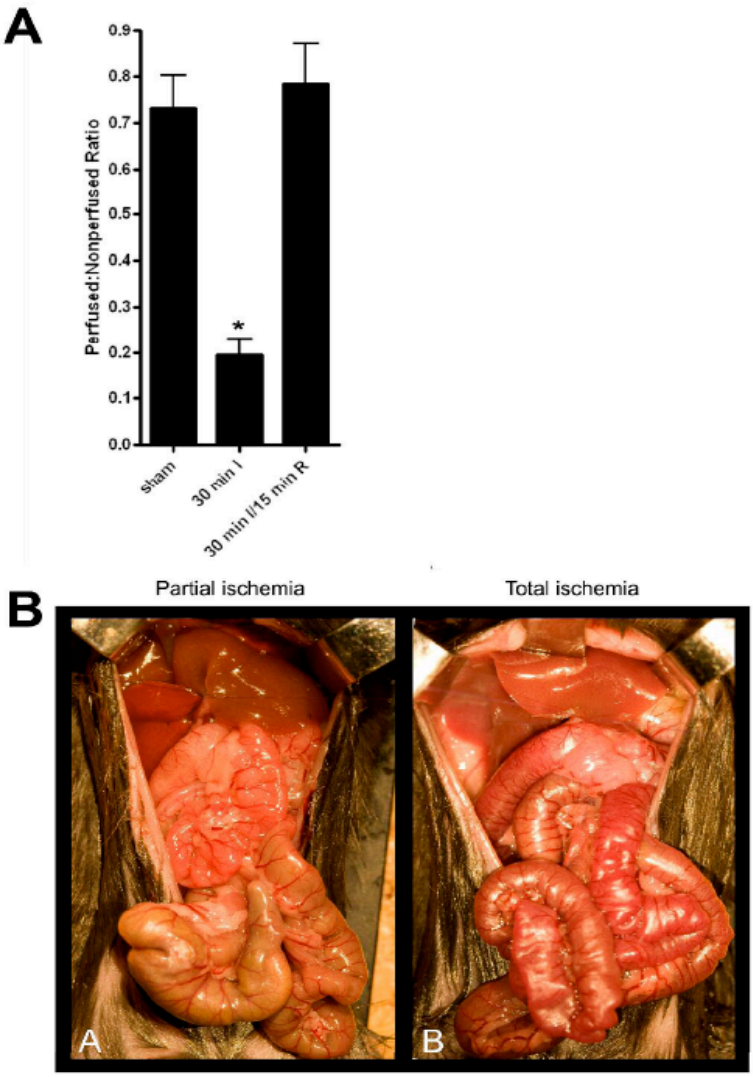

Fig. 5: Changes in hepatic perfusion and intestinal congestion. *(A)* Mice were given FITC-dextran via a carotid artery catheter prior to ischemia (sham), at 30 min ischemia (30 min I) or following 30 min ischemia and 15 min reperfusion (30 min I/15 min R) and the perfused: nonperfused FITC-dextran ratio of the liver lobes was calculated. Results are expressed as the mean ± SEM of 3-4 mice/group. * $p < 0.05$ compared to sham or 30 min I/15 min R groups. *(B)* For partial ischemia, the portal triad was occluded above the bifurcation of the right, median, and left lobes causing blood supply to the left, median and caudate lobes of the liver to be interrupted. For total ischemia, the portal triad was occluded below the bifurcation of the right, median, and left lobes causing blood supply to all lobes of the liver to be interrupted.

Influence of ischemia time on hepatic injury

Because of the fact that previous studies in murine liver IP suggest dissimilar ischemia times [79, 83], we first tested the effect of different ischemia times on hepatic injury using the hanging-weight system model. Identification of an ischemia time resulting in a medium range of hepatic damage is important for the study of liver protection by IP, as it allows detection of changes in both directions, e.g. smaller degree of injury with hepatic IP or larger degree of injury with experimental therapeutics or a specific gene deletion. Elevated serum AST and ALT concentrations are commonly detected after hepatic injuries and therefore are reliable markers for assessing recent liver parenchymal cell membrane integrity and liver injury [105, 106]. As shown in Fig. 6, ischemia times from 0 to 50 minutes, followed by 3 h of reperfusion were associated with increased ALT and AST serum enzymatic levels. In fact, over the examined time range (0 to 50 min), hepatic ischemia time closely correlated with ALT and AST levels (R^2 = 0.941, p < 0.001, R^2 = 0.957, p < 0.001, respectively). Furthermore, our AST and ALT results compared with the results of others using relatively similar ischemia times in rodents [107, 108]. Taken together these results provide feasibility of using portal triad occlusion via a hanging-weight system for inducing highly reproducible and time-dose-dependent hepatic injury. As we observed a “medium” degree of liver injury with 30 min of ischemia time, all further studies were performed using 30 min of portal triad occlusion followed by 3 h of reperfusion.

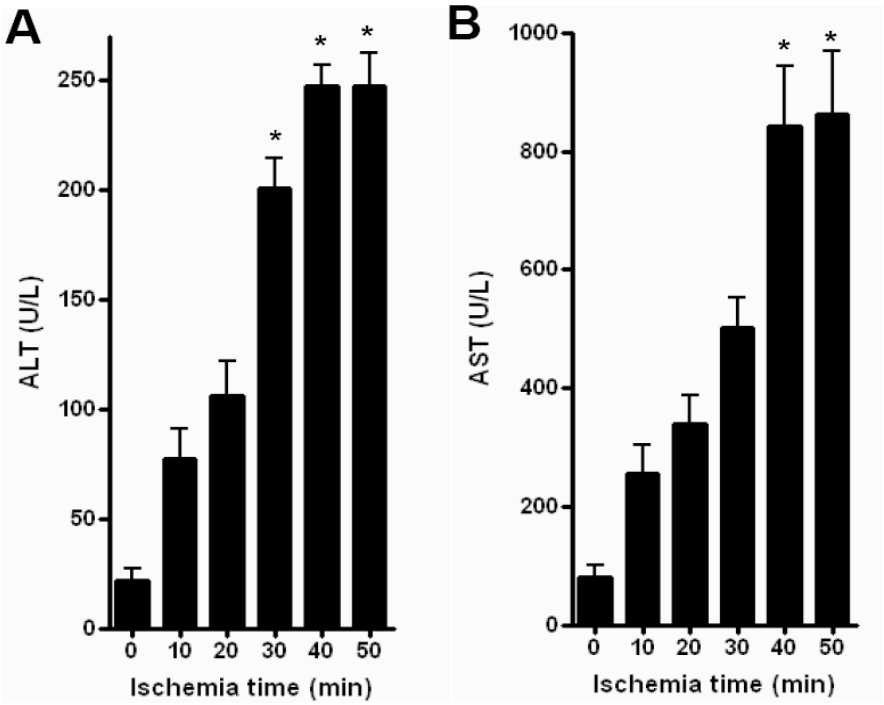

Fig. 6: Effect of different ischemia times on ALT and AST in mice. Portal triad ischemia was induced as indicated (10–50 min). After 3 h of reperfusion, *(A)* alanine aminotransferase (ALT) and *(B)* aspartate aminotransferase (AST) were measured. Results are expressed as the mean ± SEM of 6-12 mice/group. * $p < 0.05$ compared to 0 min ischemia.

Influence of different IP cycles

After having demonstrated reproducible hepatic injury with ischemia and reperfusion, we next used the hanging-weight occlusion system in experiments of liver protection by IP. To test the influence of different cycle numbers of IP, mice underwent 2-5 cycles of IP in which each cycle consisted of 3 or 5 min ischemia, followed by 3 or 5 min reperfusion. IP was followed by 30 min of ischemia and 3 h reperfusion.

There was a significant increase in ALT and AST following IR compared to sham controls (Fig. 7A and B, respectively). However, a decreasing trend in

ALT and AST was apparent in mice that underwent increasing cycles of IP consisting of 3 min ischemia, followed by 3 min reperfusion prior to IR, with a significant and maximal level of protection occurring with [(3 min ischemia/3 min reperfusion) x 4 cycles] of IP. Interestingly, ALT and AST levels appeared to increase with 5 cycles of IP [(3 min ischemia/3 min reperfusion) x 5 cycles]. As shown in Fig. 7B, mice that underwent 3-5 cycles of (5 min ischemia/5 min reperfusion) IP were also afforded a significant protection for AST. However, this protection did not occur with ALT (Fig. 7A). In fact, all of the mouse groups that underwent IP consisting of 5 min ischemia followed by 5 min reperfusion showed no significant difference in ALT when compared to the IR group.

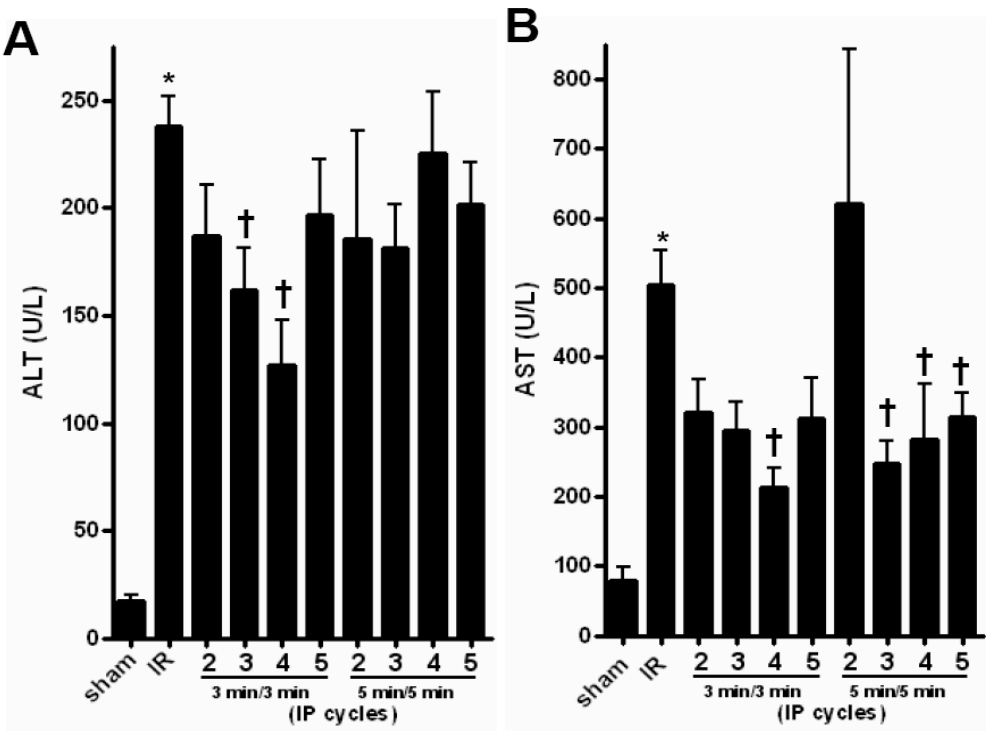

Fig. 7: Effect of different IP cycles on ALT and AST in mice. Mice underwent IR alone or 2-5 cycles of IP in which each cycle consisted of 3 or 5 min ischemia, followed by 3 or 5 min reperfusion. IP was followed by 30 min portal triad ischemia and 3 h reperfusion. Sham mice underwent the same surgical procedure but without IP or IR. Injury was assessed by measuring *(A)* ALT or *(B)* AST. Results are expressed as the mean ± SEM of 4 mice/group. * $p < 0.05$ compared to sham. † $p < 0.05$ compared to IR.

Due to the fact that both ALT and AST showed similar protective effects for IP cycles of 3 min ischemia/3 min reperfusion, but not for IP cycles of 5 min ischemia/5 min reperfusion, we further investigated the influence of different IP cycle numbers using 3 min ischemia followed by 3 min reperfusion. In agreement with our ALT and AST results, mice undergoing 3 min ischemia/3 min reperfusion showed a similar decrease in proinflammatory cytokine IL-6 and liver enzyme LDH serum levels with increasing cycles numbers (Fig. 8A and B, respectively). In these studies, one or two cycles of IP (3 min ischemia/3 min reperfusion) was not associated with a significant attenuation of LDH or IL-6 compared to unpreconditioned animals. In contrast, 3 cycles of IP resulted in a significant decrease in LDH (Fig. 8B), while 3 or 4 cycles of IP afforded significant liver protection as measured by IL-6 (Fig. 8A). As with ALT and AST levels (Fig. 7A and B, respectively), LDH and IL-6 appeared to increase with 5 cycles of IP. Collectively, these results demonstrate that a maximum level of protection using the hanging-weight system occurs when mice are subjected to 4 cycles of IP consisting of 3 min ischemia and 3 min reperfusion.

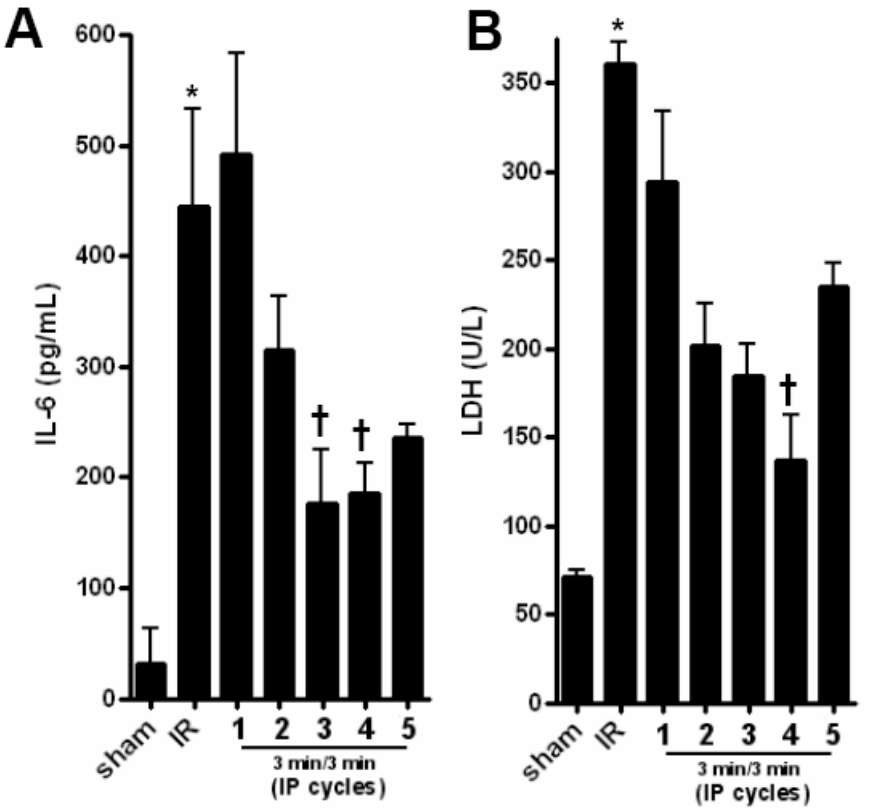

Fig. 8: Effect of 1-5 cycles of IP consisting of 3 min ischemia and 3 min reperfusion on injury markers in mice. Mice underwent IR alone or 1-5 cycles of IP consisting of 3 min ischemia and 3 min reperfusion, followed by 30 min portal triad ischemia and 3 h reperfusion. Sham mice underwent the same surgical procedure but without IP or IR. Injury was assessed by measuring *(A)* IL-6 or *(B)* lactate dehydrogenase (LDH). Results are expressed as the mean ± SEM of 6-12 mice/group. * $p < 0.05$ compared to sham. † $p < 0.05$ compared to IR.

Liver protection by IP

Since maximal protection was observed with 4 cycles of IP (3 min ischemia/3 min reperfusion), further analysis of liver injury was assessed using this method, followed by 30 min ischemia and 3 h reperfusion (Fig. 9A). Results demonstrated that this robust improvement in hepatic injury by IP was also observed in additional tests. Thus, as shown in Fig. 9B, mice subjected to IP prior to 30 minutes of ischemia also showed a significant decrease in the

percent infarction. Since leukocytes have been shown to be responsible for the acute inflammatory response during IR [109, 110], we measured myeloperoxidase as an indicator of neutrophil infiltration. Mice subjected to IP demonstrated a significant decrease in infiltration of neutrophils into the ischemic lobes compared to IR mice (Fig. 9C). These studies demonstrate that the degree of damage as measured by percent infarct and MPO are reliable readouts for liver injury and protection by IP in this model.

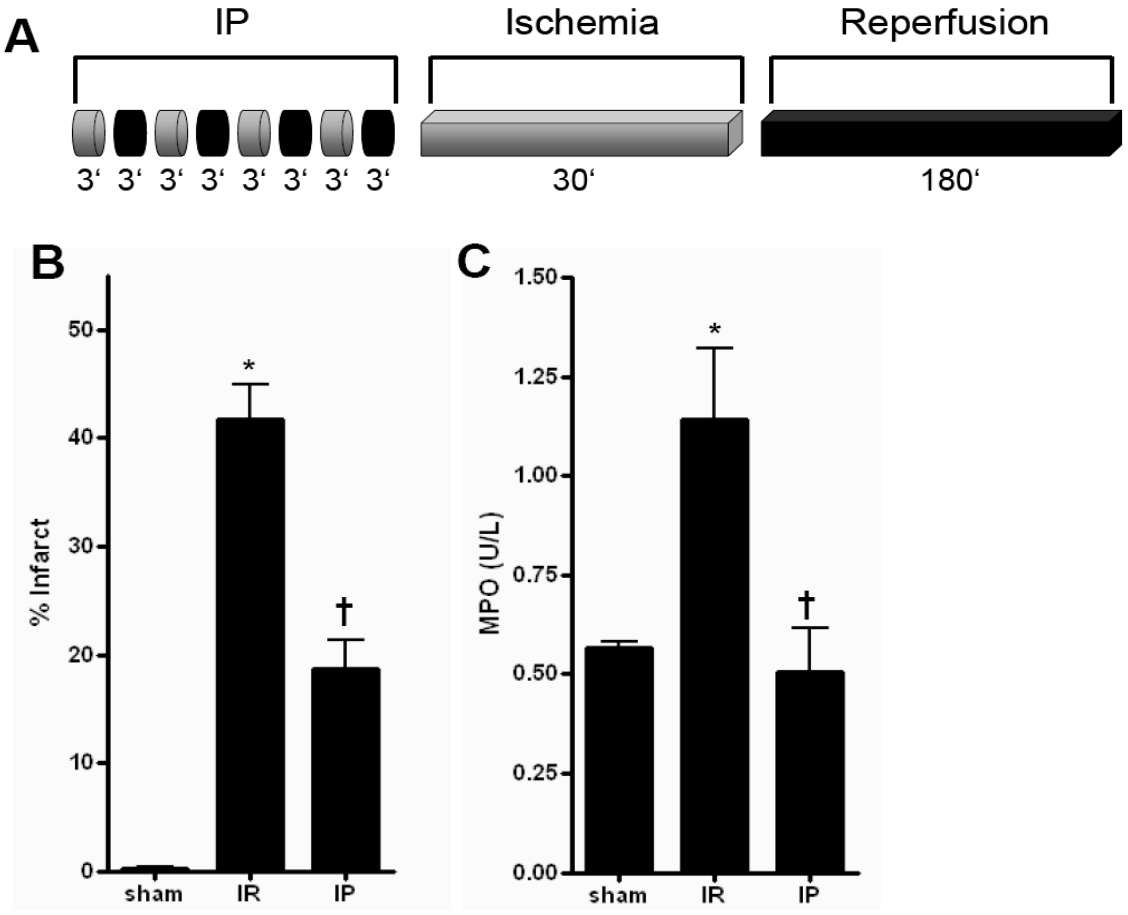

Fig. 9: IP model and IP protective effects on infarct size and MPO. *(A)* Schematic illustration of the experimental protocol for hepatic ischemic preconditioning (IP). One IP cycle consisted of 3 min (‘) ischemia followed by 3 min of reperfusion. To document hepatic protective effects of IP, *(B)* infarct size was measured using TTC staining. *(C)* Myeloperoxidase (MPO) was measured as an indicator of neutrophil infiltration. Results are expressed as the mean ± SEM of 6-12 mice/group. * $p < 0.05$ compared to sham. † $p < 0.05$ compared to IR.

As demonstrated in Fig. 10A, histological signs of ischemic injury were also attenuated by IP. Thus, 30 min of ischemia resulted in hepatocyte liver necrosis (IR). In contrast, mice with IP prior to ischemia showed only mild to moderate histological signs of injury similar to sham-operated control mice. In fact, semi-quantitative histological analysis demonstrated a reduction in the Suzuki index [97] from 3 (range 3 to 4) without IP to 2 (range 2 to 3, Fig. 10B, $p < 0.01$) with IP.

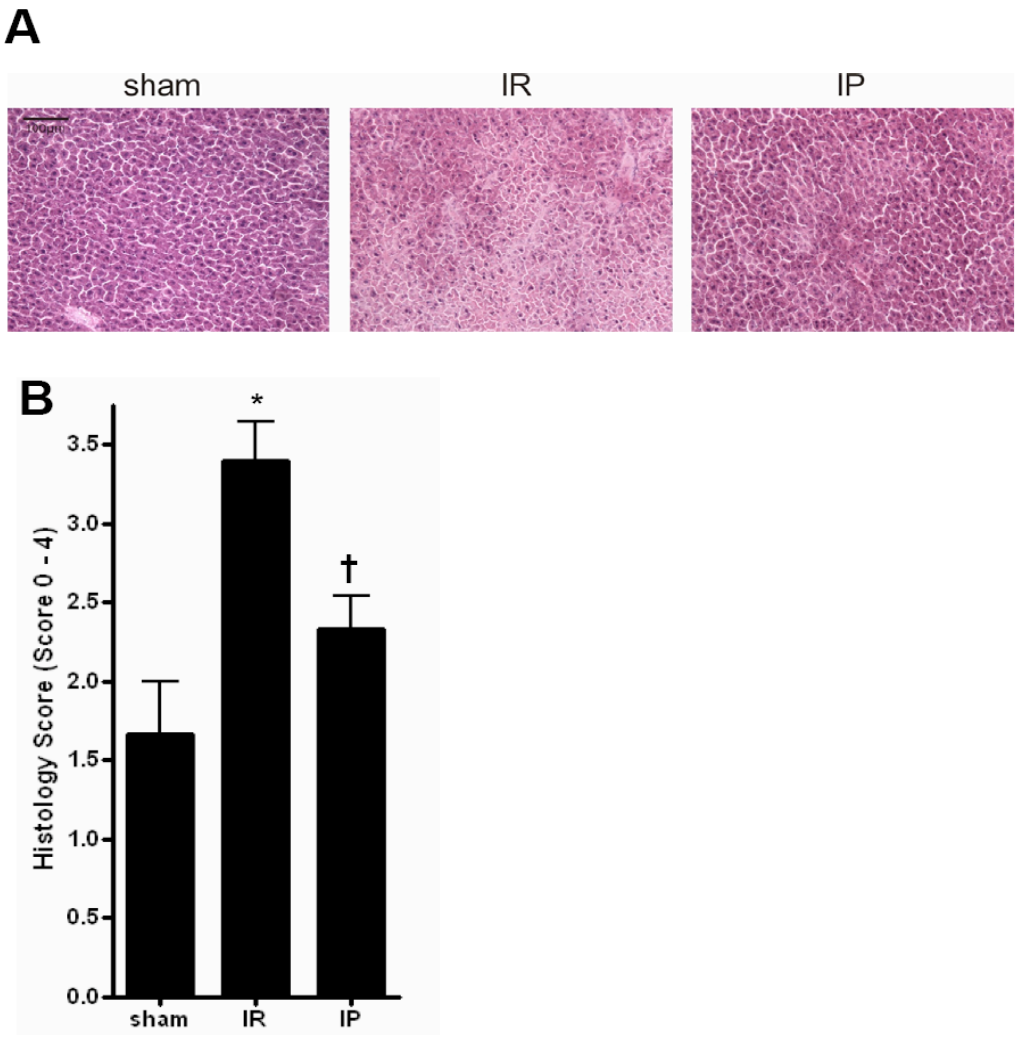

Fig. 10: Histological signs of liver injury are attenuated following IP. *(A)* Representative H&E stained sections (200X) and *(B)* quantification of ischemic injury. Results are expressed as median ± range, n = 6 mice per group. * $p < 0.05$ compared to sham. † $p < 0.05$ compared to IR.

Comparison of clamping versus hanging-weight system methods

As the next step, we compared hepatic protection by IP via portal triad occlusion using the hanging-weight system versus conventional clamping. As shown in Fig. 11, IP (4 cycles of IP consisting of 3 min ischemia and 3 min reperfusion) using the hanging-weight system resulted in robust reduction of hepatic injury. In contrast, clamping of the portal triad (using a similar IP and ischemia protocol) was not associated with a statistically significant improvement of liver function. In fact, clamping the portal triad resulted in significantly less injury compared to occlusion using the hanging-weight system with 30 min ischemia alone (IR). Furthermore, repeated clamping during IP appeared to cause more injury than IR alone. These results suggest that occlusion of the portal triad using the hanging-weight system is more reliable than occlusion with a clamp.

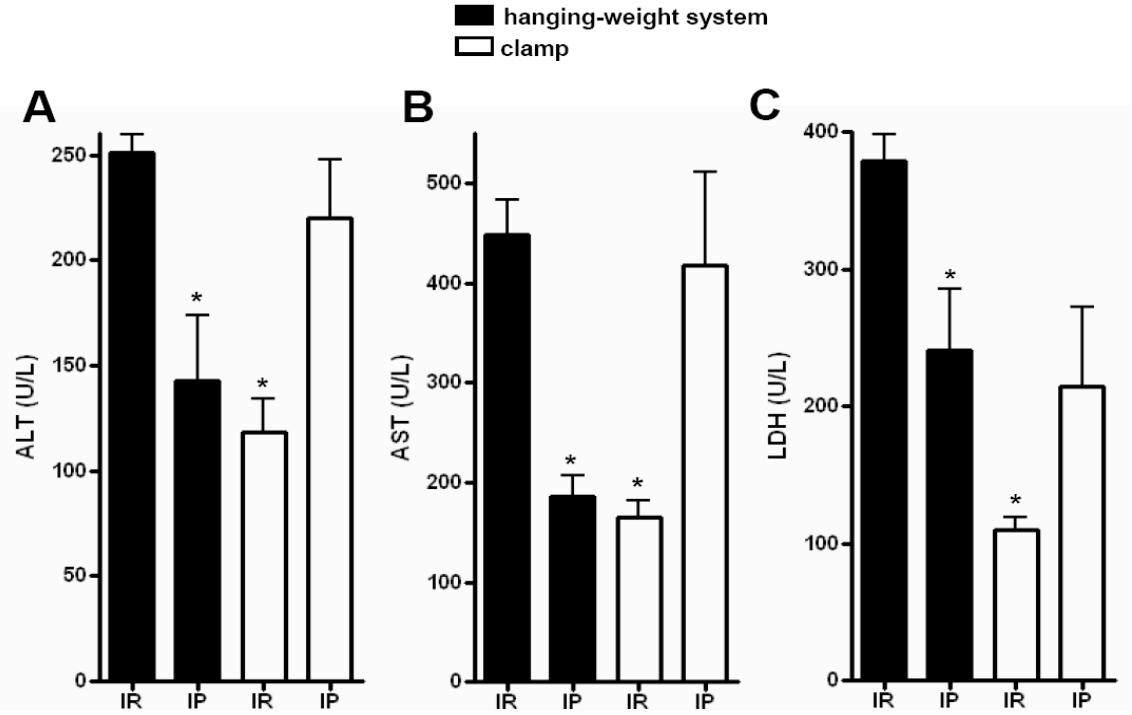

Fig. 11: Comparison of hepatic protection from ischemia by IP using the hanging-weight system versus clamping methods. IP was performed using the hanging-weight system for occlusion of the portal triad with 4 IP cycles (3 min ischemia, 3 min reperfusion) prior to 30 min of ischemia and 3 h reperfusion. Alternatively, the same IP protocol was used with clamping the portal triad. Injury was assessed by measuring *(A)* ALT, *(B)* AST and *(C)* LDH. Results are expressed as the mean ± SEM of 4-8 mice/group. * $p < 0.05$ compared to IR using the hanging-weight system.

Effect of genetic background

Based on previous reports suggesting murine strain-specific differences in sensitivity to ischemia-reperfusion injury [111-115], we compared hepatic injury using the hanging-weight system in two different mouse strains (C57BL/6 and SV129). As shown in Fig. 12, results demonstrated that 4 cycles of hepatic IP consisting of 3 min ischemia and 3 min reperfusion prior to 30 min ischemia [(3min/3min) x 4 IP] did not protect SV129 mice, as it did for C57BL/6 mice, from injury. However, 3 cycles of hepatic IP consisting of 5 min ischemia and 5 min reperfusion prior to 30 min ischemia [(5min/5min) x 3 IP] protected SV129 mice. Taken together, these data further demonstrate marked differences between different murine genetic backgrounds. In fact, these results underline the critical importance of performing control experiments in closely matched littermate controls of a similar genetic background.

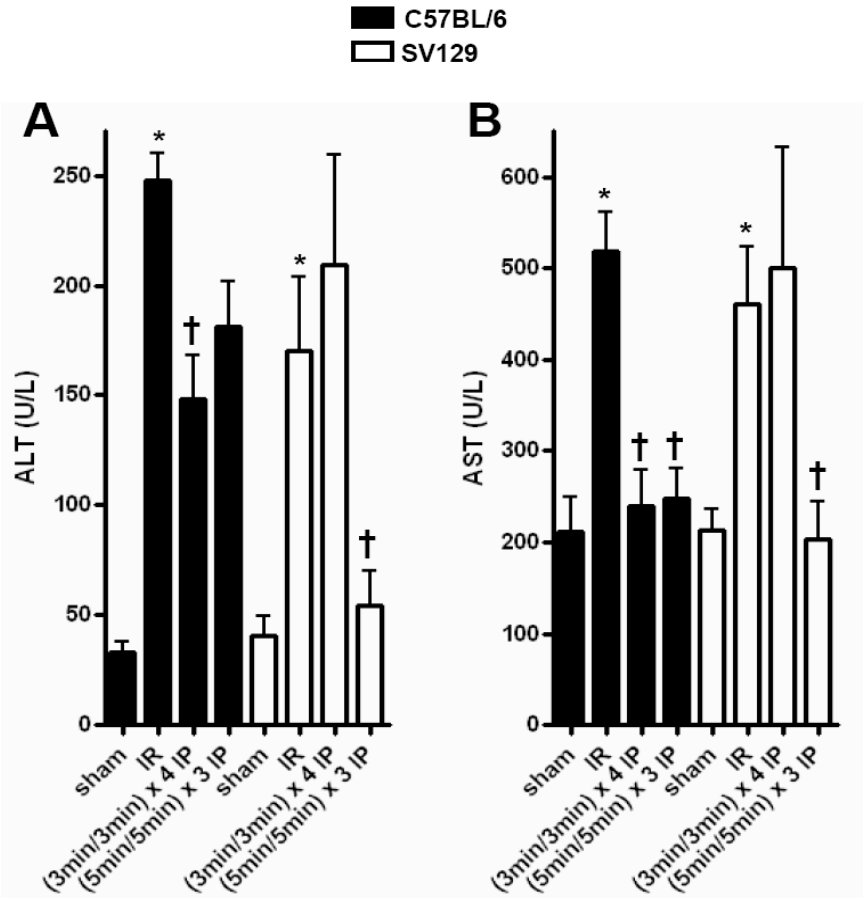

Fig. 12: Effect of genetic background on hepatic injury. To assess the influence of different murine genetic backgrounds (C57BL/6 or SV129) on hepatic injury, we compared mice underwent IR alone versus mice that underwent either 4 IP cycles consisting of 3 min ischemia/3 min reperfusion or 3 IP cycles consisting of 5 min ischemia/5 min reperfusion prior to 30 min of ischemia and 3 h reperfusion. Sham mice underwent the same surgical procedure but without IP or IR. Injury was assessed by measuring *(A)* ALT or *(B)* AST. Results are expressed as the mean ± SEM of 4-12 mice/group. * $p < 0.05$ compared to sham. † $p < 0.05$ compared to respective IR group.

Modulation of gene expression by liver IP

As the last step, we measured gene regulation by IP in this model. To test the usefulness of this model and to assess transcriptional consequences of IP, we used real-time RT-PCR to demonstrate regulation of a group of genes known as equilibrative nucleoside transporters (ENTs) which have previously been shown

to be hypoxia regulated [33]. Although ENTs are highly expressed in the liver [116], very little is known about their physiological role in the liver. Under the hypothesis that IP would also repress these genes, we performed four cycles of IP and excised the liver after 180 minutes. Similar to what is known about ENT regulation by hypoxia [33], liver ENT1-4 are transcriptionally repressed 3 h after IP (*P < 0.01 compared with control; Fig. 13A).

Complement activation following oxidative stress is an early event and inhibition of complement activation or its components may offer tissue protection [117, 118]. Tanhehco et al. demonstrated that preconditioning of the heart reduces myocardial complement gene expression [119]. To determine if preconditioning of the liver reduces hepatic complement gene expression, we transcriptionally assessed expression of mannose binding lectin (MBL)-A and MBL-C, as well as C3, C5, and C9 after four cycles of IP. All complement genes were significantly repressed 30 min after IP (Fig. 13B). Taken together, these results highlight the usefulness of this model to measure transcriptional effects of liver IP.

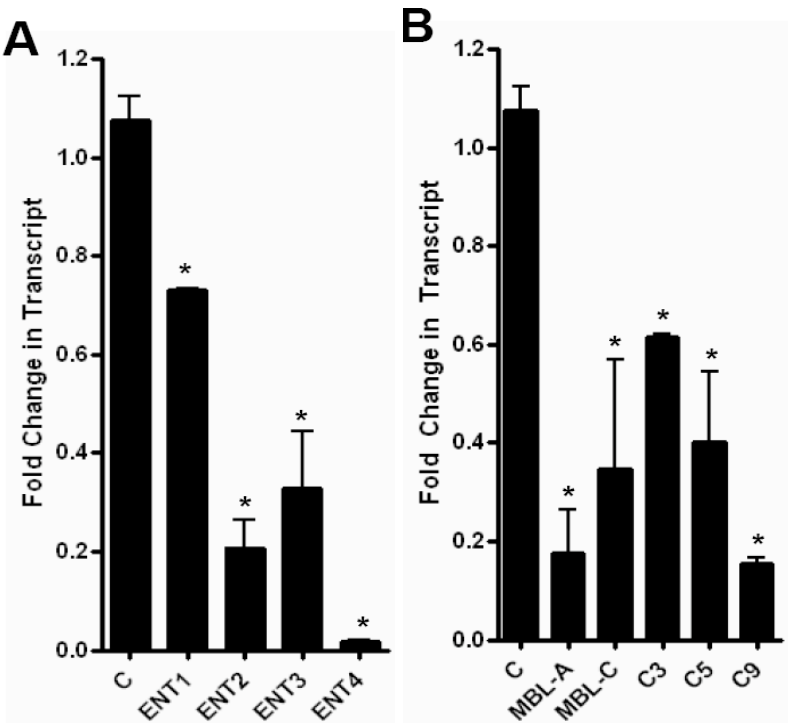

Fig. 13: Repression of the equilibrative nucleoside transporters (ENTs) and complement genes by IP. Mice were subjected to 4 cycles of IP (3 min ischemia, 3 min reperfusion), and 30 or 180 min reperfusion. Total RNA was isolated, and *(A)* ENT1-4 or *(B)* MBL-A, MBL-C, C3, C5 and C9 mRNA levels were determined by real-time PCR. Data were calculated relative to ß-actin and expressed as fold change in transcript relative to control (C) samples. Results are expressed as the mean ± SEM of 3 mice/group. * $p < 0.05$ compared to control (C).

Liver CD73 is induced by IP

Now that we established a novel reliable model for hepatic IP, we decided to further investigate its specific protective mechanisms. It has been shown that CD73 is the pacemaker enzyme of extracellular adenosine production and we sought to determine whether CD73 could also be implicated as a key mediatior in protection against hepatic IR induced injury by IP.

Based on previous studies demonstrating tissue protection via extracellular adenosine generation by hypoxia-inducible CD73 [31, 34, 36] we hypothesized

that CD73-dependent adenosine generation may play an important role during hepatic IP. We first investigated liver CD73 expression in mice subjected to four cycles of intermittent portal triad occlusion and reperfusion (3 min of ischemia/3 min of reperfusion) prior to 30 min ischemia and up to 3 hours reperfusion (Fig. 14A). A significant induction of CD73 mRNA was observed 90 and 180 min following hepatic IP (Fig. 14B). Western blot analysis confirmed CD73 protein induction with the highest level of protein appearing at 120 (2.2±0.2) and 180 min (3.1±0.1) after IP (Fig. 11C-D). These data support transcriptional and translational induction of CD73 in the liver during liver IP.

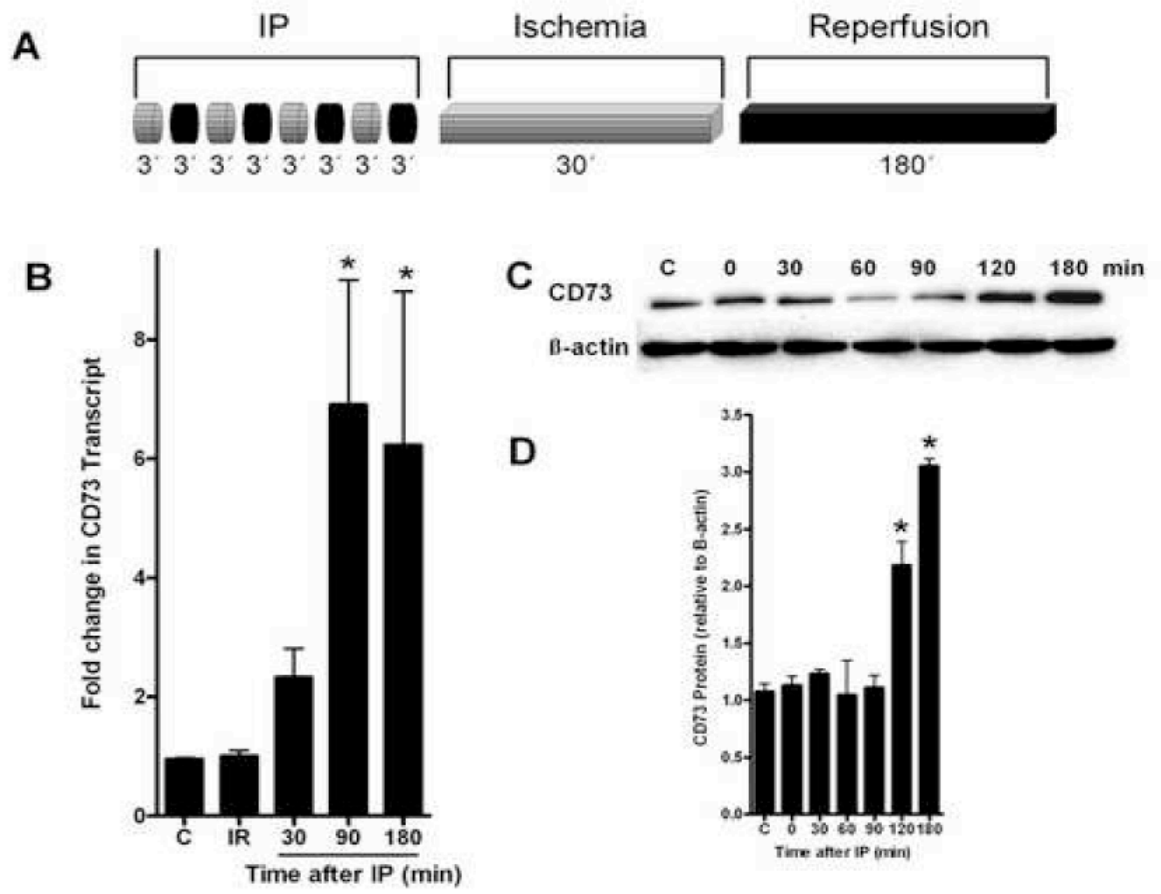

Fig. 14: CD73 is induced by liver IP. *(A)* After 4 cycles of IP [3 min ischemia (grey), 3 min reperfusion (black)] and 30 min ischemia, the median lobe was excised following

reperfusion. *(B)* Total RNA was isolated and CD73 mRNA was determined by real-time PCR. Data were calculated relative to ß-actin and expressed as fold change in transcript relative to control (C) samples. Results are expressed as the mean±SEM of 3 mice/group. *$p<0.05$ vs. control (C). *(C)* Proteins were resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-CD73 antibody and re-probed for ß-actin. *(D)* CD73 protein was calculated by densitometry relative to ß-actin.

Hepatic protection by IP is abolished in cd73$^{-/-}$ mice

To determine if deficiency of *cd73* attenuated the hepatic protective effects of IP, we performed studies in *cd73*$^{-/-}$ mice [36]. Following hepatic IR, wildtype (WT) or *cd73*$^{-/-}$ mice demonstrated significantly higher levels of LDH (Fig. 15A), AST (Fig. 15B) and ALT (Fig. 15C) compared to their respective sham controls. However, in contrast to the results with WT mice, LDH, AST or ALT serum levels were not improved by IP in *cd73*$^{-/-}$ mice. Histological signs of ischemic injury were also not improved by IP in *cd73*$^{-/-}$ mice (Fig.15D). Thus, WT mice with IP prior to ischemia showed only mild to moderate histological signs of injury In contrast, hepatic tissue protection by IP was absent in *cd73*$^{-/-}$ mice and there was no reduction in the Suzuki index [97] (Fig. 15D and 15E, respectively). Taken together, these data provide genetic evidence for a critical role of CD73 in hepatic protection by IP.

Since Kupffer cells are important for the early immune response and production of nitric oxide is central to this function [79], we attempted to quantify iNOS via immunofluorescence to determine if CD73 regulates this process. Although there were no significant differences in iNOS between WT vs. *cd73*$^{-/-}$ mice following IR or IP (data not shown), this does not rule out the possible role of

CD73 in regulating Kupffer cells.

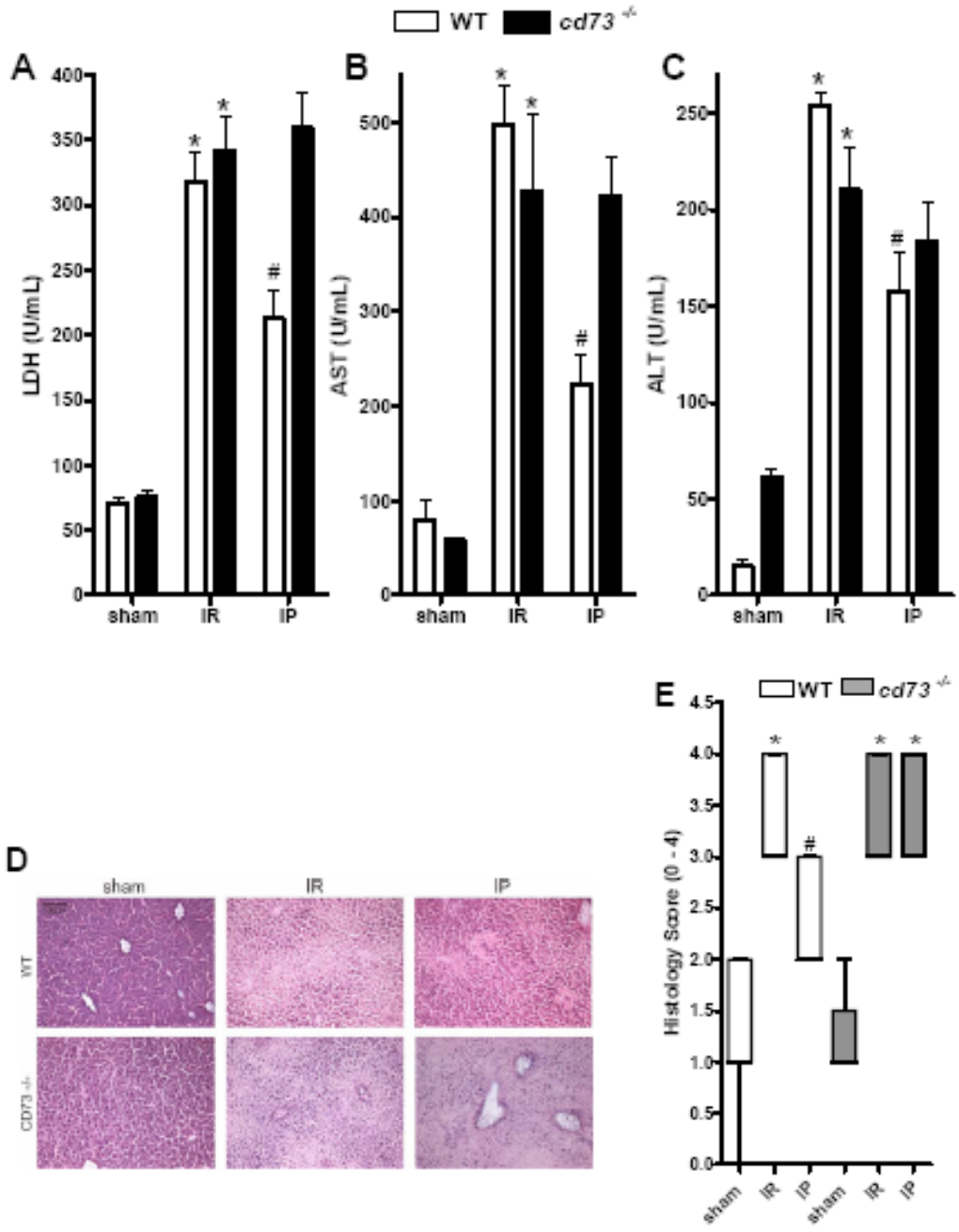

Fig. 15: Liver protection by IP is abolished in cd73-/- mice. CD73 deficient (CD73-/) or WT mice were subjected to ischemia/reperfusion alone (IR) or IP preceding IR (IP). Sham mice underwent the same surgical procedure but without IP or IR. *(A)* LDH, *(B)* AST, *(C)* ALT, *(D)* H&E staining of liver sections, and *(E)* quantification of ischemic injury (*n*=3-5 mice/group expressed as median±range). Results are expressed as the mean±SEM of 6-12 mice/group. **p<0.05* vs. respective sham. #*p<0.05* vs. respective IR.

Hepatic adenosine concentrations during IP

To confirm that CD73 induction is important for extracellular adenosine production during IP, we measured hepatic adenosine tissue levels. Liver adenosine concentrations were approximately 3.6-fold higher after hepatic IP vs. baseline (Fig. 16). Increases in extracellular adenosine with IP were significantly attenuated in *cd73*$^{-/-}$ mice compared with WT mice. Collectively these results demonstrate that CD73 plays a key role in increasing hepatic adenosine levels during IP.

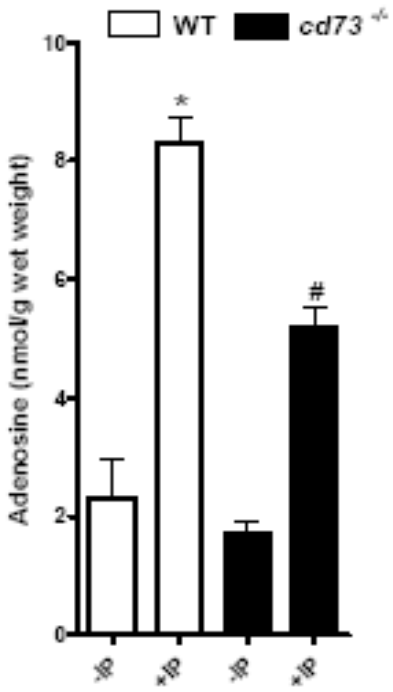

Fig. 16: Increased hepatic adenosine concentrations with IP are attenuated in cd73-/- mice. CD73-/- or WT mice were subjected to IP. The liver was snap frozen without IP (–IP) or after the last cycle of ischemia (+IP). Results are expressed as the mean±SEM of 6 mice/group. *$p<0.05$ vs. respective –IP group; #$p<0.05$ vs. WT+IP.

CD73 inhibition attenuates hepatic protection by IP

To confirm our results demonstrating that deficiency of CD73 results in an absence of hepatic protection by IP and to rule out biological compensation, we performed pharmacological studies using the specific CD73 inhibitor 5'-[αβ-methylene] diphosphate (APCP). WT mice were subjected to IR with or without prior IP following treatment with APCP (40 mg/kg/h, i.p.) or vehicle (saline). Serum LDH (Fig. 17A), AST (Fig. 17B) and ALT (Fig. 17C) were significantly improved by IP. However, APCP treatment abolished the hepatic protective effects of IP (Fig 17A-C). Furthermore, histological signs of ischemic injury were attenuated by IP in WT mice, while APCP treatment inhibited protection mediated by hepatic IP (Fig. 17D). Thus, saline-treated mice that underwent IP prior to ischemia showed only mild to moderate histological signs of injury, while APCP-treated mice demonstrated a significantly higher level of hepatocyte liver necrosis. In fact, semi-quantitative histological analysis demonstrated a significant increase in the Suzuki score [97] from 2 (range 2) without APCP treatment prior to IP to 3.5 (range 3 to 4, Fig. 17D, $p<0.05$) with APCP treatment. Furthermore, adenosine levels were significantly decreased with APCP treatment (Fig. 17E). Taken together, blockade of ecto-5´-nucleotidase enzyme activity provides pharmacological evidence for a critical role of CD73 in hepatic protection by IP.

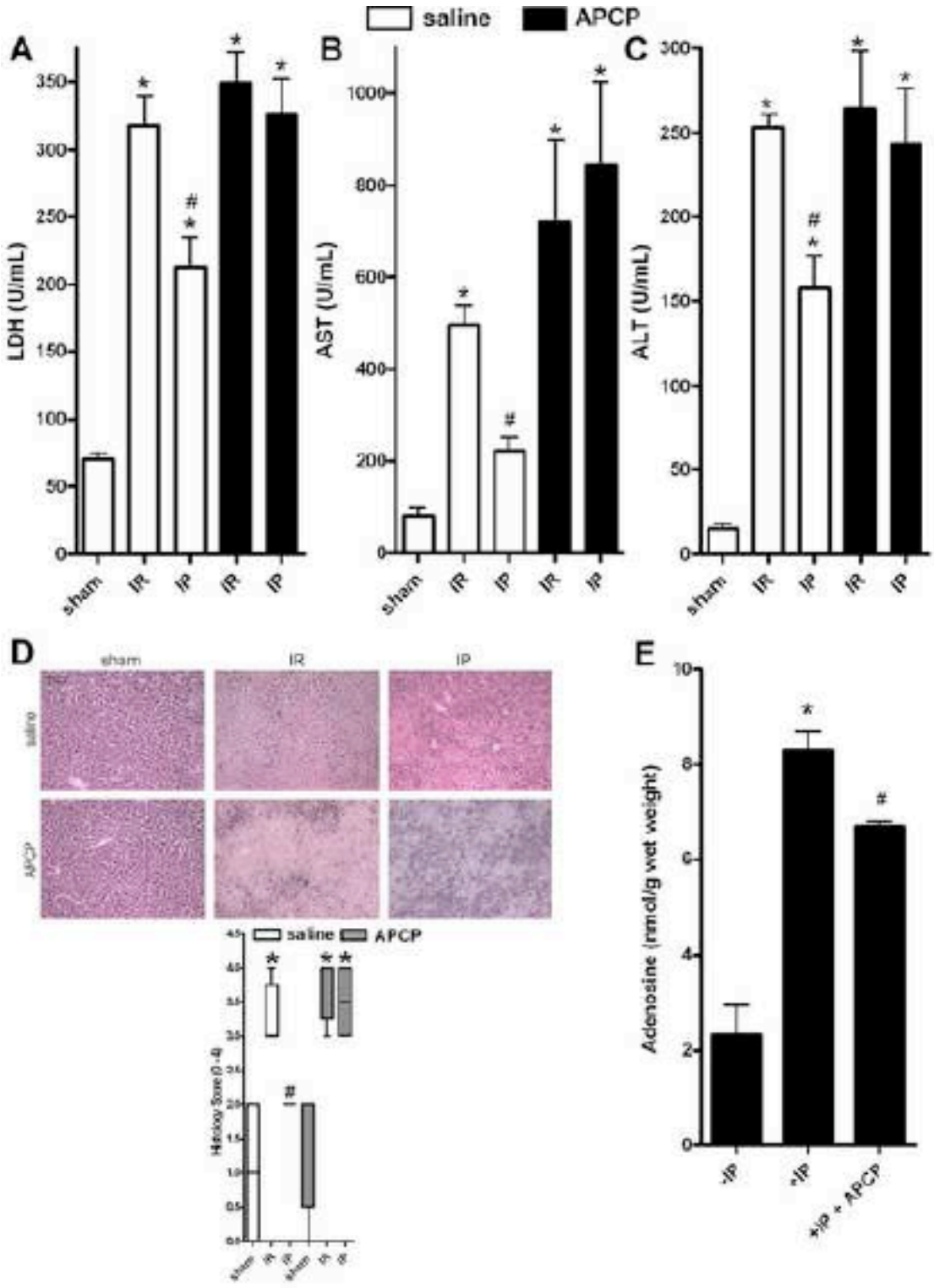

Fig. 17: CD73 inhibition abolishes liver protection by IP. Injury was measured after WT mice were pre-treated with APCP (40 mg/kg/h, i.p.) or saline prior to IR (IR) with or without prior IP (IP). Injury was measured for the following parameters: *(A)* LDH, *(B)*

AST, *(C)* ALT, *(D)* H&E staining of liver sections and quantification of ischemic injury (*n*=3-5 mice/group expressed as median±range), and *(E)* adenosine concentrations. Results are expressed as the mean±SEM of 6-12 mice/group. **p<0.05* vs. respective sham. $^{\#}$*p<0.05* vs. IR+saline, IR+APCP and IP+APCP.

Reconstitution of cd73$^{-/-}$ mice with soluble 5'-nucleotidase

As proof of principle and to demonstrate that a decrease of hepatic injury markers in *cd73*$^{-/-}$ mice reflects lack of ecto-5´-nucleotidase enzyme activity, we next reconstituted *cd73*$^{-/-}$ mice with soluble 5´-nucleotidase (5'-NT) and subjected them to hepatic ischemia with or without prior IP. As shown in Fig. 18A-C, increases in serum LDH, AST and ALT were significantly reduced following 5´-NT treatment, even without IP. Similarly, histological analysis confirmed that 5'-NT treatment resulted in a significant decrease in injury in *cd73*$^{-/-}$ mice with and without IP (Fig. 18D). Furthermore, 5'-NT treatment increased hepatic adenosine concentrations (Fig. 18E). These results confirm our genetic studies that CD73 plays a crucial role in increasing hepatic resistance to ischemia following IP.

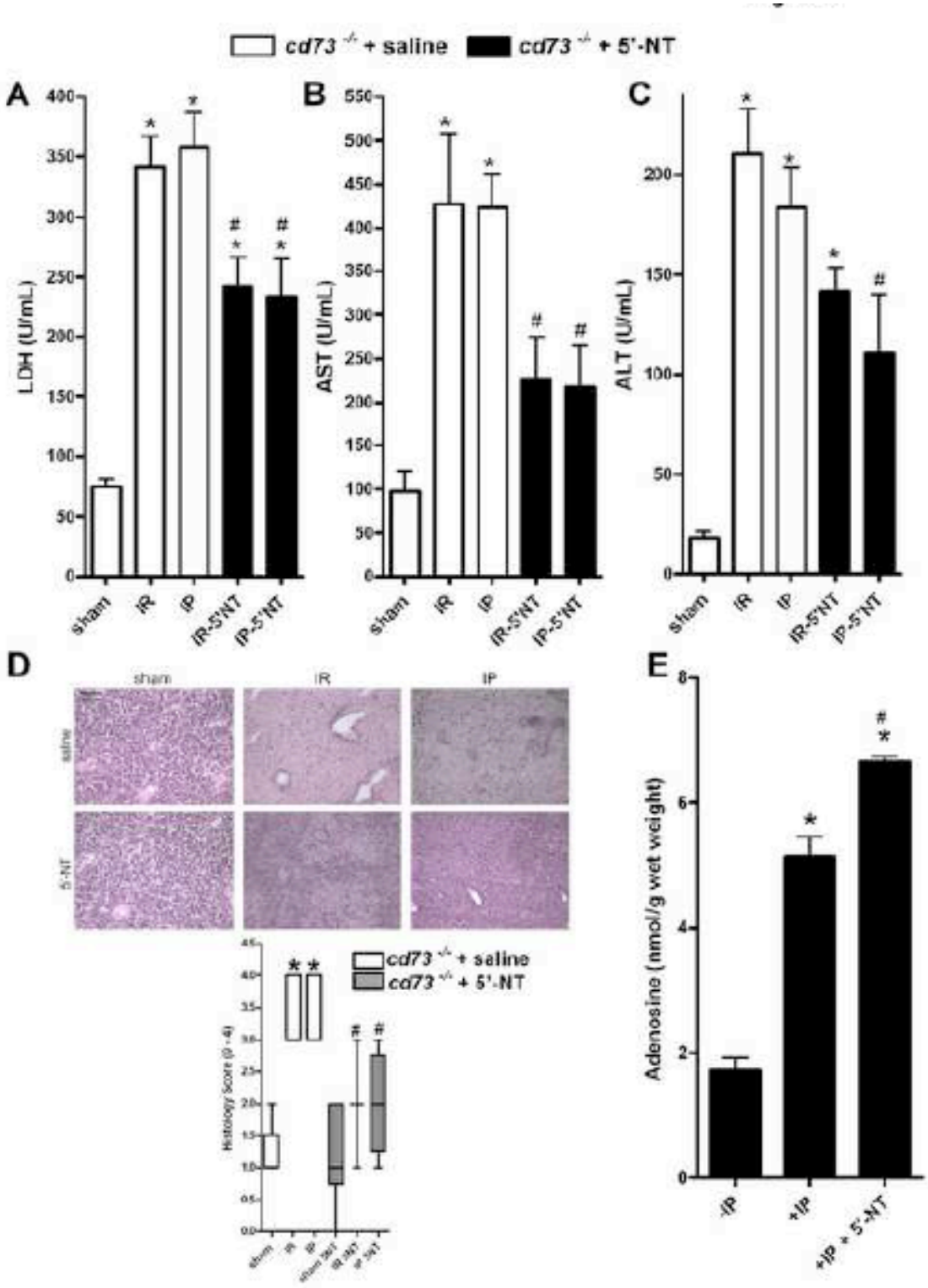

Fig. 18: Reconstitution with soluble 5'-nucleotidase improves liver protection in cd73-/- mice. CD73-/- mice were reconstituted with 5'-nucleotidase (2 U, i.v., followed by 10 U/kg/h, i.p.) or saline and subjected to IR with or without prior IP treatment. *(A)* LDH, *(B)* AST, *(C)* ALT, *(D)* H&E staining of liver sections quantification of ischemic

injury (*n*=3-5 mice/group expressed as median±range), and *(E)* adenosine concentrations. Results are expressed as the mean±SEM of 6-12 mice/group. *$p<0.05$ vs. sham. #$p<0.05$ vs. IR+saline and IP+saline.

Treatment of hepatic ischemia with soluble 5´-nucleotidase in WT mice

We next pursued 5´-nucleotidase treatment of hepatic ischemia in WT mice. WT mice were treated with 5'-NT followed by IR (30 min ischemia, 3 h reperfusion) with or without prior IP. As shown in Fig. 19A-C, 5´-NT treatment provided hepatic protection similar to that of IP or mice treated with adenosine or dipyridamole, an adenosine reuptake inhibitor. Liver histology confirmed protection from ischemia similar to what we observed previously with IP (Fig. 19D). Neutrophil infiltration as measured by MPO was also significantly inhibited with 5'-NT treatment (Fig. 19E). Furthermore, adenosine concentrations were significantly increased in WT mice treated with 5'-NT (Fig. 19F). As shown in Fig. 20A-C, 5'-NT treatment was also effective against a more severe ischemic insult (75 min ischemia, 3 h reperfusion) Taken together, these data demonstrate a therapeutic effect for treatment of hepatic ischemia with soluble 5´-nucleotidase and suggest that 5´-nucleotidase may be a novel therapy during acute hepatic ischemia.

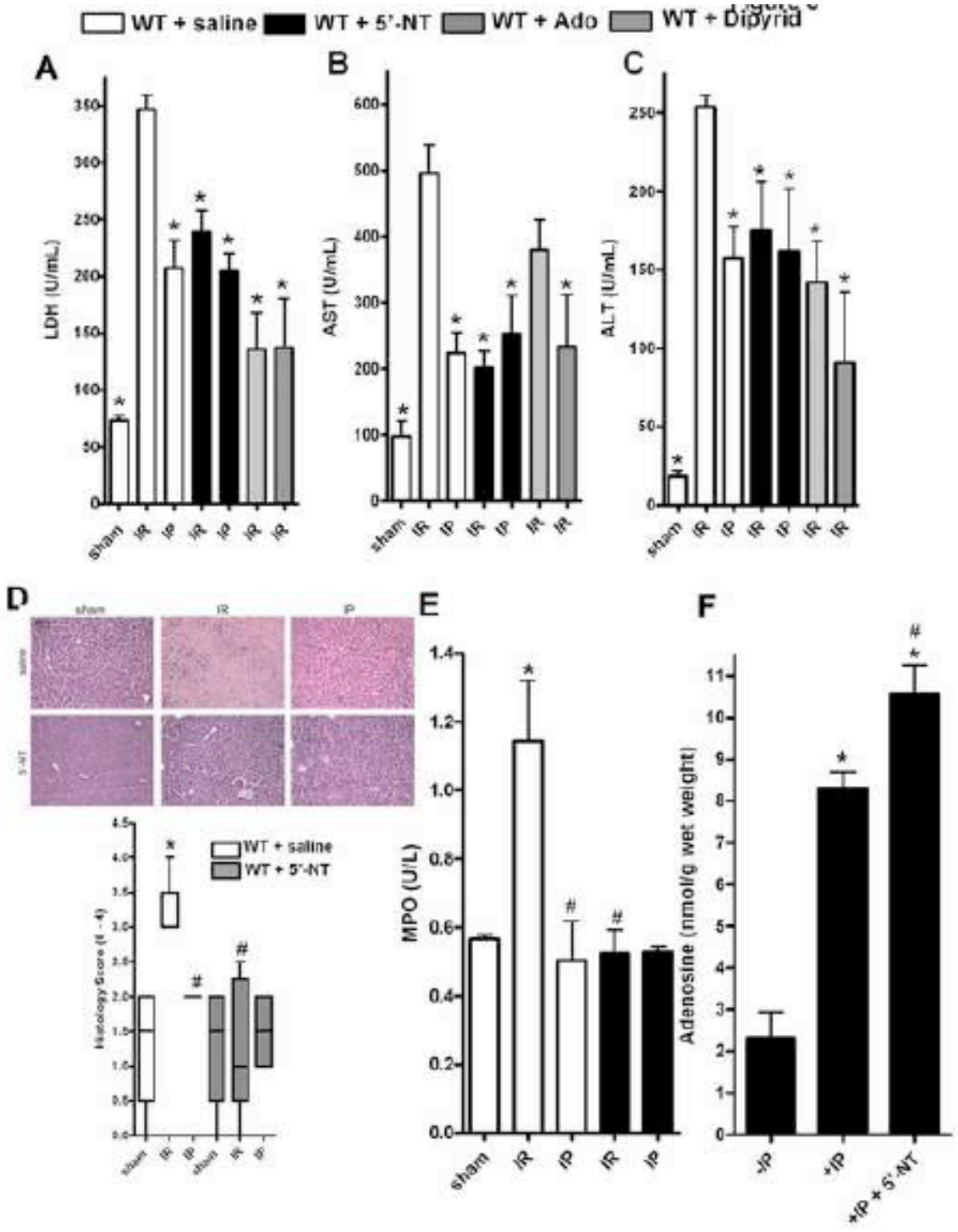

Fig. 19: Treatment with soluble 5'-nucleotidase is protective against IR in WT mice. WT mice were treated with saline, 5'-nucleotidase (2 U, i.v., followed by 10 U/kg/h, i.p.), adenosine (1 mg/kg/h, i.a.), or dipyridamole (0.5 mg/kg, i.p.) and subjected to IR (30 min ischemia, 3 h reperfusion) with or without prior IP treatment. *(A)* LDH, *(B)* AST, *(C)* ALT, *(D)* H&E staining of liver sections and quantification of ischemic injury (*n*=3-5 mice/group expressed as median±range) and *(E)* MPO. Results are expressed

as the mean±SEM of 6-12 mice/group. *$p<0.05$ vs. sham. #$p<0.05$ vs. IR+saline and IP+saline. *(F)* For adenosine measurements, the liver was snap frozen from WT mice that did not undergo IP (-IP) vs. mice subjected to IP that were pre-treated with (+IP+5'-nucleotidase) or without (+IP) 5'-nucleotidase. Results are expressed as the mean±SEM of 6 mice/group. *$p<0.05$ vs. IR+saline.

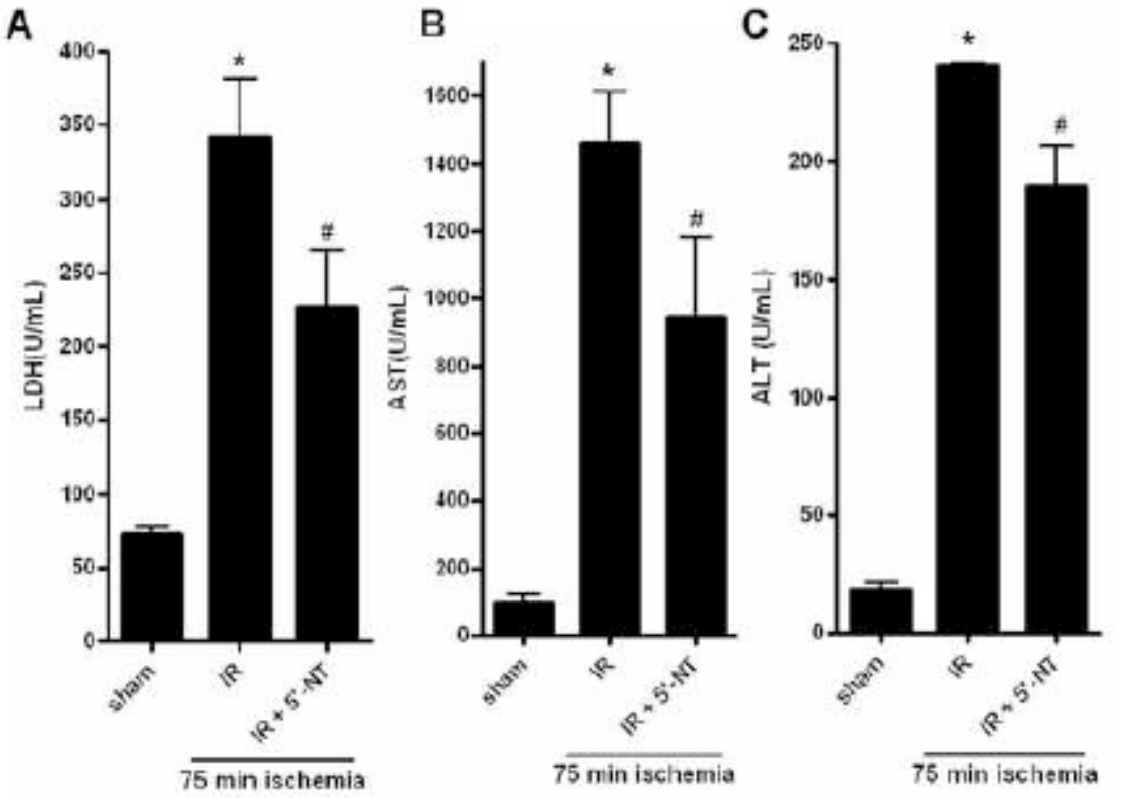

Fig. 20: Treatment with 5'-nucleotidase is protective against a longer ischemic insult. WT mice were treated with saline or 5'-nucleotidase (2 U, i.v., followed by 10 U/kg/h, i.p.) and subjected IR (75 min ischemia and 3 h reperfusion) with or without prior IP treatment. *(A)* LDH, *(B)* AST, and *(C)* ALT. Results are expressed as the mean±SEM of 4 mice/group. *$p<0.05$ vs. sham. #$p<0.05$ vs. IR.

Acute phase complement gene expression and activation is attenuated by IP in WT but not cd73$^{-/-}$ mice

To gain mechanistic insight of how extracellular adenosine generation could affect hepatic IR injury, we next looked at the consequences of IP-treatment on acute phase complement gene expression. Complement activation following

oxidative stress is an early event and inhibition of complement activation or its components may offer tissue protection [117]. Depletion of complement before hepatic ischemia was shown to attenuate superoxide generation by Kupffer cells and accumulation of neutrophils in the liver during reperfusion, thereby suppressing liver IR injury [120, 121]. Inhibition of complement components also reduced inflammatory damage during hepatic IR [121-123]. To determine the role of CD73 and thus adenosine in regulation of complement gene expression, we transcriptionally assessed expression of mannose binding lectin (MBL)-A and MBL-C, as well as C3, C5, and C9 after IP. As demonstrated in Fig. 21 (A-E), all complement genes were significantly repressed 30 min after IP in WT mice. In contrast, IP was not associated with repression of complement genes in *cd73*$^{-/-}$ mice. MBL-C was in fact, slightly upregulated, while MBL-A, C3, C5 and C9 were unchanged vs. control (C). Furthermore, IP decreased C3 deposition and thus activation of complement in WT, but not *cd73*$^{-/-}$ mice (Fig. 21F). These data demonstrate that attenuation of complement by IP is abolished following genetic deletion of *cd73* and suggest extracellular adenosine generation in modulating complement activation during IP protection.

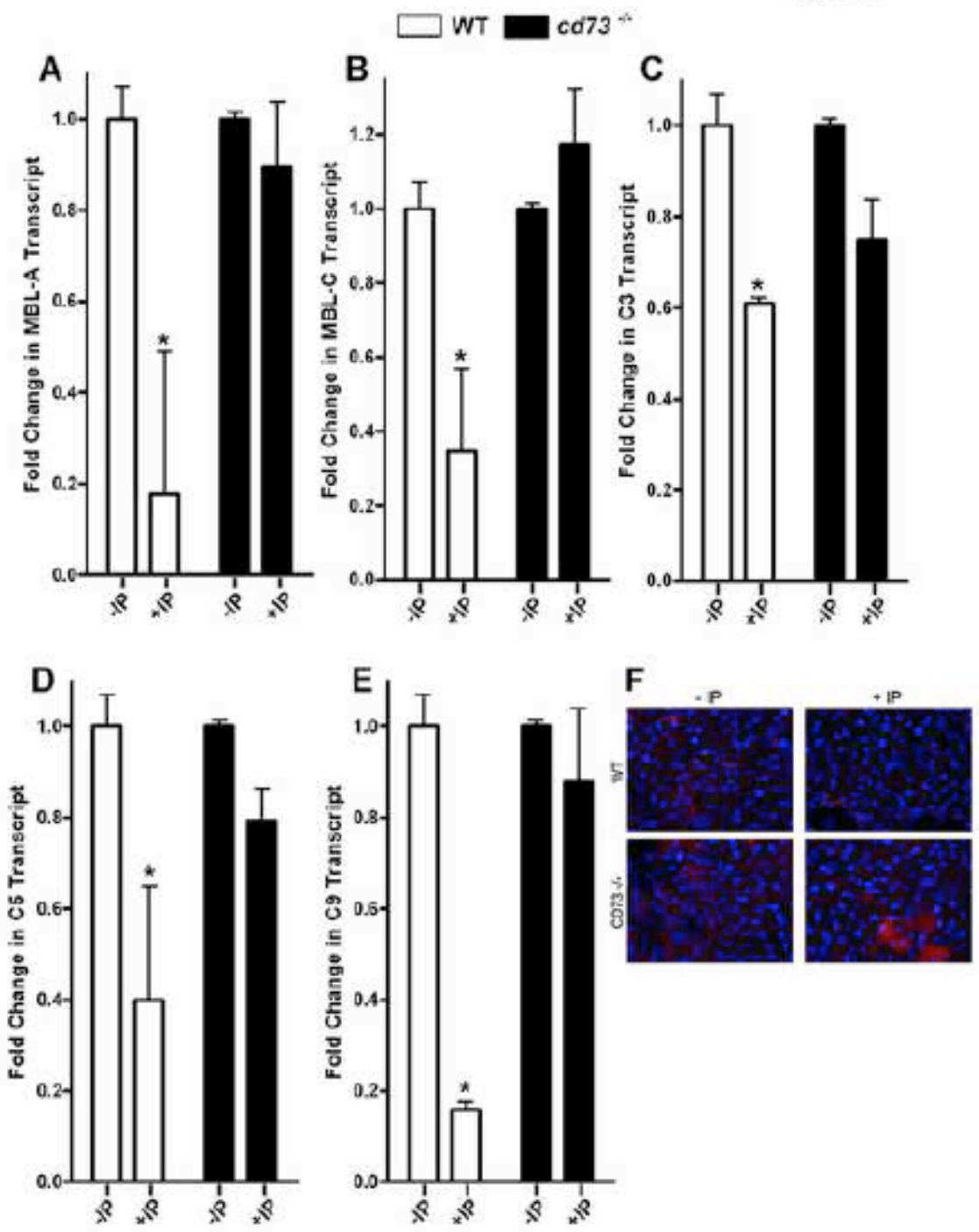

Fig. 21: Complement gene expression is decreased in WT mice but not in cd73$^{-/-}$ mice. Following 4 cycles of IP and 30 min reperfusion, the median lobe was excised. Total RNA was isolated and *(A)* MBL-A, *(B)* MBL-C, *(C)* C3, *(D)* C5 and *(E)* C9 mRNA levels were determined by real-time PCR. Data were calculated relative to ß-actin and expressed as fold change in transcript relative to control (C) samples. Results are expressed as the mean±SEM of 3 mice/group. *$p<0.05$ vs. respective control (C). *(F)*

Median lobe was harvested, sectioned and stained using C3 antibody (magnification 400x).

VI. DISCUSSION

Liver protection from ischemia by IP is an area of intense investigation. Genetically engineered mice may provide additional insight into molecular mechanisms of hepatic protection by IP. Because of the technical difficulty associated with manually clamping the portal triad, we performed a systematic evaluation using a novel model for portal triad occlusion in mice, which specifically avoids the use of a clamp. By using a hanging-weight system, the portal triad is only distressed once throughout the entire surgical procedure, causing significantly less damage to the hepatic lobes. In addition, no hepatic or intestinal congestion occurs with this technique. In the present study, we demonstrate time-dependent and highly reproducible liver injury with ischemia and protection by IP using a hanging-weight system. We also demonstrate that this model can be used for the investigation of gene regulation by IP, as ENT1-4 and complement (MBL-A, MBL-C, C3, C5, and C9) message levels were repressed by IP. Taken together, the present study provides feasibility of the hanging-weight system for portal triad occlusion during IP, minimizing the variability and limitations associated with clamping. Thus, this technique may be useful for future investigations involving the protective effects of IP in murine models.

Despite the growing number of reports investigating the mechanisms leading to hepatic protection by IP, the present understanding of the events that promote tolerance to ischemia/reperfusion damage is still quite preliminary [6]. Thus,

fundamental questions such as how IP works and which of the multiple hepatic cell types the trigger mechanism resides still remain unanswered. Similar to the present study, previous investigations have demonstrated hepatic protective effects of IP in mice [79, 83]. These studies were performed by using a clamp to occlude the portal triad. Even with these successful studies of murine liver ischemia and reperfusion, we show that using a clamp-free system of portal triad occlusion may be superior and yield more reliable and reproducible results. In fact, it has been our experience with clamp systems in mice that it is hard to guarantee reliable portal triad occlusion during ischemia. Furthermore, more tissue trauma occurs by removing and replacing the clamp, especially with reapplication of the clamp during multiple IP cycles. The use of hanging weights that are in a remote location from the liver tissue, according to our observations, provides the advantage of reliable occlusion while preventing tissue trauma due to manipulation of the liver lobes by reapplication of a clamp. Perhaps this novel hanging-weight system model can be explored in murine and other small animal models to answer fundamental questions regarding the mechanism of IP and cells responsible for protection. The recent development of cell- or tissue-specific knockout and transgenic mice will also help to answer these central questions.

Despite many advantages associated with using targeted gene deletion in mice for studying liver protection by IP, some limitations of this approach have to be pointed out. Although it is likely that the use of genetically modified mice may

yield important information about IP, biological compensation for gene deletion is known to occur [124]. Furthermore, our results show strain-specific differences in response to hepatic IP when comparing C57BL/6 and SV129 mice. Moreover, it is appreciated that different responses to liver ischemia have been observed, not only with regard to different genetic backgrounds, but also between different species [115, 125]. For example, one study showed that i.v. administration of a gas-carrier contrast reagent used in ultrasound imaging caused intravascular expansion of the gas-carrier reagent in the portal vein and therefore ischemia to the liver and subsequent midzonal patterns of hepatic necrosis [115]. Interestingly, the differences in the incidence of these lesions were highly dependent on the strain of mice and rats used. Furthermore, no lesions were found in guinea pigs and rabbits even upon repeated administrations, and dogs appeared to be a markedly less sensitive species than the rat and mouse. Of note, the tolerance of the murine liver against ischemia appears to be comparable to that of the human liver [81]. However, such studies emphasize that despite multiple similarities and molecular mechanisms, potential therapeutic targets identified in murine models cannot be directly transferred to the clinical setting, but first require further testing in other models or species.

An additional limitation of the present study is its focus on the early phase of preconditioning. In addition to the early preconditioning phase, there is a late preconditioning phase which begins 12-24 hours from the transient ischemia

and last for 3-4 days [6]. However, both phases of preconditioning can be initiated by the same stimuli and may partially share the same intracellular signaling pathways [6]. To further unravel mechanisms of hepatic protection by IP, experimental approaches may need to examine both phases of preconditioning to fully understand the molecular mechanisms of protection afforded by IP.

In summary, the present study describes a novel technique of performing IP in an intact murine model by using a hanging-weight system for occlusion of the hepatic portal triad. This study demonstrates highly reproducible injury and liver protection by IP, minimizing the variability and potential damage associated with clamping of the portal triad. Investigators who consider studying hepatic protection by IP may benefit from this model.

It is well known that inflammatory tissue damage is accompanied by accumulation of extracellular adenosine, a naturally occurring anti-inflammatory agent. Previous studies suggest that adenosine generation plays a protective role in liver protection from ischemia [53, 84]. One of the major functions of adenosine is vasodilation, resulting from its elevation in response to hypoxia and aimed at restoration of the oxygen supply [126]. A hypoxia-induced elevation of liver adenosine [127, 128] and the role of adenosine as a regulator of hepatic arterial blood flow [129, 130] have been demonstrated. Peralta et al. showed that adenosine release plays a key role in promoting liver protection by

IP [85, 131]. However, the mechanisms whereby oxygen deprivation during IP increases hepatic adenosine have not been investigated. Extracellular adenosine can be produced by dephosphorylation of AMP by membrane CD73. In the present study we investigated the contribution of CD73-dependent extracellular adenosine production to hepatic protection during IP. Transcriptional and translational profiling of ischemic liver revealed a prominent induction of CD73. This induction is consistent with previous studies that found that exposure of microvascular endothelial cells to ambient hypoxia resulted in a robust induction of CD73 transcript, protein and function [34]. Although we show that CD73 protein is upregulated due to liver IP, we did not determine cell-specificity of CD73. However, many groups have shown that CD73 is highest at the bile canalicular and sinusoidal plasma membranes of liver parenchymal cells (hepatocytes) [132-134], with the majority of enzymatic activity in the pericentral vs. periportal zones [134]. CD73 has also been reported to be present in non-parenchymal cells (Kupffer cells, sinusoidal endothelial cells and stellate/Ito cells) of the portal tracts and around the central veins [134-136].

Our results show that pharmacological inhibition or targeted gene deletion of CD73 due to increased adenosine abolished the hepatic protective effects of IP as demonstrated by LDH, serum transaminases and histology. Reperfusion causes an excessive inflammatory response including disruption of the vascular endothelium and sinusoids and subsequent endothelial and hepatocyte cell death [137]. During reperfusion, Kupffer cells are activated causing formation of

oxygen free radicals, increased phagocytosis and release of lyososomal enzymes and cytokines leading to initiation of apoptosis and death of hepatocytes [79]. Leukocytes also rapidly adhere to the denuded sinusoids and contribute to injury [79]. Our results demonstrate that mice treated with APCP or mice having a deficiency in CD73 caused an increase in hepatocyte liver necrosis, suggesting that CD73 may regulate this process.

In the present study we demonstrate that hepatic IP prior to IR attenuates the upregulation of MBL-A, MBL-C, C3, C5 and C9 complement genes and represses complement deposition/activation. Furthermore, we show novel data in which mice deficient in CD73 do not demonstrate the same degree of inhibition of complement gene expression and activation due to liver IP. Leukocyte recruitment into sinusoids during the early reperfusion phase is mediated by activation of the complement cascade [120, 121]. In addition to neutrophils, complement can also activate Kupffer cells [120, 121], suggesting that one mechanism by which CD73 exerts its hepatic protective effects is by reducing local tissue complement thus reducing subsequent leukocyte recruitment and activation of Kupffer cells. The complement system consists of three pathways: the classical, alternative and lectin pathways [117, 138]. All three pathways merge at C3 and lead to the formation of the terminal complement complex (C5b-9). Activation of these pathways depends on different molecules for their initiation. The classical pathway is triggered by antigen-bound antibody molecules binding to the C1 component, while the

alternative pathway is activated by cleavage of C3 and then C5, and the lectin pathway by formation of MBL/MBL-associated proteases 1 and 2 complexes. The liver (mainly hepatocytes) is responsible for biosynthesis of 90% of plasma complement components and their soluble regulators [139]. Although MBL, the key initiator of the lectin complement pathway, was shown to cause myocardial[140], gastrointestinal [141, 142] and renal [143, 144] IR injury, its role in hepatic IR remains unknown. Our results suggest that CD73 attenuates transcription of the main activator of the lectin complement pathway during hepatic IP. We did not measure initiators of the classical complement pathway, but other groups have shown that blocking complement activation with sCR1 or C1 inhibitor significantly ameliorated necrosis and inflammation during hepatic IR injury [121, 122, 145]. Thus perhaps CD73 regulates both lectin and classical complement pathways during hepatic IP.

Extracellular adenosine can signal through four distinct adenosine receptors (ARs): A1AR, A2AR, A2BAR and A3AR. In addition to leukocyte recruitment and activation of Kupffer cells, T cells and natural killer cells participate in hepatic injury during liver ischemia reperfusion. Interestingly, A2AAR activation using an A2A receptor agonist reduced hepatic IR injury by inhibiting natural killer cell activation [146]. Activation of the A2AAR on bone-marrow derived cells was also shown to be responsible for protecting the liver from reperfusion injury [84] . In terms of IP, blocking the A2A or A2B receptors prevented the beneficial effects of hepatic IP while stimulating the A2A/A2BARs mimicked

hepatic protection by IP [85, 86, 147]. It was also shown that IP promotes liver regeneration via stimulation of A2AARs on Kupffer cells [147]. Although it appears that A2A/A2B receptors are important in mediating protection by hepatic IP, it is still unclear which of these two receptors mediate the protective effects of hepatic preconditioning. Further studies using a combined pharmacological and genetic approach using adenosine receptor deficient mice will be required to identify adenosine signaling mechanisms involved in hepatic protection during IP.

Our results also demonstrate that reconstitution of *cd73*-/- mice with soluble 5'-nucleotidase resulted in complete restoration of hepatic protection by IP and that hepatic injury after ischemia was attenuated by treatment of WT mice with 5'-nucleotidase to a similar degree as by IP. We recently demonstrated that soluble 5'-nucleotidase treatment inhibits platelet aggregation via increasing whole blood adenosine levels through extracellular adenosine receptor signalling [148]. It was previously shown that severe vascular injury during reperfusion of the liver induces aggregation of platelets [149] and that platelets adhere to the hepatic sinusoids in conditions of ischemia [150, 151]. Thus, the inhibitory effect of 5'-nucleotidase on platelet sequestration and subsequent aggregation may be an additional mechanism by which this soluble enzyme protects against hepatic ischemic injury.

The present study reveals that IP-treatment of the liver is associated with

elevation of total adenosine concentrations. Moreover, genetic deletion of CD73 attenuates hepatic adenosine elevations following IP, suggesting that CD73 is an important source for elevating liver adenosine levels during IP. However, IP-associated increases in adenosine were not completely abolished with genetic deletion of CD73, suggesting that other mechanisms than CD73-dependent adenosine production may also contribute to elevating hepatic adenosine levels during IP. Two additional mechanisms could explain these observations. First, other metabolic pathways involving extracellular conversion of AMP to adenosine may contribute to elevating hepatic adenosine levels during IP. For example, previous studies have shown that extracellular alkaline phosphatase can elevate extracellular adenosine levels via enzymatic conversion of AMP to adenosine [152]. Secondly, elevations of intracellular adenosine due to hypoxia-dependent inhibition of adenosine kinase could result in leakage of adenosine from the intracellular to the extracellular space, thereby contributing to the elevation of hepatic adenosine levels during IP [153, 154]. However, our functional studies showing abolished liver protection with genetic deletion or pharmacological inhibition of CD73 suggest a critical role of CD73-dependent adenosine production in innate protection of the liver during IP-responses.

Collectively this study suggests that CD73 is a critical control point for endogenous adenosine generation during hepatic IP and that therapeutic strategies to increase CD73 enzymatic activity may be beneficial for the treatment hepatic ischemia. It also suggests that exploiting CD73 enzymatic

activity with soluble 5'-nucleotidase treatment to increase extracellular adenosine concentrations may represent a therapeutic strategy for treatment of liver ischemia.

VII. SUMMARY

Ischemic preconditioning (IP) represents a powerful experimental strategy to identify novel molecular targets to attenuate hepatic injury during ischemia. As a result, murine studies of hepatic IP have become an important field of research. However, murine IP is technically challenging and experimental details can alter the results. Therefore, we systematically tested a novel model of hepatic IP by using a hanging-weight system for portal triad occlusion. This system has the benefit of applying intermittent hepatic ischemia and reperfusion without manipulation of a surgical clamp or suture, thus minimizing surgical trauma. Systematic evaluation of this model revealed a close correlation of hepatic ischemia time with liver damage as measured by ALT and AST serum levels. Using different numbers of IP cycles and time intervals, we found optimal liver protection with 4 cycles of 3 min ischemia/3 min reperfusion as measured by ALT, AST, LDH, and IL-6. Similarly, ischemia associated increases in hepatic infarct size, neutrophil infiltration and histological injury were maximally attenuated with the above regimen. To demonstrate transcriptional consequences of liver IP, we isolated RNA from preconditioned liver and confirmed transcriptional modulation of known target genes (equilibrative nucleoside transporters, acute phase complement genes). Taken together, these studies confirm highly reproducible liver injury and protection by IP when using the hanging-weight system for hepatic ischemia and intermittent reperfusion.

Since the mechanisms of IP remain largely unknown, we decided to further investigate its pathways using the new HWS technique for hepatic IP. Extracellular adenosine has been implicated as an innate anti-inflammatory metabolite, particularly during ischemia, we investigated whether CD73 (ecto-5'-nucleotidase), the "pacemaker" enzyme of extracellular adenosine production, is critical for hepatic protection by IP. Using our established model, mice were subjected to four cycles of portal triad occlusion and reperfusion (3 min of ischemia/3 min of reperfusion) prior to IR or IR alone. Our results showed that hepatic IP was associated with a significant induction of CD73 transcript and protein. Targeted gene-deletion or pharmacological inhibition of CD73 abolished hepatic protection by IP as measured by LDH, AST, ALT serum levels or histological injury. Increases in extracellular adenosine with IP were significantly attenuated in *cd73*-deficient (*cd73*$^{-/-}$) mice. Reconstitution of *cd73*$^{-/-}$ mice with soluble 5'-nucleotidase resulted in complete restoration of hepatoprotection by IP and hepatic injury following ischemia was attenuated by treatment of WT mice with soluble 5'-nucleotidase. Mice deficient in CD73 did not demonstrate the same degree of IP-dependent inhibition of acute phase complement gene expression/activation as did WT mice suggesting that extracellular adenosine attenuates hepatic IR via complement regulation. In summary, extracellular adenosine production by ecto-5'-nucleotidase mediates protection during murine hepatic IP. Use of soluble 5'-nucleotidase may be a potential therapeutic for hepatic ischemia.

VIII. ZUSAMMENFASSUNG

Ischämische Präkonditionierung (IP) ist eine effektive experimentelle Technik um neue molekulare Ansatzpunkte für die Reduzierung eines ischämischen Leberschadens zu identifizieren. Als Konsequenz haben sich Studien am Mausmodell zur hepatischen IP zu einem wichtigen Forschungsbereich entwickelt. Diese sind aber technisch sehr anspruchsvoll und Details im Versuchsaufbau können die Resultate beeinflussen. Deshalb haben wir ein neues Modell der hepatischen IP entwickelt und systematisch getestet. Das „Hanging-Weight-System“ (HWS) hat im Gegensatz zu anderen Techniken den Vorteil, dass zum Setzen der intermittierenden Leberischämie keine Manipulation der Lebertrias mit einer chirurgischen Klemme oder Naht nötig ist und so mechanischer Leberschaden minimiert wird. Bei der systematischen Evaluation unseres Modells zeigte sich eine starke Korrelation der Ischämiezeit und des Leberschadens, gemessen an ALT und AST Serumkonzentrationen. Bei der Auswertung unterschiedlicher Wiederholungen der IP-Zyklen, sowie der Zeitintervalle, fanden wir eine optimale Leberprotektion mit 4 IP-Zyklen à 3 min Ischämie/3 min Reperfusion, gemessen anhand der Parameter ALT, AST, LDH und IL-6. Ebenso der ischämieassozierte Anstieg von Leberinfarktgrösse, Neutrophileninfiltration und histologischem Schaden konnte mit dem oben genannten IP-Regime maximal vermindert werden. Um den Einfluss vom IP auf die Transkription zu untersuchen, isolierten wir RNA aus präkonditionierter Leber und konnten die transkiptionelle Modulation von Zielgenen (ENTs

(equilibrative nucleoside transporters), Akute Phase Komplement Gene) bestätigen. Insgesamt zeigen unsere Studien einen in hohem Masse reproduzierbaren Leberschaden und Leberprotektion durch IP bei Anwendung des HWS zur intermittierenden hepatischen Ischämie und Reperfusion.

Da der Mechanismus der IP noch weitgehend unbekannt ist, war unser Ziel die zugrundeliegenden Signalwege mit dem neuen Hanging-Weight-System weitergehend zu untersuchen. Extrazelluläres Adenosin ist insbesondere im Zusammenhang mit Ischämie schon oft als körpereigener antiinflammatorischer Metabolit beschrieben worden. Wir untersuchten ob CD73 (Ecto-5´-Nukleotidase), das "Schrittmacherenzym" der extrazellulären Adenosinproduktion, eine entscheidende Rolle bei der Leberprotektion durch IP spielt. Mit dem Hanging-Weight-System und dem etablierten IP-Regime behandelten wir die Mäuse mit IP, Ischämie und Reperfusion bzw. die Kontrollgruppe mit Ischämie und Reperfusion. Unsere Ergebnisse zeigten, dass hepatische IP mit signifikanter Induktion des CD73-Transkripts sowie -Proteins assoziiert ist. Gezielte Genausschaltung bzw. pharmakologische Inhibition von CD73 verhinderten die Leberprotektion durch IP. Wir zeigten, dass die Zunahme des extrazellulären Adenosins durch IP in *cd73*-Knock-Out (*cd73*$^{-/-}$) Mäusen signifikant erniedrigt war und dass die Wiederherstellung von *cd73* mit löslicher 5'-nucleotidase zu einer vollständigen Wiederherstellung der Leberproduktion durch IP führte. Ischämischer Leberschaden konnte durch Behandlung von WT Mäusen mit löslicher 5'-Nukleotidase ebenfalls erniedrigt werden. Des Weiteren zeigten *cd73*$^{-/-}$-Mäuse nicht den selben Grad an IP-

abhängiger Inhibition von Akute Phase Komplement Genexpression bzw. Aktivierung wie WT Mäuse. Dies weist darauf hin, dass extrazelluläres Adenosin den hepatischen Ischämie- und Reperfusionsschaden durch Komplementregulierung erniedrigt.

Insgesamt haben wir gezeigt, dass die extrazelluläre Adenosinproduktion durch Ecto-5'-Nukleotidase ein wichtiger Mediator der Leberprotektion durch hepatische IP ist. Die Anwendung von löslicher 5'-Nukleotidase könnte potentiell eine therapeutische Option bei Leberischämie darstellen.

IX. REFERENCES

1. Strasberg SM. Nomenclature of hepatic anatomy and resections: a review of the Brisbane 2000 system. Journal of hepato-biliary-pancreatic surgery 2005;12(5):351-5.

2. Rutkauskas S, Gedrimas V, Pundzius J, Barauskas G, Basevicius A. Clinical and anatomical basis for the classification of the structural parts of liver. Medicina (Kaunas, Lithuania) 2006;42(2):98-106.

3. Kogure K, Ishizaki M, Nemoto M, Kuwano H, Makuuchi M. A comparative study of the anatomy of rat and human livers. Journal of hepato-biliary-pancreatic surgery 1999;6(2):171-5.

4. Martins PN, Theruvath TP, Neuhaus P. Rodent models of partial hepatectomies. Liver Int 2008;28(1):3-11.

5. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol 2003;284(1):G15-26.

6. Carini R, Albano E. Recent insights on the mechanisms of liver preconditioning. Gastroenterology 2003;125(5):1480-91.

7. Liu DL, Jeppsson B, Hakansson CH, Odselius R. Multiple-system organ damage resulting from prolonged hepatic inflow interruption. Arch Surg 1996;131(4):442-7.

8. Yang JC, Wang ZW, Li CL, Lin JH, Liu XG, Ji QX. Multiple organ injury at early stage of intestinal and hepatic ischemia-reperfusion in rats. Di 1 jun yi da xue xue bao = Academic journal of the first medical college of PLA 2004;24(2):198-200, 3.

9. DeLeve LD. Hepatic microvasculature in liver injury. Seminars in liver disease 2007;27(4):390-400.

10. Ramaiah SK, Jaeschke H. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. Toxicologic pathology 2007;35(6):757-66.

11. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Pina E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. J Surg Res 2008;147(1):153-9.

12. Eltzschig HK, Collard CD. Vascular ischaemia and reperfusion injury. Br Med Bull 2004;70(1):71-86.

13. Maxwell SR, Lip GY. Reperfusion injury: a review of the pathophysiology, clinical manifestations and therapeutic options. Int J Cardiol 1997;58(2):95-117.

14. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol 2000;190(3):255-66.

15. Rosser BG, Gores GJ. Liver cell necrosis: cellular mechanisms and clinical implications. Gastroenterology 1995;108(1):252-75.

16. Granger DN, ME H, DA. P. Ischemia-reperfusion injury: role of oxygen-derived free radicals. Acta Physiol Scand Suppl 1986;548:47-63.

17. Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. Hepatology 2000;32(2):169-73.

18. Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol 1991;260(3 Pt 1):G355-62.

19. Jaeschke H, Bautista AP, Spolarics Z, Spitzer JJ. Superoxide generation by Kupffer cells and priming of neutrophils during reperfusion after hepatic ischemia. Free radical research communications 1991;15(5):277-84.

20. Kaminski KA, Bonda TA, Korecki J, Musial WJ. Oxidative stress and neutrophil activation--the two keystones of ischemia/reperfusion injury. Int J Cardiol 2002;86(1):41-59.

21. Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol 2002;282(2):C227-41.

22. Toyokuni S. Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 1999;49(2):91-102.

23. Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol 2001;41:775-87.

24. Sitkovsky MV. Use of the A(2A) adenosine receptor as a physiological immunosuppressor and to engineer inflammation in vivo. Biochem Pharmacol 2003;65(4):493-501.

25. McCallion K, Harkin DW, Gardiner KR. Role of adenosine in immunomodulation: review of the literature. Crit Care Med 2004;32(1):273-7.

26. Cronstein BN. Adenosine, an endogenous anti-inflammatory agent. J Appl Physiol 1994;76(1):5-13.

27. Baxter GF. Role of adenosine in delayed preconditioning of myocardium. Cardiovasc Res 2002;55(3):483-94.

28. Mubagwa K, Flameng W. Adenosine, adenosine receptors and myocardial protection: an updated overview. Cardiovasc Res 2001;52(1):25-39.

29. Sitkovsky MV, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A, Thiel M. Physiological Control of Immune Response and Inflammatory Tissue Damage by Hypoxia-Inducible Factors and Adenosine A2A Receptors. Annual Review of Immunology 2004;22(1):657-82.

30. Cramer T, Yamanishi Y, Clausen BE*, et al.* HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell 2003;112(5):645-57.

31. Eltzschig HK, Thompson LF, Karhausen J, Cotta RJ, Ibla JC, Robson SC, Colgan SP. Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism. Blood 2004;104(13):3986-92.

32. Fredholm BB. Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell death and differentiation 2007;14(7):1315-23.

33. Eltzschig HK, Abdulla P, Hoffman E*, et al.* HIF-1-dependent repression of equilibrative nucleoside transporter (ENT) in hypoxia. J Exp Med 2005;202(11):1493-505.

34. Eltzschig HK, Ibla JC, Furuta GT, Leonard MO, Jacobson KA, Enjyoji K, Robson SC, Colgan SP. Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. J Exp Med 2003;198(5):783-96.

35. Synnestvedt K, Furuta GT, Comerford KM*, et al.* Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. J Clin Invest 2002;110(7):993-1002.

36. Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC, Colgan SP. Crucial Role for Ecto-5'-Nucleotidase (CD73) in Vascular Leakage during Hypoxia. J Exp Med 2004;200(11):1395-405.

37. Weissmuller T, Eltzschig HK, Colgan SP. Dynamic purine signaling and metabolism during neutrophil-endothelial interactions. Purinergic Signalling 2005;1(3):229-39.

38. Gomez G, Sitkovsky MV. Differential requirement for A2a and A3 adenosine receptors for the protective effect of inosine in vivo. Blood 2003;102(13):4472-8.

39. Montesinos MC, Desai A, Chen JF, Yee H, Schwarzschild MA, Fink JS, Cronstein BN. Adenosine promotes wound healing and mediates angiogenesis in response to tissue injury via occupancy of A(2A) receptors. Am J Pathol 2002;160(6):2009-18.

40. McPherson JA, Barringhaus KG, Bishop GG*, et al.* Adenosine A2A Receptor Stimulation Reduces Inflammation and Neointimal Growth in a Murine Carotid Ligation Model. Arterioscler Thromb Vasc Biol 2001;21(5):791-6.

41. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature 2001;414(6866):916-20.

42. Boucher M, Wann BP, Kaloustian S, Masse R, Schampaert E, Cardinal R, Rousseau G. Sustained cardioprotection afforded by A2A adenosine receptor stimulation after 72 hours of myocardial reperfusion. J Cardiovasc Pharmacol 2005;45(5):439-46.

43. Cohen SB, Gill SS, Baer GS, Leo BM, Scheld WM, Diduch DR. Reducing joint destruction due to septic arthrosis using an adenosine2A receptor agonist. J Orthop Res 2004;22(2):427-35.

44. Day YJ, Huang L, Ye H, Linden J, Okusa MD. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. Am J Physiol Renal Physiol 2005;288(4):F722-31.

45. Day YJ, Marshall MA, Huang L, McDuffie MJ, Okusa MD, Linden J. Protection from ischemic liver injury by activation of A2A adenosine receptors during reperfusion: inhibition of chemokine induction. Am J Physiol Gastrointest Liver Physiol 2004;286(2):G285-93.

46. Gallos G, Ruyle TD, Emala CW, Lee HT. A1 adenosine receptor knockout mice exhibit increased mortality, renal dysfunction, and hepatic

injury in murine septic peritonitis. Am J Physiol Renal Physiol 2005;289(2):F369-76.

47. Harada N, Okajima K, Murakami K, Usune S, Sato C, Ohshima K, Katsuragi T. Adenosine and selective A(2A) receptor agonists reduce ischemia/reperfusion injury of rat liver mainly by inhibiting leukocyte activation. J Pharmacol Exp Ther 2000;294(3):1034-42.

48. Jordan JE, Thourani VH, Auchampach JA, Robinson JA, Wang NP, Vinten-Johansen J. A(3) adenosine receptor activation attenuates neutrophil function and neutrophil-mediated reperfusion injury. Am J Physiol 1999;277(5 Pt 2):H1895-905.

49. Kadowaki M, Tokita K, Nagakura Y, Takeda M, Hanaoka K, Tomoi M. Adenosine A1 receptor blockade reverses dysmotility induced by ischemia-reperfusion in rat colon. Eur J Pharmacol 2000;409(3):319-23.

50. Khimenko PL, Moore TM, Hill LW, Wilson PS, Coleman S, Rizzo A, Taylor AE. Adenosine A2 receptors reverse ischemia-reperfusion lung injury independent of beta-receptors. J Appl Physiol 1995;78(3):990-6.

51. Norton GR, Woodiwiss AJ, McGinn RJ*, et al.* Adenosine A1 receptor-mediated antiadrenergic effects are modulated by A2a receptor activation in rat heart. Am J Physiol 1999;276(2 Pt 2):H341-9.

52. Odashima M, Bamias G, Rivera-Nieves J*, et al.* Activation of A2A adenosine receptor attenuates intestinal inflammation in animal models of inflammatory bowel disease. Gastroenterology 2005;129(1):26-33.

53. Odashima M, Otaka M, Jin M*, et al.* Selective A2A adenosine agonist ATL-146e attenuates acute lethal liver injury in mice. J Gastroenterol 2005;40(5):526-9.

54. Okusa MD. A(2A) adenosine receptor: a novel therapeutic target in renal disease. Am J Physiol Renal Physiol 2002;282(1):F10-8.

55. Okusa MD, Linden J, Huang L, Rieger JM, Macdonald TL, Huynh LP. A(2A) adenosine receptor-mediated inhibition of renal injury and neutrophil adhesion. Am J Physiol Renal Physiol 2000;279(5):F809-18.

56. Okusa MD, Linden J, Macdonald T, Huang L. Selective A2A adenosine receptor activation reduces ischemia-reperfusion injury in rat kidney. Am J Physiol 1999;277(3 Pt 2):F404-12.

57. Fredholm BB. AM, Burnstock G., Daly JW., Harden TK.,Jacobson KA., Leff P., Williams M. Nomenclature and classification of purinoceptors. Pharmacol Rev 1994;46(2):143–56.

58. Stiles GL. Adenosine receptors. J Biol Chem 1992; 267(10):6451-4.

59. Collis MG, Hourani SM. Adenosine receptor subtypes. Trends Pharmacol Sci 1993;14(10):360-6.

60. Eltzschig HK, Eckle T, Mager A*, et al.* ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. Circ Res 2006;99(10):1100-8.

61. Eltzschig HK, Weissmuller T, Mager A, Eckle T. Nucleotide metabolism and cell-cell interactions. Methods Mol Biol 2006;341:73-87.

62. Braun N. SJ, Robson SC., et al. Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. Eur J Neuroscience 2000;12(12):4357-66.

63. Enjyoji K, Sevigny J, Lin Y*, et al.* Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 1999;5(9):1010-7.

64. Zimmermann H. Nucleotides and cd39: principal modulatory players in hemostasis and thrombosis. Nat Med 1999;5(9):987-8.

65. Zimmermann H. 5'-Nucleotidase: molecular structure and functional aspects. Biochem J 1992;285 (Pt 2):345-65.

66. Lennon PF, Taylor CT, Stahl GL, Colgan SP. Neutrophil-derived 5'-Adenosine Monophosphate Promotes Endothelial Barrier Function via CD73-mediated Conversion to Adenosine and Endothelial A2B Receptor Activation. J Exp Med 1998;188(8):1433-43.

67. Madara JL, Patapoff TW, Gillece-Castro B, Colgan SP, Parkos CA, Delp C, Mrsny RJ. 5'-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. J Clin Invest 1993;91(5):2320-5.

68. Cavalieri B, Perrelli MG, Aragno M, Mastrocola R, Corvetti G, Durazzo M, Poli G, Cutrin JC. Ischemic preconditioning attenuates the oxidant-dependent mechanisms of reperfusion cell damage and death in rat liver. Liver Transpl 2002;8(11):990-9.

69. Schauer RJ, Gerbes AL, Vonier D, op den Winkel M, Fraunberger P, Bilzer M. Induction of cellular resistance against Kupffer cell-derived oxidant stress: a novel concept of hepatoprotection by ischemic preconditioning. Hepatology 2003;37(2):286-95.

70. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74(5):1124-36.

71. Downey JM, Liu GS, Thornton JD. Adenosine and the anti-infarct effects of preconditioning. Cardiovasc Res 1993;27(1):3-8.

72. Yellon DM, Alkhulaifi AM, Pugsley WB. Preconditioning the human myocardium. Lancet 1993;342(8866):276-7.

73. Pang CY, Yang RZ, Zhong A, Xu N, Boyd B, Forrest CR. Acute ischaemic preconditioning protects against skeletal muscle infarction in the pig. Cardiovasc Res 1995;29(6):782-8.

74. Heurteaux C, Lauritzen I, Widmann C, Lazdunski M. Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive K+ channels in cerebral ischemic preconditioning. Proc Natl Acad Sci U S A 1995;92(10):4666-70.

75. Matsuyama K, Chiba Y, Ihaya A, Kimura T, Tanigawa N, Muraoka R. Effect of spinal cord preconditioning on paraplegia during cross-clamping of the thoracic aorta. Ann Thorac Surg 1997;63(5):1315-20.

76. Turman MA, Bates CM. Susceptibility of human proximal tubular cells to hypoxia: effect of hypoxic preconditioning and comparison to glomerular cells. Ren Fail 1997;19(1):47-60.

77. Hotter G, Closa D, Prados M, Fernandez-Cruz L, Prats N, Gelpi E, Rosello-Catafau J. Intestinal preconditioning is mediated by a transient increase in nitric oxide. Biochem Biophys Res Commun 1996;222(1):27-32.

78. Lloris-Carsi JM, Cejalvo D, Toledo-Pereyra LH, Calvo MA, Suzuki S. Preconditioning: effect upon lesion modulation in warm liver ischemia. Transplant Proc 1993;25(6):3303-4.

79. Selzner N, Rudiger H, Graf R, Clavien PA. Protective strategies against ischemic injury of the liver. Gastroenterology 2003;125(3):917-36.

80. Clavien PA, Selzner M, Rudiger HA, Graf R, Kadry Z, Rousson V, Jochum W. A prospective randomized study in 100 consecutive patients

undergoing major liver resection with versus without ischemic preconditioning. Ann Surg 2003;238(6):843-50; discussion 51-2.

81. Clavien PA, Yadav S, Sindram D, Bentley RC. Protective effects of ischemic preconditioning for liver resection performed under inflow occlusion in humans. Ann Surg 2000;232(2):155-62.

82. Petrowsky H, McCormack L, Trujillo M, Selzner M, Jochum W, Clavien PA. A prospective, randomized, controlled trial comparing intermittent portal triad clamping versus ischemic preconditioning with continuous clamping for major liver resection. Ann Surg 2006;244(6):921-8; discussion 8-30.

83. Koti RS, Seifalian AM, Davidson BR. Protection of the liver by ischemic preconditioning: a review of mechanisms and clinical applications. Dig Surg 2003;20(5):383-96.

84. Day YJ, Li Y, Rieger JM, Ramos SI, Okusa MD, Linden J. A2A adenosine receptors on bone marrow-derived cells protect liver from ischemia-reperfusion injury. J Immunol 2005;174(8):5040-6.

85. Peralta C, Hotter G, Closa D, Prats N, Xaus C, Gelpi E, Rosello-Catafau J. The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A2 receptors. Hepatology 1999;29(1):126-32.

86. Nakayama H, Yamamoto Y, Kume M*, et al.* Pharmacologic stimulation of adenosine A2 receptor supplants ischemic preconditioning in providing ischemic tolerance in rat livers. Surgery 1999;126(5):945-54.

87. Yoshizumi T, Yanaga K, Soejima Y, Maeda T, Uchiyama H, Sugimachi K. Amelioration of liver injury by ischaemic preconditioning. Br J Surg 1998;85(12):1636-40.

88. Bouma MG, van den Wildenberg FA, Buurman WA. The anti-inflammatory potential of adenosine in ischemia-reperfusion injury: established and putative beneficial actions of a retaliatory metabolite. Shock 1997;8(5):313-20.

89. Keller MW, Geddes L, Spotnitz W, Kaul S, Duling BR. Microcirculatory dysfunction following perfusion with hyperkalemic, hypothermic, cardioplegic solutions and blood reperfusion. Effects of adenosine. Circulation 1991;84(6):2485-94.

90. Cronstein BN, Naime D, Ostad E. The antiinflammatory effects of methotrexate are mediated by adenosine. Adv Exp Med Biol 1994;370:411-6.

91. Nolte D, Lehr HA, Messmer K. Adenosine inhibits postischemic leukocyte-endothelium interaction in postcapillary venules of the hamster. Am J Physiol 1991;261(3 Pt 2):H651-5.

92. Hori M, Kitakaze M. Adenosine, the heart, and coronary circulation. Hypertension 1991;18(5):565-74.

93. Arai M, Thurman RG, Lemasters JJ. Contribution of adenosine A(2) receptors and cyclic adenosine monophosphate to protective ischemic preconditioning of sinusoidal endothelial cells against Storage/Reperfusion injury in rat livers. Hepatology 2000;32(2):297-302.

94. Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC, Colgan SP. Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. J Exp Med 2004;200(11):1395-405.

95. Bourdi M, Reilly TP, Elkahloun AG, George JW, Pohl LR. Macrophage migration inhibitory factor in drug-induced liver injury: a role in susceptibility and stress responsiveness. Biochem Biophys Res Commun 2002;294(2):225-30.

96. Fishbein MC, Meerbaum S, Rit J, Lando U, Kanmatsuse K, Mercier JC, Corday E, Ganz W. Early phase acute myocardial infarct size quantification: validation of the triphenyl tetrazolium chloride tissue enzyme staining technique. Am Heart J 1981;101(5):593-600.

97. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. Transplantation 1993;55(6):1265-72.

98. Delabar U, Kloor D, Luippold G, Muhlbauer B. Simultaneous determination of adenosine, S-adenosylhomocysteine and S-adenosylmethionine in biological samples using solid-phase extraction and high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl 1999;724(2):231-8.

99. Suzuki S, Nakamura S, Sakaguchi T, Mitsuoka H, Tsuchiya Y, Kojima Y, Konno H, Baba S. Pathophysiological appraisal of a rat model of total hepatic ischemia with an extracorporeal portosystemic shunt. J Surg Res 1998;80(1):22-7.

100. Kaya Y, Aral E, Coskun T, Erkasap N, Var A. Increased intraabdominal pressure impairs liver regeneration after partial hepatectomy in rats. J Surg Res 2002;108(2):250-7.

101. Kobayashi M, Takeyoshi I, Yoshinari D, Matsumoto K, Morishita Y. The role of mitogen-activated protein kinases and the participation of intestinal congestion in total hepatic ischemia-reperfusion injury. Hepatogastroenterology 2006;53(68):243-8.

102. Ochiai H, Nakamura S, Suzuki S, Baba S. Pancreatic damage resulting from temporary portal triad interruption during partial hepatectomy: protective effect of a prostaglandin I2 analogue. J Surg Res 1997;73(2):129-36.

103. Lemaire LC, van Wagensveld BA, van Gulik TM, Dankert J, van Lanschot JJ, Gouma DJ. Bacterial translocation to the thoracic duct in a setting of ischemia, partial resection and reperfusion of the porcine liver. Dig Surg 1999;16(3):222-8.

104. Carrick JB, Martins O, Jr., Snider CC, Means ND, Enderson BL, Frame SB, Morris SA, Karlstad MD. The effect of LPS on cytokine synthesis and lung neutrophil influx after hepatic ischemia/reperfusion injury in the rat. J Surg Res 1997;68(1):16-23.

105. Iu S, Harvey PR, Makowka L, Petrunka CN, Ilson RG, Strasberg SM. Markers of allograft viability in the rat. Relationship between transplantation viability and liver function in the isolated perfused liver. Transplantation 1987;44(4):562-9.

106. Lemasters JJ, Ji S, Stemkowski CJ, Thurman RG. Hypoxic hepatocellular injury. Pharmacol Biochem Behav 1983;18 Suppl 1:455-9.

107. Singh I, Zibari GB, Brown MF*, et al.* Role of P-selectin expression in hepatic ischemia and reperfusion injury. Clin Transplant 1999;13(1 Pt 2):76-82.

108. Howell JG, Zibari GB, Brown MF, Burney DL, Sawaya DE, Olinde JG, Granger DN, McDonald JC. Both ischemic and pharmacological preconditioning decrease hepatic leukocyte/endothelial cell interactions. Transplantation 2000;69(2):300-3.

109. Ferguson D, McDonagh PF, Biewer J, Paidas CN, Clemens MG. Spatial relationship between leukocyte accumulation and microvascular injury during reperfusion following hepatic ischemia. Int J Microcirc Clin Exp 1993;12(1):45-60.

110. Edens HA, Parkos CA. Neutrophil transendothelial migration and alteration in vascular permeability: focus on neutrophil-derived azurocidin. Curr Opin Hematol 2003;10(1):25-30.

111. Dodd-o JM, Hristopoulos ML, Welsh-Servinsky LE, Tankersley CG, Pearse DB. Strain-specific differences in sensitivity to ischemia-reperfusion lung injury in mice. J Appl Physiol 2006;100(5):1590-5.

112. Majid A, He YY, Gidday JM*, et al.* Differences in vulnerability to permanent focal cerebral ischemia among 3 common mouse strains. Stroke 2000;31(11):2707-14.

113. Wellons JC, 3rd, Sheng H, Laskowitz DT, Burkhard Mackensen G, Pearlstein RD, Warner DS. A comparison of strain-related susceptibility in two murine recovery models of global cerebral ischemia. Brain Res 2000;868(1):14-21.

114. Barone FC, Knudsen DJ, Nelson AH, Feuerstein GZ, Willette RN. Mouse strain differences in susceptibility to cerebral ischemia are related to cerebral vascular anatomy. J Cereb Blood Flow Metab 1993;13(4):683-92.

115. Dirven HA, Rasmussen H, Johnsen H, Videm S, Walday P, Grant D. Intestinal and hepatic lesions in mice, rats, and other laboratory animals after intravenous administration of gas-carrier contrast agents used in ultrasound imaging. Toxicol Appl Pharmacol 2003;188(3):165-75.

116. Lu H, Chen C, Klaassen C. Tissue distribution of concentrative and equilibrative nucleoside transporters in male and female rats and mice. Drug Metab Dispos 2004;32(12):1455-61.

117. Hart ML, Walsh MC, Stahl GL. Initiation of complement activation following oxidative stress. In vitro and in vivo observations. Mol Immunol 2004;41(2-3):165-71.

118. Arumugam TV, Shiels IA, Woodruff TM, Granger DN, Taylor SM. The role of the complement system in ischemia-reperfusion injury. Shock 2004;21(5):401-9.

119. Tanhehco EJ, Yasojima K, McGeer PL, McGeer EG, Lucchesi BR. Preconditioning reduces myocardial complement gene expression in vivo. Am J Physiol Heart Circ Physiol 2000;279(3):H1157-65.

120. Jaeschke H, Farhood A, Bautista AP, Spolarics Z, Spitzer JJ. Complement activates Kupffer cells and neutrophils during reperfusion after hepatic ischemia. Am J Physiol 1993;264(4 Pt 1):G801-9.

121. Chavez-Cartaya RE, DeSola GP, Wright L, Jamieson NV, White DJ. Regulation of the complement cascade by soluble complement receptor type 1. Protective effect in experimental liver ischemia and reperfusion. Transplantation 1995;59(7):1047-52.

122. Heijnen BH, Straatsburg IH, Padilla ND, Van Mierlo GJ, Hack CE, Van Gulik TM. Inhibition of classical complement activation attenuates liver ischaemia and reperfusion injury in a rat model. Clin Exp Immunol 2006;143(1):15-23.

123. Arumugam TV, Woodruff TM, Stocks SZ*, et al.* Protective effect of a human C5a receptor antagonist against hepatic ischaemia-reperfusion injury in rats. J Hepatol 2004;40(6):934-41.

124. Moghadaszadeh B, Albrechtsen R, Guo LT*, et al.* Compensation for dystrophin-deficiency: ADAM12 overexpression in skeletal muscle results in increased alpha 7 integrin, utrophin and associated glycoproteins. Hum Mol Genet 2003;12(19):2467-79.

125. Higashi N, Ueda H, Yamada O, Oikawa S, Koiwa M, Tangkawattana P, Takehana K. Micromorphological characteristics of hepatic sinusoidal endothelial cells and their basal laminae in five different animal species. Okajimas Folia Anat Jpn 2002;79(5):135-42.

126. Berne RM. The role of adenosine in the regulation of coronary blood flow. Circ Res 1980;47(6):807-13.

127. Belloni FL, Elkin PL, Giannotto B. The mechanism of adenosine release from hypoxic rat liver cells. Br J Pharmacol 1985;85(2):441-6.

128. Arnold ST, Cysyk RL. Adenosine export from the liver: oxygen dependency. Am J Physiol 1986;251(1 Pt 1):G34-9.

129. Lautt WW, Legare DJ, d'Almeida MS. Adenosine as putative regulator of hepatic arterial flow (the buffer response). Am J Physiol 1985;248(3 Pt 2):H331-8.

130. Ezzat WR, Lautt WW. Hepatic arterial pressure-flow autoregulation is adenosine mediated. Am J Physiol 1987;252(4 Pt 2):H836-45.

131. Peralta C, Hotter G, Closa D, Gelpi E, Bulbena O, Rosello-Catafau J. Protective effect of preconditioning on the injury associated to hepatic ischemia-reperfusion in the rat: role of nitric oxide and adenosine. Hepatology 1997;25(4):934-7.

132. Matsuura S, Eto S, Kato K, Tashiro Y. Ferritin immunoelectron microscopic localization of 5'-nucleotidase on rat liver cell surface. J Cell Biol 1984;99(1 Pt 1):166-73.

133. Frederiks WM, Marx F. A quantitative histochemical study of 5'-nucleotidase activity in rat liver using the lead salt method and polyvinyl alcohol. Histochem J 1988;20(4):207-14.

134. Song J, Bosch KS, Tigchelaar W, Van Den Munckhof RJ, Schellens JP, Van Noorden CJ, Frederiks WM. Demonstration of 5'-nucleotidase activity in unfixed cryostat sections of rat liver using a combined light- and electron-microscope procedure. Histochem J 1995;27(11):914-22.

135. Schmid TC, Loffing J, Le Hir M, Kaissling B. Distribution of ecto-5'-nucleotidase in the rat liver: effect of anaemia. Histochemistry 1994;101(6):439-47.

136. Bachmann S, Ramasubbu K. Immunohistochemical colocalization of the alpha-subunit of neutrophil NADPH oxidase and ecto-5'-nucleotidase in kidney and liver. Kidney Int 1997;51(2):479-82.

137. Kohli V, Selzner M, Madden JF, Bentley RC, Clavien PA. Endothelial cell and hepatocyte deaths occur by apoptosis after ischemia-reperfusion injury in the rat liver. Transplantation 1999;67(8):1099-105.

138. Qin X, Gao B. The complement system in liver diseases. Cell Mol Immunol 2006;3(5):333-40.

139. Morgan BP, Gasque P. Extrahepatic complement biosynthesis: where, when and why? Clin Exp Immunol 1997;107(1):1-7.

140. Walsh MC, Bourcier T, Takahashi K*, et al.* Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. J Immunol 2005;175(1):541-6.

141. Hart ML, Ceonzo KA, Shaffer LA, Takahashi K, Rother RP, Reenstra WR, Buras JA, Stahl GL. Gastrointestinal Ischemia-Reperfusion Injury Is Lectin Complement Pathway Dependent without Involving C1q. J Immunol 2005;174(10):6373-80.

142. McMullen ME, Hart ML, Walsh MC, Buras J, Takahashi K, Stahl GL. Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo. Immunobiology 2006;211(10):759-66.

143. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in

complement activation in the course of renal ischemia-reperfusion injury. Am J Pathol 2004;165(5):1677-88.

144. Moller-Kristensen M, Wang W, Ruseva M*, et al.* Mannan-binding lectin recognizes structures on ischaemic reperfused mouse kidneys and is implicated in tissue injury. Scand J Immunol 2005;61(5):426-34.

145. Lehmann TG, Heger M, Munch S, Kirschfink M, Klar E. In vivo microscopy reveals that complement inhibition by C1-esterase inhibitor reduces ischemia/reperfusion injury in the liver. Transpl Int 2000;13 Suppl 1:S547-50.

146. Lappas CM, Day YJ, Marshall MA, Engelhard VH, Linden J. Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. J Exp Med 2006;203(12):2639-48.

147. Arai M, Tejima K, Ikeda H*, et al.* Ischemic preconditioning in liver pathophysiology. J Gastroenterol Hepatol 2007;22 Suppl 1:S65-7.

148. Hart ML, Kohler D, Eckle T, Kloor D, Stahl GL, Eltzschig HK. Direct Treatment of Mouse or Human Blood With Soluble 5'-Nucleotidase Inhibits Platelet Aggregation. Arterioscler Thromb Vasc Biol 2008.

149. Yadav SS, Howell DN, Steeber DA, Harland RC, Tedder TF, Clavien PA. P-Selectin mediates reperfusion injury through neutrophil and platelet sequestration in the warm ischemic mouse liver. Hepatology 1999;29(5):1494-502.

150. Cywes R, Packham MA, Tietze L, Sanabria JR, Harvey PR, Phillips MJ, Strasberg SM. Role of platelets in hepatic allograft preservation injury in the rat. Hepatology 1993;18(3):635-47.

151. Cywes R, Mullen JB, Stratis MA, Greig PD, Levy GA, Harvey PR, Strasberg SM. Prediction of the outcome of transplantation in man by platelet adherence in donor liver allografts. Evidence of the importance of prepreservation injury. Transplantation 1993;56(2):316-23.

152. Ohkubo S, Kimura J, Matsuoka I. Ecto-alkaline phosphatase in NG108-15 cells : a key enzyme mediating P1 antagonist-sensitive ATP response. Br J Pharmacol 2000;131(8):1667-72.

153. Decking UKM, Schlieper G, Kroll K, Schrader J. Hypoxia-Induced Inhibition of Adenosine Kinase Potentiates Cardiac Adenosine Release. Circ Res 1997;81(2):154-64.

154. Morote-Garcia JC, Rosenberger P, Kuhlicke J, Eltzschig HK. HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak. Blood 2008;111(12):5571-80.

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Henn M, **Much C**, Eltzschig HK and Hart ML. Role of ecto-5'-nucleotidase in protection against gastrointestinal ischemia/reperfusion injury. 7th World Congress on Trauma, Shock, Inflammation and Sepsis, Munich, Germany, March 2007.
Hart ML*, **Much C***, Kohler D, Schittenhelm J, Gorzolla IC, Stahl GL, Eltzschig HK. Use of a hanging-weight system for liver ischemic preconditioning in mice. Am J Physiol Gastrointest Liver Physiol 2008;294:G1431-40.
Hart ML, **Much C**, Gorzolla IC, Schittenhelm J, Kloor D, Stahl GL, Eltzschig HK. Extracellular Adenosine Production by Ecto-5'-Nucleotidase Protects during Murine Hepatic Ischemic Preconditioning. Gastroenterology. 2008 Nov;135(5):1739-1750

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