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Role of Ecto-5'-nucleotidase in Protection Against Gastrointestinal Ischemia/Reperfusion Injury

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Für meine Eltern und Brüder in Liebe und Dankbarkeit.

I. ABBREVIATIONS

$\alpha\beta$-methylene-ADP	Alpha-Beta-Methylene-Adenosine Diphosphate (APCP)
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
HIF	Hypoxia-inducible factor
GI/R	Gastointestinal ischemia-reperfusion injury
IL	Interleukin
KO	Knock-out
MPO	Myeloperoxidase
mRNA	Messanger Ribonucleic Acid
NO	Nitric Oxide
5-NT	Ecto-5'- nucleotidase
PBS	Phosphat Buffert 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
SMA	Superior mesenteric artery
TNF-α	Tumor Necrosis Factor-alpha
WT	Wildtype

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III. INTRODUCTION

Definition of intestinal ischemia/reperfusion injury and epidemiology

Acute intestinal ischemia is a gastrointestinal emergency that generally stems from interruption of blood flow within the superior mesenteric artery or vein, and leads to small intestinal hypoperfusion, the clinical outcome of stroke, hemorrhagic shock or organ transplantation. 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. Cellular damage after reperfusion of previously viable ischemic tissue is defined as ischemia-reperfusion (I/R) injury. Gastrointestinal I/R (GI/R) is encountered in a variety of clinical conditions such as hemorrhagic shock, strangulation-obstruction of the intestine, sepsis, vascular surgery, small bowel transplantation, abdominal aortic surgery, and multiple organ failure (1-6). A rare but severe complication of open-heart surgery also can result in acute mesenteric ischemia (7). Although its incidence is quite low (0.2%-0.4%), the mortality rate of mesenteric ischemia secondary to open-heart surgery is quite high (70%-100%) (7).Due to the aging population, the incidence is predicted to increase (8). Furthermore, injury from GI/R not only results in local intestinal injury, but also can result in a secondary or multiple-organ injury (9, 10). Thus, I/R of the intestine is a systemic phenomenon that may result in bacterial translocation, endotoxemia, acute respiratory distress syndrome (ARDS) and acute hepatic injury (6, 10). Despite optimal management consisting of treatment of the initiating cause and vigilant supportive care, morbidity and mortality associated with GI/R are high (approximately 70%) (9, 10), and therefore, specific therapeutic interventions are needed.

Pathophysiology of intestinal ischemia reperfusion

The arteries most compromised by obstruction are the celiac trunk, superior mesenteric artery (SMA) and inferior mesenteric artery (11). However, the sources of the collateral flow between the mesenteric blood vessels themselves or with adjacent circulation are numerous and may compensate the blood flow to the tissues (11). Intestinal ischemia results in small intestinal hypoperfusion and leads therefore to impediment of aerobic energetic metabolism. Ischemia-induced decreases in cellular oxidative phosphorylation result in a failure to resynthesize energy-rich phosphates including ATP (10). Thus, membrane ATP-dependent ionic pump function is altered, supporting the entry of calcium, sodium and water into the cell (10).

Ischemia also promotes expression of certain proinflammatory gene products (e.g. leucocyte 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) (2, 12).The regulation of these metabolites cause cellular damage and as a result lead to progressive cellular alterations, culminating in necrosis (13). Another important contributor to cellular death after I/R injury is apoptosis (14). Several studies have evaluated apoptosis in intestinal I/R. Noda et al. reported apoptosis in the jejunum and ileum, after SMA occlusion and reperfusion in rats (15). Furthermore, adenine nucleotide catabolism during ischaemia results in the intracellular accumulation of hypoxanthine, which is subsequently converted into toxic reactive oxygen species (ROS) when molecular oxygen is reintroduced (16). Thus ischemia induces a proinflammatory state that increases tissue vulnerability to further injury on reperfusion. Furthermore, Parks & Granger reported that tissue lesions produced during reperfusion were greater than those produced during ischemia, in mesenteric I/R in felines (17).

ROS can cause tissue injury through several mechanisms including direct damage of cellular membranes through lipid peroxidation (18, 19) 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) (19). Furthermore these substances can cause vasoconstriction, vasodilatation, increased vascular permeability and stimulate platelet aggregation(20). Finally, ROS increase leucocyte adhesion molecule and cytokine gene expression (19).

Adenosine signaling

It is well known that inflammatory tissue damage is accompanied by accumulation of extracellular adenosine, a naturally occurring anti-inflammatory agent (21-24). Local tissue hypoxia or ischemia in inflamed areas represents one of the most important conditions leading to adenosine release and accumulation (21, 22, 24-28). Recent in vitro and in vivo studies clearly confirm the beneficial role of adenosine as an immune modulator (25) and the adenosine receptors have been studied for their capacity to modulate inflammation (29). Furthermore several murine models of inflammation provide evidence for adenosine receptor signaling as a mechanism for regulating inflammatory responses in vivo (22, 29-55).

Originally, the receptors were classified based on their affinities for adenosine analogues and methylxanthine antagonists (56). Four subtypes of G protein-coupled adenosine receptors exist, A2, A2a, A2b and A3. Presently these receptors are classified according to utilization of pertussis toxin–sensitive pathways (A1 and A3) or adenylate cyclase activation pathways (A2A and A2B) (57) and are widely expressed on target cell types as diverse as leukocytes, vascular endothelia, and mucosal epithelia (58). Thus adenosine controls the function of virtually every organ and tissue (58). 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 platelets, mast cells and endothelial cells (21, 22) are metabolized to adenosine via surface expressed ecto-nucleotidases (CD39 and CD73). Ectoapyrase (CD39) converts ATP/ADP to AMP and ecto-5'-nucleotidase (CD73) subsequently converts AMP to adenosine (21). Adenosine then binds to surface expressed PMN adenosine receptors to limit excessive accumulation of PMN within tissues, and as such, functions as a feedback loop to attenuate potential tissue injury (29).

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 (59). 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. This catalytic function impacts purinergic signalling mechanisms to convert a nucleotide-mediated purinergic-type 2 (P2) response to a nucleoside/adenosine P1-receptor-type response (60-62). P1 receptors are a family of G protein-coupled receptors that signal through multiple intracellular effects in response to nucleoside activation, primarily via adenosine (adenosine receptors), whereas the P2 receptors are mainly activated by specific diphosphate or triphosphate nucleotides (e.g. ATP). Such transformations of extracellular nucleotide-mediated responses tend to be beneficial in dampening inflammation and limiting thrombosis (63).

Functional CD39 expression by the vasculature is rapidly lost in the setting of acute inflammation and oxidative stress and is important in the progression of organ damage (64). Previous studies revealed an increase in CD39 in hypoxic endothelial and epithelial cells (31, 32). Using CD39-null animals it was shown

that extracellular adenosine, produced through adenine nucleotide metabolism during hypoxia, is a potent anti-inflammatory signal for PMN *in vitro* and *in vivo* (31). In addition CD39 has been considered have high thromboregulatory potential (65) and to play a functional role in promoting endothelial permeability during hypoxia (31). Guckelberger *et al.* confirmed that treatment with NTPDase abrogates the increased vascular permeability, modulates platelet activation and vascular leakage during intestinal I/R injury *in vivo* and thus improves outcome after intestinal I/R injury (66). These findings demonstrate that CD39 is a critical regulatory element in the control of inflammatory response by providing increased adenosine concentrations. Furthermore, supplementation of this ecto-enzymatic function is assumed to be potentially therapeutic in any condition characterized by vascular inflammation and thrombosis (e.g., after organ transplantation or in the setting of a stroke) (64, 67, 68).

Ecto-5'nucleotidase (CD73)

Ecto-5'-nucleotidase is a glycosyl phosphatidylinositol (GPI)-linked, membrane-bound glycoprotein which functions to hydrolyze extracellular nucleotides into bioactive nucleoside intermediates (69, 70). Surface bound CD73 produces adenosine via enzymatic conversion of AMP. Adenosine then activates one of four types of G-protein coupled, seven transmembrane spanning adenosine receptors or can be internalized through dipyridamole-sensitive carriers (21). 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 (71), attenuation of neutrophil adhesion (29) and stimulation of epithelial electrogenic chloride secretion (72). 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, some studies show that extracellular adenosine produced through adenine nucleotide metabolism during hypoxia is a potent anti-inflammatory signal for PMN *in vitro* and *in vivo* (29, 30, 32-34). 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 (31-33) and preventing intestinal barrier dysfunction during hypoxia (32).

Nucleotide signalling and tissue protection in the intestine during inflammation

A number of studies suggest that adenosine signalling may modulate tissue protection of the intestine during inflammation. For example, early studies showed that adenosine applied to the topical surface of the intestine inhibits intestinal I/R-induced neutrophil infiltration, oxidative damage, and mucosal destruction of the intestine (73-76). Rats treated with inosine, a purine nucleoside formed from the breakdown of adenosine by adenosine deaminase, demonstrate significantly less intestinal barrier dysfunction and secondary lung injury in response to intestinal I/R (77). Additionally, $CD39^{-/-}$ mice developed more profound intestinal I/R injury and demonstrated increased mortality, and CD39 supplementation inhibited increased vascular permeability associated with I/R (66). These studies suggest that adenosine modulates intestinal I/R injury.

Objective and experimental setting

To date, the role that CD73 plays in inflammation during ischemia and reperfusion of the intestine is not clear. In the present study we therefore sought to determine whether CD73 (CD73, AMP-conversion to adenosine) can be implicated as a key mediator in production of extracellular adenosine and thus in protection against intestinal I/R-induced injury in order to gain a better understanding of natural inhibitors of inflammation during intestinal I/R.

IV. MATERIAL AND METHODS

Mice

Mice that were deficient in CD73 (CD73-/-) on the C57BL/6 strain have been described previously (33). CD73 +/+ littermates (WT) were used as controls.

Gastrointestinal ischemia/reperfusion injury

All animal protocols were in accordance with the German guidelines for Use of Living Animals and were approved by the Institutional Animal Care and Use Committee of the Universtity Hospital and the Regierungspräsidium Tübingen. All mice were matched by sex, age and weight 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.

$CD73^{-/-}$ or WT mice were anesthetized with isoflurane. After a midline laparotomy, intestinal ischemia was produced by clamping the superior mesenteric artery for 15 min, followed by unclamping for 3 hours of reperfusion as previously described (78-80). In some experiments, WT mice were treated with a specific inhibitor of ecto-5'-nucleotidase, 5'-[αβ-methylene] diphospate (APCP, 40mg/kg/h i.a., Sigma), prior to ischemia. Briefly, after anesthesia, the carotic artery was exposed via blunt dissection of the paratracheal muscles and careful avoidance of tissue trauma (particularly of the vagal nerve). A catheter was inserted into the vessel by using two sutures and a small clamp. Afterwards the mice received an infusion of APCP into the carotid artery while ischemia and reperfusion was performed. For reconstitution experiments, CD73 -/- mice were reconstituted with a bolus of soluble 5'-nucleotidase purified from *Crotalus atrox* venom (5'-NT, 2 U i.a., Sigma) (33), followed by 40 U/kg/h i.a. during I/R. In additional experiments, WT mice were treated with a bolus of 5'-NT (1 U i.a.), followed by 20 U/kg/h i.a. during I/R. Sham-operated controls underwent the same surgical procedures but without I/R. After 3 hours of reperfusion mice

were euthanized and intestinal and lung samples were immediately frozen in liquid nitrogen for further investigations. Blood was collected, transferred to a 2 ml tubed and stored overnight at 4°C. Blood samples were centrifuged the next day (10 min at 3000 rpm) and serum was transfered to a new tube and frozen at –80°C.

Myeloperoxidase (MPO) activity

MPO activity, an index of neutrophil infiltration, was measured in intestinal and lung tissue homogenates as described previously (78, 79, 81). In short, 0.05g of tissue was homogenized in 500ul extraction buffer (0.5% HTAB in 10% working stock buffer). Homogenates were freeze/thawed three times, sonicated for 30 seconds and centrifuged for 15 min at 4°C at 13.2 rpm. The supernatant was then assayed for MPO activity using a spectrophotometric reaction with o-dianisidine dihydrochloride (Sigma-Aldrich). Absorbance at 450 nm was measured and reported as difference in optical density over 60 seconds (90 sec – 30 sec) x 35.4 and expressed in U/100mg protein.

Alanine aminotransferase (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) (82). Biefly, serum samples were added to ALT or AST Substrate Solution in a 96- well microtiter plate. After incubation the appropriate Colour-Reagent was put onto it. The reaction was stopped with ALT Colour-Developer or 2 N HCl for AST. The optical density was measured at 510 nm using a photometer.

Interleukin-1 (IL-1) and 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-1 or 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 streptavidin-HRP application 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.

RT-PCR

To examine the influence of intestinal I/R on CD73 transcript levels, C57BL/6 mice underwent intestinal ischemia followed by reperfusion. Mice were euthanized at indicated time points and mucosal scrapings were performed. Total RNA was isolated using the total RNA isolation NucleoSpin RNA II Kit according to the manufacture's instructions (Macherey & Nagel, Düren, Germany). cDNA synthesis was performed using reverse transcription according to the manufacturer's instructions (i-script Kit, Bio-Rad Laboratories, Inc., München, Germany). The primer sets for the PCR reaction contained 1 μM sense and 1 μM antisense with SYBR Green I (Molecular Probes Inc.). Primer sequences for murine CD73 were (sense/antisense, respectively) 5'-CAAATCCCACACAACCACTG-3' and 5'-TGCTCACTTGGTCACAGGAC-3'. Murine ß-actin sense primer, 5'-ACATTGGCATGGCTTTGTTT-3' and antisense primer, 5'-GTTTGCTCCAACCAACTGCT-3 were used to control for the starting template. Levels and fold change in mRNA were determined as described previously (83).

Western Blot

Western blotting technique was used to examine total CD73 protein level. Briefly, protein extracts 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 2h 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 2h 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.

Histological sections

Mice were euthanized at the indicated time points and intestine was removed and fixed in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, NL). Cryostat sections (5 μm) were mounted on glass slides, air dried and post-fixed in acetone/methanol (1:1) for 10 minutes at RT. Sections were stained with H&E and the extent of the mucosal damage was quantified using a semi-quantitative grading system adapted from Chiu *et al.* (32). Injury was classified using the following 5 point scale where a numerical score was assigned based on the degree of mucosal and submucosal damage. The 5 point scale progresses from normal [0] to development of apical supepithelial space [1], epithelial lifting [2-3] and cellular infiltration [4], to disintegration of lamina propria, hemorrhage and ulceration [5].

Adenosine measurement

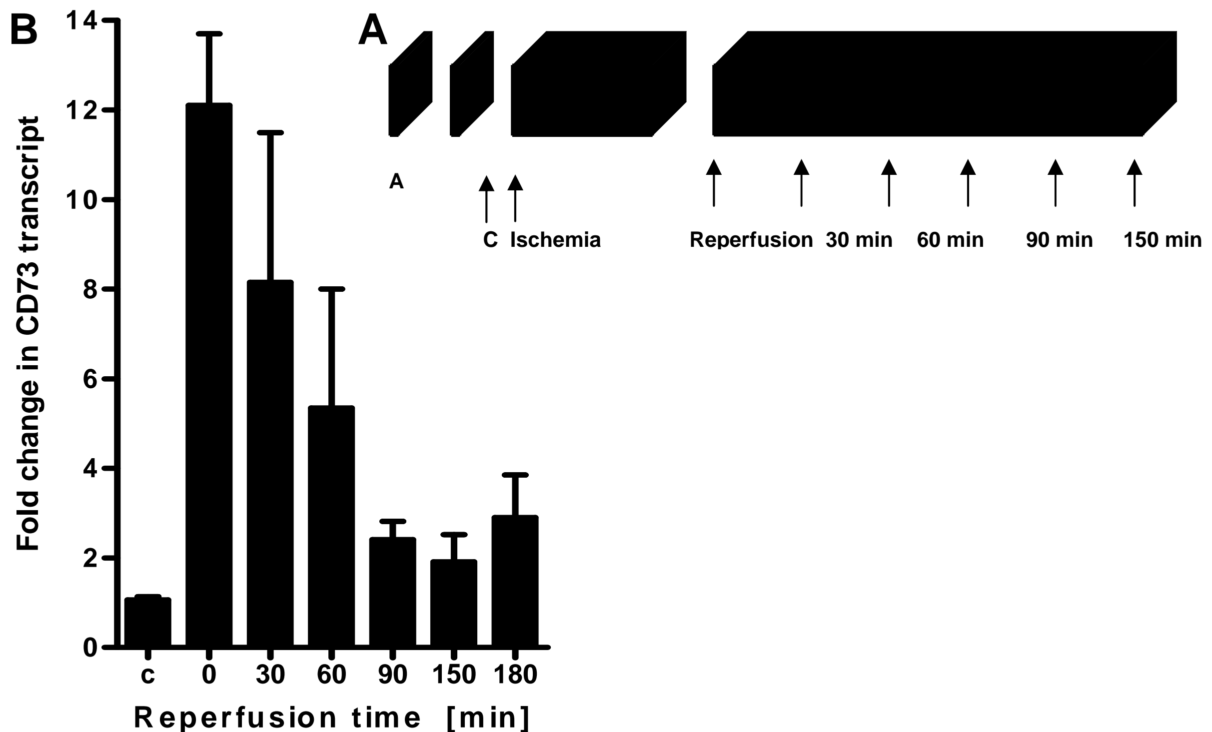
Jejunum from WT and CD73-/- mice with and without I/R was removed and immediately snap frozen with clamps that were pre-cooled to the temperature of liquid nitrogen within a time lag of 3 to 5 s. The frozen jejunum was pulverized under liquid nitrogen, and the tissue protein was precipitated with ice-cold 0.6 N

perchloric acid. Tissue adenosine levels were determined as described previously (84).

V. RESULTS

Intestinal CD73 is induced by intestinal I/R

Based on previous studies showing tissue protection by extracellular adenosine generated via hypoxia-inducible CD73 (29, 31, 33), we hypothesized that CD73-dependent adenosine generation may play an important role during intestinal I/R. Therefore, we investigated intestinal CD73 expression using our previously described model of intestinal I/R (73-75). To define transcriptional effects of intestinal I/R, intestinal epithelial scrapings were harvested at the indicated reperfusion time points following 15 min ischemia (Fig. 1a) and assessed via real-time RT-PCR. A strong induction of CD73 mRNA was observed immediately following ischemia (immediately after ischemia, 12.1±1.6-fold, $p<0.001$) with a steady decrease over the reperfusion time period (Fig. 1b).



As the next step, we measured CD73 by western blot analysis from intestinal epithelial scrapings and confirmed increases in CD73 protein after intestinal I/R compared with sham controls (Fig. 1c).

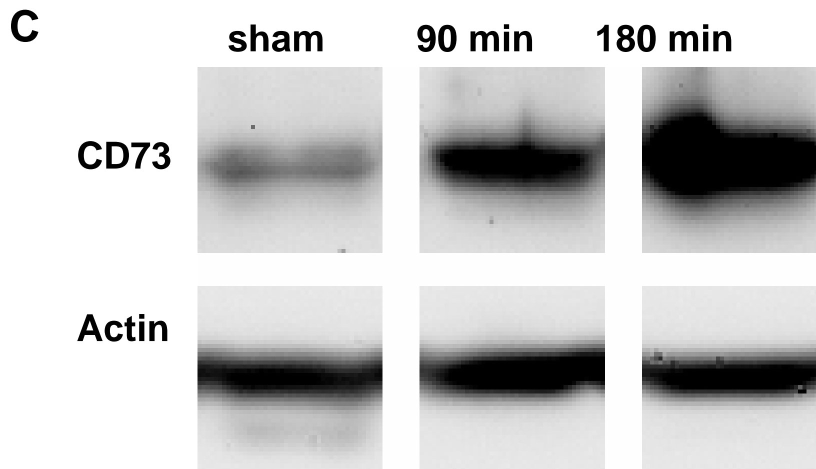
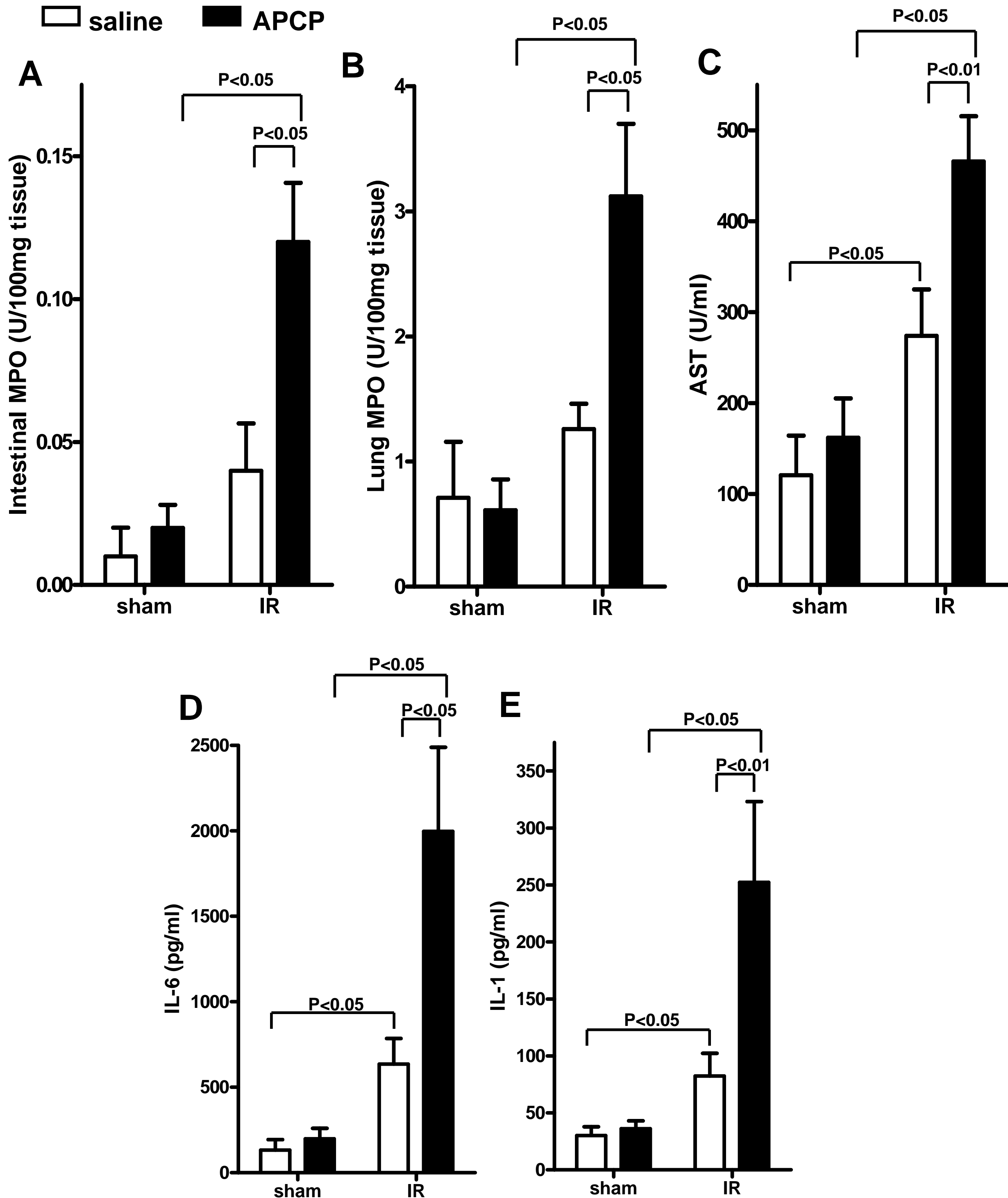


Figure 1. *CD73 is induced by intestinal IR.* ***(a)*** *Transcriptional responses to intestinal IR were measured following isolation of RNA from intestinal epithelial scrapings from WT mice that were anesthetized (A) and after a midline laparotomy (L) were subjected to 15 minutes ischemia, followed by the indicated reperfusion times. RNA was also isolated from control (C) mice that did not undergo IR.* ***(b)*** *CD73 mRNA levels were determined by real-time RT-PCR. Data were calculated relative to a internal housekeeping gene (ß-actin) and expressed as fold change compared to sham (C) mice ± SEM of 4 mice per group.* ***(c)*** *Intestinal scraping from WT mice that were subjected to 15 minutes ischemia, followed by 90 or 180 min reperfusion were flash frozen, lysed, proteins were resolved by SDS-PAGE, transferred to nitrocellulose and membranes were probed with anti-CD73 antibody. A representative western blot of two is shown. The same blot was re-probed for ß-actin as a control for protein loading.*

CD73 inhibition accentuates injury following intestinal I/R

After having shown a robust induction of CD73 during intestinal IR injury, we next performed pharmacological studies to determine the functional role of CD73 in this model. WT mice were subjected to 15 minutes of superior mesenteric artery occlusion and 3 hours of reperfusion following treatment with the specific CD73 inhibitor APCP (40 mg/kg/h, i.a.) or vehicle (saline). Consistent with previous studies (78-80) IR was associated with a significant increase in intestinal MPO activity (Fig. 2A, $p<0.05$). However, neutrophil tissue infiltration was dramatically increased following APCP-inhibition of CD73 (Fig. 2A, $p<0.05$). Similarly, increases in pulmonary MPO activity were significantly

enhanced in mice exposed to IR injury following CD73 inhibition (Fig. 2B, $p<0.05$). As shown in Fig. 2C, APCP treatment prior to intestinal IR also resulted in a significant increase in secondary hepatic injury as measured by serum AST. Additionally, inhibition of CD73 caused a significant increase in serum IL-6 and IL-1β following IR (Fig. 2D and 2E, respectively).



Histological analysis revealed that intestinal IR also significantly increased the intestinal injury score in APCP treated mice compared to WT mice that were treated with vehicle (Fig. 2F and 2G). Taken together, these studies provide pharmacological evidence of CD73 in tissue protection during intestinal IR.

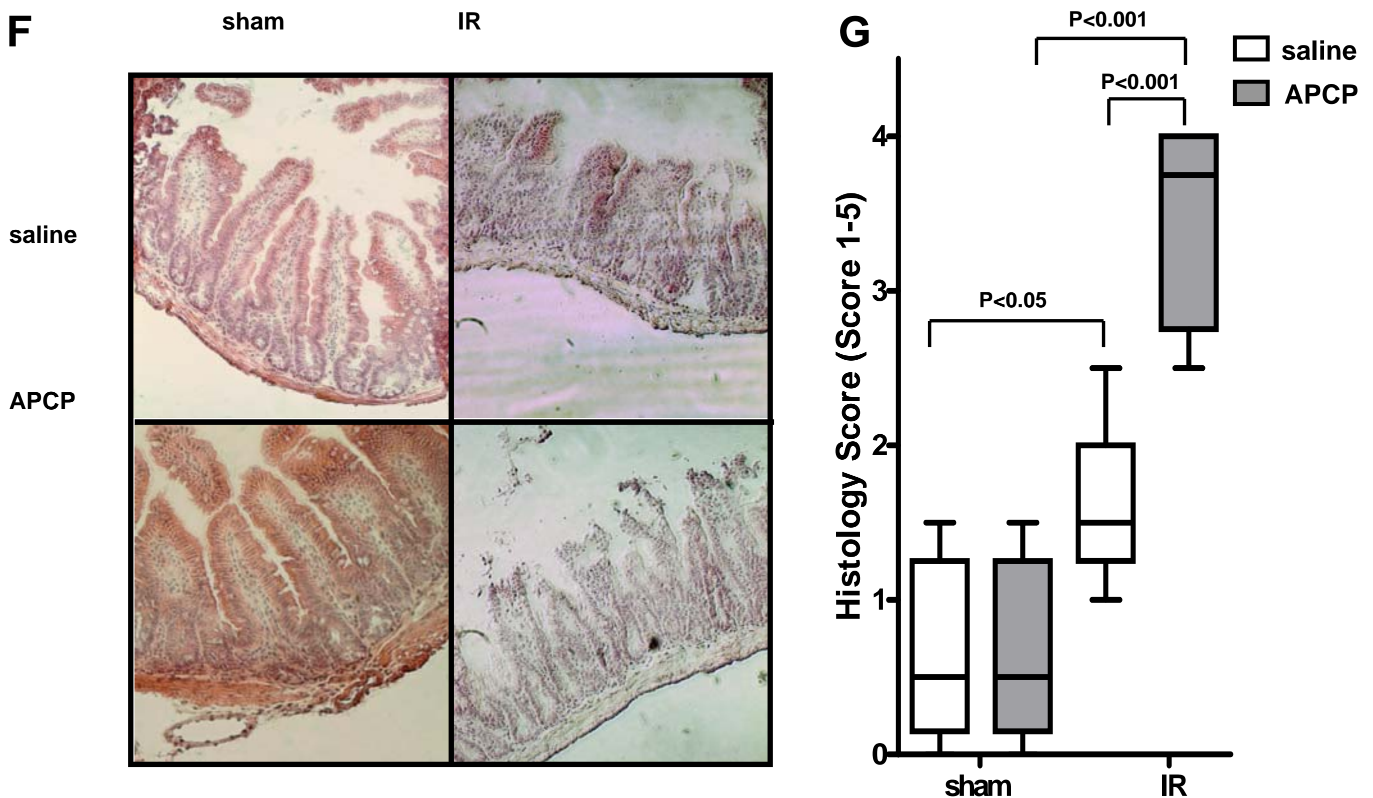
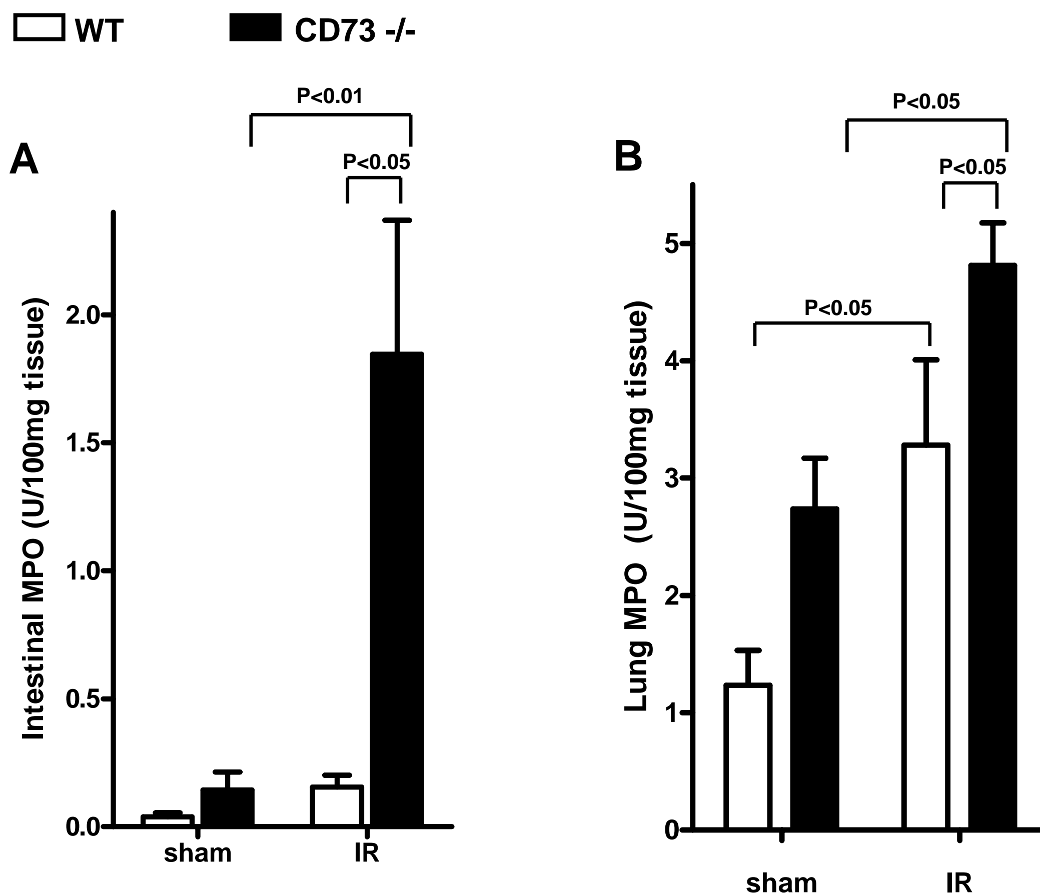


Figure 2. *Inhibition of CD73 by APCP increases injury. Injury was measured after WT mice were treated with APCP (40 mg/kg/h, i.a.) or sterile saline and subjected to 15 minutes ischemia, followed by 3 h reperfusion. Injury was measured for the following parameters:* ***(a)*** *Intestinal myeloperoxidase (MPO),* ***(b)*** *lung MPO,* ***(c)*** *AST,* ***(d)*** *IL-6,* ***(e)*** *IL-1,* ***(f)*** *H&E staining of jejunum sections (200X), and (g) quantification of ischemic injury (n= 3-5 mice per group and expressed as median ± range). All other results are expressed as the mean ± SEM of 8-12 mice per group.*

Intestinal IR injury is increased in cd73$^{-/-}$ mice

Based on these pharmacological findings demonstrating that CD73 inhibition enhances IR-induced injury, we next investigated the role of CD73 in protection against intestinal IR in *cd73* gene-targeted mice (33). Following intestinal IR, *cd73*$^{-/-}$ mice demonstrated significantly higher intestinal MPO levels as compared to littermate control (WT) mice (Fig. 3A, $p<0.01$). Similarly, pulmonary MPO levels were increased in *cd73-/-* mice (Fig. 3B, $p<0.05$). Biochemical analysis revealed that intestinal IR significantly increased serum AST (Fig. 3C) and ALT (Fig. 3D) concentrations in *cd73*$^{-/-}$ mice compared to littermate controls. *Cd73*$^{-/-}$ mice also demonstrated significantly higher IL-6 (Fig. 3E) and IL-1β (Fig. 3F) cytokine concentrations in the serum than WT control mice following intestinal IR. Taken together, these data reveal, for the first time, genetic evidence for CD73-dependent protection against intestinal IR.



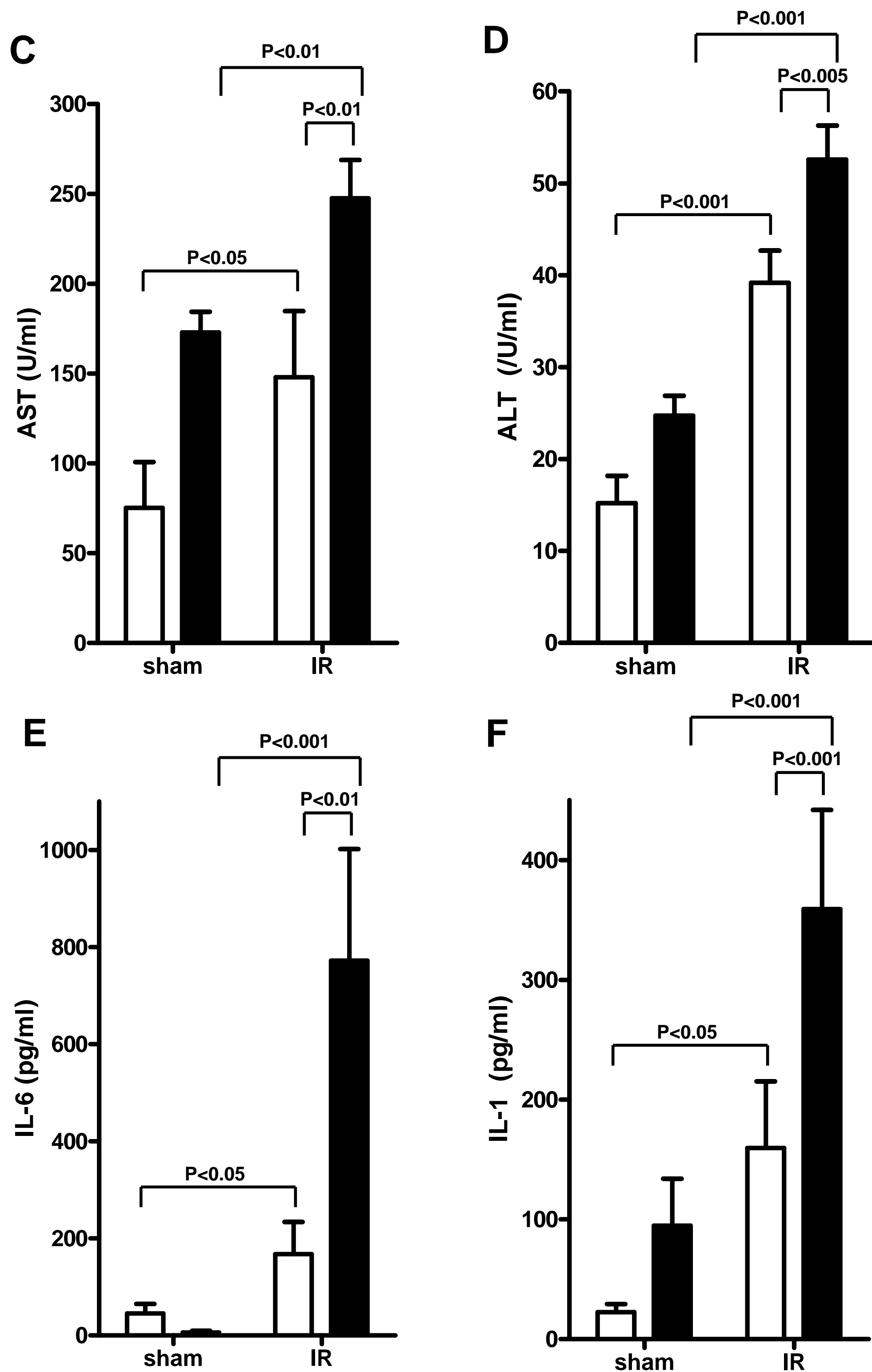


Figure 3. *Injury is increased in cd73*$^{-/-}$ *mice. Cd73 (cd73*$^{-/-}$*) deficient mice or age, weight and gender matched littermate controls (WT) were subjected to 15 minutes ischemia, followed by 3 h reperfusion. Injury was measured for the following parameters:* ***(a)*** *Intestinal MPO),* ***(b)*** *lung MPO,* ***(c)*** *AST,* ***(d)*** *ALT,* ***(e)*** *IL-6 and* ***(f)*** *IL-1. All results are expressed as the mean ± SEM of 8-12 mice per group.*

Histological signs of intestinal injury by intestinal IR are increased in $cd73^{-/-}$ mice

To confirm enhanced injury observed in $cd73^{-/-}$ mice, we examined jejunum from WT littermate controls or $cd73^{-/-}$ mice. Intestinal IR resulted in loss and shortening of intestinal villi and marked intestinal epithelial cell denudation (Fig. 4A). However the observed injury was significantly increased in $cd73^{-/-}$ mice compared to WT controls after intestinal IR as semi-quantitative histological analysis of these sections demonstrated an increase in the Chiu index (85) from 2 in WT mice to 3.5 in $cd73^{-/-}$ mice (Fig. 4A and 4B, $p<0.01$). Taken together, these data provide strong evidence for a role of CD73 in attenuating intestinal IR injury.

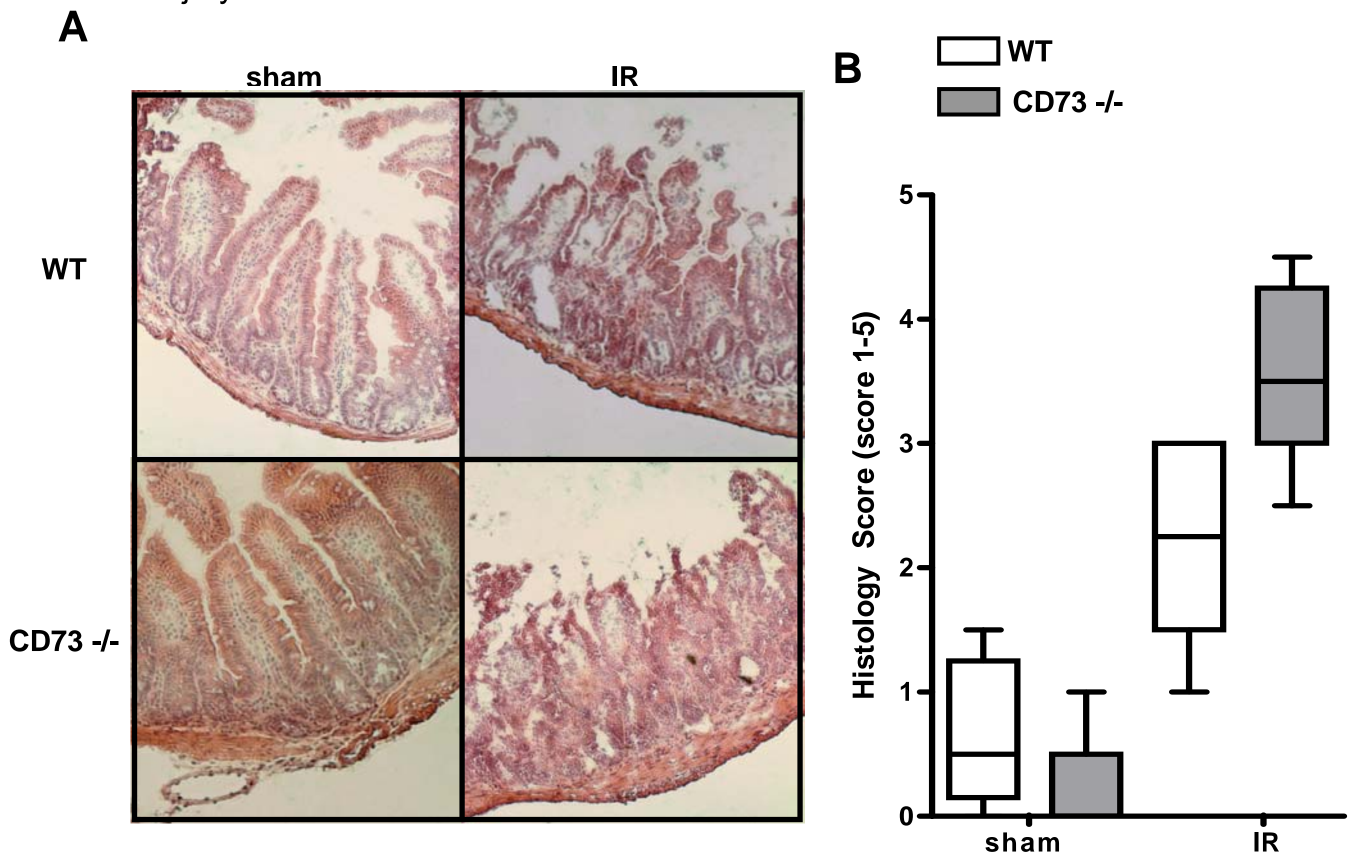


Figure 4. *Histological signs of intestinal IR injury are increased in $cd73^{-/-}$ mice.* ***(a)*** *H&E staining of jejunum sections (200X) and* ***(b)*** *quantification of ischemic injury. Results are expressed as median ± range, n=3-5 mice per group.*

Intestinal adenosine concentrations are decreased in cd73$^{-/-}$ mice

For the next step, we performed studies to confirm that the functional role of CD73 in intestinal IR injury is associated with differences in adenosine tissue concentrations. Here, we measured jejunal adenosine levels in tissues shock frozen immediately following 15 min of intestinal ischemia or in sham operated controls. In fact, intestinal adenosine concentrations were significantly increased following IR compared to sham controls (Fig. 5, p<.001). In contrast, baseline intestinal adenosine levels of *cd73$^{-/-}$* were significantly lower than littermate controls (Fig. 5; 2.71±0.2-fold, p<0.01) and did not increase with intestinal ischemia (Fig. 5). Taken together, these data demonstrate that increases in intestinal adenosine during ischemia require CD73 activity.

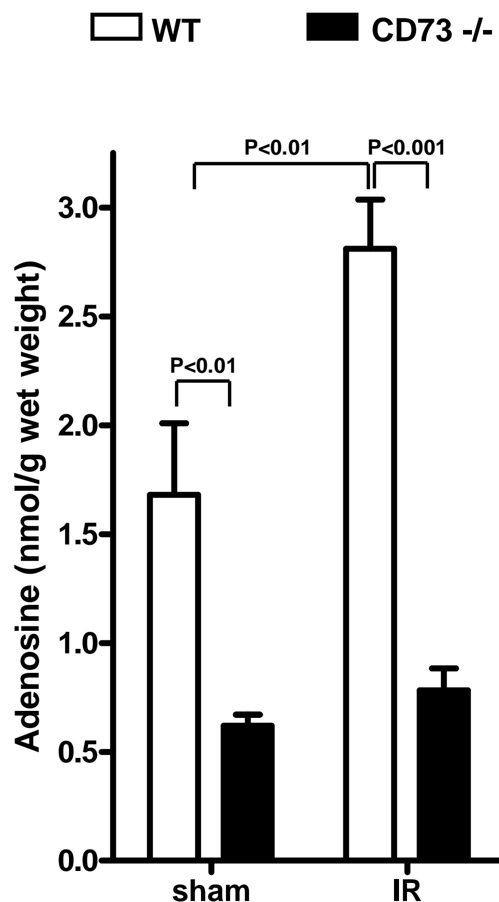
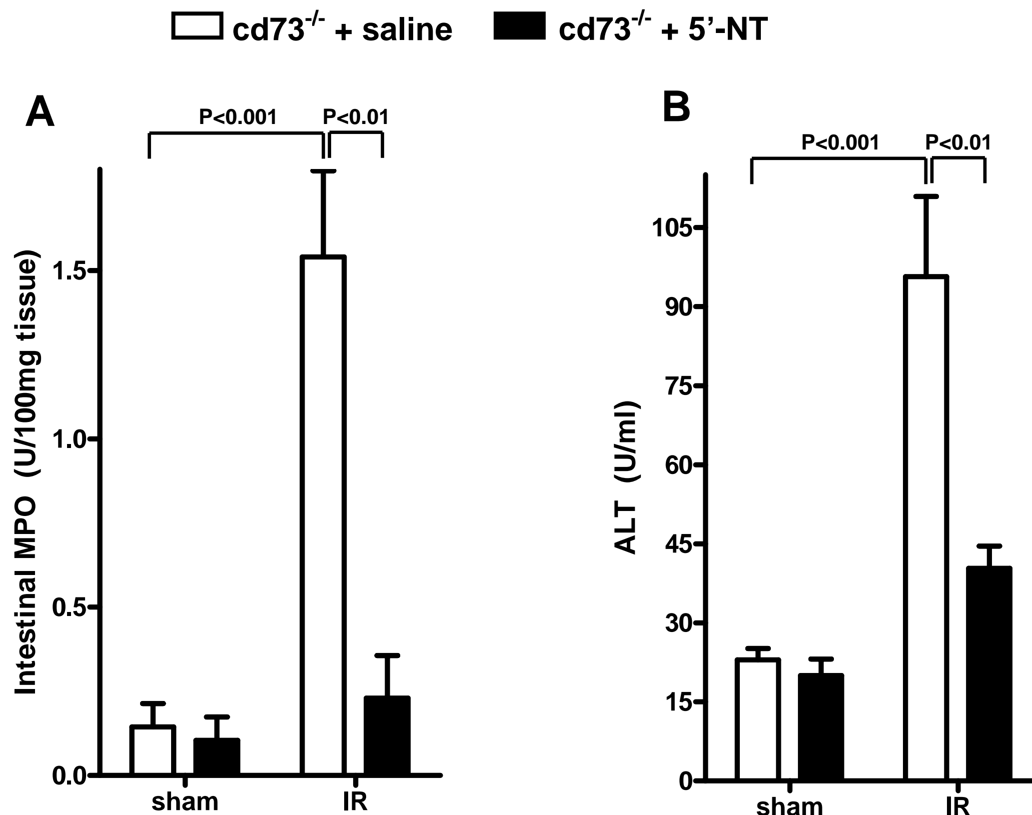


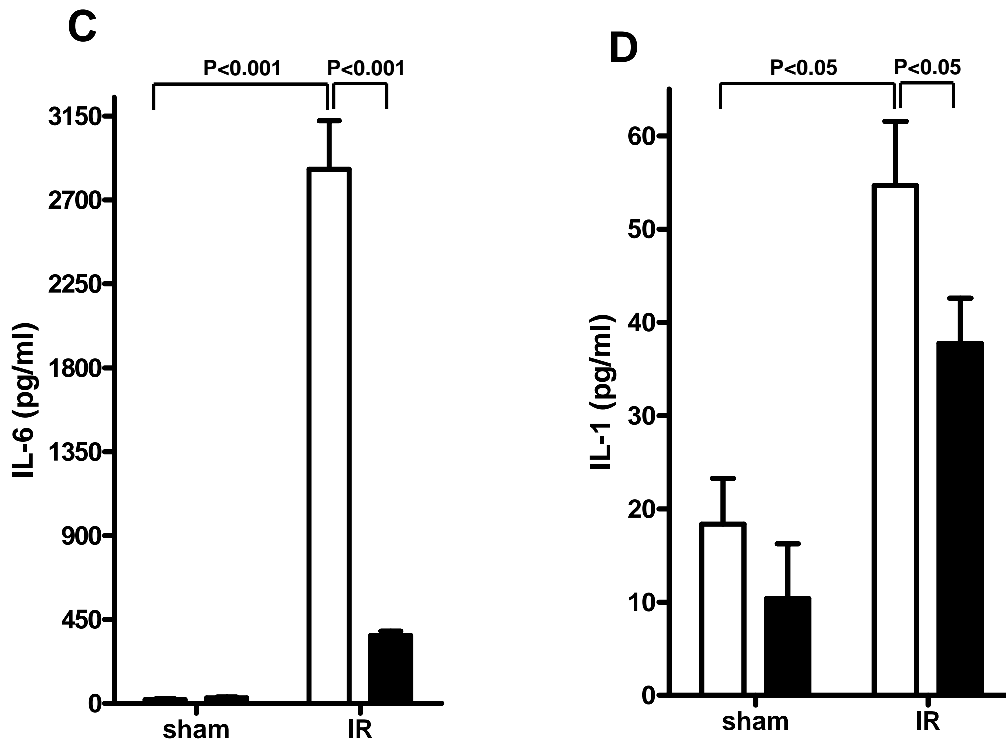
Figure 5. *Increased intestinal adenosine concentrations with IR are attenuated in cd73$^{-/-}$ mice. WT or cd73$^{-/-}$ mice were subjected to 15 minutes ischemia. The*

jejunum was snap frozen immediately prior to the end of ischemia (IR). In control mice, the jejunum was snap frozen without clamping of the superior mesenteric artery (sham). Results are expressed as the mean ± SEM of 6 mice per group.

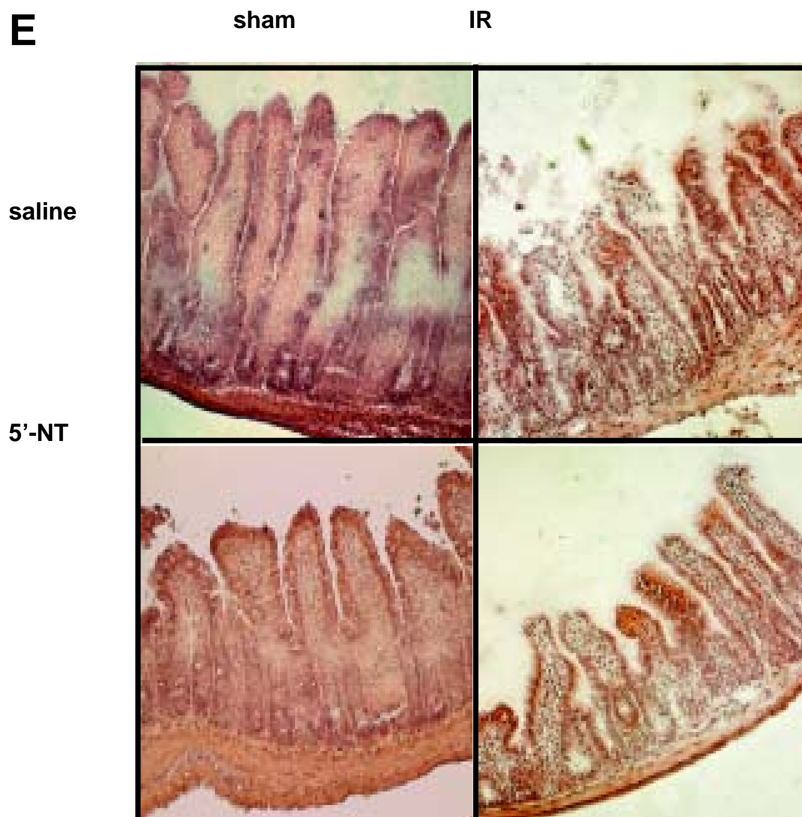
Reconstitution of cd73$^{-/-}$ mice with soluble 5'-nucleotidase

As proof of principle and to demonstrate that an increase in functional injury parameters in *cd73$^{-/-}$* mice reflects lack of ecto-5´-nucleotidase enzyme activity, we next reconstituted *cd73$^{-/-}$* mice with soluble 5´-nucleotidase (5'-NT) from *Crotalus atrox* venom (33) and subjected them to intestinal IR. As shown in Fig. 6A and Fig. 6B, reconstitution of *cd73$^{-/-}$* mice with soluble 5'-nucleotidase attenuated injury as demonstrated by a significant decrease in intestinal MPO (Fig. 6A), ALT (Fig. 6B), and pro-inflammatory cytokines IL-6 (Fig. 6C) and IL-1 (Fig. 6D).





Furthermore histological analysis confirmed that 5'-NT treatment was associated with reconstitution of a WT-phenotype (Fig. 6E and 6F). Taken together, these results confirm our genetic studies that CD73 plays a crucial role in intestinal IR-induced injury.



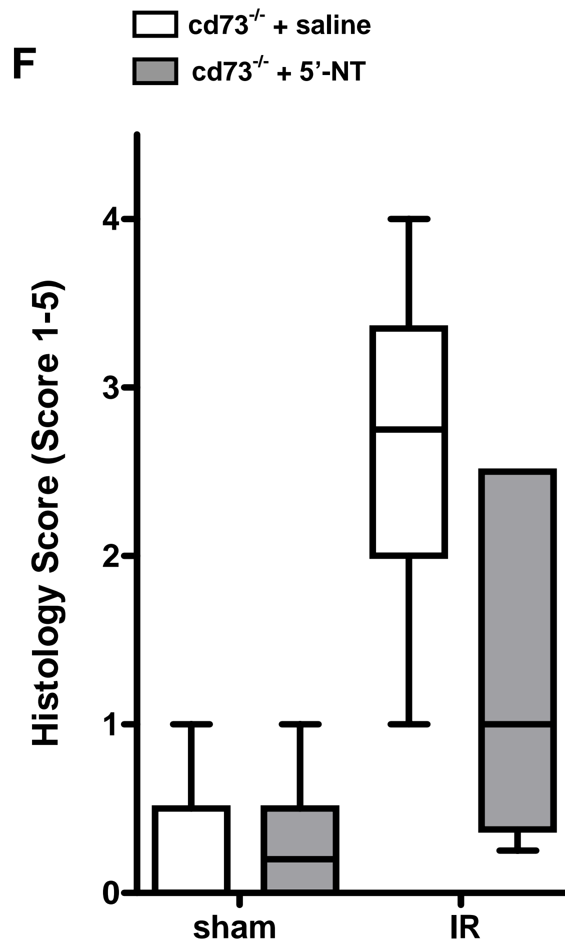
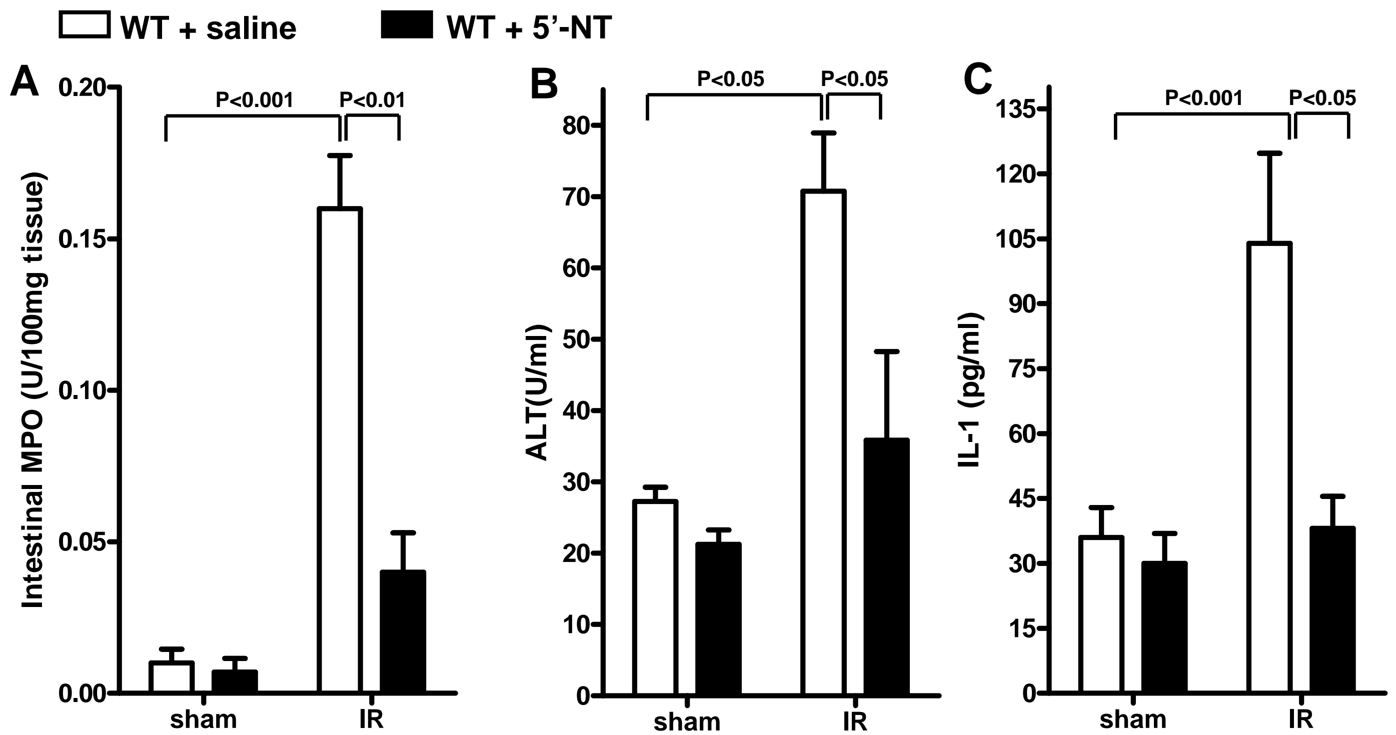


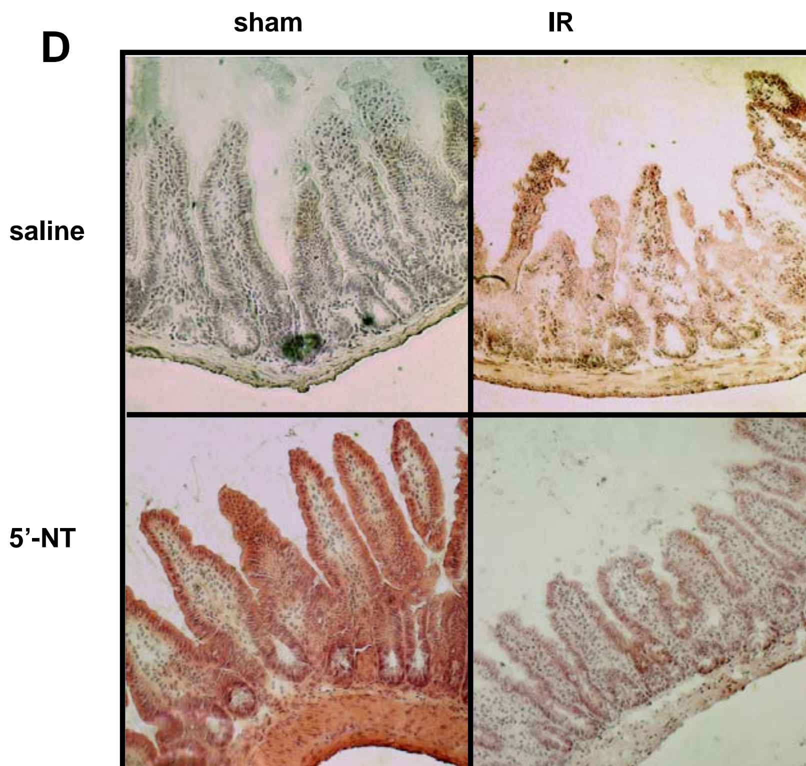
Figure 6. *Reconstitution of cd73$^{-/-}$ mice with soluble 5'-nucleotidase decreases injury in cd73$^{-/-}$ mice. Injury was measured after cd73$^{-/-}$ mice were reconstituted with 5'-nucleotidase (2 U bolus prior to ischemia, followed by 40 U/kg/h, i.a.) or sterile saline and subjected to 15 minutes ischemia, followed by 3 h reperfusion. Injury was measured for the following parameters:* ***(a)*** *Intestinal MPO,* ***(b)*** *ALT,* ***(c)*** *IL-6,* ***(d)*** *IL-1,* ***(e)*** *H&E staining of jejunum sections (200X), and (F) quantification of ischemic injury (n= 3-5 mice per group and expressed as median ± range). All other results are expressed as the mean ± SEM of 8-12 mice per group.*

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

We next pursued the usefulness of 5'-NT treatment of intestinal ischemia in WT mice. For this purpose, we treated WT mice with 5'-nucleotidase prior to intestinal IR (33). As shown in Fig. 7A-C, injury following intestinal IR was inhibited by treatment of WT mice with soluble 5'-nucleotidase.



Intestinal histological analysis confirmed this protection (Fig. 7D and 7E). Together these data demonstrate a therapeutic effect of treatment with soluble 5´-nucleotidase in intestinal IR.



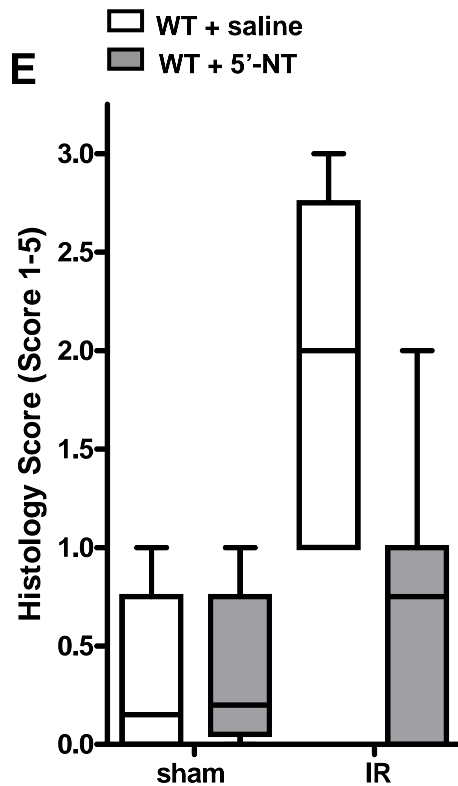


Figure 7. *Treatment with soluble 5'-nucleotidase decreases injury in WT mice. Injury was measured after WT mice were treated with 5'-nucleotidase (2 U bolus prior to ischemia, followed by 20 U/kg/h, i.a.) or sterile saline and subjected to 15 minutes ischemia, followed by 3 h reperfusion. Injury was measured for the following parameters:* ***(a)*** *Intestinal MPO,* ***(b)*** *ALT,* ***(c)****, IL-1,* ***(d)*** *H&E staining of jejunum sections (200X), and* ***(e)*** *quantification of ischemic injury (n= 3-5 mice per group and expressed as median ± range). All other results are expressed as the mean ± SEM of 8-12 mice per group.*

VI. DISCUSSION

In the present study, we pursued the contribution of extracellular adenosine production to protection against intestinal I/R-induced injury. Transcriptional and translational profiling of intestinal tissue revealed a prominent induction of CD73. Pharmacological inhibition or targeted gene deletion of CD73 significantly increased injury following intestinal I/R. Moreover, soluble 5´-nucleotidase treatment was associated with lower levels of injury. Taken together, these studies suggest that manipulation of CD73 enzymatic activity to increase extracellular adenosine concentrations may be a promising therapeutic strategy for the treatment of acute ischemic injury of the intestine.

Our results showing that intestinal CD73 is induced by intestinal I/R are consistent with previous studies that found that exposure of intestinal epithelial cells or microvascular endothelial cells to ambient hypoxia (2% oxygen) resulted in a robust induction of CD73 transcript, protein and function (31, 32, 86). Similarly, studies in $CD73^{-/-}$ mice demonstrated increased vascular leakage syndrome and pulmonary edema upon hypoxia exposure (8% oxygen over 4h), confirming a role of CD73-dependent adenosine production in multiple-organ injury during hypoxic events. Studies examining the CD73 gene promoter found a binding site for hypoxia-inducible factor (HIF)-1α and inhibition of HIF-1α expression resulted in attenuation of hypoxia-inducible CD73 expression (32), demonstrating a HIF-1α dependent pathway for CD73 induction by hypoxia. In addition to hypoxia, HIF-1α was induced in the intestinal mucosa and persisted after 1 h and 3 h of reperfusion in a rat model of intestinal I/R injury (86). Therefore, it is likely that the observed protective effects of CD73-dependent adenosine production during intestinal IR are transcriptionally coordinated by HIF-1α.

In the present study we show that pharmacological inhibition or genetic deletion of CD73 due to decreased adenosine generation significantly increased

infiltration of neutrophils into the intestinal and pulmonary tissues following intestinal I/R. Human intestinal epithelial cells express surface CD73 abundantly on their apical domain and to a lesser extent on the basolateral domain (87). *In vitro* results show that neutrophils migrate across intestinal mucosa (88, 89) and release 5'-AMP (72). 5'-AMP is subsequently degraded into adenosine at the apical pole by CD73 anchored within the intestinal epithelial luminal membrane (72, 90), where it functions as a paracrine and/or autocrine mediator of chloride secretion in intestinal epithelial cells (72, 91, 92). The ability of intestinal epithelial cells to respond to 5'-AMP with chloride secretion and hence water secretion is an important component of the epithelial barrier that protects the intestine by preventing translocation of bacteria, bacterial products and antigens to lamina propria (93, 94). A previous study showed that CD73 inhibition using APCP resulted in a significant increase in hypoxia-induced intestinal permeability (32), suggesting that hypoxia-induced CD73 *in vivo* may provide a protective mechanism for the intestinal barrier during episodes of decreased oxygen delivery. Furthermore, It has been shown that adenosine binds to surface expressed adenosine receptors on neutrophils to limit excessive accumulation of neutrophils within tissues, and as such, functions as a feedback loop to attenuate potential tissue injury (29). CD39 and CD73 have been previously identified as critical control points to attenuate excessive neutrophil accumulation during hypoxia (95). Thus, one mechanism for adenosine protection in the intestine may be due to neutrophil-intestinal epithelial cross-talk. Additional studies with mice exhibiting tissue-specific deletion of CD73 (e.g. intestinal epithelial cells, endothelial cells) will specifically determine the source of CD73 and thus adenosine in modulation of GIR injury.

Adenosine receptor expression is widespread such that adenosine controls the function of virtually every organ and tissue (fortune) (58). However adenosine receptor signaling is complex, with each of the four receptors having a unique role in different tissues (96). Activation of A_1 and possibly A_3 adenosine receptors is believed to protect heart and other tissues by preconditioning

through a pathway including protein kinase C and mitochondrial K_{ATP} channels (fortune) (21, 97, 98). Activation of A_{2A}AR limits renal (fortune) (41, 53-55, 99), liver (fortune) (42, 45) and myocardial I/R (100, 101) injury by inhibiting inflammatory processes in neutrophils, platelets, macrophages and T cells (fortune) (21). A_{2A}AR activation, via A_{2A}AR agonists, also reduces inflammation and in turn, improves survival in mouse models of sepsis (fortune) (40, 102), acute lethal liver injury (fortune) (52) and inflammatory bowel disease (fortune) (51). Previous studies on the contribution of adenosine receptor signaling in the intestine have revealed conflicting results on the role of individual adenosine receptors in this particular organ. Treatment with an A1 receptor agonist was shown to protect against intestinal I/R injury in the rat (103). However, other studies demonstrate that the A_{2B}AR mediates enhancement of chloride secretion induced by adenosine (91, 104). Additionally, we previously demonstrated that A_{2B}AR is selectively up-regulated by hypoxia, and that A_{2B}AR antagonists effectively neutralize ATP-mediated changes in post-hypoxic endothelial permeability (31). A_{2B}AR is also involved in re-sealing of endothelial cells during transendothelial migration of neutrophils (71), particularly during conditions of limited oxygen availability (31). Moreover, pharmacological inhibition of the A_{2B}AR during hypoxia exposure was associated with increased pulmonary edema and vascular leakage(33). Furthermore, the A_{2B}AR is expressed in intestinal human T84 cells and intact human intestinal epithelia (12). Interestingly, the A2b receptor appears to be the only adenosine receptor present in T84 cells and is present in both apical and basolateral membranes (12–14). Additional studies using a combined pharmacological and genetic approach using adenosine-receptor deficient mice will be required to identify adenosine signaling mechanisms involved in modulation of GIR injury.

In summary, the present study demonstrates that CD73 is a critical control point for endogenous adenosine generation and modulation of injury during intestinal I/R. Gene-targeted mice lacking the major extracellular pathway of adenosine generation (CD73) demonstrate excessive injury. The significant attenuation of

intestinal injury following ischemia by soluble 5´-nucleotidase treatment suggests possible new strategies to ameliorate the consequences of intestinal ischemia. Future challenges include the identification of cell types responsible for adenosine generation during intestinal ischemic events, thus allowing the delivery of adenosine therapeutics to specific anatomic sites.

VII. SUMMARY

Intestinal ischemia/reperfusion injury, i.e., that which occurs via occlusion, followed by reperfusion of the superior mesenteric artery, contributes to morbidity and mortality and critical illness (2, 6, 9, 10, 21, 22, 105). Extracellular adenosine has been implicated as an innate anti-inflammatory metabolite, particularly during conditions of limited oxygen availability such as ischemia. Since adenosine is primarily produced via enzymatic conversion of AMP via ecto-5'-nucleotidase (CD73), we used a mouse model of intestinal I/R injury to examine the contribution of CD73-dependent adenosine production in modulation of intestinal ischemia/reperfusion (IR) injury. Following initial transcriptional and translational profiling of intestinal tissue that revealed a prominent induction of murine CD73, we next studied the role of CD73 in protection against intestinal IR injury. Pharmacological inhibition or targeted gene deletion of CD73 significantly enhanced, not only local intestinal injury, but also secondary organ injury, following IR. To confirm that CD73 was required for adenosine generation in the intestine and is regulated by intestinal IR, we measured adenosine tissue levels and found that they were significantly higher in WT mice than CD73-deficient ($CD73^{-/-}$) mice. Moreover, reconstitution of CD73-/- mice or treatment of WT mice with soluble 5´-nucleotidase was associated with significantly lower levels of injury. These data reveal what we believe to be a previously unrecognized role of CD73 in modulation and regulation of intestinal IR-mediated injury. Taken together, these studies suggest that manipulation of CD73 enzymatic activity, to increase extracellular adenosine concentrations, may be a promising therapeutic strategy for the treatment of acute ischemic injury of the intestine.

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