

# P2X1 regulated IL-22 secretion by innate lymphoid cells is required for efficient liver regeneration

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Footnote page

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*List of Abbreviations.*

ALT, Alanine aminotransferase, AST, aspartate aminotransferase; ATP, Adenosine triphosphate; BIRC5, Baculoviral IAP Repeat Containing 5; cNK, conventional NK cell; ELISA, Enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FACS, Fluorescence-activated cell sorting; FOXM1, Forkhead Box M1; IHPBA, International Hepato-Pancreato-Biliary Association; ILC, Innate lymphoid cells, ISGLS, International Study Group of Liver Surgery; NK, Natural Killer cells; NKT, Natural Killer T cells; PBS, Phosphate-buffered saline PCNA, Proliferating cell nuclear antigen; PHLF; Posthepatectomy Liver Failure; POD, Postoperative day; PT, Prothrombin time; RAG, Recombination-activating genes; SB, Serum bilirubin; Th22 cells, T helper 22 cells; WT, wild type.

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## Abstract

Paracrine signalling mediated via cytokine secretion is essential for liver regeneration after hepatic resection, yet the mechanisms of cellular crosstalk between immune and parenchymal cells are still elusive. Interleukin-22 (IL-22) is released by immune cells and mediates strong hepatoprotective functions. However, it remains unclear if IL-22 is critical for the crosstalk between liver lymphocytes and parenchymal cells during liver regeneration after partial hepatectomy. Here we found that plasma levels of IL-22 and its upstream cytokine IL-23 are highly elevated in patients after major liver resection. In a mouse model of partial hepatectomy, deletion of IL-22 was associated with significantly delayed hepatocellular proliferation and an increase of hepatocellular injury and endoplasmic reticulum stress. Using *Rag1<sup>-/-</sup>* and *Rag2<sup>-/-</sup>γc<sup>-/-</sup>* mice we show that the main producers of IL-22 post partial hepatectomy are conventional natural killer cells and innate lymphoid cells type 1. Extracellular ATP, a potent danger molecule, is elevated in patients immediately after major liver resection. Antagonism of the P2 type nucleotide receptors P2X1 and P2Y6 significantly decreased IL-22 secretion *ex vivo*. *In vivo*, specific inhibition of P2X1 was associated with decreased IL-22 secretion, elevated liver injury and impaired liver regeneration. **Conclusion:** This study shows that innate immune cell derived IL-22 is required for efficient liver regeneration and that the secretion of IL-22 in the regenerating liver is modulated by the ATP receptor P2X1.

Cellular crosstalk including secretion of cytokines and paracrine signaling via danger-associated molecular patterns is essential for liver regeneration after hepatic resection. The cytokine IL-22 is released by immune cells and acts primarily on nonhematopoietic cells such as epithelial cells unlike most other cytokines, which target hematopoietic cells (1). In the liver IL-22 acts on hepatocytes and stellate cells and exhibits hepatoprotective properties by reducing liver fibrosis and ameliorating acute liver injury (2-5). In response to partial hepatectomy levels of IL-22 are elevated in the regenerating liver and exogenous administration of IL-22 as well as transgenic expression is associated with improved outcome (5). Yet it remains unclear what the source and triggering factors of IL-22 in response to partial hepatectomy are and whether IL-22 is essential for liver regeneration.

We hypothesize that IL-22 is an unidirectional and critical mediator between specific immune cells and hepatocytes that mediates hepatoprotection. In this study, we aimed to identify the source and the role of IL-22 in liver regeneration after partial hepatectomy.

Various sources of IL-22 have been described including  $\gamma\delta$  T cell, natural killer T cells (NKT), natural killer (NK) cells, Th17 cells and non-NK cell subsets of innate lymphoid cells (ILC), of which the first three cell types have been described to impact on liver regeneration (1, 6-8). Interestingly, the role of ILC in the context of liver regeneration remains unexplored. ILC is a collective term for innate lymphocytes that lack a specific antigen receptor yet can produce an array of effector cytokines that exhibit functions such as tissue remodeling, antimicrobial immunity and inflammation (9). ILC are further subdivided into at least three groups ILC1-3 according to transcription factors and secreted cytokines (10). Most recently, cytokine producing

ILC1 within the liver are now distinguished from conventional NK cells (cNK; that are also considered as killer ILC) by the presence of the integrin subunit CD49a (11, 12).

In the light of such new diversity of innate immune cells, one specific aim of our studies was to identify the cellular subtype that secretes IL-22 in the liver and how this secretion is being regulated.

The nucleotide adenosine triphosphate (ATP) is released at early time points after partial hepatectomy (8, 13). Extracellular nucleotides modulate cellular injury, proliferation and crosstalk (8, 14-16) via specific purinergic P2Y (G-protein coupled) and P2X (ligand-gated ion channel) receptors. Purinergic receptors are expressed on many cell types including intrahepatic lymphocytes of which cNK, NKT and ILC1 represent the largest fraction (17, 18). However, the cellular and molecular mechanisms downstream of extracellular nucleotides receptors that promote liver regeneration are still unclear. Based on our previous studies we hypothesized that purinergic signaling modulates other effector functions of hepatic immune cells including IL-22 secretion (8, 19, 20).

Here we show that IL-22 is secreted after partial hepatectomy by the ILC subsets cNK cells and ILC1 in the human and rodent liver and that IL-22 is required for efficient liver regeneration. This IL-22 production is modulated by extracellular ATP via P2X1 receptors.

## Materials and Methods

### Serum samples from patients

Plasma samples of patients undergoing liver resection were collected at the department of surgery at the medical University of Vienna, Austria. Only major resections according to the IHPBA Brisbane 2000 nomenclature (< 3 segments = minor,  $\geq 3$  segments = major) (21) were included. Samples for cytokine analysis were collected from 40 patients preoperatively and 5 days postoperatively between February 2012 and June 2014 within a prospective observational trial (ClinicalTrials.gov Identifier: NCT01700231). Groups are illustrated in supplementary table 1. Intraoperative samples for ATP measurements were collected from 27 patients between January 2013 and January 2015 (ClinicalTrials.gov Identifier: NCT02113059). Groups are illustrated in supplementary table 2. The evaluation of blood samples and patient data was approved by the Institutional Ethics Committee of Vienna (#424/2010 and #2032/2013) all patients gave written informed consent.

### Definition and Classification of Postoperative Liver Dysfunction and Morbidity

The criteria issued by the International Study Group of Liver Surgery (ISGLS) were applied to evaluate liver dysfunction i.e. post-hepatectomy liver failure (22).

Accordingly, liver dysfunction was defined by an abnormal serum bilirubin (SB) level and prothrombin time (PT) on or after postoperative day (POD) 5 based on the threshold values of the local laboratory: >1.2 mg/dl serum bilirubin (SB) and <75% prothrombin time (PT). Patients with an abnormal preoperative SB or PT, a postoperative aggravation on or after POD 5 (compared to the previous day) were defined as postoperative liver dysfunction. In patients with normal SB or PT values prior to POD 5 and discharged early due to good clinical performance, no further

blood collection could be performed on or after POD 5. These patients were considered as “no post-hepatectomy liver failure”. To evaluate postoperative morbidity, the severity of postoperative complications was recorded in grade I to V.

### ● Mice and surgical procedures

Animals were housed in specific pathogen free (SPF) conditions in accordance with Swiss veterinary office. All animals used for the experiments were on C57Bl/6 background with an age of 8 to 12 weeks and were males. C57Bl/6 wild type (WT) animals were purchased from Harlan, Netherlands. *CD39<sup>-/-</sup>* mice were provided by Simon Robson (Beth Israel Deaconess Medical Center, Boston) and *IL-22<sup>-/-</sup>* mice were provided by Jean-Christophe Renault (Ludwig Institute for Cancer Research, Catholic University of Louvain, Brussels, Belgium). *Rag1<sup>-/-</sup>* animals housed in altered Schaedler's flora (ASF) were purchased from clean mouse facility, University of Bern. *Rag2<sup>-/-</sup>γc<sup>-/-</sup>* mice were purchased from Taconic (Germantown NY). For 70% partial hepatectomy, animals were anaesthetized using isoflurane followed by ligation and resection of median and left liver lobes as described previously (23). All protocols were approved by Bernese cantonal authorities.

### Flow cytometry

Lymphocytes isolated from different organs were analyzed using flow cytometer by staining with cells with monoclonal antibodies (mAbs) according to the manufacturers protocols. Following fluorescently labeled human mAbs were used: CD3 (HIT3a), CD56 (HCD56), NKP46 (P44-8), CD4 (OKT4), TCRγδ (B1) and mouse mAbs CD3e (17A2), NK1.1 (PK136), NKp46 (29A1.4), CD4 (GK1.5), CD19 (6D5), TCRγδ (GL3), CD127 (A7R34), CD117 (2B8), CD49b (DX5) (Biolegend) and CD39 (24MDS1) (eBioscience), CD49a (Ha31/8) (BD, Biosciences). For intracellular staining of IL-22,

freshly isolated lymphocytes were incubated for 5 hours with IL-23 (10ng/ml, Peprotech) and Brefeldin A (10 $\mu$ g/ml) (Sigma) in DMEM-Glutamax (life technologies), 10% fetal bovine serum, 1% penicillin/streptomycin, 0.7%  $\beta$ -mercaptoethanol (sigma) and 1% HEPES (GIBCO). After five hours cells were fixed by fixation/permeabilization buffer (eBioscience) and stained for intracellular IL-22 (mAbs 1H8PWSR), IL-22 (hAbs-22URT1) (eBioscience) and intranuclear ROR $\gamma$ t (mAbs B2D) (BDBiosciences) according to the manufacturers protocol. Finally cell data were acquired on a SORP LSRII (BD Pharmingen). Flow cytometric analysis was done using FlowJo (Treestar).

#### **Enzyme-linked immunosorbent assay/Cytokine bead array**

Serum collected over a time course following partial hepatectomy and supernatants from *ex vivo* stimulated lymphocytes with  $\alpha$ -CD3 ((10 $\mu$ g/ml) mouse (145-2C11), human (HIT3a) Biolegend),  $\alpha$ -CD28 ((5 $\mu$ g/ml) mouse (3751), human (CD28.2), Biolegend), hIL-23 (10ng/ml, Peprotech), IL-12 (10ng/ml, Peprotech) and IL-18 ((10ng/ml), human(B001-5), mouse(B002-5), MBL) were used for measuring IL-22 levels using ELISA kit Ready-Set-Go (eBioscience) according to the manufacturers protocol using a Tecan Infinite 200 plate reader (Tecan).

Patient's samples: The cytokines IL-1 beta EXP010-10224-901 IL-17A (EPX010-12017-901), IL-18 (EPX010-10267-901) IL-22 (EPX010-12047-901) and IL-23 (EPX010-12023-901) in the human plasma samples were analyzed by ProcartaPlex multiplex Immunoassay according to the manufacturer's protocols (EBioscience) using Luminex instrument (xMAP Technology). Levels of human TGF $\beta$  were measured by ELISA according to the manufacturer's protocol (88-8350-22, eBioscience).

Following purinergic agonists and antagonists were used: Adenosine (Sigma), 2-chloroadenosine (Adenosine receptor agonist), DPCPX and ZM241385 (Adenosine



receptor antagonist), NF449 (P2X1 antagonist), NF110 (P2X3 antagonist), 5BDBD (P2X4 antagonist), A804598 (P2X7 antagonist), MRS2500 or MRS2279 (P2Y1 antagonist), ARC-118925XX (P2Y2 antagonist), MRS2578 (P2Y6 antagonist), NF157 (P2Y11/P2X1 antagonist), Plavix (P2Y12 antagonist), MRS2211 (P2Y13 antagonist) were purchased from Tocris.

### **Measurement of liver injury**

Serum samples post partial hepatectomy were collected to assess the extent of liver injury using aspartate aminotransferase (AST) and alanine transaminase assay (Modular Analytics EVO, P800; Roche, Germany).

### **Measurement of ATP levels from patients**

ATP levels in plasma samples from patients were measured by luciferin- luciferase bioluminescence assay according to the manufacturers protocol (Bio-Thema, Sweden) using a Tecan Infinite 200 plate reader (Tecan).

### **Gene expression analysis**

Primers used were: ccnb1- (Mm03053893\_gH), p21 (Mm00495793\_m1), Foxm1 (Mm00514924\_m1), BIRC5 (Mm00599749\_m1), TBP (Mm01277042\_m1) and IL-22ra1 (Mm01192943\_m1) (Applied Biosystems) with ABI PRISM 7900 (Applied Biosystems). Primer for P2X1 receptor (For. 5' CCGAAGCCTTGCTGAGAA 3', Rev. 3' GGTTTGCAGTGCCGTACA T 5' (amplicon length 87bp)), 18S (5'GTAACCCGTTGAACCCATT 3', Rev. 3' CATCCAATCGGTAGTAGCG 5' (amplicon length 148bp)), IL-22 receptor expression in human liver tissue (For. 5' GATCTGCTCCAGCACGTGAA, Rev.3' GGCTTGAGGGTAGTGTGCT5' (amplicon

length 302bp)) and 18S (For.5' GTAACCCGTTGAACCCCATT3', Rev.3' CCATCCAATCGGTAGTAGCG 5' (Amplicon length 148bp))

### **Immunohistochemistry**

Hepatic proliferation was assessed by Ki67 (DAKO) and PCNA (DAKO) immunostaining as described previously (8). Endoplasmic stress was assessed by using BIP/GRP78 immunofluorescence staining as described before (24). In brief paraffin embedded liver tissues sections were washed followed by incubating with 3% H<sub>2</sub>O<sub>2</sub> (Dr. Grogg; K45052100 407) in phosphate buffered saline with tween 20 (PBS-T). After protein blocking (PBS, 3%BSA; 5%Donkey Serum; 0,1% Triton-X-100) the slides were incubated with primary rabbit Anti-GRP78/BIP (Abcam; ab21685, 1:500) antibody in the protein block over night at 4 °C, followed by incubation for 2h with donkey anti-Rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (Life Technologies; A-21206). Liver sections were stained with hematoxylin (MERK; HX43078349) for 1 min followed by Eosin (Fluka; 45240) staining for 6 min, later sections were mounted with Eukitt (Kindler, GmbH).

### **Western blot analysis**

Proteins from the liver tissues was extracted using RIPA buffer. Proteins extracted were quantified using BCA kit (ThermoFischer scientific). Protein samples boiled in Laemmli buffer were separated by SDS-PAGE (Bio-Rad). Separated proteins were transferred on to polyvinyl difluoride (PVDF) membrane followed by incubation with the primary antibodies, overnight at 4 °C. Primary antibodies diluted in either milk or BSA rabbit Anti-GRP78/BIP (Abcam; ab21685, 1:2000) rabbit anti-Phospho-eIF2 $\alpha$  (Ser51) (1:2000, Cell signaling. #9721) and rabbit anti-GAPDH (1:10,000, Acris, ACR001P). Membranes were then washed and incubated with goat-Anti-Rabbit IgG-

HRP secondary antibody (1:10,000, Santa Cruz Biotechnology: sc-2030) for 2 hours.

The immunoblots were then washed and developed using chemiluminescence

(Luminata crescendo, Millipore, WBLUR0100) and for semi quantitative analysis Aida

2d densitometry software version 3.11 was used and signal intensities were

normalized to GAPDH.

### **Statistical Analysis**

Statistical tests as indicated were performed with Prism software (Graph Pad

Software). Statistically significant data are presented as follows. \* $p < 0.05$ ; \*\*  $p <$

0.01; \*\*\*  $p < 0.001$ ; and \*\*\*\*  $p < 0.0001$ . Tests are specifically indicated.

## Results

**IL-22 is elevated in patients that underwent liver resection.** In order to identify cytokines that mediate responses by innate immune cells and potentially contribute to liver regeneration, plasma samples of patients undergoing major liver resection were analyzed. Patient's characteristics are illustrated in supplementary table 1. When we compared preoperative cytokine levels to the fifth postoperative day, we found that plasma levels of IL-22 were consistently increased, whereas no changes for IL-17a were observed (Fig. 1A, B). Cytokines that are upstream of IL-22 secretion such as IL-1 $\beta$  and IL-23 were significantly elevated after liver resection but IL-18 and TGF $\beta$  levels were not elevated (Fig. 1C-F) (1). In order to determine if the elevation of IL-22 is specific in the regenerating liver, we measured IL-22 levels in patients that underwent major pancreatic surgery. In these patients no significant difference in IL-22 levels was observed (Suppl. fig 1A). These findings in humans confirm studies performed in mice, in which levels of IL-22 are elevated in the regenerating liver following partial hepatectomy (5).

**Hepatocellular proliferation in response to partial hepatectomy is delayed in the absence of IL-22.** In order to test the functional relevance of IL-22 in the regenerating liver, partial hepatectomy was performed in *IL-22*<sup>-/-</sup> mice and compared to WT mice. Liver regeneration as assessed by Ki67 and PCNA staining was significantly delayed in *IL-22*<sup>-/-</sup> mice (Fig. 2A, B) and liver weights at postoperative day are decreased in *IL-22*<sup>-/-</sup> mice compared to WT mice (Suppl. fig. 2A). In parallel, IL-22 receptor is upregulated in the regenerating liver (Suppl. fig. 2B,C). At 48 hours post partial hepatectomy, positive cell cycle regulators (Cyclin b1; Foxm1) were down regulated, whereas a negative cell cycle regulator (p21) is increased in partially hepatectomized *IL-22*<sup>-/-</sup> mice compared with WT mice (Fig. 2C). Moreover,

expression of Survivin/Birc5 that is required for hepatocellular proliferation post partial hepatectomy (25) was decreased in *IL-22*<sup>-/-</sup> mice. Thus, liver regeneration is decreased in *IL-22*<sup>-/-</sup> mice compared to WT controls.

**Elevated liver injury and endoplasmic reticulum stress in *IL-22*<sup>-/-</sup> mice.** Liver injury as assessed by analysis of AST and ALT and by histology was significantly increased in *IL-22*<sup>-/-</sup> mice after partial hepatectomy compared to WT controls (Fig. 3 A, B, Suppl. fig. 2D). Recent studies showed an association of IL-22 administration with reduced cellular endoplasmic reticulum (ER) stress (26). Therefore, we assessed levels of ER stress in the regenerating liver by staining for BiP/Grp78 and its down stream protein phospho-eIF2 $\alpha$  (P-eIF2 $\alpha$ ) (Fig. 3C-E). Hepatocellular ER stress was slightly elevated at baseline and significantly increased after partial hepatectomy in *IL-22*<sup>-/-</sup> mice compared with WT mice.

**IL-22 in the regenerating liver is secreted by innate lymphoid cell subsets.** IL-22 is primarily secreted by lymphatic cells (10). To identify the cellular source of IL-22 in the liver under homeostatic conditions, hepatic lymphocytes were isolated from patients and stimulated *ex vivo* with IL-23. We found that CD56<sup>dim</sup>CD3<sup>-</sup> NK cells were the main producers of intracellular (Fig. 4A) and secreted (Fig. 4B) IL-22 in the human liver. In the mouse, hepatic IL-22 producing cells also include NKT and  $\gamma\delta$  T cells in addition to ILC1, cNK (Suppl. fig. 3A, B). In order to further determine the source of IL-22 during liver regeneration, two third partial hepatectomy was performed in WT, *Rag1*<sup>-/-</sup> mice (lacking T and B cells) and *Rag2*<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> mice (lacking T, B, cNK cells and ILC) (27). Compared to WT mice, IL-22 secretion 48 hours after partial hepatectomy was increased in *Rag1*<sup>-/-</sup> mice but was absent in *Rag2*<sup>-/-</sup> $\gamma$ c<sup>-/-</sup> (Fig. 4C, Suppl. fig.4A). Thus, cNK cells and ILC1 are the main producers of IL-22 in WT

and *Rag1*<sup>-/-</sup> mice. The fraction of ILCs in *Rag1*<sup>-/-</sup> mice is higher percentage compared to WT mice that may explain the increase of IL-22 levels (Suppl. fig. 4B). Such decreased systemic levels of IL-22 in *Rag2*<sup>-/-</sup>*γc*<sup>-/-</sup> mice after partial hepatectomy correlated with elevated liver injury (Fig. 4D, Suppl. fig. 4C, D). Furthermore, liver regeneration is also delayed in these mice as shown previously (8). ILCs have been recently classified into the three populations ILC1, ILC2 and ILC3 according to activating and effector cytokines as well as associated transcription factors (10). However, subpopulations of ILC within the liver remain largely unexplored. In the rodent liver we observed mainly CD49a<sup>+</sup> and CD49b<sup>+</sup> subsets of NK1.1<sup>+</sup>CD3<sup>-</sup> cells which represent classical ILC1 (CD49a<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup>) and cNK (CD49b<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup>) cells that represent a subgroup of ILCs. In addition we show only small fractions of ILC2 and RORγt positive ILC3 in the liver (Suppl. fig. 5A). CD49a<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup> cells are resident in the hepatic sinusoids and bone marrow and correspond to ILC1 (12). These cells, however, are found only at very low levels in other lymphoid organs. Conversely CD49b<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup> cells correspond to circulating and primarily cytotoxic cNK cells (11, 12). In the regenerating liver we observed a strong shift of ILC fractions with increased levels of CD49a<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup> (ILC1) cells and reduced CD49b<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup> (cNK) cells after partial hepatectomy (Fig. 4E). Together, these data suggest that IL-22 is secreted by ILC1 and cNK cells after partial hepatectomy.

**Extracellular nucleotides modulate IL-22 secretion via P2 receptors.** We next investigated soluble factors released after partial liver hepatectomy, which may promote IL-22 secretion by focusing on extracellular nucleotides. Experiments in rodents revealed that the release of extracellular nucleotides is an early event following partial hepatectomy and impacts on various immune functions via the activation or inhibition of P2-type receptors (8, 13, 14, 20). To validate these findings

in humans, samples were obtained from patients undergoing major liver surgery.

Levels of extracellular nucleotides were measured in different venous compartments prior to and after liver resection. Patient's characteristics are shown in supplementary table 2. Extracellular ATP levels significantly increased immediately after liver resection in the portal and hepatic vein compared to the vena cava (Fig. 5A). To determine an association of extracellular ATP levels and clinical outcome, patients with post hepatectomy liver failure as assessed by the ISLGS (international Study Group of Liver Surgery) score were compared to patients without postoperative liver failure. Interestingly, clinical outcome significantly correlates with intraoperative ATP levels in the serum (Fig. 5B). Levels of extracellular ATP in the hepatic vein of patients with post hepatectomy liver failure were significantly lower compared to patients without post hepatectomy liver failure. These results show that the release of extracellular ATP occurs immediately in response to major liver resection and potentially is associated with patient's outcome.

Next we aimed to determine whether altered P2 receptor activation effects IL-22 secretion. Analysis of gene expression data in GEO database (GEO accession number GSE43339) revealed that various P2 receptors are expressed in mouse cNK and ILC1 (Suppl. fig 6A). In order to test the functional relevance of P2 receptors on IL-22 secretion we induced IL-22 secretion by primary hepatic and splenic lymphocytes using cytokines (anti-CD3, anti-CD28, IL-12, IL-18 and IL-23) with and without suramin, a non-selective P2 receptor antagonist. Secretion of IL-22 was significantly decreased in response to suramin in hepatic and splenic lymphocytes (Fig. 6A, Suppl. fig. 6B). No impact on adenosine signaling was observed (Fig. 6B, Suppl. fig. 7A-C). In order to identify the specific P2 receptor controlling IL-22 secretion, a screen was performed using various P2X and P2Y receptor antagonists

in naive WT splenic lymphocytes stimulated *ex vivo*. We identified significant reduction of IL-22 secretion in response to highly selective antagonists of the receptors P2X1 and P2Y6 and to a lesser extent to antagonists of P2X4, P2Y1 and P2Y13 in a dose dependent manner (Fig. 6C, D). In order to test whether altered activation or desensitization of P2-type receptors modulates IL-22 secretion, we assessed IL-22 secretion in mice deficient of NTPDase1/CD39, the major ectonucleotidase that hydrolyses ATP to AMP, which is expressed in various subsets of hepatic lymphocytes including ILC (Suppl. fig.8A). Secretion of IL-22 *ex vivo* was significantly decreased in hepatic lymphocytes and splenocytes isolated from *CD39*<sup>-/-</sup> mice compared to WT mice (Fig. 6E, Suppl. fig. 8B-E). *In vivo*, IL-22 levels in *CD39*<sup>-/-</sup> mice were significantly decreased and liver injury was increased as assessed by AST and ALT levels and by Hematoxilin and Eosin staining post partial hepatectomy compared to WT controls (Fig. 6F, Suppl. fig. 9A, B). Thus, these results show that disordered purinergic signaling modulates IL-22 secretion *in vitro* and *in vivo*.

### **Liver regeneration post partial hepatectomy is impaired in response to P2X1**

**inhibition.** To identify whether P2 receptor signaling modulates IL-22 secretion *in vivo* and if this receptor is relevant in the regenerating liver, partial hepatectomy was performed in WT mice with and without P2X1 and P2Y6 receptor antagonists. *In vivo*, IL-22 levels were significantly decreased post partial hepatectomy in response to P2X1 receptor inhibition using a highly specific antagonist (NF449) (Fig. 7A) but not in response to a specific P2Y6 antagonist (MRS2578) (Fig. 7B). P2X1 receptor is expressed in hepatic NK, ILC1, NKT, CD4, CD8 and gamma delta T cells that are cellular fractions that typically secrete IL-22 (Suppl. fig. 10A). Liver proliferation as assessed by Ki67 and PCNA staining was not impaired in response to P2Y6 inhibition (Fig. 7C), but significantly decreased in response to P2X1 antagonism (Fig.



7D, E). Liver injury is increased in response to P2X1 antagonism but not with P2Y6 antagonism (Fig. 7F, Suppl. fig. 9B, 10B, C). Together, these data reveal that inhibition of P2X1 decreases IL-22 secretion and is associated with impaired liver regeneration post partial hepatectomy.

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## Discussion

In this study we identified that the secretion of IL-22 by hepatic ILC subsets is required for efficient liver regeneration and is modulated by extracellular ATP mainly via the receptor P2X1. The hepatoprotective properties of IL-22 have been well established in models of liver injury (4, 6, 28, 29). Moreover, artificially elevated IL-22 in transgenic mice or via exogenous administration enhances hepatocellular proliferation (2, 29-31). Our current study now reveals that constitutive IL-22 secretion is required for optimal liver regeneration and its secretion is mediated via hepatic ILC subsets, mainly cNK cells and ILC1. The absence of these cell types in *Rag2<sup>-/-</sup>γc<sup>-/-</sup>* mice is associated with abrogated IL-22 production that explains at least in part impaired liver regeneration and elevated liver injury after partial hepatectomy in these mice (8). In other models such as drug induced liver injury, cells of the adaptive immune system (mainly Th22 cells) are important sources of IL-22 (31). Thus, the source of IL-22 depends on the context of injury.

As a potential mechanism of IL-22 secretion on liver regeneration we observed increased ER stress in the regenerating liver of *IL-22<sup>-/-</sup>* mice. It is interesting to note that *IL-22<sup>-/-</sup>* livers have elevated ER stress responses already at basal level as observed by BiP staining and phospho-eIF2α Western blot. Thus, IL-22 seems to be required to achieve homeostatic balance. In a recent study, using hepatocyte-specific deletion of IRE1α (one branch of the ER stress response pathway), impaired liver regeneration after partial hepatectomy was described (32). Therefore, some ER stress after partial hepatectomy is required as an adaptive response. However, ER stress seems to be counterproductive at high, potentially excessive levels as observed in the *IL-22<sup>-/-</sup>* mice.

We next investigated the impact of extracellular nucleotides on IL-22 secretion in the regenerating liver. By showing elevated ATP levels after major liver resection we support previous findings in which elevation of extracellular ATP levels has been observed after living liver donation (13). Further we provide clinical evidence for data obtained in mice and rats that revealed an early increase of extracellular ATP levels after partial hepatectomy (8, 13). However, we found that extracellular ATP levels were not only increased in the hepatic venous blood but also in the portal blood. In a previous study in which extracellular ATP was found to be only elevated downstream of the liver, we measured ATP levels at the end of the operation and not immediately after portal clamping. Thus, extracellular ATP may be not only derived from the liver but potentially also from the systemic circulation in patients undergoing major surgery. Extracellular nucleotides exhibit pleiotropic effects on parenchymal and non-parenchymal cell types such as resident or circulating immune cells via P2-type receptors (17). We found that the ATP receptor P2X1 and the UDP receptor P2Y6 modulate IL-22 secretion *in vitro*. However, only antagonism of P2X1 *in vivo* showed an impact on liver regeneration. Administration of P2Y6 antagonists was associated with elevated mortality after partial hepatectomy (not shown), potentially because of known effects on glucose metabolism (33). The results of this study suggest that ATP modulates the activation of lymphocytes, in particular cNK and ILC1 in the regenerating liver via P2X1 receptor. P2X1 is mainly expressed on circulating immune and inflammatory cells but not on parenchymal cells (34, 35). This pattern of expression parallels sources of IL-22. Thus, modulation of P2X1 has mainly indirect effects via the activation of circulating but not parenchymal cells. This finding is supported by recent studies that have shown the relevance of P2X1 in other models of inflammation and injury such as neutrophil migration during endotoxemia (36, 37). Desensitization is an inherent property of P2X1 receptor (38) and explains decreased

secretion of IL-22 *ex vivo* and *in vivo* in the absence of CD39 on liver lymphocytes (39). Thus, regulated activation of P2X1 is required for optimal secretion of IL-22.

Future studies need to establish whether pharmacological strategies that alter IL-22 levels in the postoperative period may improve the clinical outcome in patients undergoing major liver resection or full or partial liver transplantation. In light of the present study, targeting of IL-22 levels seems to be possible in several ways, first by direct administration of recombinant proteins, second via the modulation of P2 receptor signaling by direct activation of P2X1 or third, indirect modulation via ectonucleotidases such as CD39.

This study reveals the interaction of “danger signals” such as extracellular ATP, hepatoprotective cytokines and tissue regeneration. ATP-dependent modulation of the unidirectional crosstalk between liver infiltrating lymphocytes and hepatocytes via IL-22 may represent an evolutionarily ancient pathway initiating repair and regeneration.

## Legends

### **Figure 1. Increased levels of IL-22 in patients after major liver resection.**

Analysis of cytokine levels in plasma samples from patients (n=40) five days after major liver resection. (A, B) IL-22 but not IL-17a is elevated after resection. (C-F)

Upstream cytokines IL-23 and IL-1beta that stimulate IL-22 secretion are significantly elevated post liver resection, while IL-18 and TGFβ are not elevated. Each data point represents an individual patient, the line represents the mean (t-test).

### **Figure 2. IL-22 is required for optimal hepatocellular proliferation post liver resection.**

Liver regeneration was assessed in a mouse model of partial hepatectomy using *IL-22*<sup>-/-</sup> and WT mice (n=5 per time point). (A, B) Hepatocellular proliferation was assessed by using Ki67 and PCNA staining (counts of Ki67 or PCNA positive cells per high-power field (HPF) with a magnification of 40x). Each data point represents a single mouse, the line represents the mean. Percentages of Ki67 and PCNA positive cells were lower in *IL-22*<sup>-/-</sup> mice at early time points post partial hepatectomy compared to WT. (C) Positive cell cycle regulators (Cyclin 1; Foxm1) were down regulated whereas negative cell cycle regulators (p21) were increased in *IL-22*<sup>-/-</sup> mice compared to WT mice. Survivin (Birc5) an anti-apoptotic gene is down regulated in *IL-22*<sup>-/-</sup> mice (t-test).

### **Figure 3. Elevated liver injury and endoplasmic reticulum (ER) stress in *IL-22*<sup>-/-</sup> livers post partial hepatectomy.**

(A, B) Hepatocellular injury was assessed by aspartate aminotransferase (AST) and alanine transaminase (ALT) levels post partial hepatectomy in *IL-22*<sup>-/-</sup> mice and compared to WT mice. Each data point represents a single mouse, the line represents the mean (t-test). (C) Hepatocellular ER stress as assessed by immunofluorescent staining for BiP in *IL-22*<sup>-/-</sup> and WT mice before and

48h post partial hepatectomy. (D) Quantification of immunofluorescence revealed elevated expression of BIP/GRP78 *IL-22*<sup>-/-</sup> mice post liver resection at 48h. (E) Analysis of Phospho-eIF2 $\alpha$  (P-eIF2 $\alpha$ ) as a downstream protein by western blotting. Expression of Phospho-eIF2 $\alpha$  is higher at base line (0h) and increased at 6h and 24h post liver resection in *IL-22*<sup>-/-</sup> mice compared to WT mice.

**Figure 4. IL-22 is secreted by subsets of innate lymphoid cells in humans and mice.** IL-22 secretion was assessed by FACS (A) and ELISA (B) in different cellular subsets from human hepatic lymphocytes (n=5, t-test). Main producers of IL-22 are CD56<sup>dim</sup>CD3<sup>-</sup> NK cells. (C, D) Levels of IL-22 and liver injury was assessed 48h after partial hepatectomy in WT, *Rag1*<sup>-/-</sup> (lacking T, and B cells) and *Rag2*<sup>-/-</sup> *$\gamma$ c*<sup>-/-</sup> (lacking T, B, cNK and innate lymphoid cells) mice (n=5-6, t-test). (D) Lack of cNK cells and ILC is associated with significantly increased liver injury (n=5, each data point represents a single mouse, the line indicates the mean, t-test). (E) Analysis of rodent liver resident CD49a<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup> (ILC1) and CD49b<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup> (cNK) cell fractions pre and post partial hepatectomy (n=3, t-test).

**Figure 5. Elevated levels of extracellular ATP in patients post liver resection.**

(A) Extracellular ATP was assessed using luciferin luciferase assay in samples of 27 patients that underwent major liver resection in different venous compartments. Each data point represents a single patient. ATP levels were significantly elevated post liver resection compared to pre-operative levels. (B) Samples were dichotomized based on the presence of post hepatectomy liver failure according to the international Study Group of Liver Surgery (ISGLS) score. Levels of extracellular ATP were significantly lower in the hepatic vein in patients with post hepatectomy liver failure

compared to patients without liver failure (each data point represents a single patient; line represents the mean, t-test).

**Figure 6. Dysregulated purinergic signaling results in abrogated IL-22 secretion**

(A) Hepatic infiltrating lymphocytes and splenocytes from WT mice were stimulated using anti-CD3, anti-CD28, IL-12, IL-18 and IL-23. Incubation with the unspecific P2 receptor antagonist suramin is associated with significantly decreased IL-22 secretion (n=3, t-test). Screening was performed using various P1 and P2 receptor agonists and antagonists. (B) Activation of adenosine receptors in splenocytes is not associated with altered IL-22 secretion. (C) Antagonism of P2X1 (NF449) or P2Y6 (MRS2578) using specific inhibitors was associated with a significant and (D) dose dependent reduction of IL-22 secretion in human hepatic lymphocytes. (E) Disordered purinergic signaling in response to deletion of NTPDase1/CD39 (major ectonucleotidase) is associated with significantly reduced IL-22 secretion in isolated liver lymphocytes *in vitro* and (F) *in vivo* post partial hepatectomy in *CD39*<sup>-/-</sup> mice compared to WT mice. Liver injury in *CD39*<sup>-/-</sup> mice is significantly increased compared to WT mice.

**Figure 7. Inhibition of P2X1 is associated with decreased secretion of IL-22 and impaired liver regeneration *in vivo*.** WT mice were injected with PBS or with P2X1

(NF449) or P2Y6 (MRS2578) antagonists 1 hour prior liver resection. (A, B) Inhibition of P2X1 via NF449 is associated with significant reduction of IL-22 levels 24 hours post liver resection whereas P2Y6 inhibition is only associated with IL-22 reduction at very high levels. (C-E) Liver regeneration as assessed by Ki67 and PCNA staining is not altered in response to P2Y6 (MRS2578) inhibition whereas it is significantly decreased in response to P2X1 (NF449) inhibition. (F) Significant elevation of liver

injury in response to P2X1 (NF449) antagonism but not in response to P2Y6 (MRS2578 (50 $\mu$ g)) antagonism *in vivo* (n=5, mean, standard deviation, t-test).

Accepted Article



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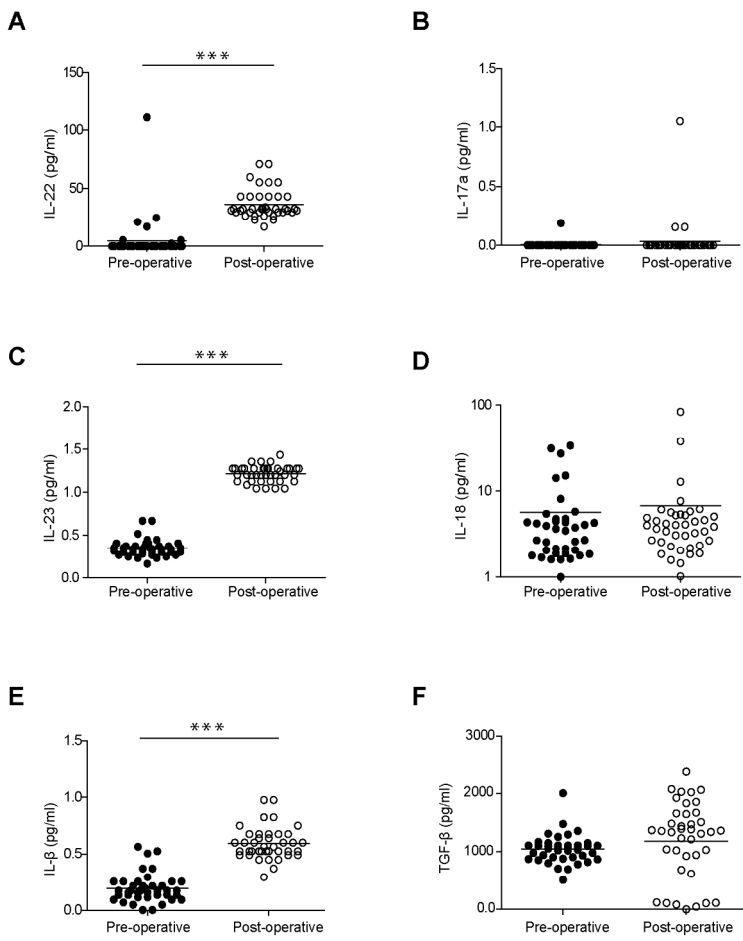
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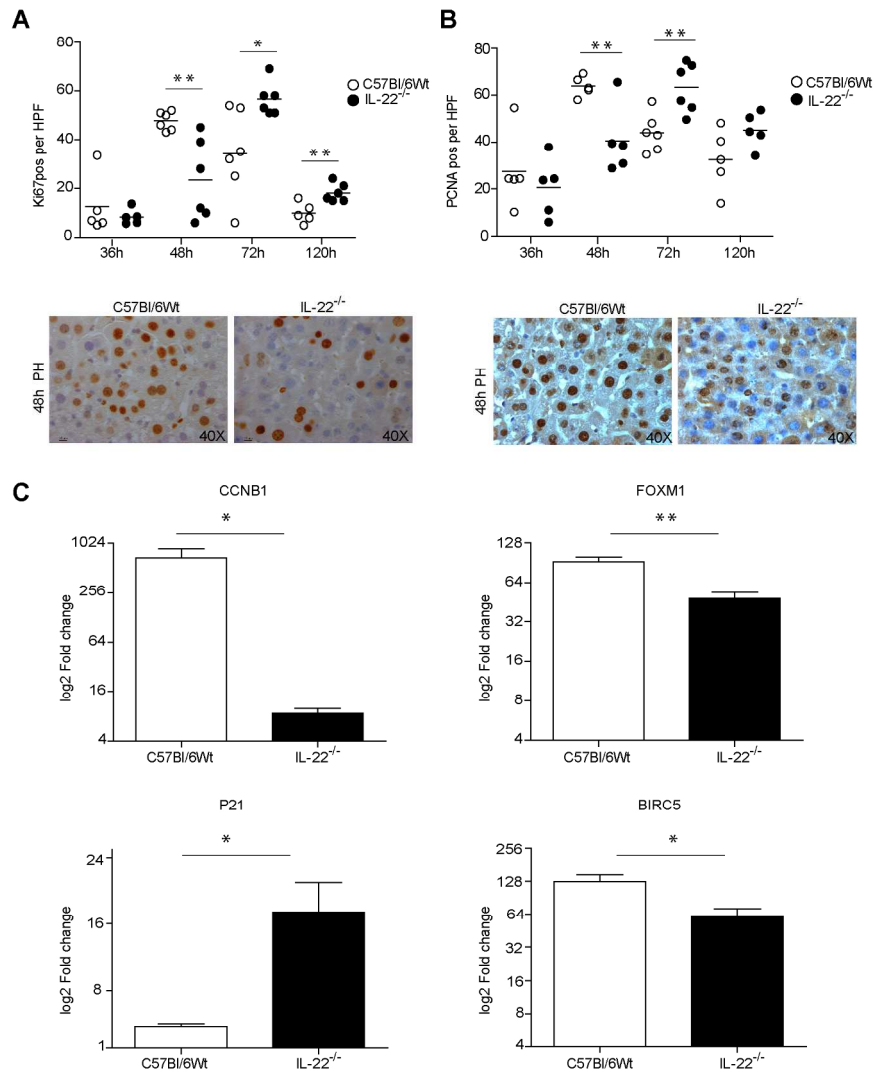
Figure 1



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Figure 2



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Figure 3

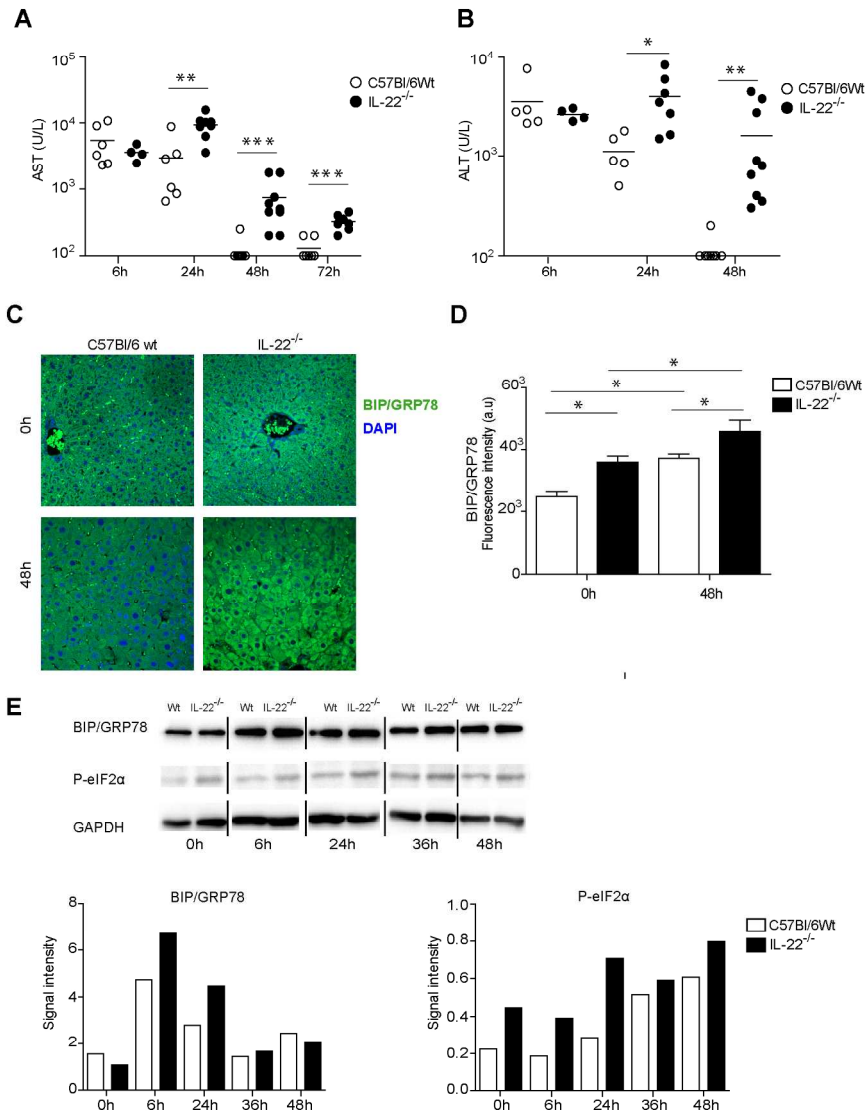
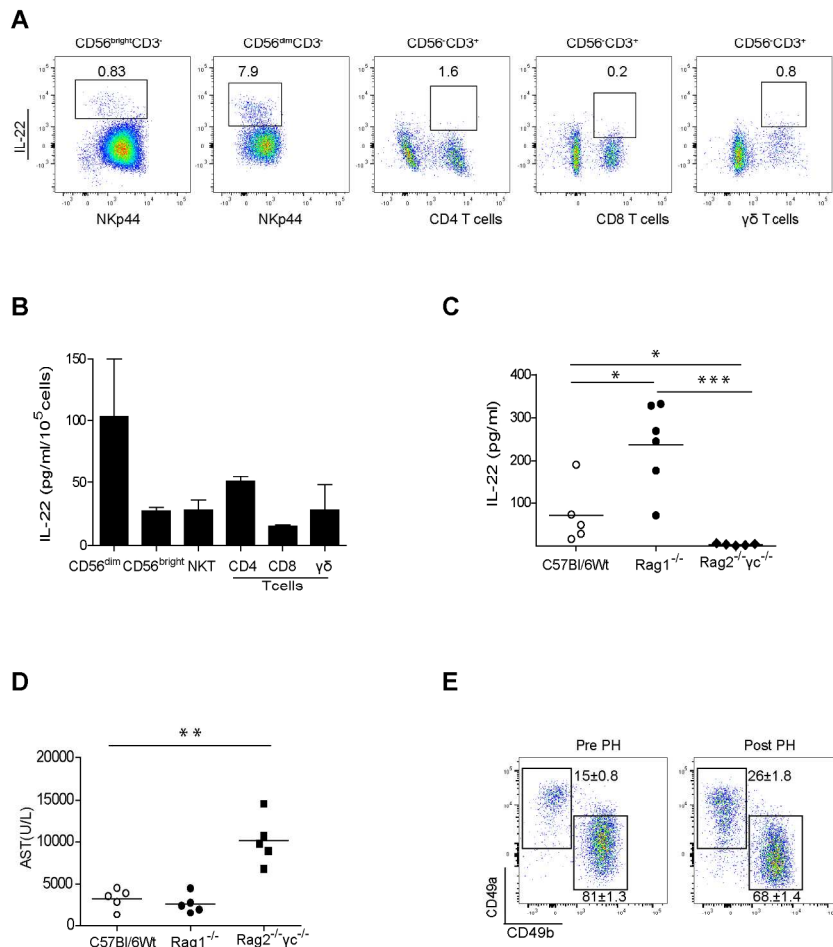




Figure 4

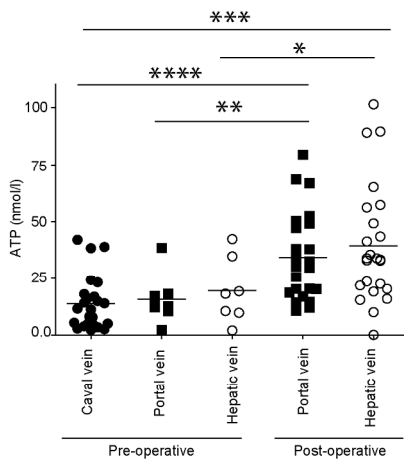


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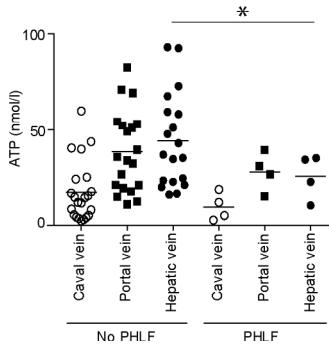
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Figure 5

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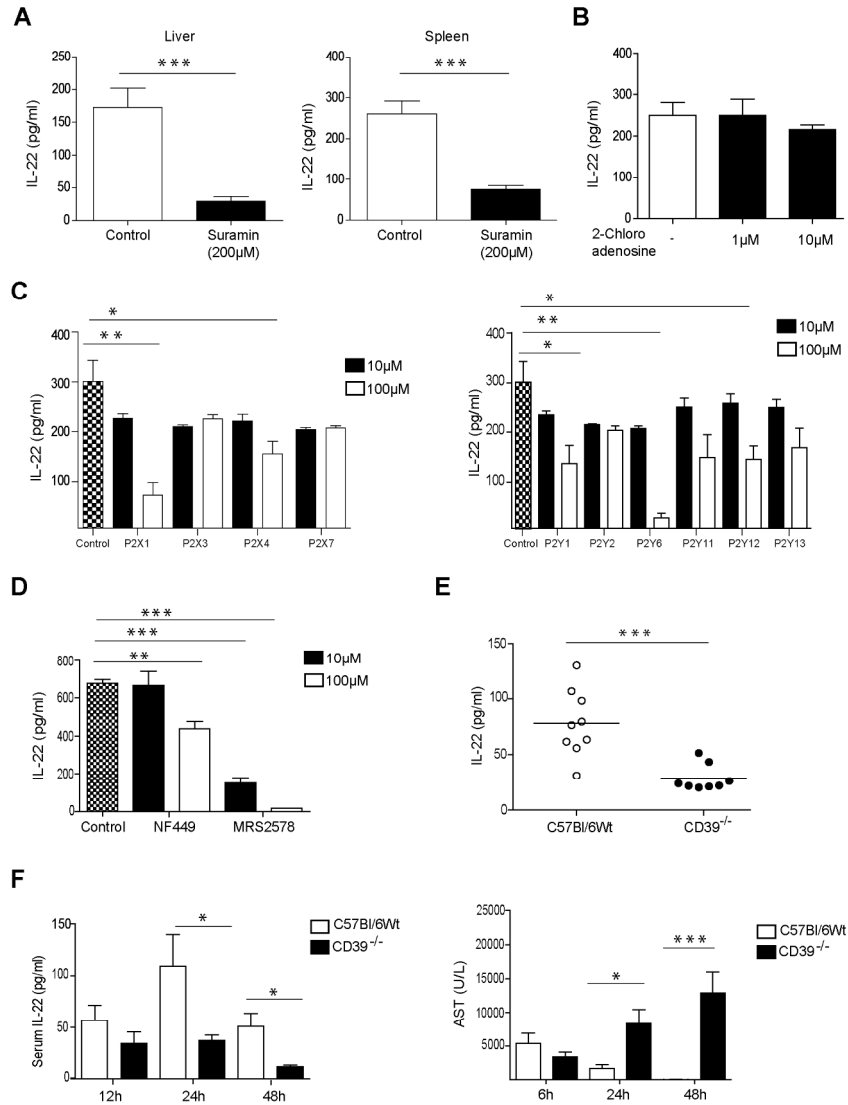
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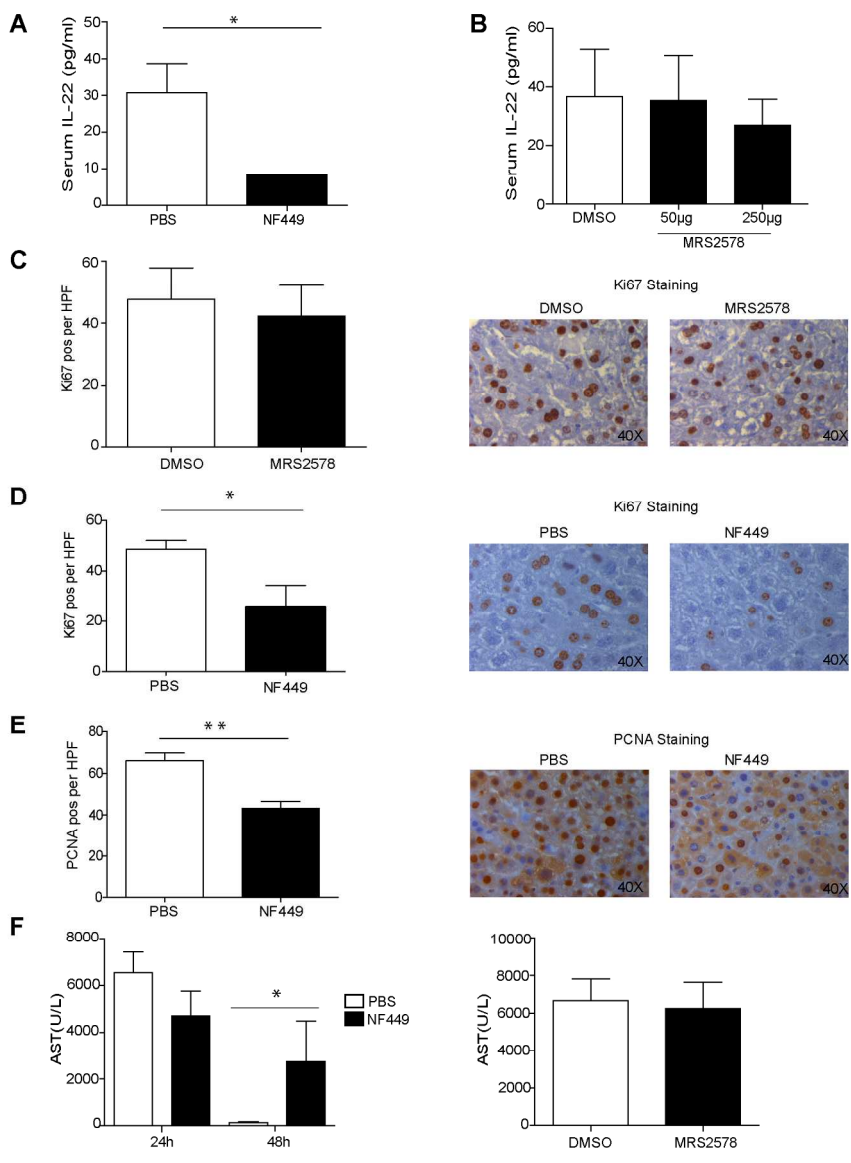
Figure 6



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Figure 7



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### Supplementary figure 1

(A) Levels of IL-22 patients (n=18) before and after major pancreatic surgery. No elevation of IL-22 levels was observed. Each data point represents an individual patient, the line represents the mean.

### Supplementary figure 2

(A) Body and liver weights from WT and *IL-22<sup>-/-</sup>* mice were measured at 0h, 48h, and 120h post liver resection. Liver to body weight ratio was significantly decreased in *IL-22<sup>-/-</sup>* compared to WT mice at 48h post liver resection. (B) Expression of IL-22 receptor in WT mice was assessed at different time points post liver resection by qPCR and compared to naïve mice (n=5 per time point) (mean±SD, t-test). (C) Expression of IL-22 receptor in human resected liver tissue was assessed by RT-PCR. (D) Hematoxylin and Eosin (H&E) staining in liver tissue section in WT and *IL-22<sup>-/-</sup>* mice 48h post liver resection (n=5 mice). Increased hepatocellular injury and focal liver necrosis occurred in *IL-22<sup>-/-</sup>* mice.

### Supplementary figure 3

Lymphocyte subsets from the liver (B) and spleen (C) of WT mice were FACS sorted and stimulated for 24 hours with  $\alpha$ -CD3,  $\alpha$ -CD28, IL-23, IL-12 and IL-18 *ex vivo*, supernatants were analyzed for IL-22 concentrations using ELISA. This experiment was done at least 3 times with three replicates/each time (mean±SD).

### Supplementary figure 4

(A) Serum levels of IL-22 were assessed in samples taken 24 hours post partial hepatectomy from WT, *Rag1<sup>-/-</sup>* and *Rag2<sup>-/-</sup>γc<sup>-/-</sup>* mice using ELISA (n=5, mean±SD, t-test). (B) ILC fractions of hepatic lymphocytes of untreated WT and *Rag1<sup>-/-</sup>* mice (n=5

mice, mean $\pm$ SD). In *Rag1*<sup>-/-</sup> mice, fractions of ILC1 (CD49<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup>), and cNK (CD49b<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>-</sup>) are increased when compared to WT mice. (C) Liver injury 24 hours post liver resection in WT mice, *Rag1*<sup>-/-</sup> and *Rag2*<sup>-/-</sup>*γc*<sup>-/-</sup> mice as measured by ALT (at least two individual experiments (5 animals/experiment, mean $\pm$ SD, t-test)). (D) H and E staining was done in liver tissues obtained 24h post hepatectomy from WT mice, *Rag1*<sup>-/-</sup> and *Rag2*<sup>-/-</sup>*γc*<sup>-/-</sup> mice (representative of 5 mice per group).

### Supplementary figure 5

(A) Hepatic liver lymphocytes were isolated and analyzed for various subsets of ILC (ILC1, ILC2, ILC3 and cNK cells) by FACS. The predominant populations in the mouse liver are ILC1 and cNK whereas ILC2 and ILC3 represent only minor fractions (representative of at least 3 experiments).

### Supplementary figure 6

(A) Expression analysis retrieved from GEO database (GEO accession number GSE43339) of purinergic P2X and P2Y receptors in isolated hepatic lymphocytes. (B) Liver lymphocytes and splenocytes from WT and *IL-22*<sup>-/-</sup> mice were isolated stimulated with  $\alpha$ -CD3,  $\alpha$ -CD28, IL-23, IL-12 and IL-18 *ex vivo* and supernatants were further analyzed using ELISA. Each figure represents at least three individual experiments (mean $\pm$ SEM, t-test).

### Supplementary figure 7

Splenocytes from WT mice were isolated and stimulated with  $\alpha$ -CD3,  $\alpha$ -CD28, IL-23, IL-12 and IL-18 *ex vivo* with (A) broad adenosine agonists and specific inhibition of adenosine receptor (B) A1 by DPCPX and (C) A2A by ZM241385 for 24 hours.

Supernatants were further analyzed using ELISA. Each figure represents at least three individual experiments (mean±SEM, t-test).

### Supplementary figure 8

Expression of CD39 on lymphocytes: (A) Hepatic infiltrating lymphocytes from WT mice and *CD39*<sup>-/-</sup> mice were isolated and analyzed by FACS. Expression of CD39 on NK, T cell subsets (CD4+ T Cells, CD8+T cells and Gamma Delta T cells) and B cells. All the data in the figure represent at least 3 individual experiments.

Impact of CD39 on IL-22 secretion: (B) Hepatic infiltrating lymphocytes from WT mice were isolated and stimulated with IL-23 (10ng/ml) and with Brefeldin (10µg/ml) *ex vivo* for 5 hours and were further analyzed by FACS and reveal the expression of

CD39 on IL-22 positive cells in the stimulated samples. Representative values in

FACS plot are an average from 3 individual experiments (t-test). (C) Splenic lymphocytes from WT and CD39 null mice were isolated and stimulated with IL-23, IL-12 and IL-18 *ex vivo* and supernatants were further analyzed using ELISA.

Impact of CD39 on IL-22 secretion of various subsets of lymphocytes: (D, E) Liver lymphocytes from WT mice and CD39 null mice were isolated and stimulated with IL-23 (10ng/ml) and with Brefeldin (10µg/ml) in *ex vivo* for 5 hours and were further analyzed by FACS. Representative FACS plots show the decreased expression of IL-22 overall and also in individual cells subsets (cNK cells) in CD39 null mice compared to WT mice (3 individual experiments, t-test).

### Supplementary figure 9

(A) Liver injury after different time points (6h, 24h and 48h) post liver resection in WT mice and CD39 null mice as assessed by ALT. Liver injury is increased in CD39 null mice at 24h and 48h post liver resection (n=5, mean±SEM, t-test). (B) H and E staining of liver tissues obtained from WT mice, *CD39*<sup>-/-</sup> mice and WT mice injected

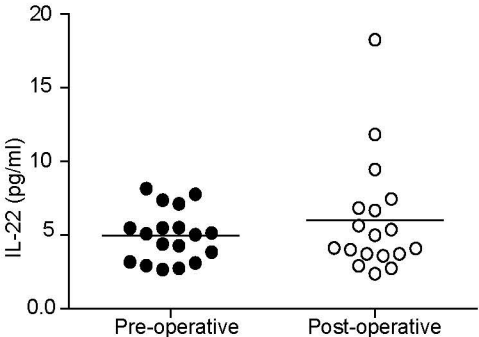
either with P2X1 antagonist (NF449) or with P2Y6 antagonist (MRS2578) 48h post hepatectomy (n=5 mice).

### Supplementary figure 10

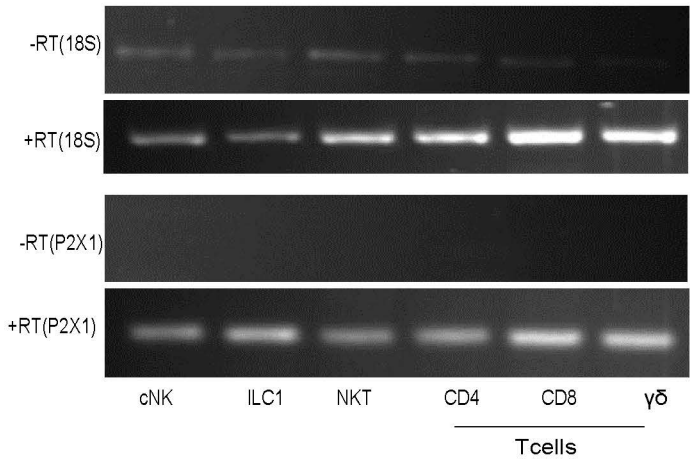
(A) Analysis of expression of P2X1 receptor of FACS sorted liver lymphocytes by RT-PCR. P2X1 is expressed in NK, ILC1, NKT, CD4, CD8 and gamma delta T cells. This experiment was repeated at least three times and genomic DNA contamination was ruled out by running negative reverse transcription. (B) WT mice were injected with saline or with NF449 a P2X1 antagonist one hour prior liver resection. In mice injected with NF449, liver injury as assessed by ALT is significantly increased 48 hours post partial liver resection (n=5, mean± SEM, t-test). (C) WT mice were injected with DMSO or with MRS2578 a P2Y6 antagonist one hour prior liver resection. No difference in liver injury was observed (n=5, mean± SEM, t-test).



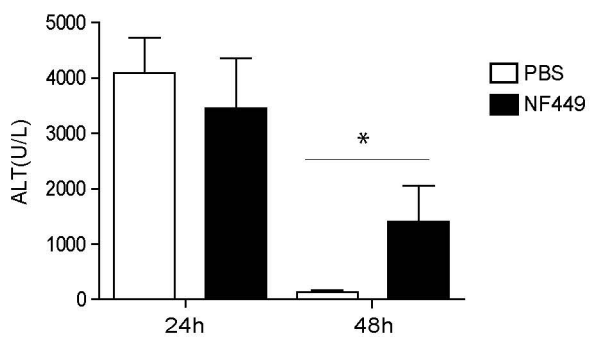
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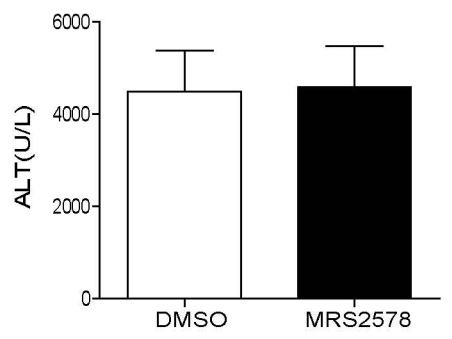
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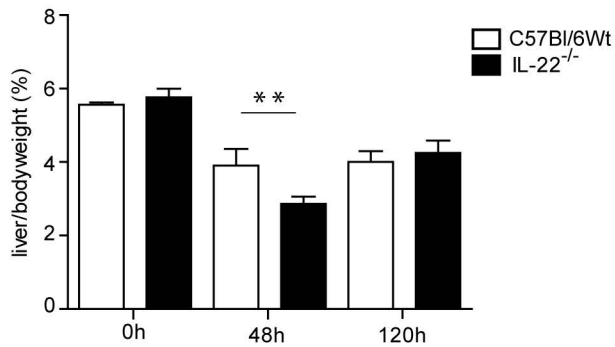
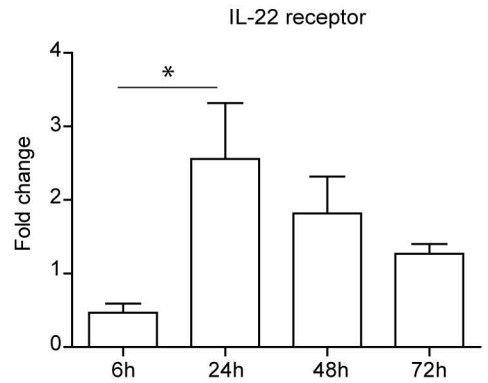


**B**

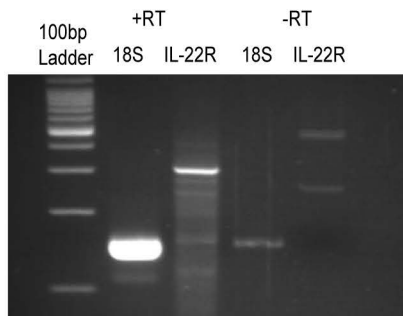
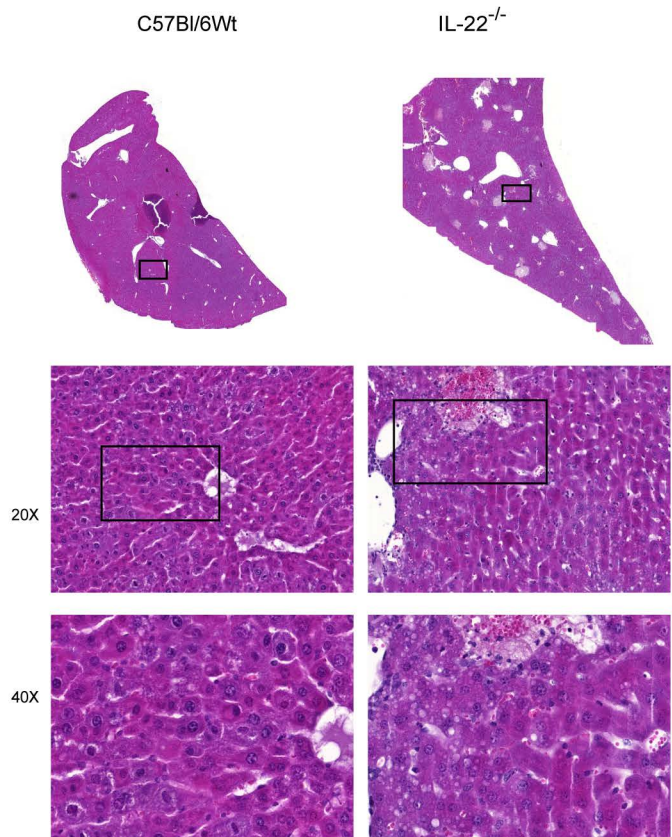


**C**



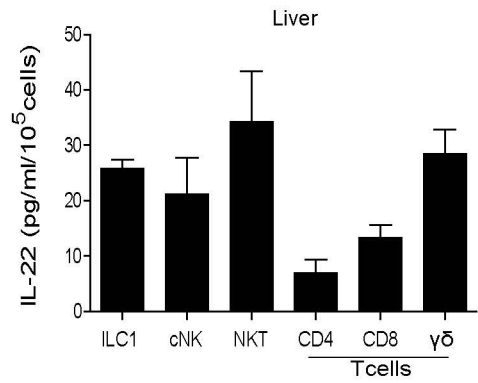
**A****B****C**

IL-22R expression in human resected liver

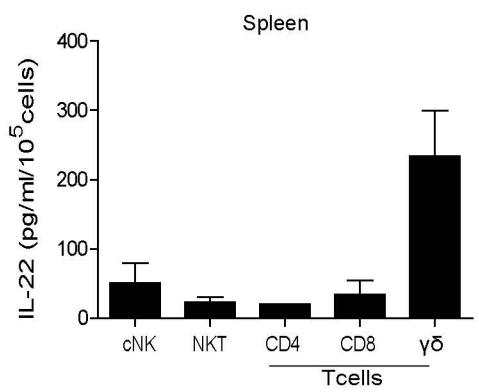
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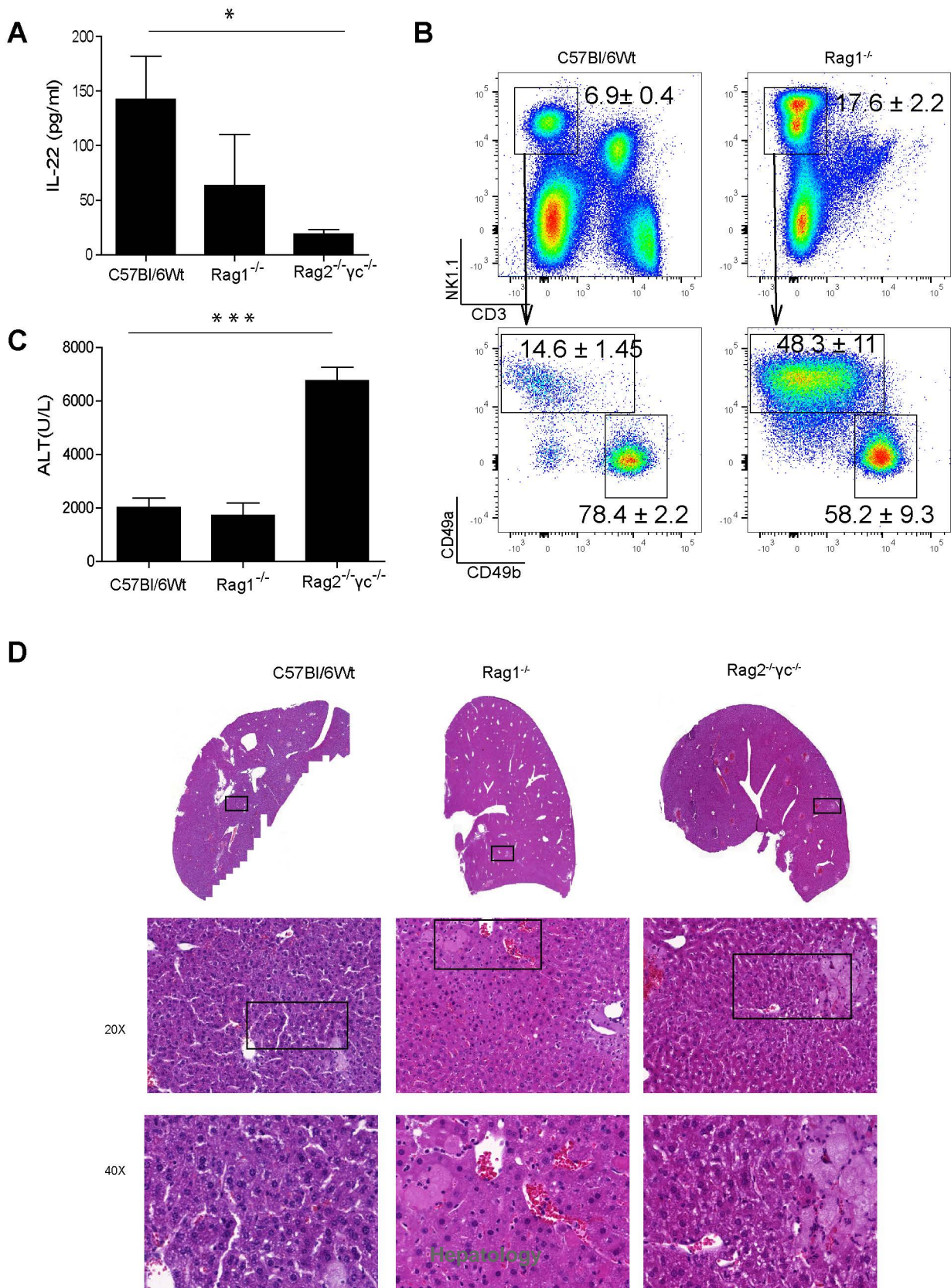
Hepatology

**A**

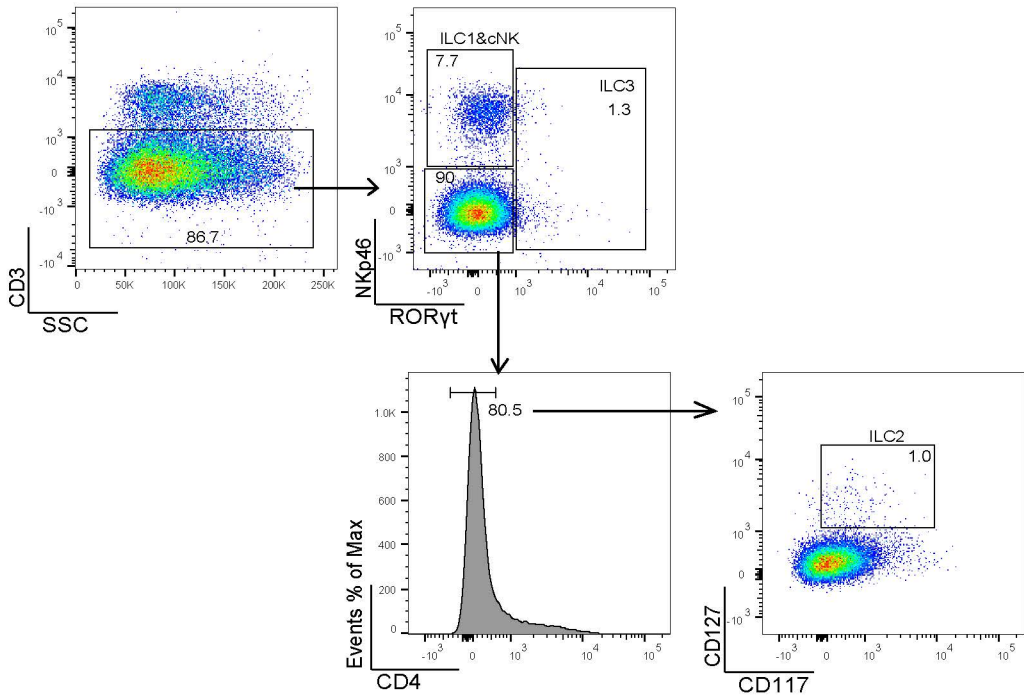


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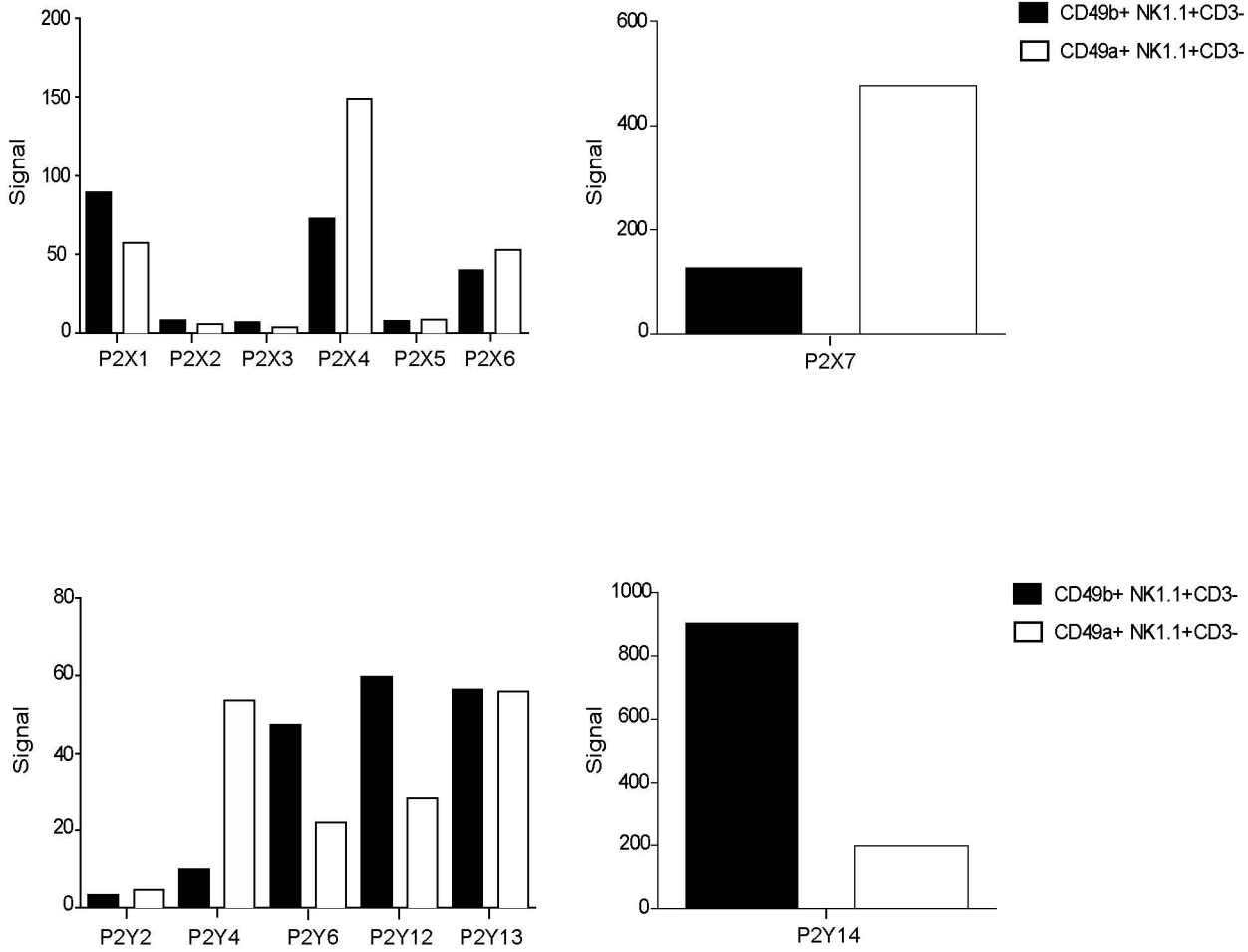




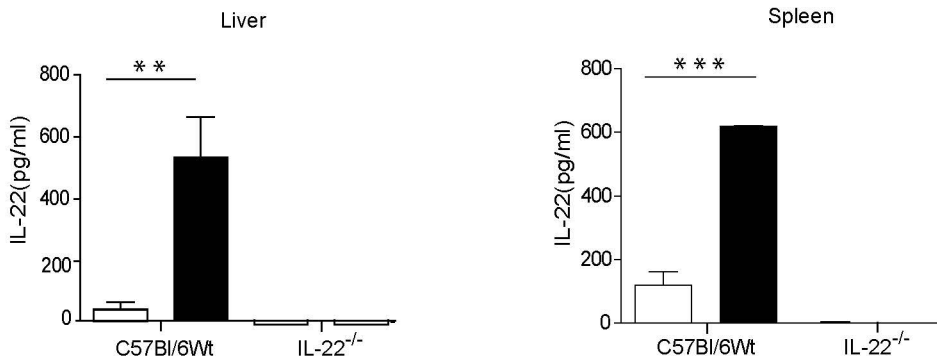
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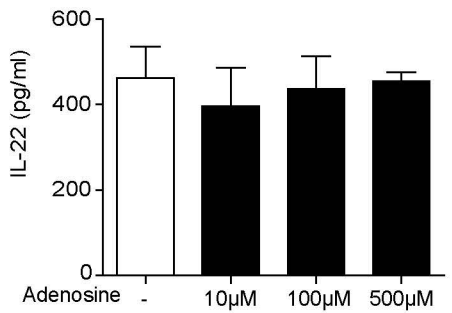
**A**



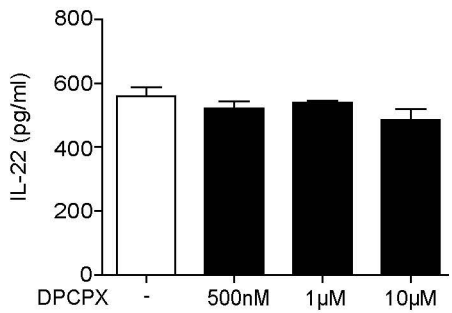
**B**



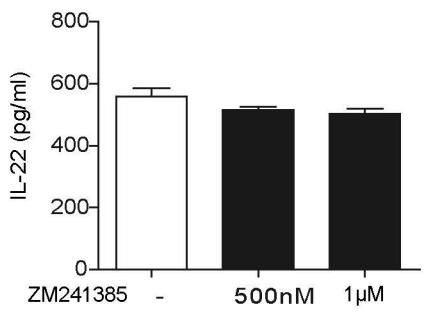
**A**



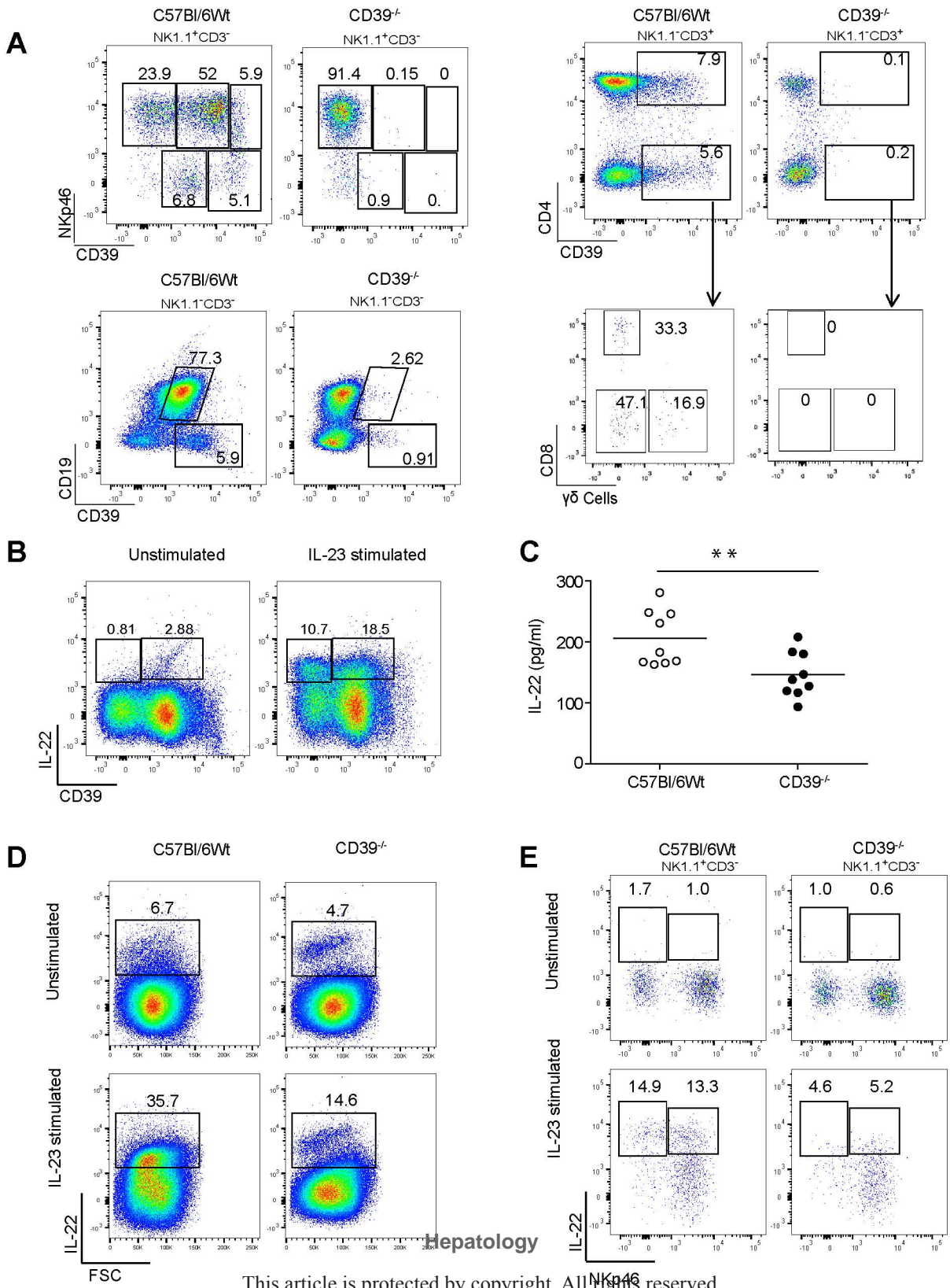
**B**



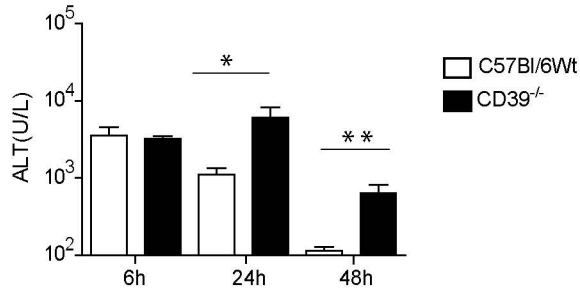
**C**





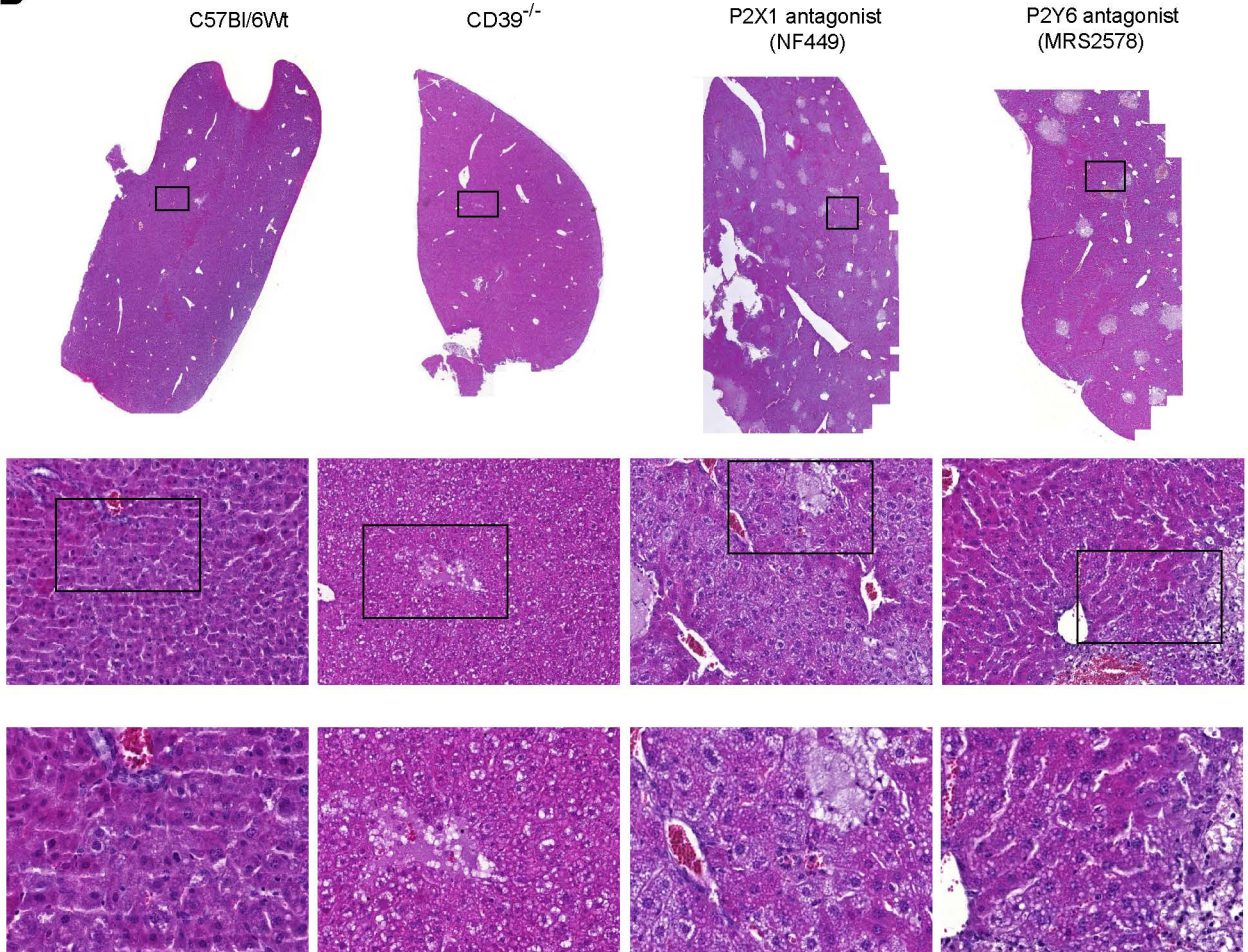


A



B

48PH



**P2X1 regulated IL-22 secretion by innate lymphoid cells is required for efficient liver regeneration**

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**Supplementary table 1****Patient Characteristics (n=40)**

<b>Variable</b>	
Age, yr, mean (SD)	64 (12)
Cases with extended resection, n	12 (30%)
Surgery for Hepatocellular Carcinoma, n	13 (32.5%)
Surgery for Cholangiocellular Carcinoma, n	11 (27.5%)
Surgery for Metastasis, n	14 (35%)
Surgery for Benign Lesions, n	2 (5%)
Cases with complications, n	26 (65%)
Cases with severe complications, n	14 (35%)
Cases with Posthepatectomy Liver Failure	8 (20%)

Major resection:  $\geq 3$  segments, Extended resection:  $\geq 5$  segments, Severe complications:  $\geq$  Grade 3 according Clavien-Dindo classification, Posthepatectomy Liver Failure: definition according ISGLS criteria

**Supplementary table 2****Patient's characteristics**

<b>Variable</b>	<b>No PHLF (n = 22)</b>	<b>PHLF (n = 5)</b>
Age, yr, mean (SD)	55 (12)	60 (13)
Units RBCs intraoperatively, mean (SD)	0.1 (0.4)	0 (0)
Length of ICU stay, days, mean (SD)	1.3 (1.1)	4 (6)
Length of hospital stay, days, mean (SD)	10.3 (7.1)	18 (19)
Cases with complications, n	8 (36%)	4 (80%)
Cases with severe complications, n	2 (9%)	1 (20%)

PHLF: Posthepatectomy Liver Failure (according ISGLS criteria), Major resection:  $\geq 3$  segments, Severe complications:  $\geq$  Grade 3 according Clavien-Dindo classification