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Purinergic $P2Y_2$ Receptors Promote Neutrophil Infiltration and Hepatocyte Death in Mice with Acute Liver Injury

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Abbreviations: P2R: purinergic P2 receptor; ConA: concanavalin A; PMH: primary mouse hepatocyte; GalN: galactosamine; WT: wild-type; KO: *$P2Y_2R$* knockout; PBS: phosphate buffered saline; JNK: Jun N-terminal kinase; BW: body weight; HPVF: high power visual field; IHIC: intrahepatic immune cells; APAP: acetaminophen.

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ABSTRACT

Background & Aims: During progression of liver disease, inflammation affects survival of hepatocytes. Endogenous release of ATP in the liver activates purinergic P2 receptors (P2R), which regulates inflammatory responses, but little is known about the roles of these processes in development of acute hepatitis.

Methods: We induced acute hepatitis in C57BL/6 mice by intravenous injection of concanavalin A and then analyzed liver concentrations of ATP and expression of P2R. We assessed P2R knockout ($P2Y_2R^{-/-}$) mice and C57BL/6 wild-type mice injected with suramin, a pharmacologic inhibitor of P2YR. Toxic liver failure was induced in mice by intraperitoneal injection of acetaminophen. Hepatocyte-specific functions of P2R signaling were analyzed in primary mouse hepatocytes.

Results: Induction of acute hepatitis in wild-type C57BL/6 mice released large amounts of ATP from livers and induced expression of $P2Y_2R$. Liver damage and necrosis were greatly reduced in $P2Y_2R^{-/-}$ mice and C57BL/6 mice given injections of suramin. Acetaminophen-induced liver damage was reduced in $P2Y_2R^{-/-}$ mice. Analysis of liver-infiltrating immune cells during acute hepatitis revealed that expression of $P2Y_2R$ in bone marrow-derived cells was required for liver infiltration by neutrophils and subsequent liver damage. Hepatic expression of $P2Y_2R$ interfered with expression of genes that regulate cell survival, and promoted tumor necrosis factor (TNF)α-mediated cell death, in a cell-autonomous manner.

Conclusions: Extracellular ATP and $P2Y_2R$ have cell-type specific, but synergistic functions during liver damage that regulate cellular immune responses and promote hepatocyte death. Reagents designed to target $P2Y_2R$ might be developed to treat inflammatory liver disease.

Keywords: liver disease, immune regulation, mouse model, apoptosis

INTRODUCTION

Release of endogenous ATP to the extracellular compartment and subsequent activation of purinergic nucleotide receptors (P2R) is increasingly recognized as a major signalling pathway involved in the regulation of inflammation and cellular stress responses.[1] Extracellular concentrations of ATP are very low under physiological conditions (in the nanomolar range) and tightly regulated by ectonucleotidases (e.g. CD39 and CD73), which dephosphorylate ATP to ADP, AMP and adenosine.[2] However, extracellular ATP concentrations can be markedly increased under cellular stress conditions such as inflammation, infection, hypoxia or trauma due to active or passive release from a number of cell types including hepatocytes and inflammatory cells.[3] Moreover, ATP metabolism under these conditions may be altered by concomitant down-regulation of nucleotidases. The autocrine and paracrine effects of extracellular ATP are mediated through interaction with members of the P2R family consisting of ligand gated ion channels ($P2X_1R$-$P2X_7R$) and G protein-coupled P2Y receptors ($P2Y_1R$, $P2Y_2R$, $P2Y_4R$, $P2Y_6R$, $P2Y_{11}R$-$P2Y_{14}R$) and subsequent activation of diverse intracellular signaling pathways.[4]

Expression of several P2R subtypes has been reported in human and rat hepatocytes and activation of these receptors was linked to various cellular functions such as hepatocyte metabolism and proliferation.[5-7] Interestingly, ATP also appears to be an important determinant of cell survival in the liver since hepatocyte apoptosis during fulminant hepatitis is strikingly reduced by the P2R antagonist Suramin in mice.[8]

Besides these functions on hepatocyte fate, extracellular ATP is also an important danger signal and regulates the recruitment and function of inflammatory cells such as dendritic cells, neutrophils and T lymphocytes.[3, 9] These findings suggest that extracellular ATP and P2R may serve as key regulators of inflammatory liver disease. In keeping with this notion, *$P2X_7R^{-/-}$* mice are protected against Concanavalin A (ConA)-mediated hepatitis, a well-established model of fulminant TNFα- and T-cell-mediated liver inflammation.[10] $P2X_7R$ is an important mediator of IL-1β release and critically regulates many inflammatory cell responses.[11] It was proposed that $P2X_7R$ receptor function is required for the activation of

NKT cells during ConA-mediated hepatitis.[10] However, this function is likely concentration-dependent since increased ATP concentrations in *CD39*$^{-/-}$ mice result in increased NKT cell apoptosis thereby protecting against ConA-hepatitis.[12] Besides $P2X_7R$, other P2 receptors are likely to play an important role in inflammation, especially in chemotaxis and release of inflammatory mediators. In this context, overwhelming evidence points to $P2Y_2R$ as a main chemotactic receptor for neutrophils.[9] These chemotactic functions of $P2Y_2R$ are of particular interest given the key pathogenic role of neutrophils in acute hepatitis.[13]

Here we demonstrate that ConA-mediated hepatitis results in increased release of endogenous ATP and subsequent induction of *$P2Y_2R$* expression. Of note, these alterations appeared to be of major pathophysiological relevance since hepatitis severity was substantially alleviated in *$P2Y_2R^{-/-}$* knockout mice. Mechanistically, $P2Y_2R$ mediated cell type specific but synergistic functions by regulating neutrophil infiltration as well as hepatocyte death.

MATERIALS AND METHODS

Cell culture

Primary mouse hepatocytes (PMH) were obtained and cultured as described.[14] PMH viability was determined by Trypan blue staining and exceeded 75-80%. To study inflammation-associated hepatocyte damage *in vitro*, PMH were incubated with recombinant TNFα (20ng/ml, R&D Systems, Wiesbaden, Germany) and Galactosamine (GalN, 5 mM, Sigma, Schnelldorf, Germany) or antibody against CD95 (Jo-2, 0.5 µg/ml, BD, Heidelberg, Germany). P2YR function was also inhibited by co-incubation with Suramin (200µM, Tocris, Ellisville, USA).

Animal experiments

$P2Y_2R^{-/-}$ knockout mice have been described [15] and were backcrossed for more than 8 generations on a C57BL/6 genetic background and housed under specific pathogen-free conditions at Freiburg University Hospital. Age- and sex-matched C57BL/6 wild-type mice were used as controls. Acute hepatitis was induced in 6-12 week old mice by intravenous injection of ConA (Type IV, Sigma, 15 µg/g body weight (BW)) and mice were sacrificed at the indicated timepoints. To inhibit P2Y receptors, animals were co-injected with Suramin (200µg/g body weight i.p.). Bone marrow chimeric mice were generated by injection of $5x10^6$ bone marrow cells into sublethally irradiated recipients (2x 4.5 Gy) followed by ConA injection 4-6 weeks later. Toxic liver failure was induced by intraperitoneal injection of acetaminophen (APAP, Sigma, 450 mg/kg BW). All animals received humane care and experiments were performed in accordance with local and institutional regulations.

Determination of extracellular ATP *in vivo*

Extracellular ATP was determined upon tail vein injection of PME-Luc cells (5×10^6) [16] with plasma membrane-targeted luciferase 30 min prior to ConA. Animals were intraperitoneally injected with luciferin at the indicated timepoints and imaging was performed for 5 min using

an IVIS100 system and Living Image Software v4.0 (both Xenogen, Caliper Life Sciences, Mainz, Germany) upon anesthesia with isoflurane.

Cytotoxicity assays and ELISA

Hepatocyte damage was determined by analysis of serum transaminases in cell culture supernatant or mouse sera using semi-automated clinical routine methods. ELISA for CXCL1, CXCL2, IL-1β, IL-2 and IL-6 (all R&D Systems) was performed according to the manufacturer's instructions.

Isolation of IHICs (Intrahepatic immune cells)

Livers were minced into small pieces and forced gently through a 70 µm-cell strainer (BD) using a sterile syringe plunger with addition of DMEM (Gibco, Karlsruhe, Germany) containing 5% FCS. The preparation was centrifuged at 1500 rpm, for 7 min at 4°C. The resulting pellet was re-suspended in 40% Percoll (Sigma) and layered over a 60% Percoll solution and centrifuged at 2500 rpm (900g) with the no-brake setting for 20 min at room temperature. After centrifugation, IHICs were collected from the inter-phase, transferred to a new tube and centrifuged to discard residual Percoll. Next, erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 µM EDTA pH 8.0) was applied to the pellet. Cells were washed once with FACS buffer before counting and flow cytometric analysis.

Flow cytometric analysis of IHICs

Cells were washed and incubated for 15 min with unlabelled CD16/32 antibody (2.4G2) to block Fc receptors. For the identification of different immune cell populations, cells were washed and extracellular marker proteins have been stained with fluorophore-conjugated antibodies for 30 min on ice: CD19 (1D3), CD11b (M1/70), CD11c (N418), NKp46 (29A1.4), NK1.1 (PK136), Ly6-C (HK1.4), Ly6-G (1A8), Gr-1 (RB6-8C5), TCRß (H57-597), TCRγδ (GL3) and MHC class II (M5/114.5.2; all from eBioscience, Frankfurt. Staining with fluorescently labelled murine CD1d-tetramer loaded with PBS57 (NIH tetramer facility)

diluted in FACS buffer was performed for 1 h at room temperature. Cells were washed twice and acquisition was performed on a FACS Canto II flow cytometer (BD). Data was analyzed using FlowJo software (Tree Star, Inc., Ashland, USA).

Histology, immunohistochemistry and immunofluorescence

For histology, livers were fixed in 3.7% neutral buffered formaldehyde at 4°C and embedded in paraffin. Confluent liver necrosis was quantified on H&E stained liver sections using ImageJ software (NIH, Bethesda, USA). Immunohistochemistry was performed using antibodies for neutrophils (sc-71674, Santa Cruz, Heidelberg, Germany) and CD3 (sm1754pt, Acris, Herford, Germany). TUNEL assays were performed using the *in situ* cell death detection kit (Roche, Mannheim, Germany).

Quantitative PCR (qPCR)

Upon isolation of total RNA using Qiazol (Qiagen, Hilden, Germany), cDNA synthesis was performed using the First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). qPCR was performed with either SYBR Green (Invitrogen, Karlsruhe, Germany) or Taqman on a 480 Lightcycler (Roche, Mannheim, Germany). Loading was normalized to $ß_2$-Microglobulin, Gapdh, Actin or Hprt. Primers and dual labeled hybridization probes were designed using Primer3 [17] and Beacon Designer v7.50 (Premier Biosoft, Palo Alto, USA), all primer sequences are available upon request.

Western blot analysis

Hepatocyte and total liver lysates were analyzed by immunoblot using antibodies for myeloperoxidase (sc16128, Santa Cruz), c-Jun (#9165; Cell Signaling), JNK (#9258, Cell Signaling), phospho-JNK (pT183/pY185 #700031, Invitrogen), p-Akt (Ser473 #4060, Cell Signaling), Akt (# 4685, Cell Signaling) and Actin (A2066, Sigma).

Statistics

Data in bar graphs represent mean ± S.D. Statistical analysis was performed by using the nonparametric Mann-Whitney test, analysis of variance followed by Bonferroni comparison test or nondirectional two-tailed Student's *t* test as appropriate. Standard deviation of gene expression in qPCR experiments was calculated as described.[18]

RESULTS

Increased expression of $P2Y_2R$ during ConA-mediated hepatitis

Extracellular ATP is an important danger signal and regulator of inflammatory responses. To address the question whether endogenous ATP is released during acute hepatitis, wild-type (*WT*) mice were injected with ConA and ATP release was monitored with an extracellular luciferase-based ATP detection system (PME-Luc cells).[16] Compared to untreated controls, significant ATP release from the liver occurred as early as 2 hours after ConA injection and remained elevated thereafter (Fig. 1A,B). Extracellular ATP activates purinergic P2 receptors (P2R) and P2R isoform expression was therefore assessed by Taqman qPCR and revealed that $P2X_4R$ and $P2Y_2R$ were the most abundantly expressed P2R isoforms in untreated livers (Fig. 1C). Moreover, $P2Y_2R$ was the only P2R isoform which was significantly induced during ConA-mediated hepatitis, while expression of $P2X_4R$, $P2X_7R$, $P2Y_4R$, $P2Y_6R$ and $P2Y_{14}R$ was rather reduced (Fig. 1C). Extracellular ATP is further metabolized to AMP and adenosine by the action of CD39 and CD73. Interestingly, CD73 expression was rather reduced in ConA-treated livers.

Since P2Rs are expressed in hepatocytes as well as in nonparenchymal liver cells, wild-type primary mouse hepatocytes (PMH) were isolated in order to analyze the P2R isoform expression in hepatocytes in more detail. Considerable expression of $P2X_4R$, $P2Y_2R$, $P2Y_{14}R$ and CD73 was observed in untreated PMH, while expression of other P2 receptors and CD39 was rather low or undetectable (Fig. 1D). $P2Y_2R$ expression in PMH was also significantly increased following stimulation with TNFα (Fig. 1D). Moreover, qPCR analysis revealed that $P2Y_2R$ was also expressed by unstimulated mouse neutrophils and lymphocytes (Suppl. Fig. 1), which is consistent with previous reports of broad $P2Y_2R$ expression in various immune cell types including neutrophils, monocytes, macrophages, dendritic cells, T-cells, B-cells and NK-cells.[3] These findings indicate that acute hepatitis in mice is characterized by release of extracellular ATP and induced expression of $P2Y_2R$, the most abundant P2YR isoform in the liver.

ConA-mediated hepatitis is alleviated in *P2Y$_2$R$^{-/-}$* livers

To further analyze the mechanistic role of P2Y$_2$R during acute hepatitis, ConA was injected into *P2Y$_2$R$^{-/-}$* mice and *WT* controls. Liver necrosis and prominent vascular congestion was apparent in *WT* livers as early as 8 hours after ConA injection (Fig. 2A). In contrast, ConA-treated *P2Y$_2$R$^{-/-}$* livers were macroscopically almost indistinguishable from untreated controls (Fig. 2A and data not shown). Importantly, histological analysis revealed that liver necrosis was six-fold reduced in *P2Y$_2$R$^{-/-}$* mice (Fig. 2B, C). Hepatic necrosis was also partially rescued upon co-injection of *WT* mice with Suramin, an established P2YR antagonist (Fig. 2B, C). Moreover, alleviated liver damage in *P2Y$_2$R$^{-/-}$* or Suramin-treated *WT* mice was also evident by markedly reduced serum transaminase concentrations (Fig. 2D). To further substantiate these differences in cell death, TUNEL assays revealed confluent areas of TUNEL positive hepatocytes in *WT* livers while only scattered apoptotic cells were apparent in *P2Y$_2$R$^{-/-}$* livers (Fig. 2E). To address the question whether P2Y$_2$R also promotes liver damage in other models of liver injury, acute toxic liver failure was induced by injection of APAP. Liver damage as determined by serum transaminase concentrations was indeed also significantly reduced in *P2Y$_2$R$^{-/-}$* mice (suppl. Fig. 2). These findings indicate that P2Y$_2$R expression strongly promotes liver injury.

P2Y$_2$R regulates neutrophil infiltration

Extracellular ATP and P2Y$_2$R have earlier been identified as essential mediators of neutrophil infiltration In contrast, iNKT and B cell numbers during ConA-mediated hepatitis were comparable in both genotypes, while NK cell numbers were slightly reduced in the absence of *P2Y$_2$R* [9, 19], raising the question whether P2Y$_2$R regulates inflammatory responses in the liver. To further address this issue, liver infiltrating immune cells were isolated and analyzed by flow cytometry. Interestingly, numbers of liver-infiltrating neutrophils, B cells, T cells, NK as well as NKT cells were comparable in *WT* and *P2Y$_2$R* livers under resting conditions (Fig. 3A, Suppl. Fig. 2A, B). By contrast, the amount of infiltrating neutrophils was not substantially altered in ConA-treated *P2Y$_2$R$^{-/-}$* livers as determined by flow cytometry (Fig. 3A, B). This

finding was also confirmed by immunohistochemistry and correlated with reduced myeloperoxidase expression (Fig. 3C, D and suppl. Fig. 2C). In contrast, $CD3^+$ T-cell infiltration was not affected in ConA-treated *$P2Y_2R^{-/-}$* livers as determined by immunohistochemistry (Fig. 3C), but absolute αβ-T cell numbers were slightly increased as determined by FACS analysis. In contrast, iNKT and B cell numbers during ConA-mediated hepatitis were comparable in both genotypes, while NK cell numbers were slightly reduced in the absence of *$P2Y_2R$* (Suppl. Fig. 3). Besides the established direct effect of ATP and $P2Y_2R$ on neutrophil migration, release of chemoattractants such as CXCL1, CXCL2 and IL-6 was deregulated in *$P2Y_2R^{-/-}$* mice and might also contribute to impaired neutrophil infiltration in *$P2Y_2R^{-/-}$* livers (Fig. 3E). In contrast, release of IL-2, a master regulator of T cell function, was not affected (Fig. 3E). To elucidate whether $P2Y_2R$ signaling is required for release of these chemo-attractants, mice were injected with bacterial lipopolysaccharide to induce TNFα-dependent hepatitis without causing significant cell death. Interestingly, induction of CXCL1, CXCL2 and IL-1β was not different in *$P2Y_2R^{-/-}$* mice under these conditions while IL-6 concentrations were only marginally reduced (data not shown). These findings suggest that impaired chemokine/cytokine expression in ConA-treated *$P2Y_2R^{-/-}$* mice rather reflects differences in the inflammatory response and liver damage.

Next, bone marrow chimeric mice were generated to elucidate the impact of $P2Y_2R$ expression in the bone marrow compartment on neutrophil infiltration and subsequent liver damage. As positive control, *WT* mice reconstituted with *WT* bone marrow resulted in increased liver damage in comparison to the other chimera (Fig. 4A,B). Most importantly, increased liver damage in *WT* mice appeared to be strongly reduced upon reconstitution with *$P2Y_2R^{-/-}$* bone marrow (Fig. 4A, B), which also correlated with reduced neutrophil infiltration in these livers (Fig. 4C, D). It should be noted that these differences in liver damage were only marginally significant due to two *WT* mice with very limited inflammation and without any major liver damage and would have been highly significant otherwise. In contrast, liver damage and neutrophil infiltration in *$P2Y_2R^{-/-}$* mice reconstituted with *WT* bone marrow was not clearly different from the other groups. Infiltration of NKT, NK, T and B cells was again

comparable in all groups (Suppl. Fig. 4), again suggesting that infiltration of these cells was independent of $P2Y_2R$. These data suggest that $P2Y_2R$ expression in the bone marrow compartment indeed regulates neutrophil infiltration thereby contributing to ConA-mediated liver damage.

Hepatocyte survival in *$P2Y_2R^{-/-}$* mice correlates with increased expression of hepatoprotective genes

$P2Y_2R$ is the most abundantly expressed P2YR isoform in the liver, raising the question whether $P2Y_2R$ expression, besides regulating neutrophil infiltration, also directly determines hepatocyte fate during acute hepatitis. Hepatocyte survival during hepatitis is regulated by several signaling pathways including Jun N-terminal kinase (JNK) and subsequent expression of the AP-1 transcription factor c-Jun.[20] Since $P2Y_2R$ was previously shown to activate JNK [6], immunoblot analysis was performed to analyze whether increased hepatocyte survival in *$P2Y_2R^{-/-}$* was mediated by deregulated JNK/c-Jun signaling. JNK phosphorylation (i.e. activation) was rather induced in some *$P2Y_2R^{-/-}$* livers at 4h after ConA injection. Moreover, expression of c-Jun and its target gene inducible nitric oxide synthetase (Nos2), two mediators of hepatocyte survival during ConA-mediated hepatitis,[20] was induced to the same extent at early timepoints in both genotypes. However, hepatic expression of these genes was rather reduced at later stages in *$P2Y_2R^{-/-}$* mice, most likely reflecting impaired liver damage in these animals (Fig. 5A,B). These findings indicate that the observed differences in hepatocyte survival are likely not mediated by deregulated JNK/c-Jun signaling. In contrast, increased liver damage in WT mice correlated with reduced phosphorylation of AKT, a major regulator of hepatocyte survival (Fig. 5A). In addition, expression of Gadd45α, Gadd45β and A20 was increased in *$P2Y_2R^{-/-}$* livers at early timepoints after ConA injection (Fig. 5B). These genes are established targets of the transcription factor NF-κB, which is a master regulator of hepatocyte survival (Fig. 5B).[21] These findings provide evidence that increased hepatocyte survival observed in *$P2Y_2R$* deficient livers may be mediated by hepatoprotective NF-κB signaling.

$P2Y_2R$ expression in hepatocytes promotes cell death *in vitro*

The relative contribution of $P2Y_2R$ expression in hepatocytes to the pathogenesis of ConA-mediated hepatitis is difficult to dissect in bone marrow chimeric mice since these animals fail to mount comparable inflammatory responses as demonstrated above. Therefore, to elucidate whether $P2Y_2R$ expression indeed contributes to hepatocyte fate in a cell autonomous manner, PMH isolated from *WT* and $P2Y_2R^{-/-}$ mice were incubated with TNFα and Galactosamine (GalN) or an agonistic antibody against CD95 (Jo2), two *in vitro* models of inflammation-related hepatocyte damage and apoptosis. Increased hepatocyte damage occurred in *WT* PMH treated with TNFα/GalN and was significantly reduced in the absence of $P2Y_2R$ or upon inhibition of P2YR function by Suramin as determined by morphological criteria as well as transaminase release to the culture medium (Fig. 6A,B). $P2Y_2R^{-/-}$ PMH were also partially resistant to FasL-mediated apoptosis (Fig. 6B). Moreover, expression of CXCL1 and CXCL2 was strikingly reduced in $P2Y_2R^{-/-}$ hepatocytes, likely reflecting more limited damage in these cells (Fig.6C). These findings indicate that hepatic $P2Y_2R$ expression promotes TNFα-related hepatocyte death and subsequent chemokine release in a cell autonomous manner, which may in turn further promote liver inflammation.

DISCUSSION

Compelling evidence points out to an important role of the ATP/P2R signaling pathway in regulating inflammatory responses.[3] Many of these pro-inflammatory functions are considered to be mediated by $P2X_7R$-dependent inflammasome activation and subsequent IL-1β release[11] and disease severity is consistently reduced in *$P2X_7R^{-/-}$* mice in several disease models such as ConA-mediated hepatitis, asthma bronchiale, chronic obstructive pulmonary disease, rheumatoid arthritis, graft versus host disease and allergic dermatitis.[10, 22-26] However, also other P2 receptors are likely to play a role in the orchestration of a complex phenomenon such as inflammation. Among them, $P2Y_2R$ has been recognized as an essential chemotactic receptor for neutrophils.[9] Here we demonstrate that endogenous ATP is released during inflammatory liver disease in mice and that subsequent signaling through $P2Y_2R$ critically determines the severity of acute hepatitis by distinct but synergistic mechanisms. First, $P2Y_2R$ signaling regulates neutrophil infiltration in a cell autonomous manner, but seems to be dispensable for infiltration of other immune cells such as NKT cells. These findings are consistent with previous observations that $P2Y_2R$ regulates neutrophil chemotaxis during bacterial peritonitis and chronic lung disease and is involved in the clearance of apoptotic cells.[9, 19, 27] Endogenously released ATP is therefore likely to regulate hepatic inflammatory responses in several ways by promoting $P2Y_2R$-dependent neutrophil infiltration as well as $P2X_7R$-dependent NKT cell function.[10]

Second, activation of $P2Y_2R$ in hepatocytes, which is the predominant P2YR isoform expressed in these cells, promotes cell death and adds yet another level to this paracrine signaling network. Previous studies suggest that $P2Y_2R$ rather mediates pro-survival functions in hepatocytes such as proliferation.[6, 7] However, $P2Y_2R$ may also promote cell death since activation of the death-inducing signaling complex and subsequent hepatocyte apoptosis are prevented by the P2YR inhibitor Suramin.[8] $P2Y_2R$-mediated hepatocyte damage in turn results in increased chemokine release from hepatocytes, which may further promote neutrophil infiltration. However, although bone marrow chimeric mice were generated, it was difficult to extrapolate the precise contribution of $P2Y_2R$ signaling in

inflammatory cells versus hepatocytes by using this approach for the following reason. Neutrophil infiltration is essential for the pathogenesis of ConA-mediated hepatitis and liver damage is consistently rescued upon neutrophil depletion.[13] The hepatocyte-specific effects of $P2Y_2R$ are therefore likely underestimated in *WT* mice reconstituted with $P2Y_2R^{-/-}$ bone marrow, since these animals fail to mount a comparable inflammatory response. The relative cell-type specific contributions of $P2Y_2R$ during acute hepatitis therefore need to be addressed in tissue-specific conditional knockout mice, which are currently generated.

At the molecular level, the links between $P2Y_2R$ and hepatocyte death could be several-fold. $P2Y_2R$ activation causes a large calcium release from intracellular stores,[4, 28] thereby possibly promoting mitochondria overload with calcium and eventual cell death.[29] In addition, hepatic P2YR function activates Jun N-terminal kinase (JNK) and subsequent expression of the AP-1 transcription factor c-Jun.[6, 7] Sustained JNK activation has been closely linked to TNFα-dependent cell death,[29, 30] although recent genetic evidence suggests that JNK1/2 expression in hepatocytes is dispensable for ConA-mediated hepatitis.[31] In contrast, hepatocyte survival during ConA-mediated hepatitis is strongly promoted by the prototypic JNK target c-Jun.[20] Interestingly, JNK phosphorylation and c-Jun expression were not consistently deregulated at early stages in $P2Y_2R^{-/-}$ livers and are therefore unlikely to contribute to the observed phenotype. These differences are rather mediated by the transcription factor NF-κB, a well-established regulator of hepatocyte survival and liver inflammation.[21, 29] This was evident by the increased expression of many hepatoprotective NF-κB target genes including Gadd45α, Gadd45β and A20, all of which protect against apoptosis.[32-34] In keeping with this notion, it was recently shown that $P2Y_2R$ inhibits the activity of NF-κB in cardiomyocytes.[35] NF-κB in turn also regulates the transcription of $P2Y_2R$, at least in intestinal epithelia.[36] However, it remains to be determined whether similar molecular interactions are also present in the liver. Increased expression of hepatoprotective genes in $P2Y_2R^{-/-}$ mice was only transient and occurred early after ConA injection, suggesting that these alterations may also be due to differences in the acute phase

response. The intracellular molecular pathways activated by hepatic ATP/$P2Y_2R$ signaling therefore need to be further dissected in the future.

There is increasing evidence that ischemia and subsequent hypoxia essentially contribute to inflammation-related organ damage.[37] In keeping with this notion, inflamation-mediated liver hypoxia and damage can be efficiently rescued upon liver-specific delivery of vasoactive substances such as nitric oxide.[20] It was also shown that ischemia reperfusion injury results in strong release of ATP and subsequent inflammation while signaling through adenosine receptors appear to be rather protective.[38] It is therefore possible that $P2Y_2R$ function may turn out to have similar functions in both, acute hepatitis and vascular liver disease.

Importantly, ConA-mediated liver damage was partially rescued upon administration of the P2YR antagonist Suramin, which has been used to treat trypanosomiasis for many decades and is under clinical investigation to target prostate and lung cancer.[39-41] These findings point out to a novel pharmacological approach to treat acute hepatitis. $P2Y_2R$ agonists have been used in phase III trials to increase the secretory functions of epithelia in patients with cystic fibrosis.[42] However, although $P2Y_{12}R$ inhibitors are well established in the clinics to inhibit thrombocyte function, specific $P2Y_2R$ inhibitors are not yet available but currently developed by pharmaceutical companies in order to treat sepsis and other inflammatory disorders.[42]

In conclusion, our data elucidating the important role of *$P2Y_2R$* in promoting neutrophil infiltration as well as hepatocyte death during acute hepatitis may pave the path towards novel strategies to treat inflammatory liver disease.

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FIGURE LEGENDS

Figure 1: Increased expression of $P2Y_2R$ during ConA-mediated hepatitis. (A) Release of endogenous ATP in ConA-treated livers was assessed in untreated or ConA-treated *WT* mice using an extracellular luciferase-based ATP detection system (PME-Luc cells) and *in vivo* imaging. Luciferase activity in representative mice treated with PBS and ConA (15µg/g for 4h) is shown. **(B)** Luciferase activity was quantified in the right upper quadrant of the abdomen at the indicated timepoints following ConA injection (n = 4-6/group; **, $P \leq 0.01$). **(C)** P2R RNA expression in PBS or ConA-treated *WT* livers (4h) was determined by Taqman qPCR. Relative expression compared to the housekeeping gene β_2-microglobulin is given in [%] ± S.D., n = 8 mice/genotype and treatment; *, $P \leq 0.05$, n.d., not detectable. **(D)** P2R RNA expression in PBS or TNFα-treated primary mouse hepatocytes (PMH, 20ng/ml for 4h) as compared to β_2-microglobulin expression in [%], n = 3; *, $P \leq 0.05$, n.d., not detectable.

Figure 2: ConA-mediated hepatitis is alleviated in $P2Y_2R^{-/-}$ livers. (A) Macroscopical appearance of representative *WT* and $P2Y_2R^{-/-}$ (*KO*) livers following ConA treatment (8h, 15µg/g BW). Note the congestion and appearance of necrosis in *WT* livers. **(B)** Confluent liver necrosis (yellow lines) following ConA treatment for 8h were identified on H&E stainings. In some experiments, *WT* mice were co-injected with ConA and Suramin (SUR, 200µg/g BW), an established P2YR antagonist. **(C)** Necrotic areas were quantified on H&E stained histological sections and are given in [%] of liver area ± S.D. n ≥ 4 livers/genotype and treatment. *, $P \leq 0.05$; **, $P \leq 0.01$. **(D)** Concentrations of serum transaminases ALT and AST in individual ConA-treated mice (8h) with the indicated genotypes are depicted. n = 7-26 mice /genotype and treatment, *, $P \leq 0.05$; §, $P \leq 0.0001$. **(E)** Representative TUNEL stainings of livers from ConA-treated mice with the indicated genotypes (green), nuclei were counterstained with DAPI (blue).

Figure 3: $P2Y_2R$ regulates neutrophil infiltration. (A,B) Groups of mice were injected with ConA or PBS and sacrificed after 8h. Intrahepatic leucocytes were isolated, stained with

fluorescently labeled antibodies and analyzed by flow cytometry. **(A)** Dot plots are electronically gated on living cells and numbers indicate frequency of neutrophils (Ly-6G^{+} Ly-6C^{int} cells). **(B)** Bar diagrams show average of absolute numbers of neutrophils within the liver **(C)** Inflammation in PBS and ConA-treated livers of the indicated genotypes was also analyzed by immunohistochemistry for neutrophils and CD3^{+} T-lymphocytes. Cell numbers per high power visual field (HPVF, 400x) were counted and are given as mean ± S.D., n = 8 livers/genotype and treatment; **, $P \leq 0.01$; n.s., not significant. **(D)** Myeloperoxidase (MPO) expression in livers from mice with the indicated genotypes was analyzed by immunoblot. **(E)** Serum concentrations of the indicated cytokines and chemokines were analyzed by ELISA in PBS and ConA-treated (4h) mice with the indicated genotypes. Results are shown as mean ± S.D., n ≥ 4 mice/genotype and treatment, *, $P \leq 0.05$.

Figure 4: P2Y$_2$R expression in the bone marrow compartment contributes to neutrophil infiltration and subsequent liver damage. (A) ConA-mediated hepatitis was induced in bone marrow chimeric mice. Necrotic liver areas (yellow lines) following ConA treatment for 8h were identified on H&E stainings only in *WT* mice reconstituted with *WT* bone marrow. **(B)** Serum transaminase concentrations of the individual chimeric mice are depicted (8h).**(C)** Intrahepatic leucocytes were isolated, stained with fluorescently labeled antibodies and analyzed by flow cytometry. Dot plots are electronically gated on living cells and numbers indicate average (±SD, n≥3) frequency of neutrophils (Ly-6G^{+} Ly-6C^{int} cells). **(D)** Scatter blot shows the frequency of neutrophils within liver leucocytes in the different chimeras.

Figure 5: Increased expression of hepatoprotective genes in *P2Y$_2$R$^{-/-}$* livers. (A) c-Jun expression as well as JNK and AKT phosphorylation was analyzed by immunoblot in ConA-treated livers of the indicated genotypes at the indicated timepoints. Actin was used as control. **(B)** RNA expression of cell survival-related genes was analyzed in ConA treated

livers after 4h and 8h by qPCR. Expression was normalized to untreated *WT* livers (mean ± S.D., n = 4 livers/genotype and treatment); ***, $P \leq 0.001$; *, $P \leq 0.05$.

Figure 6: $P2Y_2R$ expression in hepatocytes promotes cell death *in vitro.* PMH from mice with the indicated genotypes were isolated and incubated with TNFα and GalN (20 ng/ml and 5 mM, respectively) or Jo2 (0,5 µg/ml) for 6h. In some experiments, P2YR function was inhibited pharmacologically by incubation with Suramin (200µM). Phase contrast microscopy revealed the occurrence of cell death in TNFα/GalN-treated *WT*, but not *KO* PMH **(A)**. **(B)** Release of AST to the supernatant was analysed as a measure of hepatocyte death and is depicted as mean ± S.D., n = 3-10 /genotype and treatment, *, $P \leq 0.05$. **(C)** RNA expression of the indicated chemokines was analysed by qPCR. Expression was normalized to expression on PBS-treated *WT* PMH. n = 3 /genotype and treatment, ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

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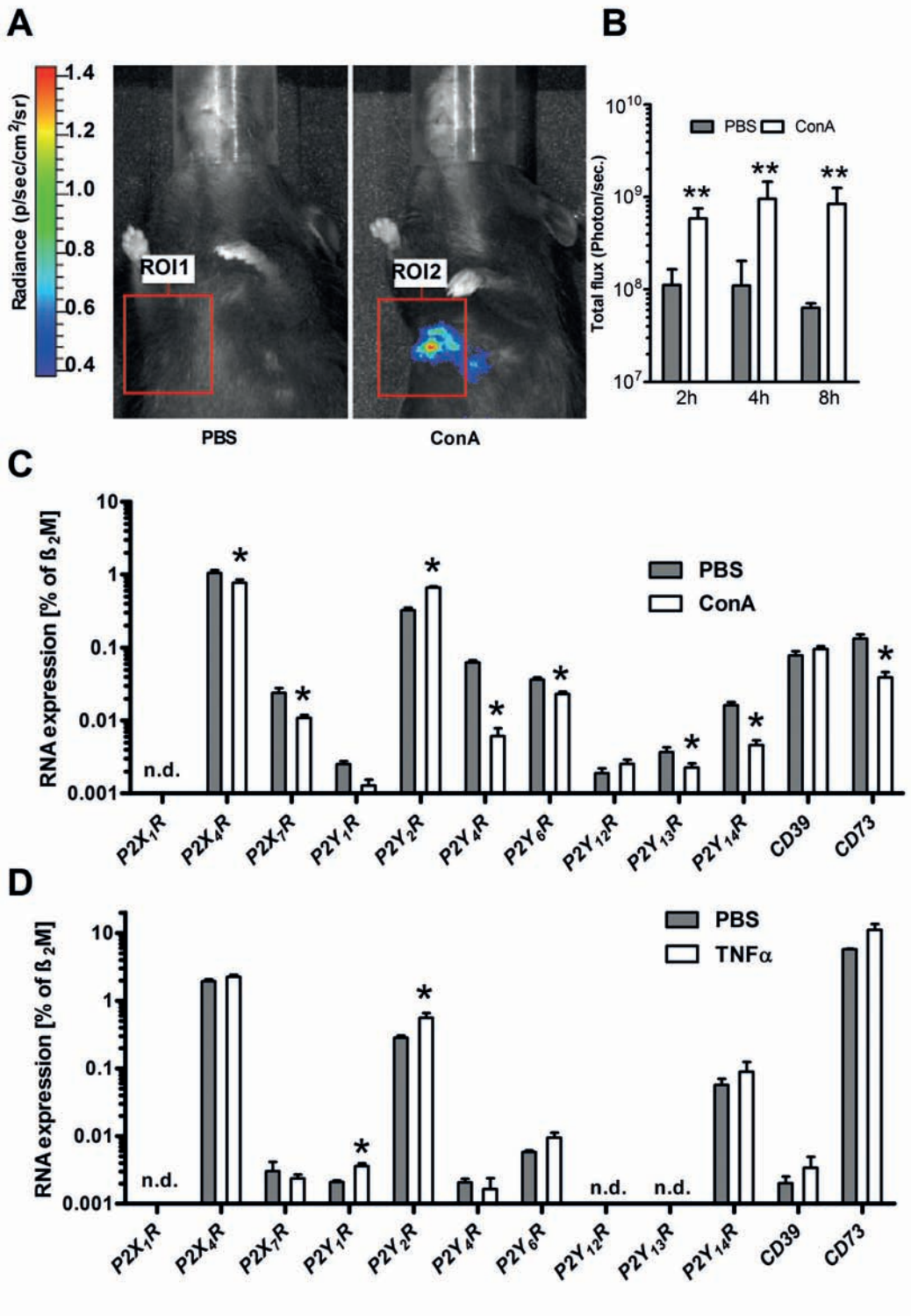
Fig. 1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ROI2
PBS
ConA
B
Total flux (Photon/sec.)
PBS
ConA
2h
4h
8h
C
RNA expression [% of ß2M]
n.d.
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P2Y1R
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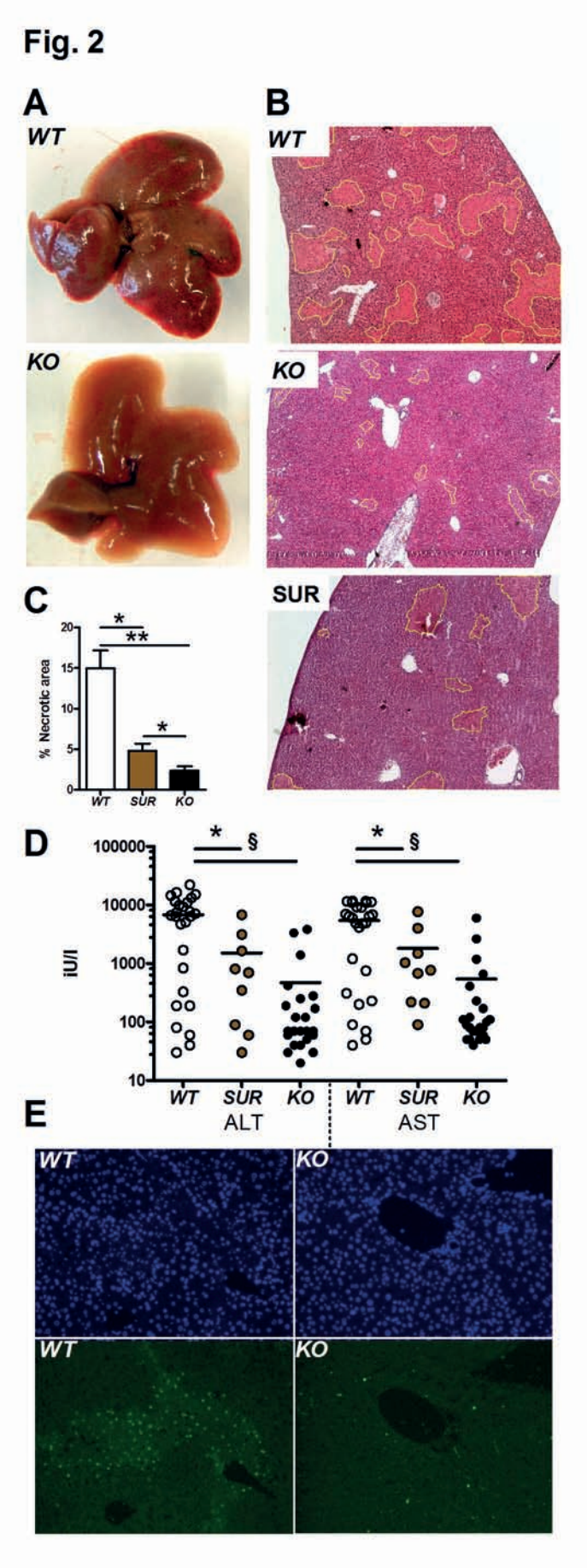
CD73
D
TNFα

Fig. 2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B
WT
KO
SUR
C
% Necrotic area
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15
10
5
0
*
**
*
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SUR
KO
D
iU/l
100000
10000
1000
100
10
*
§
*
§
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SUR
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SUR
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ALT
AST
E
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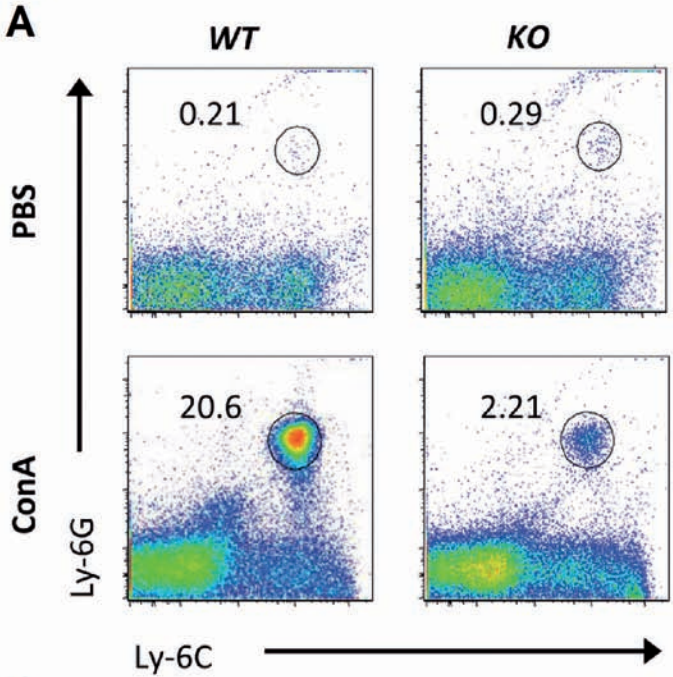
KO
WT
KO

Fig. 3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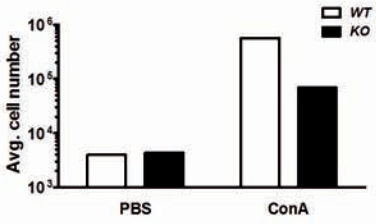
Ly-6G
Ly-6C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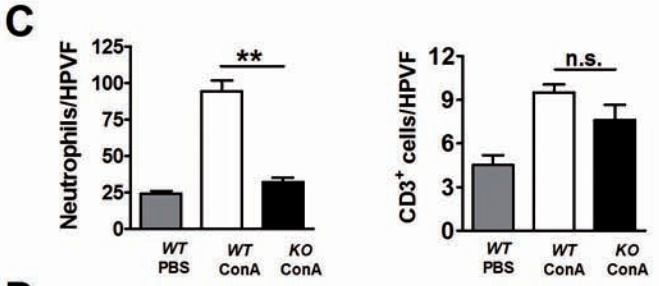
C
Neutrophils/HPVF
**
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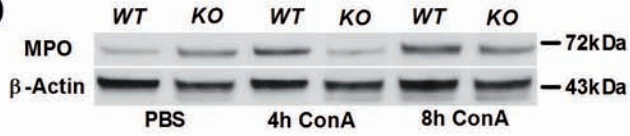
WT ConA
KO ConA
CD3+ cells/HPVF
n.s.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KO
MPO
β-Actin
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43kDa
PBS
4h ConA
8h ConA

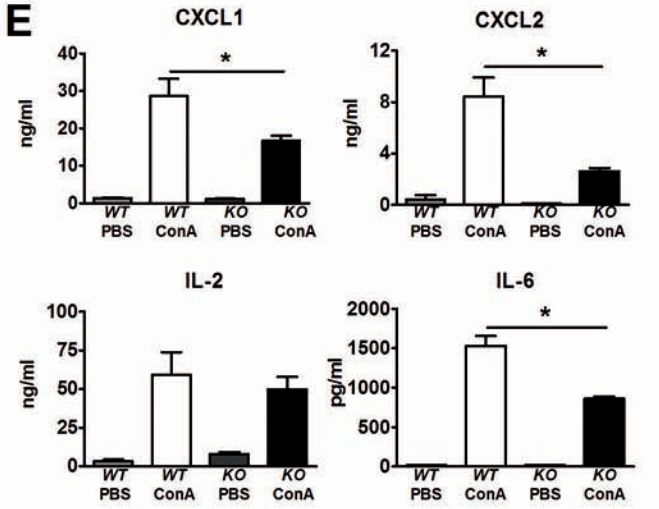
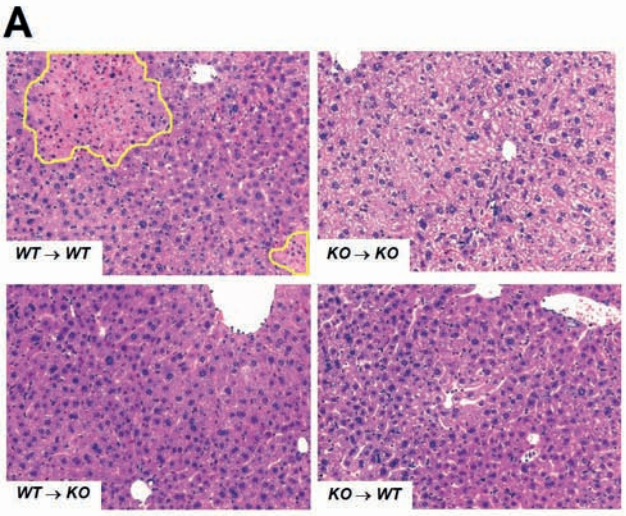

E
CXCL1
CXCL2
IL-2
IL-6
ng/ml
pg/ml
*
WT PBS
WT ConA
KO PBS
KO ConA

Fig 4.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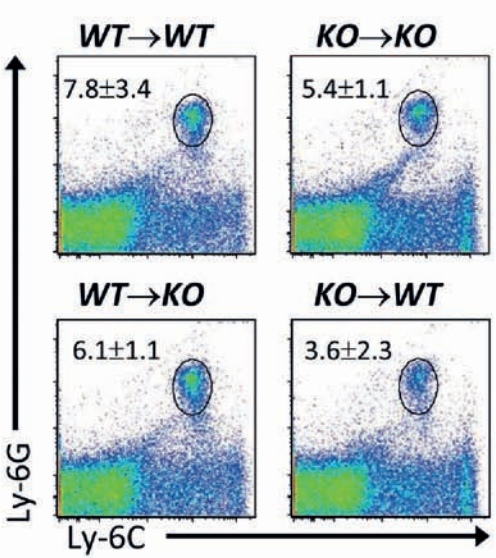
KO → KO
WT → KO
KO → WT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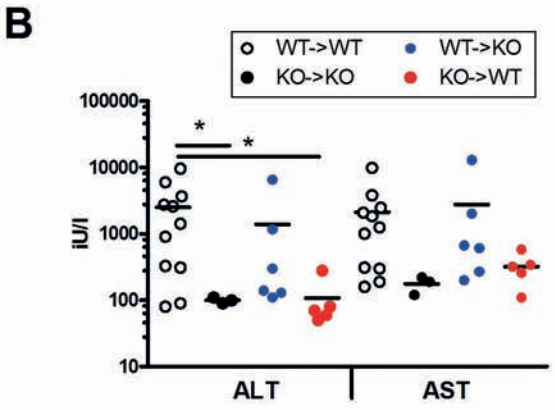
5.4±1.1
WT→KO
KO→WT
6.1±1.1
3.6±2.3
Ly-6G
Ly-6C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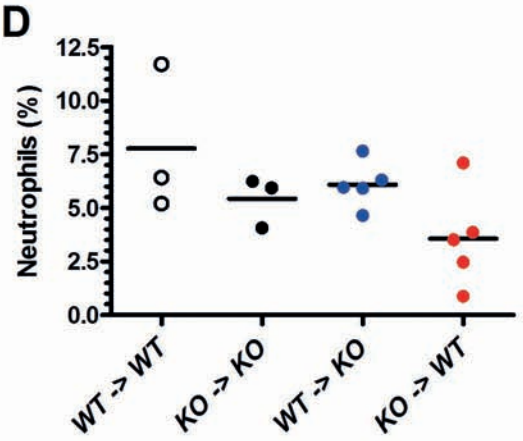
KO->KO
WT->KO
KO->WT
100000
10000
1000
100
10
iU/l
*
*
ALT
AST


D
Neutrophils (%)
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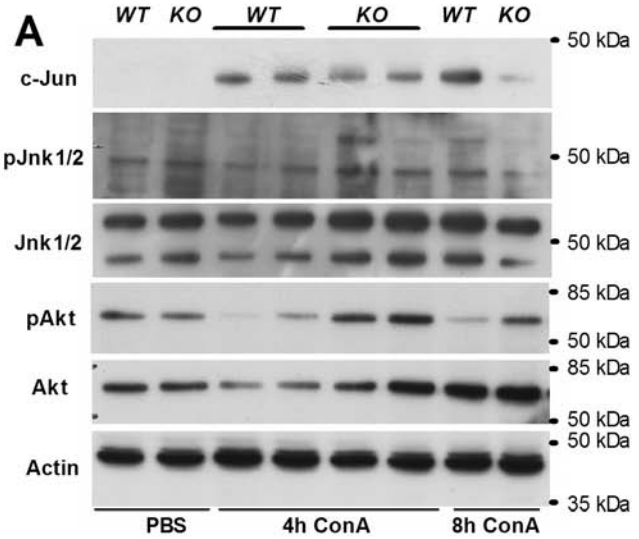
7.5
5.0
2.5
0.0
WT -> WT
KO -> KO
WT -> KO
KO -> WT

Fig.5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KO
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KO
WT
KO
c-Jun
pJnk1/2
Jnk1/2
pAkt
Akt
Actin
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50 kDa
85 kDa
50 kDa
85 kDa
50 kDa
50 kDa
35 kDa
PBS
4h ConA
8h ConA

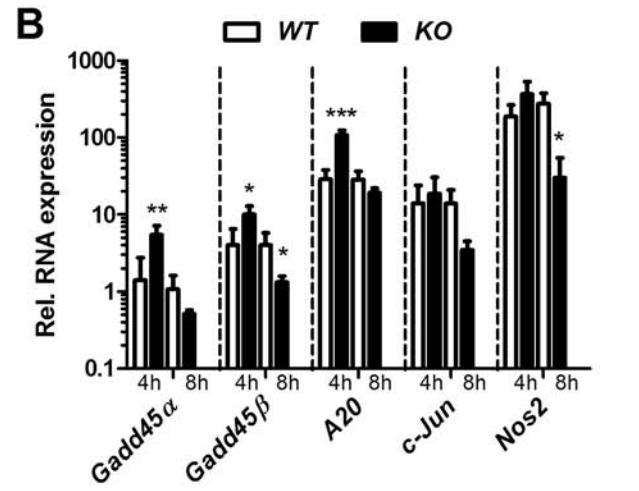
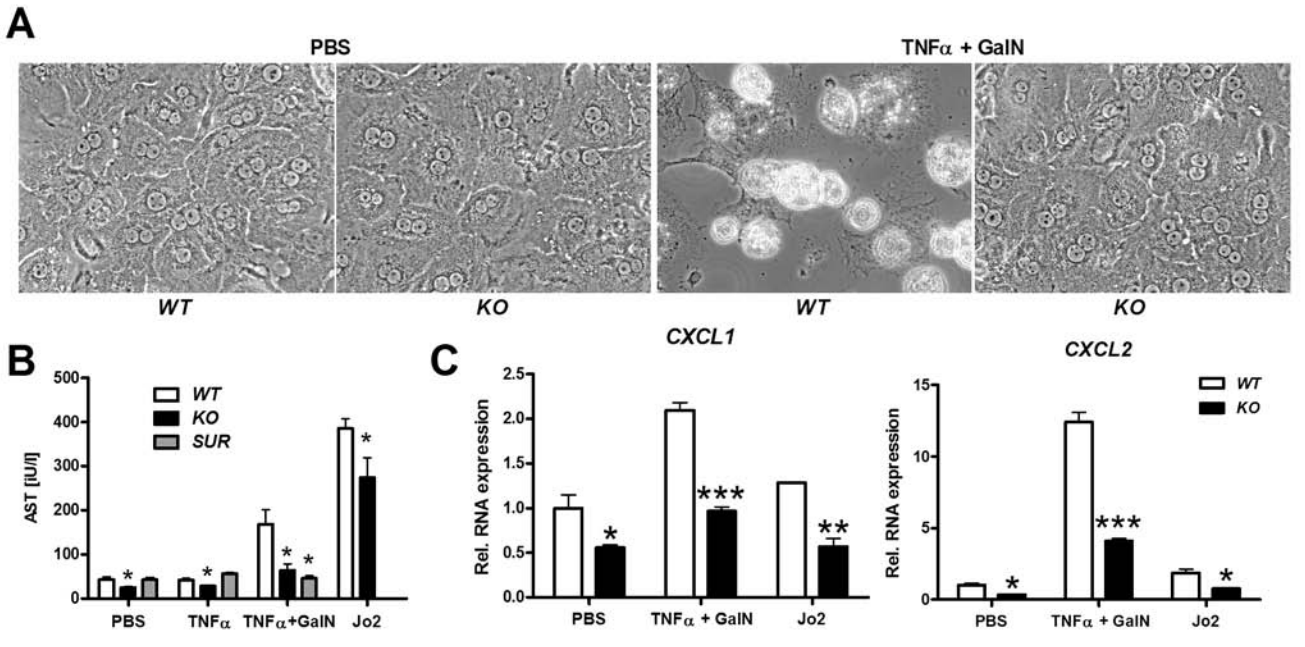

B
WT
KO
Rel. RNA expression
1000
100
10
1
0.1
**
*
*
***
*
4h
8h
4h
8h
4h
8h
4h
8h
4h
8h
Gadd45α
Gadd45β
A20
c-Jun
Nos2

Fig. 6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TNFα + GalN
WT
KO
WT
KO
B
AST [iU/l]
WT
KO
SUR
PBS
TNFα
TNFα+GalN
Jo2
C
CXCL1
Rel. RNA expression
PBS
TNFα + GalN
Jo2
CXCL2
WT
KO
Rel. RNA expression
PBS
TNFα + GalN
Jo2