Ductular reaction-associated neutrophils promote biliary epithelium proliferation in chronic liver disease

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Supplementary Material and Methods

Animal models

10-12-week-old male and female C57BL/6J mice (Charles River) were fed with a standard rodent chow diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydro-collidin (DDC) (Sigma-Aldrich, St. Louis, MO). This model has been previously reported to mimic histological and pathophysiological characteristics of chronic liver diseases in humans [1]. After one week of DDC treatment, DDC-treated and control female and male animals were sacrificed and liver, blood and bone marrow samples were collected for further neutrophil phenotype and function characterization.

As a neutrophil depletion model, 12-week-old male mice were treated with anti-Ly6G clone 1A8 antibody (500µg) or isotype (500µg) (BioXcell) every 2 days and simultaneously fed with DDC for 3 weeks. First antibody or isotype administration was performed 2 days before starting with DDC diet.

To inhibit CXCR1/2 receptor activity, the inhibitor SCH-527123 (MedChemExpress) was used. SCH-527123 was suspended in 20% 2-Hydroxypropyl-beta-cyclodextrin (HPβCD) (Sigma Aldrich). SCH-527123 inhibitor or vehicle were daily administered to 12-week-old male mice by oral gavage at a dose of 50mg/kg body weight for 3 weeks together with DDC diet. After this time, animals were sacrificed, and liver and blood samples were collected.

Neutrophil elastase and protein arginine deiminase 4 deficient mice ($NE^{-/-}$ and $PAD4^{-/-}$) were obtained from Jackson Laboratory. 12-week-old $NE^{-/-}$ and $PAD4^{-/-}$ male mice were fed with DDC diet for 3 weeks.

As secondary injury mice models, carbon tetrachloride (CCl4) was injected every two days for four weeks in 12-week-old female and male mice. For the bile duct ligation model (BDL), the bile duct was ligated for 14 days in 8-10 weeks C57BL6/J males.

All animal experiments were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Mouse serum biochemical assays

Blood from mice was collected in heparinized tubes and analyzed by the Biomedical Diagnostic Center of Hospital Clinic, Barcelona. Serological analysis included the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (AP) levels.

Spinning disk intravital microscopy

Image acquisition was carried out with Olympus IX81 inverted microscope, associated with an Olympus focus drive and a monitorized stage (Applied scientific Instrumentation) and fitted with a monitored objective turret equipped with 4x/0.16 UPLANSAPO, 10x/0.40 UPLANSAPO, and 20x/0.70 UPLANSAPO objective lenses and linked to a confocal light path (WaveFX; Quorum Technologies™). Briefly, mice were anesthetized with Ketamine and xylazine (200mg/kg and 10mg/kg, respectively). Next, the animals were catheterized through the tail vein and after abdominal surgery, the liver was appropriately exposed. Mice were intravenously injected with 4μL αEpCAM-APC

(eBioscience™) and 4µL αLy6G-PE (eBioscience™) monoclonal antibodies (0.5mg/mL) 4 hours prior SD-IVM. Velocity software (Perkin Elmer) was used to drive the confocal microscope. Acquired images were analyzed or exported as TIF images using Velocity software. Exported images were analyzed by ImageJ.

Human liver tissue clearing immunofluorescence

Liver tissue clearing and immunofluorescence from cirrhotic patients was performed following iDISCO protocol [2] with minor modifications. Briefly, liver tissue samples around 1mm thick were fixed in 4% paraformaldehyde at 4ºC overnight with shaking. Then, samples were washed three times in DPBS-/- with shaking at room temperature for 30 minutes. Samples were then dehydrated with methanol/water series and further bleached with 5% peroxide hydrogen in methanol overnight at 4ºC. Samples were then rehydrated with methanol/water series, washed and permeabilized at 37ºC for 2 days. After incubation in blocking solution for additional 2 days at 37ºC, samples were incubated with primary antibodies KRT7 (1:50, DAKO, M7018) and MPO (1:50, Abcam, ab9535) for 5 days at 37ºC. After one day of serial washes, secondary antibodies donkey anti-rabbit Cy3 (1:500, Jackson ImmunoResearch, 711-165-152) and goat anti-mouse Alexa Fluor 488 (1:200, Invitrogen, A11001) were added for 4 days at 37ºC. Samples were washed throughout a day, dehydrated with methanol/water series and incubated in 66% and 100% DCM (Sigma 270997-12) solutions for 3 hours and 15 minutes. Finally, after incubation with DiBenzyl Ether (DBE, Sigma 108014), sample images were acquired with Confocal LSM880 Zeiss Microscope using Imaris Cell Software.

Neutrophil isolation

Liver samples were mashed through a 70µm cell strainer and digested with collagenase solution containing 50 mg/mL of collagenase A (Sigma-Aldrich, 37170821) and 0.01 mg/mL of DNaseI (Sigma-Aldrich, 10104159001) for 30 minutes at 37ºC. Digested samples were centrifuged at 50g for 3 minutes without break to precipitate hepatocytes. Supernatants were collected and pelleted. Pellets were suspended in 8 mL of 40% percoll solution and layered onto 3 mL of 70% percoll. After 30minutes centrifuge at 800g without break, the cell layer containing granulocytes was collected and washed twice with DPBS-/-.

Blood samples were washed with 2ml of FACS buffer (2% heat-inactivated FBS, 1% sodium azide in DPBS-/-) at 1500rpm for 5 minutes. Erythrocytes were lysed with Lysing Buffer (BD PharmaLyse) for 10 minutes at 37ºC.

Neutrophil isolation from bone marrow was performed as previously described [3]. Femurs and tibias were cut out and muscles removed. Bones were placed in a petri dish with HBSS-/- supplemented with 0.5% inactivated FBS (HBSS-prep). The ends of the bones were cut, and bone marrow was flushed into a 50 mL conical tube with a 25G needle and a 10 mL syringe filled with HBSS-prep. Suspension was pelleted at 400g for 5 minutes and resuspended in 10 mL of 0.2% NaCl for 30-40 seconds to lyse. Osmolarity was restored with 10 mL of 1.6% NaCl and filtered through a 70µm cell strainer. Lysed suspension was resuspended in 5 mL of HBSS-prep layered over 62.5% of percoll in HBSS-/- and centrifuged at 1000g for 30 minutes without break. A cloudy pellet containing neutrophils was collected after centrifugation.

Collected cells from each organ were then used for flow cytometry analysis or immunomagnetic isolation.

RNA Sequencing of liver and blood neutrophils

Liver and circulating neutrophils from 1-week DDC-treated mice and liver neutrophils from control mice (n=3 mice per group) were isolated by Ly6G-immunomagnetic separation (Anti-Ly-6G MicroBeads UltraPure kit, Miltenyi Biotec, Gladbach, Germany) after liver suspension percoll gradient and blood lysis. RNA was isolated using the commercial kit RNeasy Micro Kit (Qiagen) following manufacturer's instructions. The quantity and quality of the RNAs were evaluated using Nanodrop and Agilent RNA 6000 Pico Chips (Agilent Technologies, Cat.# 5067-1513). Sequencing libraries were prepared using "SMARTer Stranded Total RNA-seq Kit v2 – Pico Input Mammalian" kit (Takara Bio USA, Cat.# 634411), following "SMARTer Stranded Total RNA-seq Kit v2 – Pico Input Mammalian User Manual (Rev. 063017)". Paired-ended sequencing was performed on Illumina platform. STAR program [4] against Rattus norvegicus genome (Rn6.0) was used for mapping the reads followed by the quantification of genes with the RSEM [5] program using Ensembl reference annotation v-91. After excluding genes with at least an expected value greater than ten, we used TMM method and limma-voom transformation [6] to normalize the non-biological variability. Differential expression between different groups was assessed using moderated t-statistics [7]. Principal component Analysis plots, Gene Ontology analysis and normalized expression heatmaps were built using R statistics software. Protein-protein interaction network from top 50 differentially expressed genes ($p<0.05$) in circulating versus intrahepatic neutrophils was generated by string-db.org platform. Disconnected nodes and networks were removed. Resulting genes were analysed using the pathway analysis option from string-db.org.

Immunohistochemistry and immunofluorescence analysis

Paraffin embedded liver sections (3 μm) were stained for KRT7 (1:50, Dako, M701801), KRT19 (1:100, TROMA III, AB_2133570), SOX9 (1:500, Millipore, ab5535), EpCAM (1:200, Abcam, ab71916), CCND1 (1:100, Abcam, ab134175), CD31 (1:50, Abcam, ab28364), KI67 (1:50, abcam, ab16667), KRT7 (1:500, Thermofisher, 15539-1-AP), cleaved Caspase 3 (1:100, Cell signaling, 9661S), MPO (1:100, RyD, AF3667), P21 (1:5, HUGO 291, CNIO), S100A8/9 (1:100, Abcam, Ab22506), CD177 (1:50, Abcam, Ab203025), KI67 (1:50, Thermofisher, 14-5698-82), Ly6G (1:500, Biolegend, 127602), citrullinated histone H3 (1:1000, Abcam, Ab5103) and MPO (1:50, Abcam, ab9535).

Sections were deparaffinized and incubated in Target Retrieval Solution (Citrate Ph6, DAKO, Glostrup, Denmark), heated in a pressure cooker for 20 minutes, or rehydrated and antigen retrieved with EnVision Flex Target Retrieval Solution Low or High PH in a DAKO PT Link. Samples were incubated with primary antibody overnight at 4ºC. After washing in PBS, sections were incubated with secondary antibody and Diaminobenzidine (DAB, Dako) was used as a chromogen. Sections were then counterstained with hematoxylin.

For immunofluorescence staining, secondary antibodies donkey anti-rabbit Cy3 (1:500, Jackson ImmunoResearch, 111-165-003), donkey anti-rat Alexa Fluor 488 (1:200, Jackson ImmunoResearch, 712-546-153), donkey anti-goat Alexa Fluor 488 (1:200, Jackson ImmunoResearch, 705-545-003) and goat anti-mouse Alexa Fluor 488 (1:200, Invitrogen, A11001) were incubated for 30 minutes at room temperature and eventually, sections were mounted with Mounting Medium for Fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) for nuclear staining.

For detection of liver fibrosis, Sirius red staining was performed. Liver sections (5 µm) were deparaffined and hydrated in distilled water. Afterward, sections were incubated in thiosemicarbazide 0.5% for 10 minutes prior to incubation for 1 hour in a 0.1% Sirius Red solution. Finally, sections were dehydrated in alcohol and mounted with DPX Mounting Medium. Stained area quantification was performed by taken 10-15 random images at 20x magnification. Subsequently, percentage of stained area versus total image area was quantified with ImageJ. The number of positive CD31 vessels, CCND1 positive nuclei, citH3 and Ly6G positive neutrophils per field of view were manually counted at 20x or 40x magnification images using Fiji. Minimum distance between neutrophils (MPO+) and biliary cells (KRT7+) was calculated using QuPath software.

Mouse biliary organoid culture and conditioned medium collection

Liver organoids were obtained from control and 1-week DDC-treated mice. Mouse biliary organoids were generated following the stablished protocol by Broutier et al. [8] with minor modifications. Organoids were cultured in the defined expansion biliary medium consisting of Advanced DMEM/F12 (Thermo Fisher Scientific) supplemented with 1% HEPES (Gibco), 1% GlutaMax (Gibco), 1% Pen-Strep, 1x N2 supplement and 1x B27 supplement (without vitamin A) from Gibco; 250ng/mL recombinant human RSPO1, 50 ng/mL recombinant human HGF and 100 ng/ml recombinant human FGF10 from Preprotech; 50 ng/mL mouse EGF (RyD), 1,25mM N-acetylcysteine, 10nM human [Leu^{15}]-gastrin I, and 10mM Nicotinamide from Sigma and 10µM Rho Inhibitor γ-27632 (Axon Medchem). Medium was changed every 2 days and passaged every week as previously described [8]. When organoids reached 80-90% confluency, expansion biliary medium was replaced by basal medium consisting in Advanced

DMEM/F12 (Thermo Fisher Scientific) supplemented with 1% HEPES (Gibco), 1% GlutaMax (Gibco), 1% Pen-Strep. After 18 hours, organoid conditioned medium was collected and centrifuged for debri removal before using in neutrophil *in vitro* assays. All organoid lines used in this study has been confirmed to be negative for mycoplasma.

Neutrophil and biliary organoid co-culture

Mouse liver organoids were digested using Tryple Express (Gibco) for 30 minutes at 37ºC and neutrophils from WT and $NE^{-/-}$ bone marrows were isolated as described in the Neutrophils Isolation methods section. Organoid cells were stained with Vybrant CFDA Cell Tracer (Thermofisher, V12883) and neutrophils were labelled with WGA-AF594 dye (Thermofisher, W11262) following manufacturer's protocol. Number of viable cells was counted and 65.000 organoid cells were mixed with 325.000 WT or $NE^{-/-}$ neutrophils (ratio 1:5) per condition. Mixed organoid-neutrophils conditions and control conditions (only organoids and only neutrophils) were stimulated with 50nM PMA (MedChemExpress) and plated in a 24-well ultralow-attachment microcavities plate (Corning). As a culture medium, 20% of organoid expansion medium diluted in organoid basal medium (Advanced DMEM/F12, 1% HEPES, 1% GlutaMax, 1% Pen-Strep) was used. WT and $NE^{-/-}$ bone marrow neutrophils and PMA were refreshed every 3 days. Organoid area after 7 days of co-culture was measured in at least 40 microcavities per condition and percentage was obtained after normalizing organoid area per microcavity area.

Neutrophil migration assay

Circulating neutrophils were isolated from 1-week male and female DDC mice by Ly6G-immunomagnetic separation (Anti-Ly-6G MicroBeads UltraPure kit, Miltenyi Biotec, Gladbach, Germany) after blood lysis. 600uL of organoid conditioned medium or vehicle (organoid basal medium) were added to low-attachment 24 well-plate. 3.0 µm-pore transwells (CellQuart, #9323002) were placed on the top of the added medium and after pre-treatment of neutrophils with 50µM SCH-527123 (MedChemExpress) or vehicle for 45 minutes, 250.000 neutrophils per condition were added within the insert. Migration was allowed for 2 hours and migrated neutrophils were manually quantified using Neubauer Chamber and Trypan-blue dead cell exclusion.

In vitro stimulation of neutrophils with LPS and biliary organoid conditioned medium

$Ly6G^+$ neutrophils were isolated from liver and blood samples from DDC mice (n=4 mice per group) using the Anti-Ly-6G MicroBeads UltraPure kit (Miltenyi Biotec, Gladbach, Germany) following the manufacturer's instructions. Enriched neutrophil bone marrow fraction was isolated by percoll gradient. 250.000 neutrophils were plated per well in a 48 well-plate. For LPS stimulation, liver and blood neutrophils were incubated with RPMI supplemented with LPS (100 ng/ml) or vehicle. After 6 hours, neutrophils for RNA extraction and supernatants were collected. For organoid conditioned medium stimulation and CXCR1/2 inhibitor treatment, bone marrow neutrophils were pre-treated for 45 minutes with 50µM SCH-527123 (MedChemExpress) or vehicle. Then, neutrophils were centrifuged and vehicle or organoid conditioned medium with or without SCH-527123 (50µM) were added. After 6 hours, neutrophils were collected for RNA extraction or flow cytometry analysis.

Cytokine detection on cell culture supernatant

Interleukin (IL) IL-6, IL-10 and tumor necrosis factor alpha (TNFα) concentrations were determined in the supernatants of cultured neutrophils after LPS stimulation (250.000 neutrophils/condition) using a Milliplex® MAP Mouse high sensitivity Cytokine/Chemokine Detection Panel (a multiple immunoassay based on Luminex Technology) following the manufacturer's instructions (EMD Millipore, Germany).

RNA Isolation and mRNA Expression Analysis

Total RNA was extracted from total liver tissue of mice models using Trizol (Life Technologies, Carlsbad, CA) and from cultured neutrophils using the commercial kit RNeasy Micro Kit (Qiagen) following manufacturer's instructions. Total RNA extracted was assessed by Nanodrop (ThermoScientific) . Gene expression quantification was accomplished using Taqman gene expression assay probe and- primers and Platinum SYBR Green qPCR SuperMix (Thermofisher, Ref. 11733046) and PrimeTime® qPCR Primers (IDT). Quantitative real-time PCR (qPCR) was performed with an ABI 7900 HT cycler (Life Technologies) and QuantStudio 7 Pro (Applied Biosystems). Expression values were calculated based on the ΔΔCt method. The results were expressed as $2^{\wedge\text{-}\Delta\Delta Ct}$.

Flow cytometry analysis for phenotypic characterization

Neutrophils from 1-week DDC-fed mice (n=3-6 mice per group) were isolated from liver and bone marrow using a percoll gradient whereas blood was only lysed for further processing. Prior to surface staining, isolated neutrophils were incubated with Mouse BD Fc Block™ (BD; #553141; 1:200) to block non-antigen-specific staining. Blocking was performed in a total volume of 50 uL of FACS

buffer (2% heat-inactivated FBS, 1% sodium azide in DPBS-/-) for 10 minutes at 4ºC. Following, cells were stained for 30 minutes at 4ºC in a final volume of 100 uL with the following fluorescent antibodies: Rat anti-mouse Ly6G-FITC (BD; #551460; 1:100), rat anti-mouse CD62L (L-selectin)-PE (eBioscience™; #12-0621-82; 1:100), rat anti-mouse CD11b-Alexa Fluor 647® (BD; #557686; 1:100), rat anti-mouse CD192 (CCR2)-BUV395 (BD, #747972, 1:200) and rat anti-mouse CD184 (CXCR4)-eFluor 450 (eBioscience™; #48-9991-82; 1:100). Cells were then washed and fixed in 4% paraformaldehyde for 15 minutes at room temperature. Afterwards, cells were washed and resuspended in 200 uL of FACS buffer before acquisition. The samples were evaluated by flow cytometry using a LSR Fortessa (BD). Positive gating was performed using a fluorescence minus one (FMO) control for each surface staining and calculations of median intensity fluorescence (MIF) were based on Ly6G+ cells. Data were analysed using BD FACSDIVA™ Software (BD) and FlowJo v10.8.1 Software.

Phagocytic and oxidative respiratory burst assays

The phagocytic and oxidative burst capacity of blood granulocytes from control mice (n=5 mice per group) compared to blood and liver granulocytes from 1-week DDC mice (n=5 mice per group) were analysed using Phagotest™ (BD; #341060) and Phagoburst™ (BD; #341058) kits, respectively. A density of $1x10^6$ liver granulocytes or 100uL of heparinized whole blood were used per tube. For phagocytosis measurement, 5uL of opsonized FITC-labelled E. coli were added per tube and samples were incubated on ice (control samples) or at 37°C (test samples) for 60 minutes. In the case of burst analysis, neutrophils were incubated with reconstituted DHR125 solution (1:10) at 37ºC for 5 minutes followed by an incubation with wash buffer (control samples) or 675nM phorbol 12-myristate 13-

acetate (PMA) (test samples) for 30 minutes at 37ºC. After incubation, phagocytosis-related samples were placed on ice and quenching solution was added to discriminate between surface-bound and internalized bacteria fluorescence. Blood and liver granulocytes were stained with rat anti-mouse Ly6G-APC (BD; #560599; 1:100) and rat anti-mouse Ly6G-PE (BD; #551461; 1:100). Blood was further lysed and fixed. Finally, cells were resuspended in 10% heat inactivated FBS in RPMI containing DNA staining solution for flow cytometry analysis (FACS Canto II). Positive gating was performed based on control samples. Phagocytic and burst index measures: (% of DHR^+/FITC-E. $coli^+$ cells * MFI of DHR^+/FITC-E. $coli^+$ cells)/100. Data were analysed using BD FACSDIVA™ Software (BD).

EdU staining assay

To evaluate the retention time of liver neutrophils in response to 1 week of DDC diet injury, 5-ethynyl-2′-deoxyuridine (EdU) was injected intraperitoneally into mice (n=4 mice per time point) at 50mg/kg. After 1, 2, 3, 4, 5, 6, 7, 8 or 9 days of EdU injuection, all mice were sacrified. Neutrophils from BM, liver and peripheral blood were isolated by Ly6G-immunomagnetic separation and were stained for EdU following the manufacturer's protocol of Click-iT™ Plus EdU Flow Cytometry Assay Kit (Thermofisher; #C10646) with minor modifications. Briefly, cells were resuspended in 1% BSA in DPBS-/- at $1x10^7$cells/mL. Cells were stained with rat anti-mouse Ly6G-FITC (BD; #551460; 1:100) for 30 min at 4ºC and washed. Following, cells were fixed and permeabilized and EdU was detected in a final volume of 600uL with a cocktail containing Alexa Fluor™ 350 picolyl azide (1:1000), copper protectant (1:5) and 1X Click-iT™ EdU buffer additive (1:10) in D-PBS. Finally, cells were washed, resuspended in 200uL of FACS buffer and

analysed by flow cytometry (LSR Fortessa; BD). Neutrophils isolated from a DDC-fed mouse without EdU injection were used as a negative control. Percentage of EdU^+ cells was determined based on $Ly6G^+$ cells. Data were analysed using BD FACSDIVA^{TM} Software (BD).

Statistical Analysis

Experimental data are presented as mean values ± SEM. All parameters analyzed followed normal distribution by Shapiro-Wilk test unless indicated in the figure legend. For normal distributed data, unpaired two-tailed t test was used when 2 groups were compared, and comparison of more than two datasets was done using one-way analysis of variance (ANOVA) with Turkey's post-test or two-way ANOVA with Dunnett's post-test. For non-normal distributed data, Mann-Whitney test was used when 2 groups were compared, Kruskal-Wallis with Dunn's post-test or two-way ANOVA with Sidak's post-test were used when more than two groups were compared. Statistical analyses were performed using GraphPad software.

Supplementary Figures

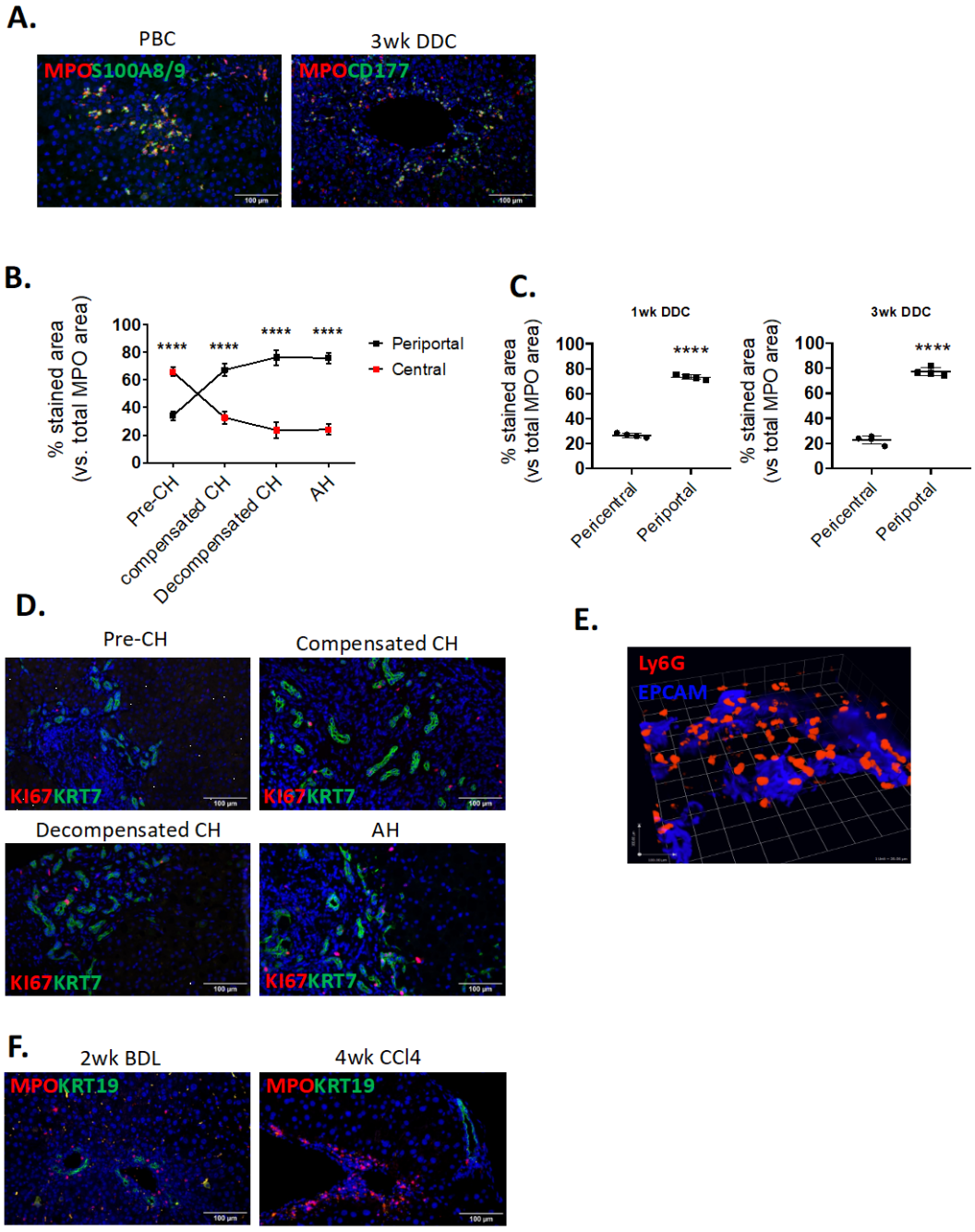


Fig. S1. DRANs are recruited to biliary progenitor cells. **(A)** Representative images of neutrophils recruited to periportal areas co-stained for neutrophil markers MPO and S100A8/9 in human (primary biliary cholangitis, PBC) or MPO and CD177 in DDC-fed mice. **(B)** Percentage of periportal and central neutrophils throughout alcohol-related liver disease (ALD) progression: early (n=5), compensated cirrhosis (CH) (n=5), decompensated cirrhosis (n=5) and alcohol-related hepatitis (AH) (n=5). **(C)** Neutrophil quantification in pericentral and periportal (DRANs) areas in DDC-treated mice**. (D)** Representative images of

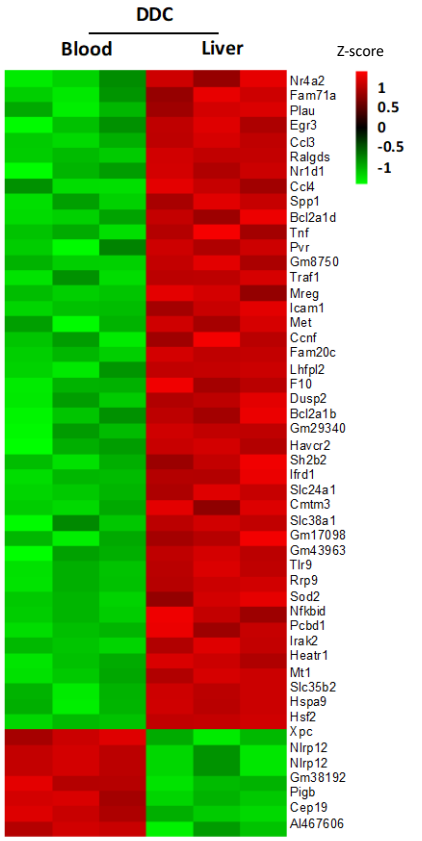
KI67 and KRT7 staining in ALD. Scale bar: 100 μm. **(E)** Representative intra-vital images showing DRANs (Ly6G, red) recruitment at DR cells (EpCAM, blue) in a mouse subjected to bile duct ligation for 5 days. **(F)** Representative immunofluorescence of neutrophil (MPO) distribution in relation to biliary cells (KRT7) in bile-duct ligation (BDL) and CCl4 liver injury mice models. Scale bars: 100 μm. Data presented as mean ± SEM. ****p<0.0001 as determined by Two-way ANOVA with Sidak's multiple comparison test (B) or Unpaired t-test (C).

A.

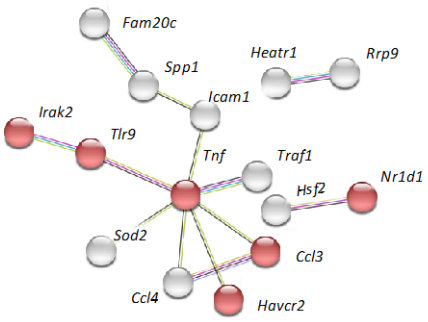
	Bone marrow				Blood				Liver			
	Absolute % EdU+ Ly6G+ cells	SEM	Normalized % EdU+ Ly6G+ cells	SEM	Absolute % EdU+ Ly6G+ cells	SEM	Normalized % EdU+ Ly6G+ cells	SEM	Absolute % EdU+ Ly6G+ cells	SEM	Normalized % EdU+ Ly6G+ cells	SEM
Day 1	5,267	0,994	10,523	1,986	0,450	0,210	1,010	0,472	0,625	0,217	1,832	0,637
Day 2	38,075	2,790	76,074	5,575	0,550	0,155	1,235	0,349	2,675	1,776	7,839	5,204
Day 3	**50,050**	0,811	**100,000**	1,620	39,633	11,362	66,723	28,634	20,473	9,365	59,993	27,444
Day 4	39,875	3,095	79,670	6,184	**44,550**	4,699	**100,000**	10,548	**34,125**	6,173	**100,000**	18,089
Day 6	16,525	3,333	33,017	6,660	21,000	8,104	47,138	18,191	29,575	5,551	86,667	16,268
Day 7	9,450	3,080	18,881	6,154	15,467	6,899	34,720	15,487	26,600	5,550	77,949	16,264
Day 8	1,550	1,022	3,097	2,042	2,575	1,120	5,780	2,514	9,225	3,926	27,033	11,506
Day 9	1,100	0,248	2,198	0,496	2,950	0,868	6,622	1,949	5,950	1,206	17,436	3,534

Fig. S2. Liver neutrophils remain in the liver for 3.5 days before decaying. **(A)** Absolute percentage (%) of EdU+ Ly6G+ cells and normalized % of EdU+ Ly6G+ cells (versus the day of maximum percentage per organ) are shown for every day and for the bone marrow, blood and liver neutrophils. Maximum normalized % of cells and absolute % used for normalization are shown in bold. Data is presented as mean of 3-4 mice and SEM is shown.

A.



B.



C.

	Gene ontology pathways	p. adjusted
GO:0030155	regulation of cell adhesion	1.99E-11
GO:0050900	leukocyte migration	3.44E-09
GO:0001819	positive regulation of cytokine production	1.72E-14
GO:0002237	response to molecule of bacterial origin	5.65E-13
GO:0032611	interleukin-1 beta production	2.44E-11
GO:0045321	leukocyte activation	2.78E-11
GO:0042110	T cell activation	3.44E-09
GO:0002224	toll-like receptor signaling pathway	3.52E-08
GO:0006909	phagocytosis	3.88E-08
GO:1903409	reactive oxygen species biosynthetic process	2.03E-07
GO:1903555	regulation of tnf superfamily cytokine production	2.99E-07
GO:0032635	interleukin-6 production	2.65E-06
GO:0045428	regulation of nitric oxide biosynthetic process	2.87E-06
GO:0048661	positive regulation of smooth muscle cell proliferation	3.06E-06
GO:1904018	positive regulation of vasculature development	6.27E-06
GO:0045766	positive regulation of angiogenesis	1.69E-05

D.

	Gene Ontology pathways	p. adjusted
GO:0016054	organic acid catabolic process	4.8E-06
GO:0009063	cellular amino acid catabolic process	2.1E-05
GO:0033539	fatty acid beta-oxidation using acyl-CoA dehydrogenase	6.9E-03
GO:0009083	branched-chain amino acid catabolic process	1.1E-02
GO:0055114	oxidation-reduction process	1.3E-02
GO:0006635	fatty acid beta-oxidation	1.3E-02
GO:0071346	cellular response to interferon-gamma	3.6E-03
GO:0035458	cellular response to interferon-beta	3.9E-03
GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	6.9E-03
GO:0050870	positive regulation of T cell activation	3.1E-02

Fig. S3. Liver neutrophils acquire an altered phenotype in chronic liver damage. (A) Heat map of the top-50 differentially expressed genes between liver and circulating neutrophils in DDC mice. **(B)** String analysis of top-50 differentially expressed genes. Only connected nodes are represented in the network. Genes corresponding to *Cellular response to polysaccharide* (GO 0071222) are shown in red. **(C).** Gene ontology enriched pathways in DDC-liver neutrophils when compared to control liver neutrophils. **(D).** Gene ontology enriched pathways in control liver neutrophils when compared to DDC-liver neutrophils.

A

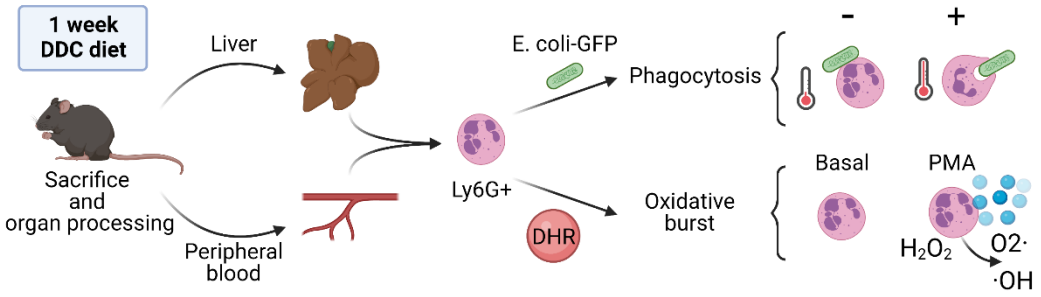


Fig. S4. Liver neutrophils present an altered functionality. (A) Graphical scheme of phagocytosis and oxidative burst assessment in liver and circulating neutrophils isolated from 1-week treated DDC mice.

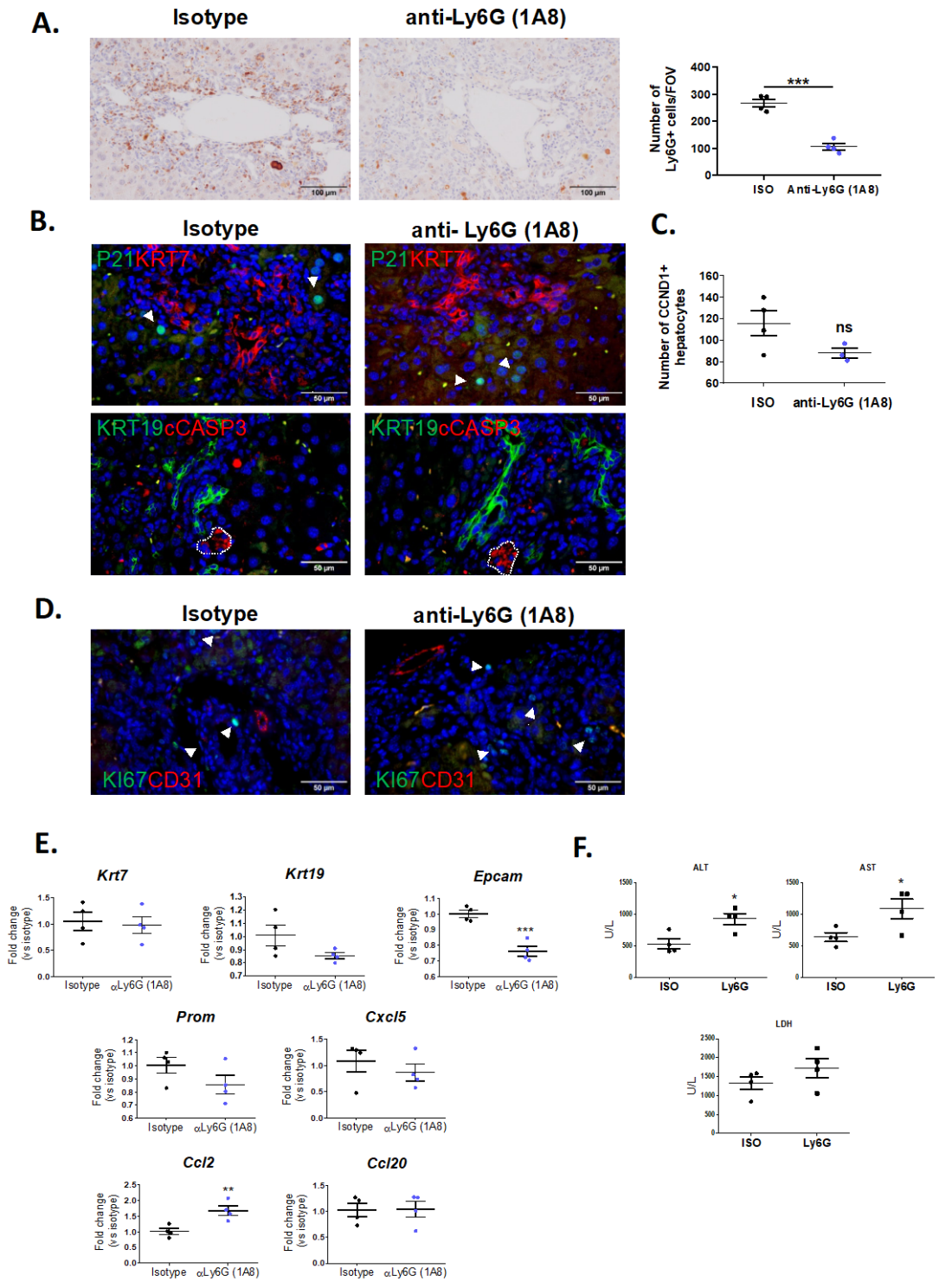


Fig. S5. Neutrophil depletion impacts ductular reaction expansion in chronic liver injury. As a depletion model, mice were treated with αLy6G (1A8) antibody or isotype (500µg each, n=4 mice per group) together with DDC diet for 3 weeks. **(A)** Representative immunohistochemistry of Ly6G. Quantification of manually counted Ly6G+ cells per field of view (FOV) is shown. **(B)**

Representative immunofluorescence images of senescence (P21) and apoptosis (cleaved caspase 3 (cCASP3)) markers together with KRT7 in anti-Ly6G and isotype treated groups. Arrows point senescent nuclei (P21) and dashed lines marks apoptotic areas (Ccasp3). Scale bars: 50 μm. **(C)** Quantification of the number of Cyclin D1 (CCND1) positive hepatocytes per FOV. **(D)** Representative staining of KI67 and the endothelial cell marker CD31. Scale bars: 50 μm. **(E)** Gene expression analysis of progenitor cells (*Krt7, Krt19, Epcam, Prom*) and inflammation (*Ccl2, Ccl20, Cxcl5*) markers. **(F)** Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Data presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.0001, ns: non-significant, as determined by Unpaired t-test.

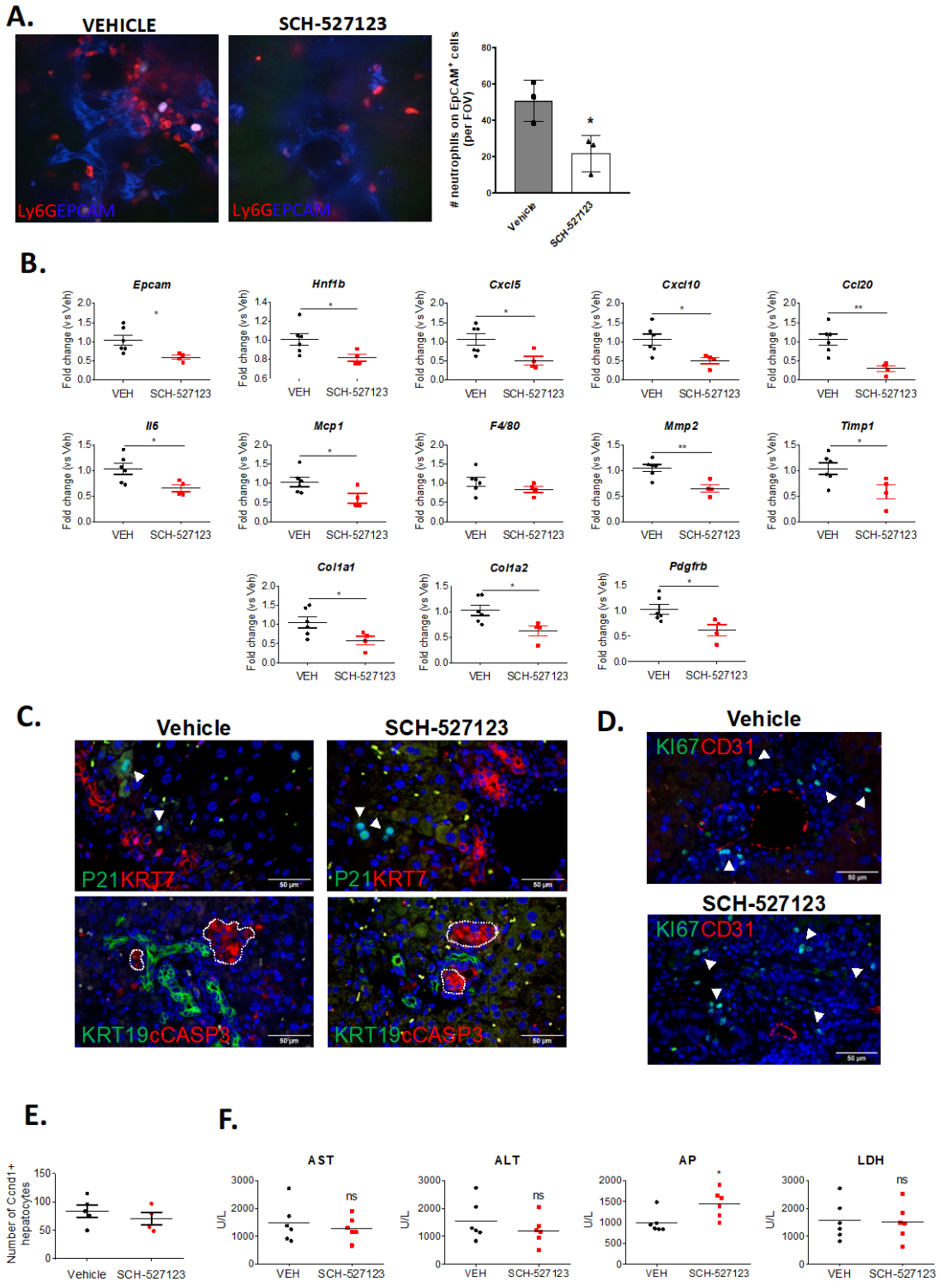


Fig. S6. Long-term inhibition of neutrophil recruitment attenuates liver injury in DDC-induced chronic injury. First, mice were simultaneously fed with DDC and daily treated with vehicle or CXCR1/2 inhibitor (50mg/Kg) for 3 weeks (n=4-6 mice per group). **(A)** Representative SD-IVM images of DRANs recruited at the portal injury site (EpCAM$^+$ cells) in mice receiving DDC for 1 week and

treated with CXCR1/2 inhibitor (SCH-527123) or vehicle. Quantification of neutrophils recruited at biliary epithelium in both groups is shown. Each symbol represents a field of view (FOV). **(B)** Whole liver gene expression analysis of key markers of inflammation (*Cxcl5, Ccl20, Il6, Mcp1, F4/80, Cxcl10*), fibrosis (*Timp1, Col1a1, col1a2, Mmp2, Pdgfrb*) and DR (*Epcam, Hnf1b*) from mice treated with the inhibitor or vehicle (n=4-6 mice per group) for 3 weeks. **(C)** Representative immunofluorescence images of senescence (P21) and apoptosis (cleaved caspase 3 (cCASP3)) markers together with KRT7 in SCH-527123 and vehicle groups. Arrows point senescent nuclei (P21) and dashed lines marks apoptotic areas (cCASP3). Scale bars: 50 μm. **(D)** Representative staining of KI67 and the endothelial cell marker CD31. Scale bars: 50 μm. **(E)** Quantification of the number of Cyclin D1 (CCND1) positive hepatocytes per FOV. **(F)** Serum levels of ALT, AST, AP and LDH in both groups. Data presented as mean ± SEM. *p<0.05, **p<0.01, ns: non-significant, as determined by Unpaired t-test (A, B, AST, ALT and LDH in F) and Mann-Whitney test (AP in F).

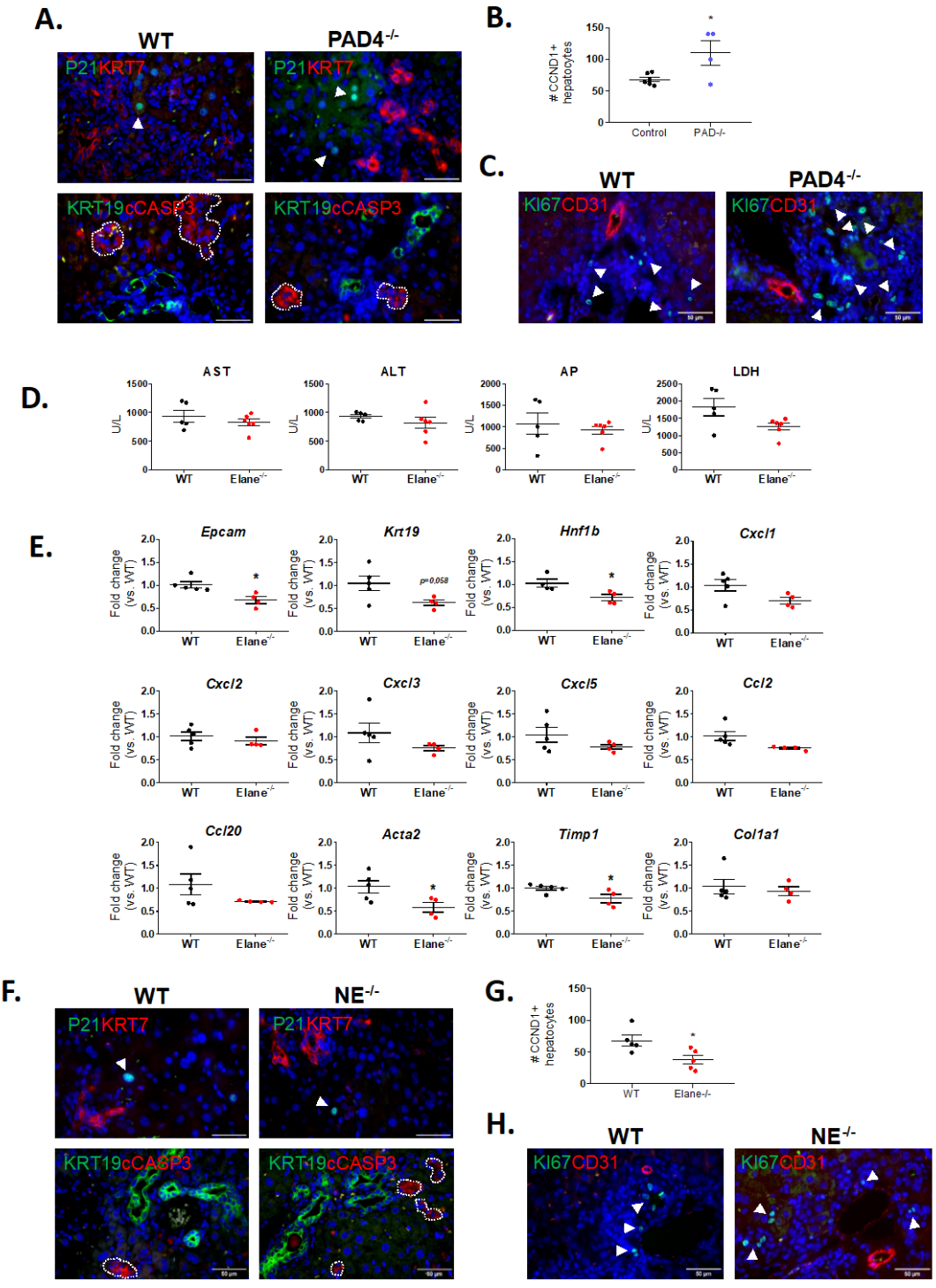


Fig. S7. Absence of elastase release ameliorates biliary progenitor cell expansion. (A) Representative immunofluorescence images of senescence (P21) and apoptosis (cleaved caspase 3 (cCASP3)) markers together with KRT7 in WT and PAD4$^{-/-}$ mice treated with DDC for 3 weeks. Arrows point senescent nuclei (P21) and dashed lines marks apoptotic areas (cCASP3). Scale bars: 50 µm. **(B)** Quantification of the number of Cyclin D1 (CCND1) positive hepatocytes

per FOV. **(C)** Representative staining of KI67 and the endothelial cell marker CD31. Scale bars: 50 µm. **(D)** Serum levels of ALT, AP, AST and LDH. **(E)** Whole liver gene expression analysis of biliary/progenitor (*Hnf1β, Krt19, Epcam*), inflammation (*Ccl2, Ccl20, Cxcl5, Cxcl3, Cxcl2, Cxcl1*) and fibrosis (*Acta2, Timp1, Col1a1*) markers. **(F)** Representative immunofluorescence images of senescence (P21) and apoptosis (cleaved caspase 3 (cCASP3)) markers together with KRT7 in WT and $NE^{-/-}$ mice treated with DDC for 3 weeks. Arrows point senescent nuclei (P21) and dashed lines marks apoptotic areas (cCASP3). Scale bars: 50 µm. **(G)** Quantification of the number of Cyclin D1 (CCND1) positive hepatocytes per FOV. **(H)** Representative staining of KI67 and the endothelial cell marker CD31. Scale bars: 50 µm. Data is presented as mean ± SEM. *$p<0.05$ as determined by Unpaired t-test.

Supplementary Videos data legends

Supplementary movie 1. Chronic liver injury results in neutrophil recruitment at human biliary epithelium. Representative video of neutrophils (MPO) recruited to biliary epithelium cells (KRT7) in a cleared liver sample of a patient with chronic liver injury.

Supplementary movie 2. Interaction of DRANs with ductular reaction cells in mice receiving DDC for 1 week. 3D reconstruction demonstrates that neutrophils (Ly6G) directly interact with ductular reaction cells (KRT7).

Supplementary movie 3. Static behavior of DRANs in mice receiving DDC for 1 week: Intra-vital visualization of the static behavior of neutrophils (Ly6G) recruited at the biliary epithelium (EpCAM).

Supplementary movie 4. Static behavior of DRANs attached to biliary epithelium in mice receiving DDC for 1 week and subjected to focal thermal injury: Intravital visualization showing that neutrophils retained at ductular reaction structures are not mobilized towards a sterile (acute) injury. Video was recorded in DDC mice at 24 hours after focal thermal injury.

Supplementary references

[1] Swamydas M, Luo Y, Dorf ME, Lionakis MS. Isolation of Mouse Neutrophils. Curr Protoc Immunol 2015;110:3.20.1-3.20.15. https://doi.org/10.1002/0471142735.IM0320S110.

[2] Renier N, Wu Z, Simon DJ, Yang J, Ariel P, Tessier-Lavigne M. iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging. Cell 2014;159:896–910. https://doi.org/10.1016/J.CELL.2014.10.010.

[3] Mócsai A, Zhang H, Jakus Z, Kitaura J, Kawakami T, Lowell CA. G-protein–coupled receptor signaling in Syk-deficient neutrophils and mast cells. Blood 2003;101:4155–63. https://doi.org/10.1182/blood-2002-07-2346.

[4] Dobin A, Gingeras TR. Mapping RNA-seq Reads with STAR. Curr Protoc Bioinformatics 2015;51:11.14.1. https://doi.org/10.1002/0471250953.BI1114S51.

[5] Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 2011;12:1–16. https://doi.org/10.1186/1471-2105-12-323.

[6] Law CW, Chen Y, Shi W, Smyth GK. voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol 2014;15:1–17. https://doi.org/10.1186/GB-2014-15-2-R29.

[7] Smyth GK. limma: Linear Models for Microarray Data. Bioinforma Comput Biol Solut Using R Bioconductor 2005:397–420. https://doi.org/10.1007/0-387-29362-0_23.

[8] Broutier L, Andersson-Rolf A, Hindley CJ, Boj SF, Clevers H, Koo BK, et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. Nat Protoc 2016;11:1724–43. https://doi.org/10.1038/nprot.2016.097.