Supplemental material and methods

Mice and drug treatment

All experimental procedures were carried out in accordance with the UK laws, following the three Rs and with approval of the Home Office and local ethics committees (PPL P8999BD42 and PPL 7007578) in an unblinded manner. C57BL/6 wild-type (WT) mice were used to establish the experimental model of CPI-hepatitis. The role of lymphocytes in mediating hepatocyte damage was assessed in $Rag2^{-/-}$, lacking all mature lymphocytes, and mice treated with anti-CD4 or anti-CD8 depletion antibodies (all BioXCell). The contribution of monocytes to this pathology was assessed in Ccr2 knockout mice expressing red fluorescent protein ($Ccr2^{rfp/rfp}$) and mice treated with Cenicriviroc (CVC) (MedchemExpress, USA). CVC was dissolved in vehicle containing 0.5% methylcellulose (400cps) (VWR International Ltd, UK) + 1% Tween-80 (Fisher Scientific Ltd, UK) in water and administered in drinking water at 100mg/kg/day.

Stimuli of hepatic inflammation were trialled for the induction of experimental CPI-hepatitis by the i.p. administration of 20 µg/mouse TLR4 ligand (TLR4-L) [Monophosphoryl Lipid A (MPLA) VacciGrade, InvivoGen] or 20 µg/mouse TLR9 agonist (TLR9-L) CpG oligodeoxynuleotide 1668: 5-S-TCCATGACGTTC CTGATGCT-3) (TIB Molbiol, Germany) on D1, one day post the first administration of CPI or PBS. D1 mice served as baseline control, as these mice did not receive TLR4-L or TLR9-L for hepatic priming.

Blood and liver tissue sampling

Mice were sacrificed by terminal anaesthesia receiving 0.2 ml of Pentoject (Centaur Services, UK) i.p. on D1, D4, D7, D10 and D14 post administration of CPIs or PBS. Deep anaesthesia was confirmed by checking paddle and eye reflexes and blood was subsequently collected by cardiac puncture of the right ventricle in blood collection tubes (Microvette, Sarstedt, Germany) to prevent clotting. Mice were then perfused using PBS. Following perfusion, liver tissue was excised and fixed in 10% formalin (Sigma-Aldrich, USA) or Optimal cutting temperature compound (OCT compound; VWR, USA) for histological examination, snap frozen in liquid nitrogen for mRNA analysis or kept on ice cold PBS for fresh cell staining. Plasma was collected by centrifugation of collected blood from the right ventricle.

Histology

4 µm thick liver sections from formalin-fixed paraffin-embedded (FFPE) liver tissue were stained with haematoxylin and eosin (H&E) and provided by the Research Histology Facility, Imperial College London.

Immunofluorescence microscopy

OCT fixed liver tissue was cut into 15 µm thick cryosections and stored at -80ºC. Before staining, slides were defrosted and fixed in ice cold acetone for 10 minutes. Slides were subsequently incubated with PBS and 5% BSA for 45 minutes to block unspecific binding. Anti-mouse CD8, F4/80, CD11b, CCR2, GZMB and albumin fluorescently labelled monoclonal antibodies (**Supplementary Tab.1**) were diluted in PBS, 0.1% Triton X-100 and 5% BSA at optimised concentrations and incubated for 1.5 hours at room temperature in a humidity chamber in the dark. Slides were

washed with PBS and 0.1% Triton X-100 three times for 5 minutes and once with PBS for 5 minutes before mounting with fluoroshield with DAPI (Sigma-Aldrich, USA). Slides were then stored in the dark at 4ºC.

Supplementary Table 1. List of monoclonal antibodies for immunofluorescent staining of cryosections.

Antibody	Fluorochrome	Clone	Working concentration	Cat. Number
anti-mouse F4/80	AF488	BM8	10 µg/ml	BioLegend #123120
anti-mouse CD8	APC	53-6.7	8 µg/ml	BioLegend #100712
anti-mouse Granzyme B	PE	QA16A02	4 µg/ml	BioLegend #372208
anti-mouse CD11b	PE	M1/70	4 µg/ml	BD #553311
anti-mouse CCR2	PE	SA203G11	2 µg/ml	BioLegend #150610
anti-albumin	AF647	n/a	10 µg/ml	Invitrogen # A34785

Immunohistochemistry

Double heat-induced epitope retrieval (HIER) immunohistochemistry (IHC) on FFPE tissue was performed to assess the expression of CD8 and F4/80. Tissue was stained manually overnight at 4ºC in a humidity chamber using anti-mouse monoclonal antibodies (**Supplementary Tab.2**). Antigen retrieval was carried out by HIER using EDTA Tris buffer pH 9. Staining was then performed using the EnVision™ G|2 doublestain system – rabbit/mouse (DAB+/permanent red) (Dako, Agilent Technologies, USA) and visualized with DAB and permanent red according to

the manufacturer's instructions. Slides were counterstained with haematoxylin (Agilent Technologies, USA). Images were captures with Leica DM4 B microscope (Leica Camera AG, Germany).

Supplementary Table 2. List of monoclonal antibodies for immunohistochemistry staining of FFPE sections

Antibody	Clone	Working concentration	Incubation	Cat. Number
anti-mouse F4/80	SP115	1:100	overnight at 4°C	Abcam #ab111101
anti-mouse CD8	EPR21769	1:1000	overnight at 4°C	Abcam #ab217344

Quantitative reverse transcription PCR (RT-qPCR)

Snap frozen liver tissue was thawed and homogenised using the TissueLyser II (Qiagen, Germany). RNA was extracted following manufacturer's instructions using the RNeasy Plus Mini Kit (Qiagen, Germany) and measured by nanodrop, reading the optical density at 260-280 nm. 1 µg of total RNA was reversed transcribed to cDNA using SuperScript IV reverse transcriptase with Random Hexamers (Invitrogen, USA). 100ng of cDNA and the 2xSensiMix SYBR Lo-ROX kit (Bioline, UK) were used for quantification of the genes listed in **Supplementary Tab.3** according to manufacturer's guidelines. The abundance of *Gapdh* mRNA was used for reference.

Supplementary Table 3. List of primers used for RT-qPCR

mRNA	Forward	Reverse
Cxcl9	TCGGACTTCACTCCAACACAG	AGGGTTCCTCGAACTCCACA
Cxcl10	TCTGAGTGGGACTCAAGGGAT	AGGCTCGCAGGGATGATTTC
Ccl2	CACTCACCTGCTGCTACTCA	GCTTGGTGACAAAAACTACAGC
Gapdh	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG

RNA sequencing

RNA was extracted as described for RT-qPCR. Extracted RNA was checked for sufficient quantity (Nanodrop A280, ThermoFisher, Wilmington, USA) and quality (Bioanalyzer 2100, Agilent, Santa Clara, USA). Library preparation was performed using NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs, Ipswich, USA). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers followed by the second strand cDNA synthesis. The library was ready after end repair, A-tailing, adapter ligation, and size selection. After amplification and purification, the insert size of the library was validated on a Bioanalyzer 2100 (Agilent, Santa Clara, USA) and quantified by PCR. Libraries were sequenced on Illumina NovaSeq 6000 S4 flowcell (Illumina, San Diego, SUA) using 150bp paired-end reads to a target sequencing depth of 40 million read pairs per sample.

Sequence data were evaluated for quality control issues using fastqc v0.11.9[1]. No adaptor or quality-based trimming was required. Reads were aligned to reference

genome GRCm39[2] and corresponding Ensembl genebuild release 104 using the STAR 2.7.9a aligner[3]. Transcript quantification was performed using the RSEM 1.3.3 algorithm[4]. Raw count data were analysed in R v4.0.4 (R Statisical Foundation, Vienna, Austria). Differential expression analyses were conducted using the limma-voom pipeline (limma[5] v3.46.0). Lowly expressed genes were filtered; count data were normalised and transformed and associated precision weights generated using the voom() function. Count data were modelled using a design model incorporating experimental group, blocked on batch and without an intercept (~0+Group+Batch); differential gene expression was estimated for contrasts of interest. A Benjamini-Hochberg false discovery rate correction was applied to resulting p-values. Transcription factor activity was computationally inferred using the dorothea[6] (v1.2.2) package; analyses were restricted to high quality regulons (A, B). Pathway analysis was conducted using Generally Applicable Gene-set Enrichment for Pathway Analysis (GAGE, gage[7] v2.40.2) and murine KEGG pathways (release 99.1) annotated as “signalling” or “metabolism”. A Benjamini-Hochberg false discovery rate correction was applied to resulting p-values. Differential expression results for comparisons of interest were mapped to KEGG pathways using the Pathview package (v1.0[8]).

Isolation of hepatic mononuclear cells

Liver tissue was mechanically dissociated using scalpels and passed through a 100 µm cell strainer (BD Biosciences, UK). Subsequently, the cell suspension was centrifuged at 60x*g* for 1 min at room temperature to pellet the hepatocytes. Mononuclear cells were then isolated using Optiprep (Sigma-Aldrich, USA) density gradient, according to manufacturer’s instructions. Subsequently, red blood cells

were lysed for 1 minute with ACK lysis buffer (Thermo Fisher Scientific, USA), followed by a final washing step in PBS.

Flow cytometry of liver immune cells and absolute cell counts

Isolated hepatic mononuclear cells were transferred into FACS tubes, resuspended in 100 µl FACS buffer and incubated with TruStain fcX™ (anti-mouse CD16/32) antibody (BioLegend, UK) for 10 minutes prior staining. Surface staining of cells was carried out in the presence of TruStain fcX™ using fluorochrome-labelled monoclonal antibodies listed in **Supplementary Tab.4**, for 25 minutes at room temperature in the dark. Following incubation, cells were washed in PBS and resuspended in 150 µl FACS buffer and 50 µl 123count eBeads (Thermo Fisher Scientific, UK). For intracellular staining, following the last wash, cells fixed, permeabilised and stained using the True-Nuclear™ Transcription Factor Buffer Set (BioLegend, UK), following manufacturer's instructions. Subsequently, cells were resuspended in 150 µl FACS buffer and 50 µl 123count eBeads (Thermo Fisher Scientific, UK) for acquisition. Fluorescence minus one (FMO) were used as controls.

Supplementary Table 4. List of monoclonal antibodies used for flow cytometry.

Surface marker	Fluorochrome	Clone	Cat. Number
anti-mouse F4/80	BV421	BM8	BioLegend #123137
anti-mouse IFNγ	BV421	XMG1.2	BioLegend #505830
anti-mouse Ly6G	BV605	1A8	BioLegend #127639

anti-mouse CXCR3	BV605	S18001A	BioLegend #155915
anti-mouse CD45	BV650	30-F11	BioLegend #103151
anti-mouse CD11b	BV711	M1/70	BioLegend #101242
anti-mouse CD8	BV711	53-6.7	BioLegend #100759
anti-mouse CD3	BV785	17A2	BioLegend #100232
anti-mouse CCR2	FITC	SA203G11	BioLegend #150608
anti-mouse Granzyme B	FITC	QA16A02	BioLegend #372206
anti-mouse CD8	FITC	53-6.7	Invitrogen #11-0081-85
anti-mouse Perforin	PE	S16009A	BioLegend #154306
anti-mouse CXCL9	PE	MIG-2F5.5	BioLegend #515604
anti-mouse CD64	PerCP/Cy5.5	X54-5/7.1	BioLegend #139308
anti-mouse CD19	PerCP	6D5	BioLegend #115532
anti-mouse Ly6C	PE/Cy7	HK1.4	BioLegend #128018
anti-mouse NK1.1	PE/Cy7	PK136	BioLegend #108714
anti-mouse CD4	AF647	GK1.5	BioLegend #100424
anti-mouse MHC Class II	APC-eFluor 780	M5/114.15.2	eBioscience #47-5321-82
anti-mouse TCRβ	APC/Cy7	H57-597	BioLegend #109220

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Supplementary figure legends

Supplementary figure 1. Determination of liver inflammation following CPI only, TLR9-L/CPI or TLR4-L/CPI treatment of mice. A) Measurement of ALT plasma levels in CPI or PBS only treated mice. B) Representative pictures of liver sections stained for TUNEL (red), albumin (green), and DAPI (blue) on D7 of TLR9-L/CPI and PBS-treated mice. Magnification: 100X (left). Representative pictures of liver sections stained for TUNEL (red), albumin (green), CD8 (white) and DAPI (blue) on D7 of TLR9-L/CPI and PBS-treated mice. Magnification: 200X, 400X (right). C) Experimental set-up of time course comparing CPI and PBS-treated mice across different time points and following TLR4-L administration. Measurement of ALT levels in plasma. D) Representative H&E stained liver sections of CPI or PBS-treated mice during TLR4-L time course (n=4/group/time point) compared to D7 TLR9-L/CPI (n=8). Magnification: 200X. Each symbol represents an individual mouse. ** p<0.01.

Supplementary figure 2. Liver inflammation following TLR9-L and prolonged CPI treatment of mice. A) Set-up of experimental CPI-hepatitis time course, comparing CPI (blue) and PBS (grey)-treated mice across different time points. B) Representative H&E stained liver sections of CPI or PBS-treated mice on D10 and D14 (n=4/group/time point). Magnification: 200X. C) Measurement of CK-18 levels in plasma during the prolonged time course. D) Absolute numbers of total liver $CD8^+$ T cells and $CCR2^+$ monocytes. Each symbol represents an individual mouse. * p<0.05, ** p<0.01, *** p<0.001. **** p<0.0001.

Supplementary figure 3. Determination of liver inflammation following TLR9-L and single or combination CPI treatment of mice. A) Measurement of CK-18

levels in plasma on D7 following treatments. B) Representative H&E stained liver sections of anti-CTLA-4, anti-PD-1, anti-CTLA-4+anti-PD-1, or PBS-treated mice on D7 (n=4/group/time point). Magnification: 100X. C) Representative H&E images of histological patterns in single CPI treated mice. D) Absolute numbers of total liver $CD8^+$ T cells and $CCR2^+$ monocytes on D7. Each symbol represents an individual mouse. * p<0.05, ** p<0.01, *** p<0.001.

Supplementary figure 4. Absolute number of liver lymphocytes following TLR9-L/CPI treatment. A) Representative contour plots of the gating strategy to identify liver lymphocytes. Absolute numbers of $CD4^+$ T cells (B) and NK, NKT and B cells (C). Each symbol represents an individual mouse. Results shown are representative of 1-3 independent experiments. * p<0.05, ** p<0.01.

Supplementary figure 5. Macrophage distribution in livers following TLR9-L/CPI treatment. A) Representative contour plots of the gating strategy to identify liver myeloid cells. B) Absolute numbers of liver Kupffer cells (KC), monocyte-derived macrophages (MoMF) and neutrophils in CPI and PBS-treated mice. C) Representative pictures of liver cryosections stained for CD11b (red) and DAPI (blue) (left) and F4/80 (green) and DAPI (blue) (right) on D7 of CPI and PBS-treated mice (n=4/group). Magnification: 400X. D) Representative pictures of FFPE liver sections of D7 CPI or PBS-treated mice stained for F4/80 (DAB, brown), CD8 (Permanent Red, red) and haematoxylin to identify nuclei (n=4/group). Magnification: 100X, 400X. E) Proportions of different myeloid cell populations within $CD45^+CCR2^+$ cells on D7 in CPI-treated mice. F) Representative histograms and frequency of $CCL2^+$ KCs in CPI and PBS-treated mice during the time course. Data showing fold change of $CCL2^+$ liver myeloid cells frequencies of D7 CPI normalised to D7 PBS (n=4/group). Each symbol represents an individual mouse. Results shown

are representative of 1-3 independent experiments. * p<0.05, ** p<0.01, *** p<0.001. **** p<0.0001.

Supplementary figure 6. Monocyte/CD8 distribution in livers following TLR9-L/CPI treatment. A) Representative pictures of liver cryosections stained for CD11b (red) and DAPI (blue); B) CD8 (white) and DAPI (blue); and C) CCR2 (red) and DAPI (blue) on D1, D4 and D7 of CPI and PBS-treated mice (n=4/group). Magnification: 400X.

Supplementary figure 7. Therapeutic inhibition of monocyte liver recruitment is during experimental CPI-hepatitis. A) Set-up of experimental CPI-hepatitis time course and CVC/vehicle treatment schedule. B) Representative contour plots pre-gated on $MHCII^-$ cells showing monocyte gating for D7 CVC (3 days of treatment) and D10 CVC (6 days of treatment), compared to vehicle treated mice. C) Absolute numbers of total liver $CCR2^+$ monocytes during the time course. D) Absolute numbers of total liver $CD8^+$ T cells (left) and representative liver sections of D10 vehicle vs CVC-treated mice stained for CD8 (white) and DAPI (blue) (n=4/group; magnification: 200X). E) Frequencies of total liver $GZMB^+$ and $perforin^+CD8^+$ T cells during the time course. F) Measurement of CK-18 in plasma (left) and representative H&E stained liver sections (n=4/group; magnification: 100X). Each symbol represents an individual mouse. * p<0.05, ** p<0.01, *** p<0.001. **** p<0.0001.