Supplementary Materials for

Resident Kupffer cells and neutrophils drive liver toxicity in cancer immunotherapy

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Other Supplementary Material for this manuscript includes the following:

Tables S1 to S8
Materials and Methods

Mice. All animals were bred and housed under specific pathogen free conditions at the Massachusetts General Hospital. Experiments were approved by the MGH Institutional Animal Care and Use Committee (IACUC) and were performed in accordance with MGH IACUC regulations. The following mouse strains were used in this study: Female C57BL6/J mice (7-14 week old) were purchased from Jackson Laboratories (Bar Harbor, ME); GREAT (IFN-γ-IRES-EYFP Cat #017581), IL-12p40-IRES-EYFP (Cat #006412), Batf3−/− (Cat #013755), Ifngr1−/− (Cat #003288), Rag2−/− (Cat #008449), CD45.1 (Cat #002014), μMT (Cat #002288), Il12p40−/− (Cat #002693), Zbtb46-Dtr (Cat #019506), Clec4f-Cre (Cat #033296), Lsl-Dtr (Cat #007900), Ifngr1-floxed (Cat #025394), Csf3r−/− (Cat #017838) were obtained from Jackson Laboratories. Additional B6 mice were bred/maintained in-house and congenic CD45.1STEM mice (50) were received from the Scadden lab and maintained in our facility.

Mouse tumor models. MC38 tumor cell lines were obtained from Dr. Mark Smyth (QIMR Berghofer). MC38 cells were implanted at 2 x 10^6 cells per tumor in the flank. TC-1 tumor cell lines were obtained from Dr. Sara I. Pai. TC-1 cells were implanted at 2 x 10^6 cells per tumor in the flank. Tumors were allowed to grow for one week prior to therapy. Pre-treatment tumor volumes were normalized between treatment groups. Tumor dimensions were measured using a digital caliper. On day 7 of tumor growth, tumor bearing mice were treated with 5 mg / kg (or ~100 µg) of aCD40 Clone FGK4.5 (BioXCell Cat #BE0016-2) intraperitoneally for aCD40 studies. Experimental readouts occurred two days following treatment, unless otherwise noted. For ICB, anti-PD-1 (Clone 29F.1A12) was generously provided by Gordon J. Freeman. Mice were treated with ~10 mg / kg anti-PD-1 and ~5 mg / kg anti-CTLA-4 (Clone 9D9, BioXCell Cat #BE0164) intraperitoneally on days 6, 7, and 8 of tumor growth, and tissues were analyzed on day 9.

Flow cytometry studies: Livers were excised and ~200-300 mg of tissue were taken for digestion and flow cytometry analysis. Tissue was minced using surgical scissors, then digested with 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I, 60 U/ml hyaluronidase (Sigma Aldrich), and 20 mM HEPES buffer in PBS at 37°C for 20 minutes shaking at 900 rpm. Digested tissue was then processed through a 40 µm cell strainer, centrifuged at 1500 rpm for 5 minutes, subjected to ACK red blood cell lysis, and resuspended in 0.5% BSA in PBS for staining. Tumors and Lungs and were isolated and minced using surgical scissors, then digested with 0.2 mg/ml Collagenase I (Worthington) in RPMI-1640 at 37°C for 30 minutes shaking at 900 rpm. Digested tumors were then processed through a 40 µm cell strainer, centrifuged at 1500 RPM for 5 minutes. Lungs were subject to ACK lysis for 5 minutes, and all samples were resuspended in 0.5% BSA in PBS for staining. Femurs were harvested and heads removed with a razor blade, then bones were flushed using a 26-g needle and 0.5% BSA in PBS until bones appeared white. Harvested cells were processed through a 40 µm cell strainer, subject to ACK lysis for up to 5 minutes and resuspended in 0.5% BSA for staining. Spleens were harvested and processed through a 40 µm cell strainer, subject to ACK lysis for 1-2 minutes, and resuspended in 0.5% BSA for staining. Colons were removed and flushed with buffer containing 5% FBS in PBS using a round-tipped needle. Fat layer
was removed using forceps and colons were opened longitudinally using round-tipped scissors, then cut into ~1.5 inch segments. Tissue was then manually agitated in 5% FBS to remove excess mucus, and placed into a 50 mL falcon tube with 5% FBS, 1 mM dithiothreitol (DTT) (Sigma-Aldrich) and 1.5 mM EDTA in RPMI-1640 (DTE buffer) to shake 2x at 37°C for 20 minutes, vortexing afterward to remove intraepithelial leukocytes (IEL). DTE and IEL were discarded after each incubation. Tissue was then minced using surgical scissors and digested in 1 mg / mL collagenase I (Worthington) in RPMI + 5% FBS at 37°C for 35-40 minutes shaking at 240 RPM. Digested tissue was processed through a 70 µm cells strainer and resuspended in 0.5% BSA for staining. Visceral fat pads were removed in DMEM + 2% BSA or 2% FBS and minced using surgical scissors into 1-2 mm pieces, then digested in 1.5 mg / mL collagenase I (Worthington) in DMEM+BSA buffer at 37°C for 20 minutes shaking at 240 RPM. Digested tissue was processed through a 70 µm cell strainer and resuspended in 0.5% BSA for staining. Peritoneal Lavage: Mice were anesthetized using isofluorane and cervical dislocation, then outer skin was snipped to expose peritoneal membrane. 5 mL 0.5% BSA was injected into the peritoneal cavity using a syringe + 25-27 gauge needle, gently massaged to harvest peritoneal cells, then collected by aspiration using the same needle and syringe. Cells were pelleted and resuspended in 0.5% BSA for staining. For all tissues, single cell suspensions were stained with Zombie Aqua viability dye (Biolegend), blocked with TruStain FcX (Biolegend), then stained with flow cytometry antibodies where indicated; antibodies are tabulated in table S7. Cells were quantified using Flow Cytometry Absolute Count Standard beads (Bangs Laboratories). Cells were collected on BD LSRII flow cytometer using BD FACS Diva software, then analyzed using FlowJo (Treestar). Intracellular cytokine staining: For intracellular staining of IL-12 protein, cells were stained for cell surface antigens as noted. Afterwards, cells were fixed and permeabilized using the BD Fixation / Permeabilization kit, then stained for intracellular IL-12, and finally fixed in 1% PFA before flow cytometry analysis. For comparing IL-12 production in WT vs. Batf3−/− mice (Fig. 3A), all mice were treated with 10 µg / gram of body weight of Brefeldin-A (Cayman Chemical Cat#11861) via retro-orbital injection 6h before euthanasia.

Cytokine neutralization: For IL-12p40 (Clone C17.8, BioXCell Cat# BE0051), IL-23p19 (Clone G23-8, BioXCell Cat#BE0313), and TNF-α (Clone XT3.11, BioXCell Cat#BE0058) neutralization studies, mice were dosed with 500 µg of antibody daily starting on day 7 of tumor growth and continuing for 1-2 days. For TC-1 tumor study, IL-12p40 was neutralized for 6 consecutive days. Neutralization of IFN-γ (Clone XMG1.2, BioXCell Cat# BE0055) was performed by administering 1 mg of antibody initially on day 7 of tumor growth with 500 µg of anti-IFN-γ dosed daily intraperitoneally for an additional days 1-2.

Lymphocyte depletion: T and NK cells were targeted using 100 µg / injection anti-CD4 (BioXCell Cat #BE0003), 200 µg / injection anti-CD8α (BioXCell Cat #BE0004), or 200 µg / injection anti-NK1.1 (BioXCell Cat #BE0036). Injections were initiated on day 6 of tumor growth and given every 2 days through day 10.

Neutrophil targeting: mice were injected with anti-Gr1 (BioXCell Cat #BE0075) i.p. at 100 µg per dose on day 6 and 8 of tumor growth before sacrifice on day 9. anti-Ly6G (BioXCell Cat #BP0075) was administered at 500
µg/mouse on day 7 (~2h before aCD40) and boosted with 250 µg/mouse on day 8 before sacrifice on day 9. For tumor growth studies, all mAbs were dosed one additional time on day 10 (100 µg anti-Gr1 or 250 µg anti-Ly6G). CXCR2 inhibitor SB 225002 (Tocris Cat #2725/10) was injected at 200 µg/dose following the same schedule as anti-Ly6G. For IL-12 and IFN-γ reporter mice, anti-Ly6G (Absolute Antibody Cat #Ab00295-2.0) was dosed at 200 µg/mouse daily on days 6-8. For anti-PD-L1-based targeting of neutrophils, anti-PD-L1 (BioXCell Cat #BE0101) was injected i.p. at 12.5 mg/kg, first ~10 hours after aCD40 treatment and again 1 day after treatment, each time followed by 12.5 mg/kg anti-Rat IgG2b (BioXCell Cat #BE0252) ~1 hour later.

**Histology:** Two days after aCD40 treatment, livers were excised and placed in ice cold PBS + 0.5% BSA. All tissues were fixed in 10% formalin overnight, then washed twice with PBS and placed in 70% EtOH or PBS until processing. Three days after treatment with aCD40, colons were excised, flushed with cold 5% FBS in PBS, opened longitudinally, rolled and tied loosely with a nylon suture (Ethicon). For H&E, tissues were paraffin embedded, sectioned, and stained with Hematoxylin and Eosin at the MGH Histopathology Research Core.

For MPO staining of mouse livers, fixed tissue was paraffin-embedded and sectioned (5 µm thickness) After deparaffination and rehydration, heat induced epitope retrieval (HIER) was performed using Retrievagen A (pH6.0) (550524, BD Biosciences) and the sections were permeabilized in 0.3% Triton X-100 in PBS for 10 minutes at RT. The tissue sections were treated with 1% H2O2 (H1009, Sigma-Aldrich) in dH2O for 10 minutes to block endogenous peroxidase activity and blocked with 4% normal goat serum in PBS for 1 hour at RT after rinsed with PBS. A Myeloperoxidase (MPO Ab-1, RB-373-A, 1:25; Thermo Fisher Scientific) antibody was incubated overnight at 4°C and a biotinylated goat anti-rabbit IgG secondary antibody and VECTASTAIN ABC Kit (BA-1000, 1:100 and PK-6100, Vector Laboratories) were used for color development. The reaction was visualized with AEC (3-amino-9-ethylcarbazole) substrate (K-3464, Dako/Agilent) and the sections were counterstained with Harris Hematoxylin (HHS32-1L, Sigma-Aldrich).

All the slides were scanned by using a digital slide scanner NanoZoomer 2.0RS (Hamamatsu, Japan) and the images were processed and analyzed using NDP.view software (Hamamatsu Photonics KK).

**Whole mount liver imaging:** On day 7 of tumor growth, mice were injected as described above with either unlabeled or fluorescently-labeled anti-CD40 mAb (mAb: BioXCell Cat #BE0016-2; Antibody labeling kit: Thermo Fisher Cat #S30044). Two days after aCD40 treatment, at the time of sacrifice, mice were injected retro-orbitally with fluorescently labeled Lectin to label vasculature. IL-12p40-IRES-EYFP or IL-12p40-IRES-EYFP Batf3−/− mice were euthanized and livers were excised and immediately placed in PBS between an inverted petrie dish and a microscope cover slip. Livers were imaged using an Olympus FluoView FV1000MPE confocal imaging system (Olympus America). Images were processed and analyzed using Fiji from ImageJ.

**Single cell RNAseq:** MC38 tumors were implanted into the flanks of IL-12p40-IRES-EYFP mice and allowed to grow for 7 days before being treated with aCD40 or left treated. Tumors and livers were harvested 2 days later.
Tissues were digested as above to generate single cell suspensions, cells were stained for CD45 (table S7), and labeled with 7AAD (Sigma-Aldrich), and CD45+ cells or IL-12-EYFP+ cells were sorted using a BD FACS Aria sorter. InDrops single cell RNA sequencing was performed as described before (49) with changes to DNA primers listed in table S6. Briefly, a microfluidic device was used to co-encapsulate individual cells and polyacrylamide beads carrying barcoding reverse transcription (RT) primers and lysis reagents into 2-3 nl droplets, followed by primer release and RT at 50°C. After the RT reaction, droplets were broken, and the resulting barcoded cDNA was taken through the following sequencing library preparation steps 1) second strand synthesis, 2) in vitro transcription providing linear amplification of the material, 3) fragmentation of the amplified RNA, 4) a second reverse transcription using random hexamer primers bearing a universal PCR primer annealing site, and 5) indexing PCR, yielding a sequencing-ready library. Libraries were sequenced on the NextSeq Illumina platform, paired-end mode, dual indexing, with read lengths summarized in table S6.

**p40-IRES-EYFP transgene quantification and definition of IL-12+ cells.** InDrops scRNA-seq allows for the detection of transcript sequences until ~1kb upstream of the poly-A tail. However, detection of transcripts at ~400-600 bp from the poly-A tail is most likely because inDrops v3 DNA fragments have an average length of ~400-600 bp (49). As the IRES-EYFP-bGH-Poly(A)-loxP was inserted 50 bp downstream of the TAG stop codon in the 3'UTR of p40 and has a length of ~1561 bp, it is therefore most likely that the 5’end of a DNA fragment in the inDrops library falls in the EYFP + bGH-Poly(A)-LoxP sequence of the p40-IRES-EYFP transgene (p40-IRES-EYFP transgene sequence was obtained from Richard M. Locksley and Hong-Erh Lian). To accurately quantify Il12b (p40) transcript counts, we therefore added the IRES-EYFP-bGH-Poly(A)-loxP as an artificial chromosome to the ensemble reference genome and IRES-EYFP-bGH-Poly(A)-loxP transcript and exon annotations to the ensemble gtf reference genome annotation file. After gene-expression quantification using STARSolo (see scRNA-seq read processing), we summed the Il12b (reads that mapped to Il12b exons) and EYFP (reads that mapped to IRES-EYFP-bGH-Poly(A)-loxP) UMI counts to accurately quantify Il12b transcript expression. Summation of Il12b gene and EYFP UMI counts was needed because the 5’end of some DNA fragments can still fall in the exons of the Il12b gene as inDrops DNA fragments can be primed from poly-A tail (in GTF2.2 gene annotation the 3’UTR is included in the last exon), or poly-A stretches within introns of the Il12b gene (54). We confirmed that reads mapped at expected distances with the integrative genome viewer (55). Although IL-12b+ cells were enriched using an EYFP+ FACS gate, many IL-12b– cells were also sorted through the EYFP+ FACS gate, as true EYFP+ events are very rare and therefore other more prevalent cell types were caught as contaminants in the EYFP+ FACS gate. We therefore decided to define IL-12+ cells based on mean Il12b+ transcript counts (the summed Il12b + Eyfp UMI counts) expression of 10 neighbors cells (determined by Euclidean distance after dimensionality reduction, see ‘Dimensionality reduction and visualization of single cell data’ section below), rather than on EYFP+ FACS gate annotation.

**scRNA-seq read processing.** Gene-expression counts were obtained from the raw .FASTQ sequencing-read files using STARSolo, which is part of the splice-aware mapper software package STAR (56).To increase reproducibility in read-counting between scRNA-seq studies, STARSolo was designed to match 10X
**Interactive SPRING viewer:** The single cell transcriptomes embedded in UMAP space were visualized using the interactive SPRING viewer (60).

**Differential gene expression (DGE) analysis:** We identified sets of differentially expressed genes between tumor and liver IL-12+ cells, liver IL-12+ dendritic cells and IL-12+ macrophages, treated and untreated CD45+ cells, and treated and untreated neutrophils by performing a Wilcoxon rank-sum test using the Benjamini-Hochberg method for multiple hypothesis correction as implemented in Scanpy (59). We included a pseudo value of 1 CP10K prior to computing fold changes. We used a false discovery rate (FDR) of 5% for selecting genes as significantly differentially expressed.

**Comparison of CD45+ subsets to previously reported populations:** To compare the cell populations reported in this study with those from (26), we used Naive Bayes classifiers trained on the dataset from this study and from Zilionis et al., 2019 to calculate a reciprocal likelihood score, as defined in (26). In brief, for cluster a in study A, and cluster b in study B, we defined a similarity score $S(a,b) = E_a(P_c(x=a)) E_b(P_c(x=b))$, where $E_c(f(x))$ is the mean of $f$ over all cells $x$ in cluster $c$; and $P_c(x=c)$ is the probability calculated from the Naive Bayes classifier that transcriptome $x$ is classified to cluster $c=a,b$ out of all clusters defined in study $C=A,B$. See (26) for the full definition.

**Global comparison of expression changes between human and mouse:** Our data were compared to the transcriptional changes occurring in patients who developed colitis as a result of checkpoint blockade immunotherapy (15). First, we used the DGE analysis described above to compare the transcriptomes of T cells, NK cells, DC, and monocytes/macrophages from aCD40-treated and untreated mouse livers. For each cell type, we selected genes satisfying the following criteria:

1. Be within the top 50 most upregulated or the 50 most downregulated when comparing treated and untreated livers. A pseudo value of 1 CPM was added before calculating fold-changes.


3. Display a statistically significant change in expression (FDR<0.05).

We then computed the fold changes for each of the orthologous genes between cells from patients suffering from immunotherapy-induced colitis and cells from patients not receiving immunotherapy in orthologous cell types. We computed the Pearson correlation (R) between the signs of the changes in human and mice for each set of genes and their associated cell types. Four orthologous cell types were analyzed as shown in the results. We applied the Bonferroni correction (m = 4 hypotheses tested) prior to assessing the significance of each correlation.

**Volcano Plots and Fold Change:** For visualizing results in volcano plots, fold-change values report the ratio of $(1 + \text{CP10K})$ transcript abundance between treated and untreated conditions.
**Parabiosis:** CD45.1<sup>STEM</sup> and B6.129-Il12btm1LKy/J (IL-12-EYFP) mice were anesthetized under isofluorane, shaved on one side, and a longitudinal incision in the skin was made between the elbow and knee. Elbows and knees of the parabionts were secured together with a black monofilament nylon suture (Ethicon), and the longitudinal incision was sutured closed, thus connecting the skins of the parabiont mice. Animals were provided with buprenorphine as an analgesic for 3 days following surgery. Once hematopoietic equilibrium was confirmed (3-5 weeks post-surgery), both mice from the parabiotic pair were injected with MC38 tumors on the outer flank. Tumors were allowed to grow for 7 days before treatment with aCD40 immunotherapy, and tissues were harvested 2 days following immunotherapy to analyze IL-12<sup>+</sup> populations by flow cytometry.

**Bone marrow transfer experiments:** CD45.1 (Jackson Labs Cat #002014 or CD45.1<sup>STEM</sup>) recipient mice were irradiated with a single dose of 1000 cGy using a cesium-137 irradiator. The next day, bone marrow was harvested from donor mice and processed for injection: For IFNgR1 deficiency experiments, either WT CD45.1 mice or CD45.2 <i>Ifngr1</i>–/– mice served as donors. For <i>Zbtb46</i>-DTR experiments, either WT (CD45.2), <i>Il12p40</i>–/–, or <i>Zbtb46-Dtr</i> mice served as donors. Cells from each type of donor were counted manually. For 50:50 bone marrow chimeras, cells were mixed at a 1:1 ratio before injection. Cells were injected retro-orbitally at 10-14 x 10<sup>6</sup> total cells / mouse in 200-400 µL volume, and mice were allowed to reconstitute for 5.5-7.5 weeks. Chimerism was confirmed by cheek bleed before tumor challenge and immunotherapy treatment.

**Diphtheria toxin injection:** Mice receiving diphtheria toxin (DT) (Sigma-Aldrich) were dosed at 10 ng of DT per gram of body weight to initiate depletion 0.5-1 day before aCD40 injection, and then maintained with 4 ng of DT per gram of body weight administered one day after immunotherapy treatment.

**Clodronate Liposomes:** Mice receiving clodronate or control liposome injections were dosed with 200 µl of liposomes (Liposoma Cat# P-010-010) retro-orbitally one day before immunotherapy treatment and again one day following treatment.

**Bulk RNAseq:** For RNAseq, 50:50 WT : <i>Ifngr1</i>–/– bone marrow chimeras were injected with tumors and aCD40 as usual. On day 9 after treatment, livers were processed for FACS and CD45.1 (WT) or CD45.2 (<i>Ifngr1</i>–/–) neutrophils were sorted directly into TRIzol reagent and placed on ice. RNA was extracted using TRIzol™ Plus RNA Purification Kit (Thermo Fisher Cat #12183555). Libraries were prepared in collaboration with the Harvard Biopolymer Core Facility.

The Total-RNA samples were quantified using an Agilent Bioanalyzer 2100 instrument, with a corresponding Agilent Bioanalyzer RNA Pico assay. The resulting RIN (RNA Integrity Number) scores and concentrations were taken into account for qualifying samples to proceed. The samples were normalized to 1ng of input in 9µL (~111 pg/µL), and the mRNA was captured using oligo-dt primers as part of the Takara SMARTseq V4 Ultra Low workflow. The number of PCR cycles was increased to 13 to account for difficulties associated with neutrophil-derived RNA. A Bioanalyzer High Sensitivity DNA assay was run on the cDNA to evaluate success. cDNA synthesis, adapter ligation, and amplification were conducted subsequently with the Illumina NexteraXT Library Prep kit. Following amplification, residual primers were eluted away using Aline PCR CleanDx Beads. The resulting purified libraries were run on an Agilent 4200 Tapestation instrument, with a corresponding Agilent
High Sensitivity D1000 ScreenTape assay to visualize the libraries and check that the size and concentrations of the libraries matched the expected product. Molarity values obtained from this assay were used to normalize all samples in equimolar ratio for one final pool. The pool was denatured and loaded onto an Illumina NextSeq 500 instrument, with a High-Output 75-cycle kit to obtain Single-End 75 bp reads. The pool was loaded at 2 pM, with 5% PhiX spiked in as a sequencing control. The basecall files were demultiplexed through the Harvard BPF Genomics Core's pipeline, and the resulting fastq files were used in subsequent analysis.

All samples were processed using an RNA-seq pipeline implemented in the bcbio-nextgen project (https://bcbio-nextgen.readthedocs.org/en/latest/). Raw reads were examined for quality issues using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to ensure library generation and sequencing are suitable for further analysis. If necessary, adapter sequences, other contaminant sequences such as polyA tails and low quality sequences with PHRED quality scores less than five were trimmed from reads using atropos (https://github.com/jdidion/atropos; 10.5281/zenodo.596588). Trimmed reads were aligned to UCSC build mm10 opeof the murine genome, augmented with transcript information from Ensembl release GRCm38.98 using STAR (version 2.6.1d) (56). Alignments were checked for evenness of coverage, rRNA content, genomic context of alignments (for example, alignments in known transcripts and introns), complexity and other quality checks using a combination of FastQC (version 0.11.8), Qualimap (version 2.2.2d) (61), MultiQC (version 1.9) (https://github.com/ewels/MultiQC) and custom tools. Counts of reads aligning to known genes were generated by featureCounts (version 2.0.1) (62). In parallel, Transcripts Per Million (TPM) measurements per isoform were generated by quasialignment using Salmon (version 0.14.2) (63). Differential expression at the gene level was called with DESeq2 (version 1.22.1) (64), using counts per gene estimated from the Salmon quasialignments by tximport (version 1.12.1) (65) as quantitating at the isoform level has been shown to produce more accurate results at the gene level.

**Gene Ontology:** The significantly differentially expressed genes from aCD40-treated liver neutrophils compared to untreated liver neutrophils were used to generate a list of significantly enriched biological process. Gene names were entered into the GO enrichment analysis tool for Mus musculus and enriched biological processes were determined with PANTHER Overrepresentation Test (Released 20200728) using Fisher’s exact test; Annotation Version and Release Date: GO Ontology database DOI: 10.5281/zenodo.4081749 Released 2020-10-09 (51–53).

**Human liver samples:**

**Patient selection and inclusion criteria:** Patients on immune checkpoint therapy who developed hepatic irAEs were identified through The Oncology Department of the Lausanne University Hospital and the Hospital of the University of Geneva.

The Oncology Department of the Lausanne university Hospital is a tertiary leading referral center in the field of immuno-oncology treatments and research. Patients on immune check point therapy followed at the Oncology department who had developed a Common Terminology Criteria for Adverse Events (CTCAE v. 5.0) (66) grade 2 or higher immune mediated hepatitis (IMH) that had been histologically documented between January 2015 and September 2020 were retrospectively identified using a multiparametric clinical data warehouse search engine. In all cases, the indication for liver biopsy was
retained upon expert hepatologist advice. All included patients had given their written informed consent for re-use of their medical and histopathological data, with the exception of deceased persons. Any patient that had formally opposed to the use of his/her information was excluded.

**Patient characterization:** Demographic, clinical, laboratory and histopathological data were retrieved manually from electronic medical records and medical archives. Demographic data included sex and age. The patient’s current cancer course was described according to the type of tumor, extension and applied immunotherapy. Laboratory parameters included liver function tests, such as alanine transaminase (ALT), aspartate transaminase, total bilirubin, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) and INR (international normalized ratio). Importantly, the presence of concomitant acute or chronic infections by hepatotropic viruses (Hepatitis A (HAV), B (HBV), C (HCV), E (HEV) virus, Ebstein-Barr virus (EBV), Cytomegalovirus (CMV)), responsible for abnormal LFTs was ruled out in all patients. The prescribed immunosuppressive treatments were retrieved. Based on clinical presentation as well as biological parameters patients were assigned to three different groups of disease severity named as mild, moderate and severe by an expert hepatologist (M.F.) who was directly involved in the management of immunosuppression.

**Histopathology and immunochemistry:** Liver biopsies were processed according to standard histological methods with fixation in 10% buffered formalin and paraffin embedding. Stains included namely hematoxylin & eosin (H&E) and a connective tissue stain (Masson’s trichrome). Definitive confirmation of a histological picture compatible with IMH and exclusion of other causes of abnormal LFTs was obtained by expert liver pathologist interpretation of the slides (C.S.). Immunohistochemical staining for CD15 (clone: BD Pharmingen MMA, dilution 1/1500) was performed on 3-5 µm thick sections using the automated Ventana system (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer’s protocol.

For patients 21-24, biopsy or autopsy samples were fixed and embedded as above, and immunohistochemical staining for MPO (clone: DAKO A0398, Dilution 1:1000) and CD15 (Ventana) were performed on 3-5 µm thick section using the automated Ventana system (Ventana Medical Systems, Tucson, AZ, USA). Bound primary antibody was visualized using the amplified DabMap detection kit based on the conversion of diaminobenzidine to a brown dye with horseradish peroxidase (HRP/SA complex using secondary biotinylated antibodies).

The intensity and prevalence of MPO and/or CD15 staining was assessed and each liver was assigned a CD15/ MPO-positivity evaluation between 0 and 2 according to relative MPO signal strength.

**Statistical analysis of flow cytometry, histology or tumor burden data.** All statistical analyses were performed using GraphPad Prism software, except for 1) bulk RNAseq data shown in Figure S22 and 2) all single cell RNAseq analyses; details are provided in respective supplemental methods sections above. Results were expressed as mean±SEM. Student’s two-tailed t-test were done to compare two groups. One-way ANOVA was used to compare multiple groups. p values > 0.05 were considered not significant (n.s.); p values < 0.05 were considered significant. * p value < 0.05, ** p value < 0.01, *** p value < 0.001, **** p value < 0.0001.
Fig. S1. IL-12p40-EYFP and IFN-γ-EYFP reporter mice revealed global Th1 cytokine induction following aCD40. (A) Flow cytometry plots showing IL-12-EYFP (x-axis) expression in multiple tissues from mice treated or not with aCD40. Y-axis = viability dye (Zombie Aqua). (B) Flow cytometry plots showing IFN-γ-EYFP (x-axis) expression in multiple tissues from mice treated or not with aCD40. Y-axis = viability dye (Zombie Aqua).
**Fig. S2.** Weight loss following aCD40 did not depend on IL-23p19. Changes in body weight of MC38-tumor bearing mice treated or not with aCD40, and with or without neutralization of IL-23p19, day two after immunotherapy (n = 4-5 mice/group).
**Fig. S3. Colon crypt hyperplasia following aCD40 was IL-12-dependent.** (A) Hematoxylin and eosin (H&E) staining of fixed colon tissue taken from mice treated or not with aCD40, with or without neutralization of IL-12, three days after immunotherapy treatment. Representative crypt length annotations (dashed yellow lines). (B) Quantification of colon crypt height sampled throughout the length of the rolled colon (n = 3-5 mice/group).
Fig. S4. Chimerism of selected liver immune cell populations following bone marrow reconstitution and immunotherapy treatment. (A) Quantification by flow cytometry of F4/80+ CD11b-lo/neg cells with CD45.1 (+/+ ) or CD45.2 (−/− ) origin from livers of bone marrow recipient mice 2 days after treatment with aCD40 (n = 5-6 mice/group) (B) Quantification by flow cytometry as in (A) but for CD11b+ Ly6G+ neutrophils (n = 5-6 mice/group).
Fig. S5. IL-12 and IFN-γ induction following aCD40 were interdependent in multiple tissues. (A)

Quantification of flow cytometry data for IL12-EYFP+ cells in lungs and bone marrow of mice treated or not with aCD40, with or without IFN-γ neutralization (n = 3-4 mice/group). (B) Quantification of flow cytometry data for IFN-γ-EYFP+ cells in lungs and bone marrow of mice treated or not with aCD40, with or without IL-12 neutralization (n = 5-6 mice/group).
Fig. S6. Batf3-dependent DCs were required for antitumor immunity but dispensable for toxicity following aCD40. (A) MC38 tumor volume from WT and Batf3−/− mice treated with aCD40 on day 7 of tumor growth (n = 8-10 mice/group). (B) Body weight data from mice as in (A). (C) Example of gating strategy for IL-12p40-PE+ cells by intracellular protein staining.
Fig. S7. IL-12p40-EYFP-expressing cells were found proximal to areas of structural aberration and aCD40 accumulation in the liver. (A) Whole mount imaging of livers from IL-12-EYFP reporter mice treated with aCD40, day 2 post-treatment. Lectin-rhodamine (blue); IL-12-EYFP (green). (B) Images as in (A), including fluorescently labeled aCD40-antibody (red). Dashed yellow lines indicate contours of lesions.
Fig. S8. CD8+ T cell- and Rag2-dependence distinguished anti-tumor immunity from toxicity following aCD40. (A) Body weight from WT, μMT, and Rag2−/− mice treated with aCD40 on day 7 of tumor growth (n = 5 mice/group). (B) MC38 tumor volume from WT, μMT, and Rag2−/− mice treated with aCD40 on day 7 of tumor growth (n = 5 mice/group). (C) Body weight change two days after aCD40 treatment. Mice were treated with aCD40 on day 7 of tumor growth, and given or not CD8-, CD4-, or NK1.1- depleting mAbs. Untreated mice served as controls (n = 5-7 mice/group). (D) MC38 tumor volume on day 19 from mice treated or not with aCD40 on day 7 of tumor growth, and given or not CD8-, CD4-, or NK1.1- depleting mAbs (n = 5-7 mice/group). For (C) and (D), mice in aCD40-treated and untreated groups also served as positive and negative immunotherapy controls, respectively, for figure 1F.
Fig. S9. Transcriptional data comparing IL-12-EYFP-expressing cells between tissue sites. (A) Volcano plots showing differential gene expression between IL-12+ cells from tumors and livers of aCD40-treated and untreated mice. Genes with a false discovery rate (FDR) of <5% are colored in blue and red, denoting enriched genes in the tumor and liver, respectively. Genes with an absolute log fold change of > 2 and an (FDR) of < 10^{-5}, or an absolute log fold change of > 4.7 and an (FDR) of < 10^{-7}, are annotated in their respective colors. See also table S1. (B) Volcano plots showing differential gene expression between IL-12+ liver DC and Kupffer cells from...
aCD40-treated and untreated mice. Genes with a false discovery rate (FDR) of <5% are colored in blue and red, denoting enriched genes in DC and macrophages, respectively. Genes with an absolute log fold change of > 2 and an (FDR) of < 10^{-5}, or an absolute log fold change of > 4.7 and an (FDR) of < 10^{-7}, are annotated in their respective colors. See also table S2. (C) Single-cell expression of DC3- and macrophage-associated state markers in IL-12^{+} tumor and liver cells. Colorbar saturated at 99.5^{th} expression percentile measured across all IL-12^{+} cells in tumor or liver. (D) Expression of the genes from (C) in the IL-12^{+} DC3 states in tumor (DC3 (Tu)) and liver (DC3 (Li)) and in the IL-12^{+} macrophage state in liver (Mø (Li)). Dots represent mean expression of each biological replicate (n = 2 livers, n = 3 tumors). Error bars indicate standard error of each replicate-specific mean.
Fig. S10. Examples of gating strategies for IL-12p40-EYFP+ cells. (A) Example of the gating strategy used for sorting of IL-12p40-EYFP+ cells for scRNA-seq (see also Fig. 4A). (B) Example of a gating strategy used for analyzing IL-12p40-EYFP+ cells by flow cytometry. A similar strategy was used for analyzing IL-12-PE+ cells labeled by intracellular protein staining.
**Fig. S11. KCs were the major producers of IL-12 in the liver following aCD40.**

(A) Flow cytometry-based quantification of IL-12-EYFP⁺ cell subsets from livers of aCD40-treated mice. (B) Flow cytometry-based quantification of IL-12-PE⁺ cell subsets from livers of aCD40-treated mice, based on subsets as in (A) but gated on IL-12-PE⁺ cells. (C) Relative contributions of KCs, DC-like cells and other macrophages to IL-12 production in the liver following aCD40 treatment, based on intracellular IL-12 protein staining.
Fig. S12. Clodronate Liposome- and DTR-mediated approaches efficiently depleted KCs. (A) Flow cytometry-based quantification of Kupffer cells (KCs) per mg of liver following aCD40 treatment with or without clodronate liposome treatment (n = 4-5 mice/group). (B) Diagram showing experimental scheme for studying livers in KC-depleted mice. (C) Representative flow cytometry plots showing Kupffer cells in aCD40-treated livers from control mice and Clec4f-cre+/o lsl-Dtr+/– mice; all mice received DT treatment as in (A). (D) Quantification by flow cytometry of CD11b–/lo F4/80+ cells in Clec4f-cre+/o lsl-Dtr+/– mice compared to controls (n = 8 mice per group).
Fig. S13. IFN-γ sensing determined KC activation phenotype. Flow cytometry data comparing MHCII, CD80, and CD86 expression in Ifngr1+/+ vs. Ifngr1–/- CD11b–lo F4/80+ KCs from livers of bone marrow chimeras two days after treatment with aCD40.
Fig. S14. scRNA-seq of liver CD45+ cells revealed identifiable immune cell states and perturbation following aCD40. (A) Reciprocal likelihood score for each major cell-type comparison pair from Zilionis et al. (2019) (26) and this study. The score was calculated using the probability estimates returned by a naive Bayes multinomial classifier and is non-vanishing only when two compared states show mutual correspondence. (B) UMAP visualization of CD45+ sorted cells colored by treatment condition.
Fig. S15. Diverse lymphocytes contributed to elevated \( \text{Ifng} \) expression following aCD40. (A) Single-cell expression of \( \text{Ifng} \) and \( \text{Il12rb1} \) in livers from mice left untreated or treated with aCD40. Colorbar saturated at 99.9\(^{th}\) expression percentile measured across all cells. (B) Single-cell expression of the indicated genes in T/NK cell-states in livers from mice left untreated or treated with aCD40. Colorbar saturated at 99.5\(^{th}\) expression percentile.
percentile measured across all cells. (C) Expression of *Ifng* and *Il12rb1* in major cell-types from livers of mice left untreated (grey) and treated with aCD40 (red). Dots represent mean expression of each biological replicate (n = 2). Error bars indicate standard error of each replicate-specific mean. (D) Expression of *Ifng* and *Il12rb1* in T/NK cell states from livers of mice left untreated (grey) and treated with aCD40 (red). Dots represent mean expression of each biological replicate (n = 2). Error bars indicate standard error of each replicate-specific mean. (E) Scatterplot showing changes in average *Ifng* expression and in relative abundance of NK/T cell states between livers of mice left untreated (crosses) and treated with aCD40 (circles).
Fig. S16. Flow cytometry confirmed diversity of IFN-γ-expressing lymphocytes. Flow cytometry data showing IFN-γ-EYFP expression in NK, CD4+ T, and CD8+ T cells from untreated (grey) and aCD40-treated (pink) mouse livers (top); quantification of IFN-γ-EYFP+ cells per mg of liver tissue within NK, CD4+ T, CD8+ T, and other cells (bottom).
Fig. S17. Anti-Gr1- and anti-Ly6G-based neutrophil targeting. (A) Top: Timeline of anti-Gr1 and aCD40 treatment. Bottom: Flow cytometry-based quantification of myeloid populations in circulation before or after aCD40 treatment, with or without anti-Gr1 (n = 6-7 mice/group). (B) Top: Timeline of anti-Ly6G, CXCR2 inhibitor (CXCR2i), and aCD40 treatment. Bottom: Flow cytometry-based quantification of neutrophil-like cells in the liver after aCD40 treatment, with or without anti-Ly6G or a CXCR2 inhibitor (n = 3-5 mice/group).
Fig. S18. Csf3r-deficiency reduced liver-associated neutrophils. Flow cytometry-based quantification of neutrophils, DCs, KCs, and CD11b⁺ macrophages in livers from aCD40-treated mice sufficient or deficient for CSF3R (n = 4-5 mice/group).
Fig. S19. Neutrophils up-regulated $Tnf$ among other major transcriptional changes following aCD40. (A) Volcano plot of differential gene expression between neutrophils sequenced from livers of untreated (blue) or aCD40-treated (red) mice. See also table S3. (B) Expression of $Tnf$ in major cell types in livers from mice treated or not with aCD40. Dots represent mean expression of each biological replicate ($n = 2$ mice). Error bars indicate standard error of each replicate-specific mean.
Fig. S20. TNF neutralization suppressed multiple effectors of aCD40-associated toxicity. (A) Changes in body weight from mice treated or not with aCD40, with or without TNF neutralization (n = 4-5 mice/group). (B) Flow cytometry-based quantification of IL-12-producing cells, IFN-γ-EYFP+ cells, and neutrophils from livers of mice treated or not with aCD40, with or without TNF neutralization (n = 4-5 mice/group).
Fig. S21. Liver-associated neutrophils expressed TNF receptor following aCD40. Single-cell expression of *Tnfrsf1a* and *Tnfrsf1b* in CD45+ cells sorted from livers of aCD40-treated mice. Colorbar saturated at 99.9th expression percentile measured across all cells.
Fig. S22. IFN-γ sensing determined multiple features of the neutrophil transcriptional state following aCD40. (A) Flow cytometry-based quantification of Ifngr1+/+ or Ifngr1−/− neutrophils in livers of 50/50 bone marrow chimera mice with and without aCD40-treatment (n = 6 mice/group). (B) Bulk RNAseq quantification for various genes from Ifngr1+/+ and Ifngr1−/− neutrophils sorted from livers of 50%:50% bone marrow chimera mice following aCD40 treatment (n = 8 mice). TPM, transcripts per million. Statistical significance calculated via DESeq2.
Fig. S23. PD-L1 expression by neutrophils after aCD40 treatment, and impact of PD-L1 targeting on various cell types in the liver. (A) Quantification by flow cytometry of PD-L1 expression on CD11b+ Ly6G+ neutrophils from livers of mice treated or not with aCD40 (n = 6 mice/group). (B) Quantification of PDL1-expressing neutrophils from livers of mice treated or not with aCD40 (n = 6 mice/group) (left); examples of PD-L1+ neutrophil gating, based on FMO (right). (C) Quantification of CD11b+ F4/80+ macrophages from livers of mice treated or not with aCD40, given or not PD-L1-targeting mAbs followed by anti-Rat IgG2b depleting mAbs (n = 6 mice/group). (D) Quantification of various IL-12-producing cells from livers of mice treated or not with aCD40, given or not PD-L1-targeting mAbs followed by anti-Rat IgG2b depleting mAbs (from left to right: IL-12+ KCs, IL-12+ DCs and IL-12+ CD11b+ F4/80+ macrophages; n = 6 mice/group).
Fig. S24. Checkpoint blockade treatment in normal adult mice activated inflammatory phenotypes in the liver. (A) Flow cytometry-based quantification of IL-12p40-EYFP+ cells in livers of mice with or without ICB treatment. Data from two separate experiments (n = 10-14 mice/treatment condition). (B) Flow cytometry-based quantification of MHCII expression on Kupffer cells from livers of mice treated or not with ICB (n = 8-9 mice/group). (C) Quantification by flow cytometry of neutrophils from livers of mice treated or not with ICB. Data from two separate experiments (n = 11-14 mice/treatment condition).
Fig. S25. Human ICB-associated irAEs showed multiple parallels to mouse irAEs. Scatterplots comparing changes in average expression of selected genes and respective human orthologs in T cells (A), and DCs (B), in colons from human patients (immunotherapy-associated colitis vs untreated) and livers from mice (aCD40-treated vs untreated). For each cell type, we selected up to 100 genes based on: (1) statistical significance (FDR<0.05) and magnitude of change (>2-fold) in the mouse; and (2) the existence of a 1:1 homolog in human. Outlined quadrants (red, yellow) show conserved responses to therapy; genes with conserved responses are listed. See also table S4.
Fig. S26. Human irAE hepatitis showed granulocytic inflammation associated with severity of inflammation. Representative H&E stainings of mild (left), moderate (middle), and severe (right) cases of irAE hepatitis from cancer patients following ICB therapy (top). CD15 staining in livers from each representative case (bottom). Portal tracts indicated by asterisks. Additional information is available in table S5.
Supplementary Tables

Table S1. scRNA-seq data - Differentially gene expression for IL12b+ cells from tumor vs. liver of aCD40-treated mice.
Please see separate excel document.

Table S2. scRNA-seq data - Differentially gene expression for DC3-like vs. Macrophage-like IL12b+ cells from aCD40-treated livers.
Please see separate excel document.

Table S3. scRNA-seq data - Differentially gene expression for neutrophils from untreated and aCD40-treated livers.
Please see separate excel document.

Table S4. scRNA-seq data - Human/Mouse comparison.
Please see separate excel document.

Table S5. Clinical information for human liver data.
Please see separate excel document.

Table S6. InDrops primer modifications (inDrops V3) and InDrops read lengths.
Please see separate excel document.

Table S7. Commercial antibodies used for flow cytometry studies.
Please see separate excel document.

Table S8. Raw data files.
Please see separate excel document.