Antibody-induced erythrophagocyte reprogramming of Kupffer cells prevents anti-CD40 cancer immunotherapy-associated liver toxicity

Marc Pfefferlé[1], Irina L Dubach[1], Raphael M Buzzi[1], Elena Dürst[1], Nadja Schulthess-Lutz[1], Livio Baselgia[1], Kerstin Hansen[1], Larissa Imhof[1], Sandra Koernig[2], Didier Le Roy[3], Thierry Roger[3], Rok Humar[1], Dominik J Schaer[1] and Florence Vallelian[1]

[1]Division of Internal Medicine, University Hospital and University of Zurich, Zurich, Switzerland
[2]CSL Ltd., Research, Bio21 Institute, Parkville, Vic, Australia
[3]Infectious Diseases Service, Lausanne University Hospital and University of Lausanne,

Supplementary Methods (detailed procedures and protocols)

Mice

C57BL/6J (JAX™ strain) mice were obtained from Charles River Laboratories. Ms4a3^{Cre} mice were obtained from Dr. Florent Ginhoux (SingHealth and Duke NUS, Singapore) [1] and bred with Ai14^{tdTomato} mice (The Jackson Laboratory). C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1), B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-2), and B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) mice were obtained from the Swiss Immunological Mouse repository (SwImMR). CD45.1 x OT-1 and CD45.1 x OT-2 mice were obtained by crossing OT-1 and OT-2 mice with CD45.1 mice, respectively. The CD40$^{fl/fl}$ mouse strain was generated from the ES clone EPD0901_3_A02, obtained from the KOMP repository (www.komp.org), by the Wellcome Trust Sanger Institute (WTSI) as described previously [2]. CD40$^{fl/fl}$ mice were crossed with Lysm^{Cre} mice, which were obtained from SwImMR, to generate CD40$^{fl/fl}$ LysM^{Cre} mice or with Clec4f^{Cre} mice, which were acquired from The Jackson Laboratory, to obtain CD40$^{fl/fl}$ Clec4f^{Cre} mice. Control littermates without the Cre driver were used for experiments involving these mouse strains.

All breeding colonies were housed and bred in the specific pathogen-free (SPF) animal facility at the Laboratory Animal Services Center (LASC) of the University of Zurich in individually ventilated cages. Mice were housed under a 12/12-h light/dark cycle in accordance with international guidelines. Mice that were 7-10 weeks old were used for all experiments. Only healthy, well-conditioned mice with uncompromised and groomed fur were included as

experimental animals. The mice were monitored daily for health parameters, including weight, in accordance with guidelines provided by the Swiss Federal Veterinary Office.

For all experiments, the experimental unit is defined as a single animal. Both male and female animals were included. All experimental protocols were reviewed and approved by the Veterinary Office of the Canton of Zurich (ZH044 2021). Animal experiments performed in Lausanne were approved by the Service des Affaires Vétérinaires, Direction Générale de l'Agriculture, de la Viticulture et des Affaires Vétérinaires, état de Vaud (Epalinges, Switzerland) under authorizations VD3760b. All animal experiments were performed according to Swiss and ARRIVE guidelines.

In vivo treatments

Ter119 antibodies: Rat anti-mouse Ter119 IgG2 mAb was acquired from InVivoPlus and diluted in sterile phosphate-buffered saline (PBS) before s.c. injection. Murine Ter119 (mTer119) IgG2 mAb was produced by CSL Behring [3] (non commercialized) and diluted in sterile PBS before s.c. injection.

IgG2 Isotype Control Antibody: mouse IgG2a isotype control antibodies were purchased from InVivoPlus and diluted in sterile PBS before s.c. injection. Isotype control antibodies were always injected with a similar dose as mTer119 antibodies.

Transfusion experiments: In total, 10^9 murine RBCs were stained with CFSE (Thermo Fisher, for flow cytometric analysis and histology) or IVISense 680 (PerkinElmer, for in vivo imaging system (IVIS) analysis) for 20 minutes in PBS, washed and opsonized with the rat Ter119 antibody (667 µg/ml). Approximately 150 million RBCs were then injected intravenously into C57BL/6J mice. Blood was collected every 10 minutes for flow cytometric analysis. After 45 minutes, mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg), xylazine (16 mg/kg), and acepromazine (3 mg/kg), transcardially perfused using 20 ml PBS and organs collected for IVIS analysis. Another group of mice was sacrificed after 1 hour, their liver collected and digested as indicated in the corresponding subsection for flow cytometric analysis or histology.

Prolonged mTer119 treatment: An initial dose of 1.4 mg/kg Ter119 antibody was injected subcutaneously before starting daily s.c. injections of 0.4 mg/kg Ter119 antibody 4 days later for 10 days in total. Analysis or further inflammatory challenges were performed 24 hours after the last injection.

Etanercept treatment: Etanercept was purchased from Pfizer (Enbrel) and injected intraperitoneally (i.p.) into mice at a dose of 100 mg/kg two times before anti-CD40 challenge (days -2 and 0).

Agonistic anti-CD3 antibody challenge: Anti-CD3 antibodies were purchased from BioXCell (clone 145-2C11), diluted in PBS and injected intravenously at a dose of 100 µg in a volume of 2.5 µl/g weight. The animals were sacrificed 4 hours later for plasma cytokine measurements.

LPS challenge: LPS was purchased from Sigma–Aldrich, diluted in PBS and injected i.p. at a dose of 6 mg/kg in a volume of 5 µl/g weight. Blood was sampled before LPS injection and 4 hours after injection for cytokine measurements.

Tumor experiments: MC38 tumor cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% non essential amino acids (NEAA) and 1% sodium-pyruvate (tumor cell culture medium) in 15-cm cell culture dishes (TPP). The cell line was regularly tested for mycoplasma. Once confluent, tumor cells were harvested using Versene 1x (Gibco) (4 min at 37°C), washed twice in PBS, and 3 million tumor cells in 150 µl of culture medium mixed with 150 µl Geltrex (Thermo Fisher) were injected subcutaneously into the mouse flanks (day 0). mTer119 treatment was initiated 7 days before tumor cell injection (day -7) and continued daily for the entire duration of the experiment. Anti-CD40 treatment was administered two times, at day 7 and 9 after tumor cell injection. Tumor volume was measured one day before anti-CD40 treatment and monitored regularly until the end of the experiment on day 15. Tumor dimensions were measured under light isoflurane anesthesia using a caliper, and tumor volume was calculated as $V = (W^2 \times L)/2$, where W is the width and L is the length of the tumor.

Organ and cell preparation

Spleen cell suspensions

Spleens were harvested, mechanically disrupted in PBS and passed through a 70-µm cell strainer. The cell suspensions were then centrifuged (300 x*g*, 5 min at 4°C), incubated in RBC lysis buffer (BioLegend) for 2 minutes at 37°C and centrifuged once more to obtain spleen cell populations devoid of mature erythrocytes.

Liver digestion

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg), xylazine (16 mg/kg), and acepromazine (3 mg/kg), and the abdomen was cut open to access the portal vein. A 24-G catheter was placed in the portal vein and connected to a pump. The inferior vena cava was cut open to prevent pressure build-up. The liver was perfused through the catheter with 15 ml of HBSS + 2 mM EDTA to clear the blood followed by 50 ml of HBSS + 350 mg of $CaCl_2$ + 0.4 mg/ml collagenase B buffered solution (Roche). After digestion, the liver was excised, mechanically disaggregated in a petri dish on ice, and filtered through a 70-µm-pore cell strainer. The cell suspension was centrifuged once (100 ×*g*, 3 min at 4°C), and the pellet was discarded. The supernatant was then centrifuged once more (300 ×*g*, 5 min at 4°C) to obtain a pellet consisting of nonparenchymal cells including liver macrophages.

Colon digestion

The colon was dissected out, and the feces were removed by flushing with HBSS + 10% FBS. The colon was then cut into pieces and placed in a shaker at 37°C for 15 minutes in 50 ml of HBSS + 2 mM EDTA. The supernatant was removed, and the process was repeated 2 more times to remove any adipose tissue. The tissue was then transferred to 5 ml of digestion medium, which consisted of RPMI medium + 10% FBS (Biochrome) + 125 µg/0.65 U Liberase (Roche) + 200 µg/400 U DNase I (Roche), and incubated for 30 minutes on a shaker at 37°C. The digested tissue was then passed through a 70-µm cell strainer and centrifuged (300 ×*g*, 5 min at 4°C).

Lymph node digestion

The inguinal lymph nodes were dissected out, minced into pieces of approximately 1 mm^3 and incubated in digestion medium (collagenase IV 1%, DNase I 0.04%) on a shaker for 30

minutes at 37°C. The digested lymph nodes were then passed through a 70-µm cell strainer and centrifuged (300 ×*g*, 5 min at 4°C).

Peritoneal cell isolation

Peritoneal lavage was performed by flushing fluid in the peritoneal cavity using a 24-G needle with a 5-ml syringe filled with PBS + 3% FBS (Biochrome). The peritoneal lavage fluid was then passed through a 70-µm cell strainer and centrifuged (300 ×*g*, 5 min at 4°C).

Macrophage isolation

Anti-rat IgG Dynabeads (Invitrogen) were washed and incubated with rat anti-mouse F4/80 IgG2a antibodies (BD Biosciences) at a ratio of 2.5 µg of antibody per 50 µl of Dynabeads. Single-cell suspensions isolated from the liver, colon or peritoneal cavity were incubated with anti-F4/80-coated Dynabeads on a shaker at 4°C for 30 minutes. After incubation, positive selection of Dynabead-bound single-cell suspensions was performed on a DynaMag magnet (Invitrogen) with three washing steps, as instructed by the manufacturer.

$CD4^+$ and $CD8^+$ T-cell isolation

$CD4^+$ and $CD8^+$ T cells were negatively enriched from spleen single-cell suspensions using a MagniSort CD4 or CD8 enrichment kit (Invitrogen) according to the manufacturer's instructions.

RBCs isolation

Blood was collected by heart puncture, applied over 5 ml of Percoll (Cytiva) 70% in a 50 ml Falcon tube and centrifuged at 800 x*g* 20 minutes at room temperature without brake. After centrifugation, the supernatant was discarded and the RBCs were washed with 50 ml PBS 3000 x*g* 10 min at 4°C.

CFSE labeling

RBCs, $CD4^+$ and $CD8^+$ T cells were labeled with a 1 µM CFSE (Thermo Fisher) at 37°C for 20 minutes, washed with PBS and counted before use. The final purity of T cells was confirmed to be > 95% by flow cytometry.

Pfefferlé M, *et al. J Immunother Cancer* 2023; 11:e005718. doi: 10.1136/jitc-2022-005718

Adoptive transfer experiments

In total, 10^6 CFSE-labeled $CD4^+$ T or $CD8^+$ T cells isolated from spleens harvested from CD45.1 x OT-2 or CD45.1 x OT-1 mice, respectively, were injected intravenously into chronic mTer119- or saline-treated mice. After two hours, the mice were challenged intravenously with 75 µg of OVA protein (InvivoGen) in combination with 75 µg of agonistic anti-CD40 mAbs administered subcutaneously to both flanks. After 72 hours, the mice were sacrificed, the draining iliac lymph nodes were harvested as described above, and the CFSE dilution of $CD45.1^+CD4^+$ or $CD45.1^+CD8^+$ T cells was assessed by flow cytometry.

Plasma cytokine and transaminase measurements

Blood was collected by terminal heart puncture under anesthesia or submental blood sampling and centrifuged at 3000 ×*g* for 10 minutes at 4°C to obtain the plasma. Plasma cytokine measurements were then performed with diluted plasma (1:5) by using Bio-Plex Cytokine Assays (Bio–Rad) as instructed by the manufacturer. The assays were performed with a Bio-Plex 200 system (Bio–Rad), and the results were analyzed using the Bio-Plex Data Pro software (Bio–Rad) protocol. ALT levels in diluted plasma were measured using a Reflotron (Roche) system as instructed by the manufacturer’s protocol.

Flow cytometry

In all flow cytometry experiments, except for flow cytometric analysis of RBCs, live dead staining was performed using the LIVE/DEAD Fixable Near-IR cell stain kit (Invitrogen) as described by the manufacturer. Fc receptor blockade was performed by preincubating cells with TruStain FcX™ PLUS (anti-mouse CD16/32) antibodies (BioLegend). Multiparameter analysis was performed with an LSR Fortessa analyzer (BD Biosciences) or an Aurora 5L spectral flow cytometer (Cytek). The autofluorescence of cells was subtracted in all experiments using a spectral flow cytometer. Data were analyzed using FlowJo software (version 10.7.1) and FCS Express 6 (De Novo software).

IVIS imaging

C57BL/6J mice were transcardially perfused with PBS and imaging of their liver, spleen, kidneys and heart was performed using Program Living Image 4.7.1 software with the

following parameters: excitation wavelength of 640 nm, emission wavelength of 710 nm, exposure time of 2 seconds, binning of 1 and field of view of 10.

Histology (IF and brightfield)

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg), xylazine (16 mg/kg), and acepromazine (3 mg/kg) and transcardially perfused with cold PBS. Organs were fixed by transcardial perfusion of 4% paraformaldehyde (PFA), harvested and placed in 4% PFA.

Organ fixation for paraffin embedding and microtome sectioning

After 12 hours, organs were removed from the 4% PFA solution and embedded in paraffin blocks. Microtome sections (5 µm) were cut for hematoxylin and eosin or IF staining.

Organ fixation for vibratome sections

After 4 hours, organs were removed from the 4% PFA solution, and vibratome sections (70 µm) were cut for IF staining.

IF staining for Vibratome sections

Vibratome sections were permeabilized with 0.5% Triton X-100 in PBS with 4% BSA for 4 hours and then incubated overnight at 4°C with a rabbit anti-mouse Iba1 antibody (WAKO) diluted 1:1000 in PBS containing 0.2% Triton X-100 and 2% BSA, followed by incubation for 2 hours with Alexa Fluor 555-coupled goat anti-rabbit IgG (Invitrogen) diluted 1:400 in PBS containing 0.2% Triton X-100m and 2% BSA. The sections were counterstained with Alexa Fluor 488-conjugated Phalloidin (Thermo Fisher, 1:1000) and Hoechst 33342 (Thermo Fisher, 1:2000) in 0.2% Triton X-100m and 2% BSA for 40 minutes. The sections were washed in PBS after each antibody incubation step and mounted on glass coverslips using ProLong Gold Antifade Mountant (Thermo Fisher).

Multispectral Multiplex Immunofluorescence for paraffin-embedded slides

Paraffin-embedded microtome sections were stained for IF analysis using the Opal 4-Color anti-Rabbit Manual IHC Kit (Akoya Biosciences). Briefly, after 1 h incubation at 65°C, the slides were rehydrated through successive xylol and ethanol incubation steps and further fixed in

10% neutral buffered formalin for 20 minutes. For antigen retrieval, the slides were incubated in boiling pH 6 AR buffer (PerkinElmer) for 30 minutes and then allowed to cool to room temperature for 15-30 minutes. The slides were incubated in Hydrogen Peroxide 0.3% for 10 minutes, then blocked with Antibody Block solution (PerkinElmer) for another 10 minutes and washed in Tris-buffered saline with Tween 20 (TBST). A primary rabbit anti-mouse F4/80 antibody solution (CellSignaling Technologies) was diluted 1:500 in Antibody Diluent (PerkinElmer) and incubated on the slides for 1 hour. The slides were then washed in TBST and incubated for 10 minutes with secondary Opal Polymer anti-Rabbit HRP (PerkinElmer) diluted 1:5 in Opal Polymer anti-Rabbit HRP Diluent (PerkinElmer). After another round of washing with TBST, the slides were incubated with Opal Fluorophore 570 for 10 minutes. The slides were placed in boiling pH 6 AR buffer (PerkinElmer) for 30 minutes to strip off the bound antibodies and allowed to cool for 15 minutes. For F4/80 and HMOX1 co-staining, the slides were subjected to another round of staining using a primary rabbit anti-mouse HMOX1 antibody (ENZO) diluted 1:500 with a 12 hour incubation. In this case the Opal Fluorophore used were 620 for F4/80 and 520 for HMOX1. Finally, the slides were counterstained with a DAPI spectral solution (PerkinElmer) and mounted using ProLong Gold Antifade Mountant (Thermo Fisher).

Microscopy image acquisition and analysis

Whole-liver sections processed by hematoxylin-eosin, immunohistochemical (IHC) or IF staining and embedded in paraffin were acquired using a Zeiss Axio Scan.Z1 microscope or a Akoya Phenoimager HT. Regions of liver vibratome sections subjected to IF staining were visualized with a Leica SP8 confocal laser scanning microscope. Images were analyzed using Qupath [4] (v0.3.2) and ImageJ.

Macrophage quantification in IF tissue sections

F4/80$^+$ cells were quantified in whole-liver sections using the integrated watershed cell detection plugin in Qupath. The cells were detected in the Alexa Fluor 555 channel using a requested pixel size of 0.5 µm. The background radius, median filter radius and sigma were 8, 0 and 1.5 µm, respectively. The minimum and maximum areas were 10 and 400 µm^2, respectively. The threshold was set to 150, and cell expansion to 2 µm.

Quantification of HMOX1 positive macrophages in multiplex IF tissue sections

F4/80⁺ cells and expression of HMOX1 were quantified in whole-liver sections using the integrated positive cell detection plugin in Qupath. F4/80⁺ were detected in the Opal 620 channel using the requested pixel size of 0.5 µm. The background radius, median filter radius and sigma were 8, 0 and 1.5 µm, respectively. The minimum and maximum areas were 10 and 500 μm^2, respectively. For intensity parameters split by shape was activated and the threshold was set to 25. Cell expansion was set to 1.5 µm. HMOX1 positive cells were detected using the Opal 520 channel, setting the intensity threshold parameters for score compartment to cytoplasm mean and the threshold to 20.

BMDM cell culture

BM cells were isolated by flushing the femurs and tibias of 8- to 10-week-old C57BL/6J mice, followed by straining of the BM through a 70-µm filter. The BM cells were plated at a density of 30000 cells/cm^2 on tissue culture-treated 96-well plates (TPP) in complete RPMI-1640 medium (10% fetal calf serum (FCS) and 1% L-glutamine) supplemented with 1% penicillin/streptomycin (P/S) and 100 ng/ml recombinant mouse M-CSF (PeproTech). On day 3, half of the medium was replaced. Experiments were carried out on day 7.

In vitro erythrophagocytosis assay

BMDMs were isolated and cultured on a 96-well plate at a concentration of 10^4 cells/well as described above. On day 7 of BMDM culture, donor RBCs were obtained from mice by submental vein puncture. The whole-blood samples were washed twice with PBS. RBCs ($50x10^6$) were stained using red pHrodo (pHrodo® Cell Labeling Kit for Incucyte® Phagocytosis Assays, Sartorius) according to the manufacturer's protocol. Stained RBCs were added to increasing concentrations of BMDMs. Immediately before starting image acquisition, the Ter119 antibody (400µg/ml) was added to each well. Images were acquired every 15 minutes for 8 hours using an Incucyte®live-cell analysis system.

Sequencing-based workflows and data analysis

scRNA-seq data acquisition

Multiplexed anti-CD40 experiment: Livers from anti-CD40 mAb- and saline-treated mice were digested, and leukocytes were enriched using the MagniSort™ Mouse CD45 Positive Selection

Kit (Invitrogen) according to the manufacturer's instruction . Approximately 2 million cells per condition were stained with 1.5 µg of TotalSeq™ B0301-B0304 anti-mouse Hashtag antibodies (BioLegend) in accordance with the manufacturer's instructions and pooled together at equal cell numbers. The pooled multiplexed sample was then processed according to the 10x Genomics Chromium Single Cell 3' v3.1 Reagent Kit with Feature Barcoding Technology for Cell Surface Protein instruction guide (10x Genomics).

mTer119 experiment: Livers from mTer119-treated and saline-treated mice were digested, and macrophages were enriched using a mixture of rat anti-mouse F4/80-coated (BD Biosciences) and rat anti-mouse CD11b (BioLegend)-coated Dynabeads (Invitrogen) for positive selection with direct isolation according to the manufacturer's instructions. The anti-F4/80-coated and anti-CD11b-coated Dynabeads were mixed together at a ratio of 60:40. The enriched cell population was then processed according to the 10x Genomics Chromium Single Cell 3' v3.1 Reagent Kit instruction guide (10x Genomics).

For all experiments, the sample volume was adjusted to a target capture of 10000 cells, and the sample was loaded on the 10x Genomics chromium next-GEM chip G to generate gel beads-in-emulsion (GEMs). The GEM solution was placed in an Applied Biosystems Veriti 96-well thermocycler for reverse transcription, as described by the 10x Genomics instruction guide (53 min at 53°C followed by 5 min at 85°C). The resulting barcoded cDNA was then cleaned using Dynabeads MyOne Silane and amplified for 11 cycles (as recommended by the 10x Genomics user guide for a target cell recovery of >6000 cells). After amplification, for multiplexed experiments, cDNA generated from polyadenylated mRNA for the 3' gene expression library was separated from DNA generated for the Cell Surface Protein Feature Barcode for the Cell Surface Protein library with Dynabeads MyOne Silane and SPRIselect reagents based on size. The quality and concentration of both cDNA and DNA were assessed using a High-Sensitivity D5000 ScreenTape (Agilent). All samples exhibited product sizes with a narrow distribution centered at approximately 2000 bp and yielded between 50 and 800 ng of cDNA (manually selecting products between 100-250 and 5000-6000 bp). cDNA and DNA were then subjected to enzymatic fragmentation, end repair and A-tailing. Adaptors were ligated to the fragmented cDNA and DNA, and the sample index was added during sample index PCR (set for 12 cycles, as recommended by the 10x Genomics user guide to correlate

with a cDNA/DNA input of 12-150 ng). Library quality and concentration were assessed using a High-Sensitivity D5000 ScreenTape (Agilent). All gene expression libraries showed an average fragment size of approximately 400 bp. For multiplexed runs, 3' Gene Expression and Cell Surface Protein libraries were pooled at a ratio of 4:1 and sequenced on an Illumina NovaSeq 6000 system with a sequencing depth of 50000 and 12500 reads per cell, respectively, following the recommendations of 10X Genomics (paired-end reads, single indexing, read 1 = 28 cycles, i7 = 8 cycles, i5 = 0 cycles and read 2 = 91 cycles). For nonmultiplexed runs, the 3' Gene Expression libraries for each sample were pooled at an equimolar amount and sequenced on an Illumina NovaSeq 6000 system with a sequencing depth of 50000 reads per cell, following the same recommendations of 10x Genomics.

scRNA-seq data analysis

The Cell Ranger Single-Cell Software Suite (version 6.0.2) was used for cDNA oligopeptide alignment to the reference GRCm38.p5, barcode assignment and UMI counting of fastq files obtained by Illumina sequencing. For each sample, the cell-containing droplets were filtered from the empty droplets, followed by the generation of an expression matrix using Cell Ranger Count (version 6.0.2). Demultiplexing of the cells within each sample was performed with the filtered matrix produced by Cell Ranger in R (version 3.6.3) using Seurat (version 3.2.3) [5] and the HTODemux function (positive quantile set at 0.99). The resulting gene expression matrices were further analyzed with Python (version 3.8.6) using the Scanpy (version 1.7.0) [6] library. Low-quality cells were defined as those with less than 1500 total counts, expressing more than 5000 or fewer than 350 genes or those in which mitochondrial genes accounted for more than 15% of all genes or ribosomal genes accounted for more than 15% of all genes and were excluded from downstream analyses. Genes expressed in fewer than 100 cells were also removed. Normalization was performed using the pool-based size factor estimation implemented in the scran R package (version 1.14.1) [7,8]. Size factors were determined using the function, and normalization was performed by dividing the total gene count in each cell by the cell-specific size factor, deconvoluted from the pool size factor. Data were log transformed. For the Ter119 dataset, the data from the two conditions were integrated using scanorama for batch-effect correction (version 1.7.1) based on the top 5000 DEG. Principal component analysis (PCA) was performed using the tl.pca function (Scanpy 1.7.0) with default settings followed by nearest neighbor graph construction using the pp.neighbors (Scanpy

1.7.0) function with the first 15 principal components. Further dimensionality reduction was performed using the Leiden algorithm, and cells were plotted in 2 dimensions using UMAP plots. Differential gene expression was calculated using the tl.rank_genes_group function (Scanpy 1.7.0) with the implemented Wilcoxon rank sum test. After marker gene based cell type assignment all non-phagocyte cells were excluded from downstream analysis.

RNA-velocity analysis

For the Ter119 dataset, the ratio of spliced and unspliced counts was calculated using velocyto (version 0.17.16) [9]. The resulting matrix was merged with the counts dataset, followed by RNA velocities calculation and visualization using scVelo (version 0.2.3.) [10].

Pathway and transcription factor enrichment analysis of scRNA-seq data

Pathway enrichment analysis was performed using the GSEApy library (version 0.10.7) [11] with hallmark gene sets from the Molecular Signature Database (MSigDB) [12] as a reference. For the input, we used the preranked analysis with all genes ranked according to p value*sign(log fold change). Mus musculus gene names were converted to human gene names using the python based Biomart library (0.9.2) [13]. The pathway enrichment analysis results were visualized using heatmaps, with each row corresponding to a pathway, and the running enrichment score for each gene was plotted. The rows were ordered according to their normalized enrichment score.

RT–qPCR analysis

Total RNA was isolated from liver macrophages using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. TaqMan reverse transcription reagents (Life Technologies) were used for reverse transcription with the following cycling protocol: 10 min at 25°C, 39 min at 48°C and 5 min at 95°C (Mastercycler gradient, Eppendorf, Z316083). RT–qPCR was performed using Fast SYBR™ Green Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). The primers and their corresponding target genes are listed in Table 2. Relative mRNA levels for experimental samples were calculated with 7500 Fast System Sequence Detection Software version 1.4 (Applied Biosystems) after normalization to *Hprt* levels.

Table 2: Sequences for PCR primers

Target gene	Forward sequence (5′-3′)	Reverse sequence (3′-5′)
Hprt	cctcctcagaccgcttttt	aacctggttcatcatcgctaa
Cxcl9	gctgccgtcattttctgc	tctcactggcccgtcatc
Cxcl10	cttttcctcttgggcatcat	gcatcgtgcattccttatca
Il12b	tgggagtaccctgactcctg	aggaacgcacctttctggtt
Cd74	caccgaggctccacctaaag	ttaccgttctcgtcgcactt
Hmox1	aggctaagaccgccttcct	tgtgttcctctgtcagcatca

Statistical analysis

Sample size was calculated with R (4.1.2). Data plotting and statistical analysis were performed with Prism 9 (GraphPad) and JMP 15 (SAS) or the Bioturing SingleCell browser (v3.3.15). We used ANOVA with Tukey’s posttest, t tests (two-tailed), and Fisher’s test or the chi^2 test to analyze contingency tables, as indicated in the figure legends. All data points are displayed in bar plots as the mean ± standard deviation (n.s. = not significant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

REFERENCES

1. Liu Z, Gu Y, Chakarov S, Bleriot C, Kwok I, Chen X, et al. Fate Mapping via Ms4a3-Expression History Traces Monocyte-Derived Cells. Cell. 2019;178:1509–25.e19.

2. Ingoglia G, Yalamanoglu A, Pfefferlé M, Dubach IL, Schaer CA, Valkova K, et al. Line-selective macrophage activation with an anti-CD40 antibody drives a hemophagocytic syndrome in mice. Blood Adv. 2020;4:2751–61.

3. Crow AR, Kapur R, Koernig S, Campbell IK, Jen C-C, Mott PJ, et al. Treating murine inflammatory diseases with an anti-erythrocyte antibody. Sci Transl Med [Internet]. 2019;11. Available from: https://stm.sciencemag.org/content/11/506/eaau8217

4. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017;7:16878.

5. Hao Y, Hao S, Andersen-Nissen E, Mauck WM 3rd, Zheng S, Butler A, et al. Integrated analysis of multimodal single-cell data. Cell. 2021;184:3573–87.e29.

6. Wolf FA, Angerer P, Theis FJ. SCANPY: large-scale single-cell gene expression data analysis. Genome Biol. 2018;19:15.

7. Lun ATL, McCarthy DJ, Marioni JC. A step-by-step workflow for low-level analysis of single-cell RNA-seq data with Bioconductor. F1000Res. 2016;5:2122.

8. Lun ATL, Bach K, Marioni JC. Pooling across cells to normalize single-cell RNA sequencing data with many zero counts. Genome Biol. 2016;17:75.

9. La Manno G, Soldatov R, Zeisel A, Braun E, Hochgerner H, Petukhov V, et al. RNA velocity of single cells. Nature. 2018;560:494–8.

10. Bergen V, Lange M, Peidli S, Wolf FA, Theis FJ. Generalizing RNA velocity to transient cell states through dynamical modeling. Nat Biotechnol. 2020;38:1408–14.

11. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102:15545–50.

12. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 2015;1:417–25.

13. Durinck S, Spellman PT, Birney E, Huber W. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nat Protoc. 2009;4:1184–91.