

Supporting Information

Single-cell transcriptome reveals the reprogramming of immune microenvironment during the transition from MASH to HCC

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INVENTORY

1. Supporting Experimental Section.....Page 2-5
2. Supporting References.....Page 5
3. Supporting Figures and Legends.....Pages 6-35

Supporting Experimental Section

GeneSwitches Analysis

GeneSwitches¹ (version 0.1.0) was employed to elucidate the sequential order of gene expression and functional events during the cellular state transitions. Initially, gene expression data were binarized into binary states (1 = on, 0 = off) using the ‘binarize_exp’ function within the GeneSwitches package (fix_cutoff = TRUE, binarize_cutoff = 0.05). To accomplish this, gene-specific thresholds were determined by fitting a two-component Gaussian mixture model to the gene expression data. To enhance numerical stability, Gaussian noise with a mean of zero and a standard deviation of 0.1 was introduced prior to model fitting. Genes lacking a clear bimodal "on-off" distribution were excluded.

Logistic regression was then applied to model the binary gene expression states using the ‘find_switch_logistic_fastglm’ function. For genes exhibiting high zero-inflation, random downsampling of zero expressions (downsample = TRUE) was utilized to maintain accurate predictions of switching times. The top 50 genes with the best model fit, as indicated by high McFadden's Pseudo R^2 values, were visualized along the pseudo-timeline. The activated genes were depicted above the timeline, whereas the inactivated ones were plotted below the timeline.

scVelo Analysis

RNA velocity analysis was performed by recounting spliced and unspliced reads using the Python script `velocity.py`² (<https://github.com/velocyto-team/velocyto.py>) on the output directory generated by Cell Ranger. To calculate the RNA velocities, which encompass the rates of transcription, splicing and degradation, a likelihood-based dynamical model and a velocity graph were constructed using the scVelo package³ (<https://scvelo.readthedocs.io/>). The resultant velocity fields were subsequently projected onto the UMAP-based embedding

obtained from Seurat.

Cell-cell interactions analysis

Cell-cell interactions analysis was performed with CellChat package⁴ (version 1.1.3). Briefly, we firstly imported the normalized expression matrix to create the cellchat object with the ‘createCellChat’ function. Thereafter, the data was preprocessed with ‘identifyOverExpressedGenes’, ‘identifyOverExpressedInteractions’ and ‘projectData’ functions with the default parameters. The ‘computeCommunProb’, ‘filterCommunication’ and ‘computeCommunProbPathway’ functions were then used to determine any potential ligand-receptor interactions. Finally, the cell-cell interaction networks and heatmaps were aggregated using the ‘aggregateNet’ function.

Histology

For paraffin-embedded section, livers were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were subsequently prepared and stained with haematoxylin (#G1004, Servicebio, Wuhan, China) and eosin (#G1001, Servicebio, Wuhan, China), Sirius Red (#G1018, Servicebio, Wuhan, China), or processed for immunohistochemistry (IHC) staining. For frozen section, livers were frozen in OCT compound (#4583, Tissue-Tek, Sakura Finetek, Torrance, CA, USA), sectioned and stained with Oil Red O (#G1015, Servicebio, Wuhan, China) and haematoxylin.

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using the TRI reagent (#T9424, Sigma-Aldrich, MO, USA), and was reverse transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). The subsequent qRT-PCR was carried out using 2× SYBR Green qPCR Master Mix (Biotool, Houston, TX, USA) on a LightCycler 480 thermocycler (Roche Diagnostics, Mannheim, Germany). Cycle threshold (Ct) values were analyzed with LightCycler 480 software, and raw

data were processed accordingly. The expression levels of target genes were normalized to reference genes (*Actb*) using the $2^{-\Delta Ct}$ method, where $\Delta Ct = Ct_{\text{target}} - Ct_{\text{Actb}}$. The primers used in this study are listed in Supplementary Table S4.

Western blotting

Western blotting was performed as previously described⁵. The antibodies used for western blotting are listed in Supplementary Table S1.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described⁵. The antibodies used for immunofluorescence staining are listed in Supplementary Table S1.

Isolation of primary tumor cells

The tumor tissue was minced with scissors and subjected to digestion at 37°C for 30~40 minutes with gentle shaking at 200 rpm in a digestion buffer containing 1 mg/mL collagenase/dispase (#10269638001, Roche, Basel, Switzerland). After digestion, cell suspensions were filtered through a 100 µm cell strainer and centrifuged at 1000 rpm for 5 minutes at 4°C. Thereafter, the pellet was resuspended in 10 mL of PBS/2% FBS and sequentially filtered through 70 µm and 45 µm cell strainers. The filtered suspensions were centrifuged at 1000 rpm for 5 minutes at room temperature (RT). The pellet was resuspended in Red Cell lysis buffer to get rid of red blood cells, followed by centrifugation at 1000 rpm for 2 minutes at RT. Subsequently, the tumor cell pellet was subjected to western blotting.

Measurement of cytokine and chemokine in serum samples

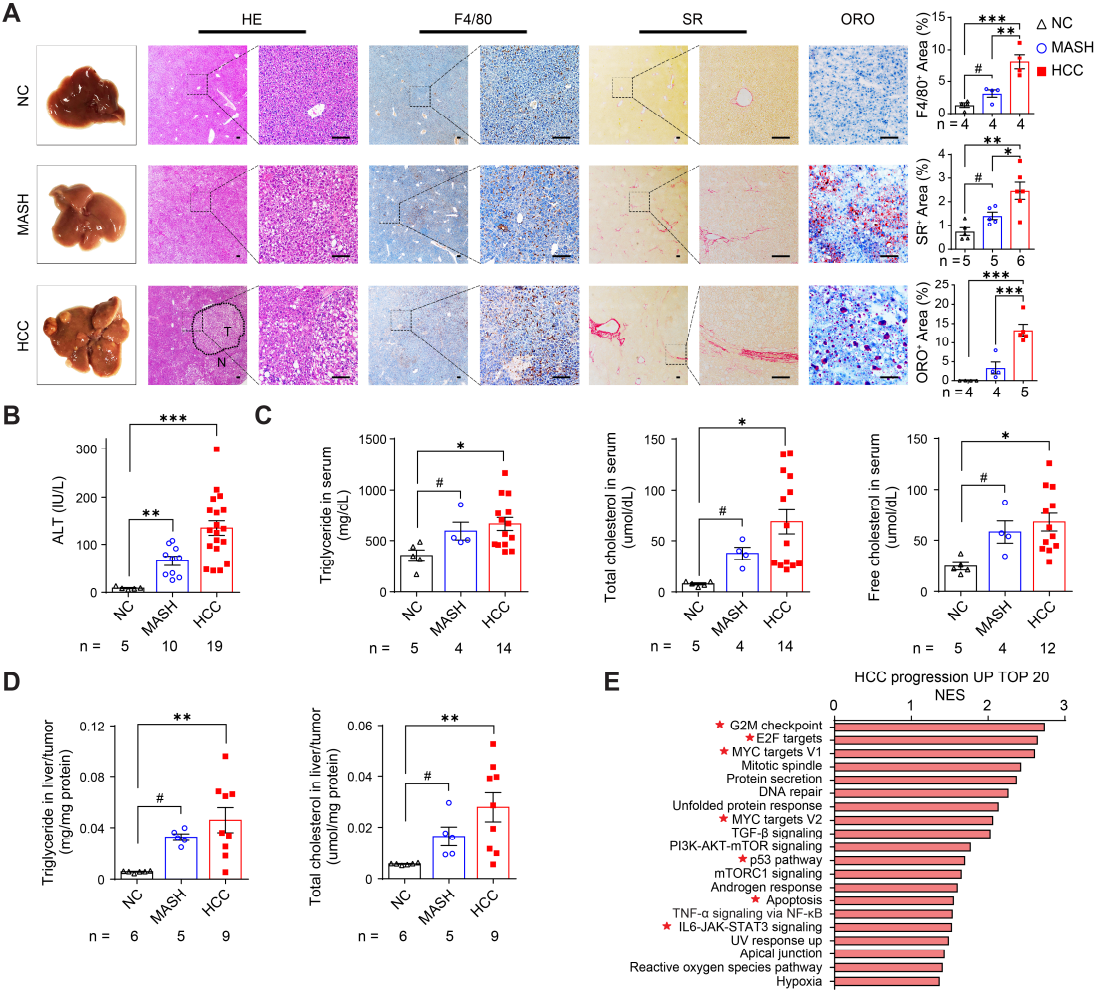
Cytokine and chemokine in serum were quantified using the LEGENDplex Mouse Inflammation Panel (13-plex) (#740150, BioLegend, San Diego, CA, USA) according to manufacturer's instructions. Specially, flow cytometry was performed on the Cytex Northern Lights Flow Cytometer (Cytex Biosciences, Fremont, CA, USA), and data analysis was

conducted with LEGENDplex Cloud Analysis Suite (BioLegend, San Diego, CA, USA).

Supporting References

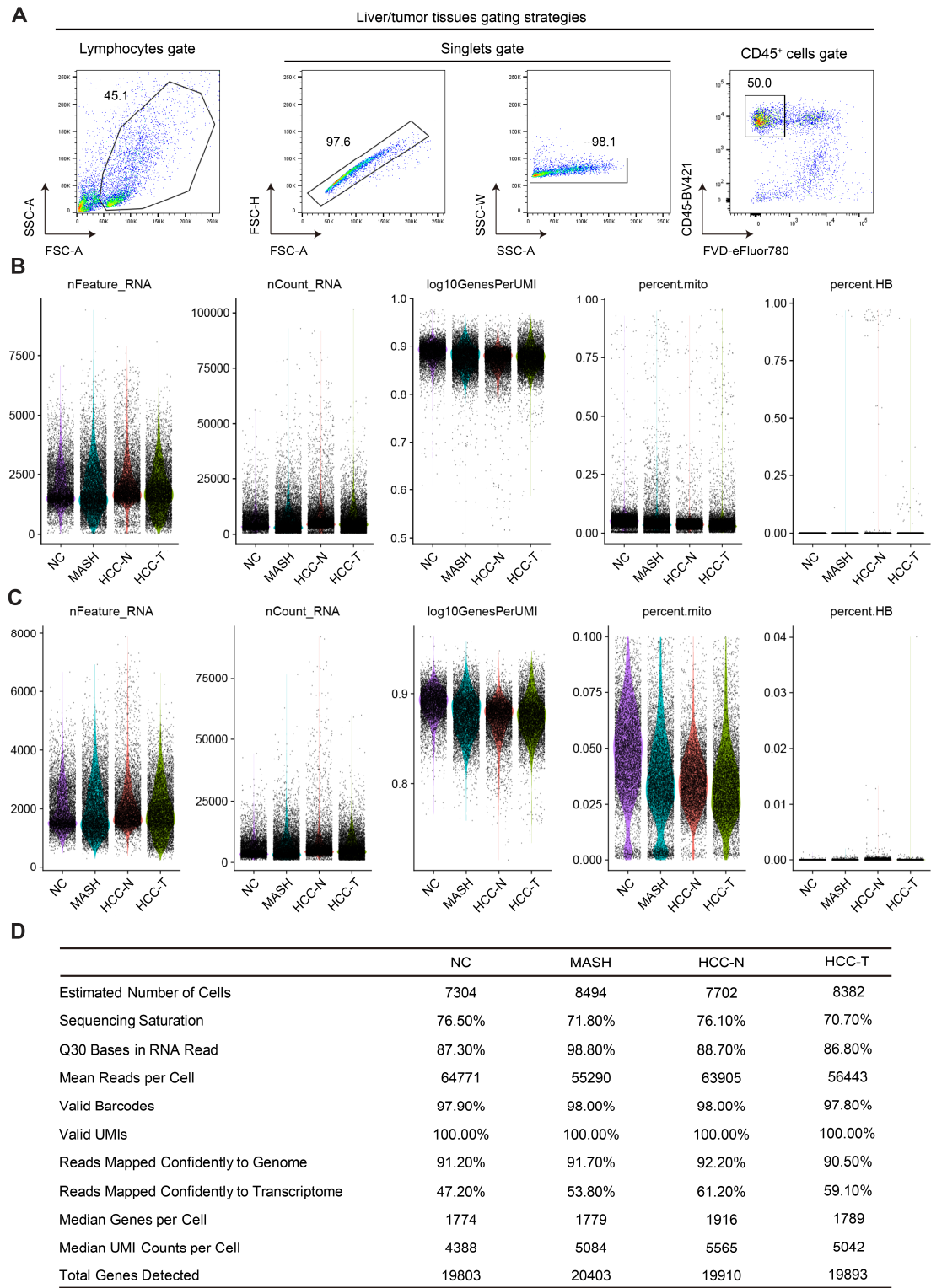
1. Cao EY, Ouyang JF, Rackham OJL. GeneSwitches: ordering gene expression and functional events in single-cell experiments. *Bioinformatics* **2020**;36:3273-5
2. La Manno G, Soldatov R, Zeisel A, Braun E, Hochgerner H, Petukhov V, *et al.* RNA velocity of single cells. *Nature* **2018**;560:494-8
3. Bergen V, Lange M, Peidli S, Wolf FA, Theis FJ. Generalizing RNA velocity to transient cell states through dynamical modeling. *Nature biotechnology* **2020**;38:1408-14
4. Jin S, Guerrero-Juarez CF, Zhang L, Chang I, Ramos R, Kuan CH, *et al.* Inference and analysis of cell-cell communication using CellChat. *Nature communications* **2021**;12:1088
5. Xie Y, Huang Y, Li ZY, Jiang W, Shi NX, Lu Y, *et al.* Interleukin-21 receptor signaling promotes metabolic dysfunction-associated steatohepatitis-driven hepatocellular carcinoma by inducing immunosuppressive IgA(+) B cells. *Molecular cancer* **2024**;23:95

Figure S1

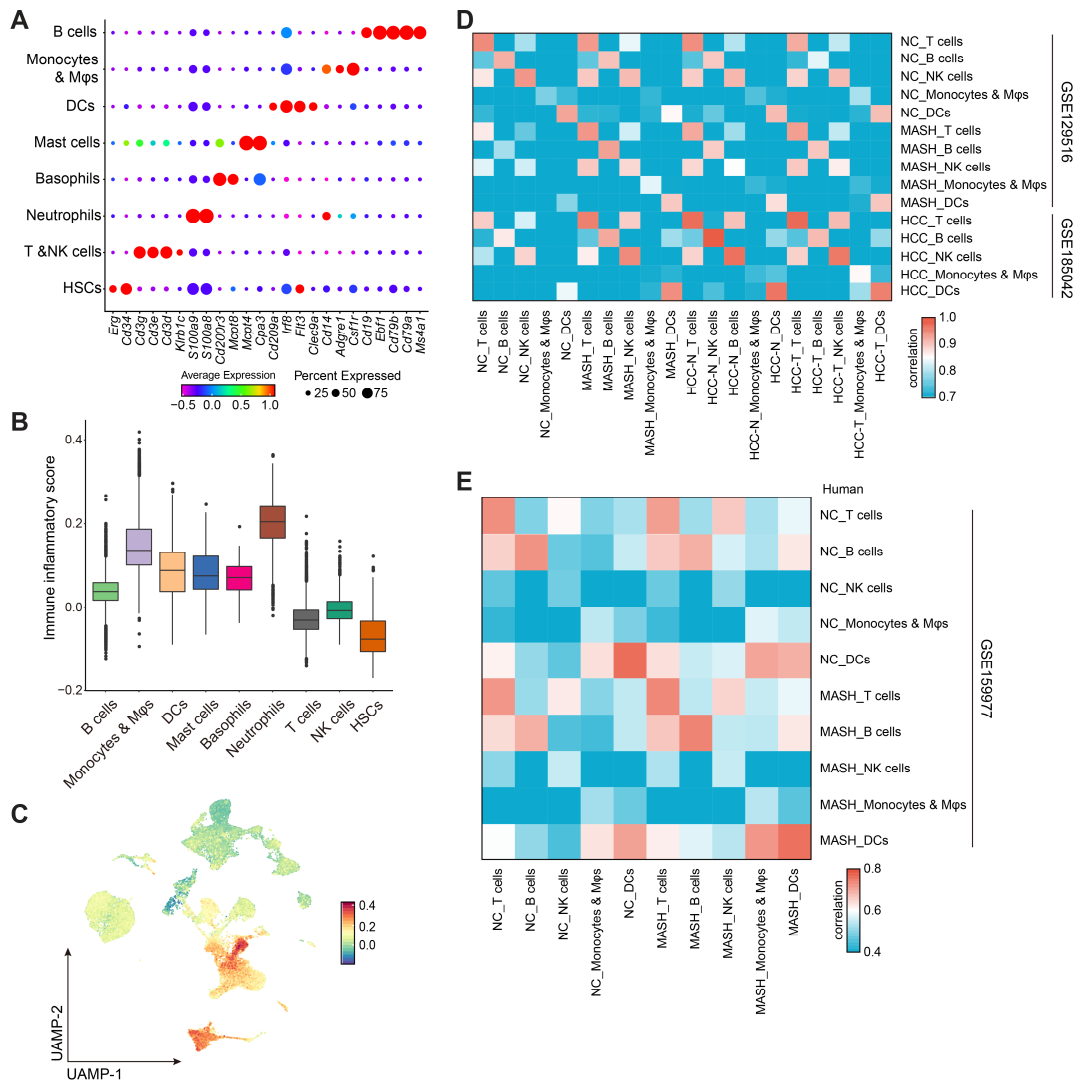


Supplementary Fig. S1. Establishment and validation of STAM model. (A) Infiltration of inflammatory cells, fibrosis and steatosis occurred in the livers of mice with metabolic dysfunction-associated steatohepatitis or hepatocellular carcinoma. Shown are the representative images of hematoxylin-eosin (HE), F4/80 immunohistochemical staining, Sirius Red (SR) and Oil Red O (ORO) in the hepatic tissues of mice with normal chow (NC), metabolic dysfunction-associated steatohepatitis (MASH) or hepatocellular carcinoma (HCC). Scale bar = 100 μ m. (B) Liver damage occurred in the mice with MASH or HCC. Serum ALT levels were detected in the mice at the above-mentioned stages. (C) The levels of triglyceride, total cholesterol and free cholesterol were elevated in the sera of mice with MASH or HCC. (D) The levels of triglyceride and total cholesterol were elevated in the liver/tumor extracts of mice with MASH or HCC. (E) Top 20 hallmark gene sets sorted by normalized enrichment score (NES) are shown to depict HCC progression from normal liver to tumor in STAM model by gene set enrichment analysis (GSEA). The gene sets previously described for human HCC are marked with a red asterisk. Each data point represents an individual mouse in A-D, and the number of mice in each group is shown in the panels accordingly. One-way ANOVA was used to determine significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. # All were significant using Student's t test. ns, not significant difference.

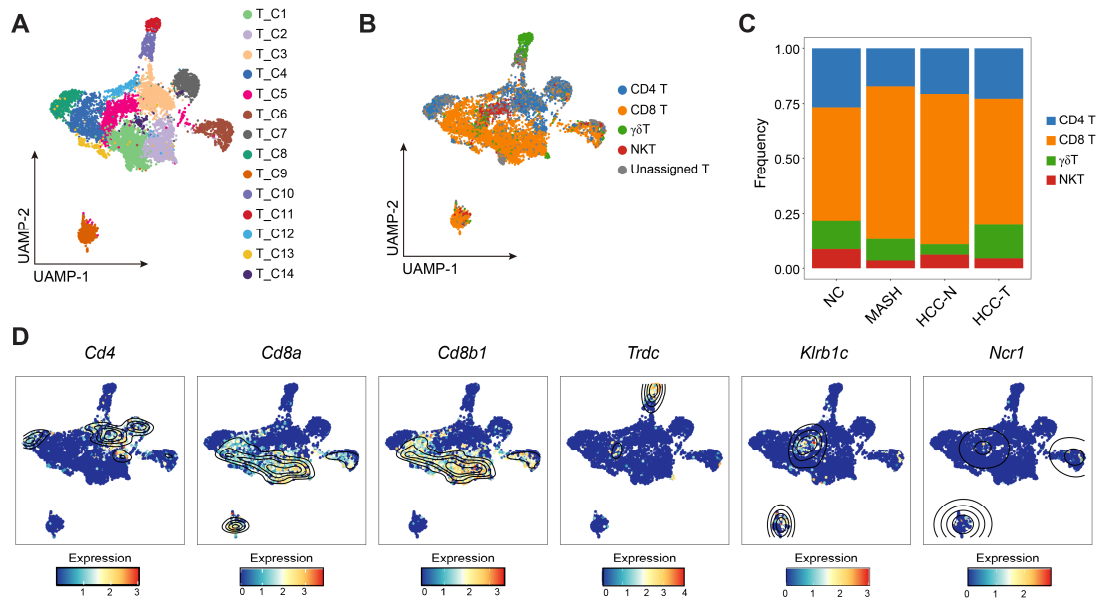
Figure S2



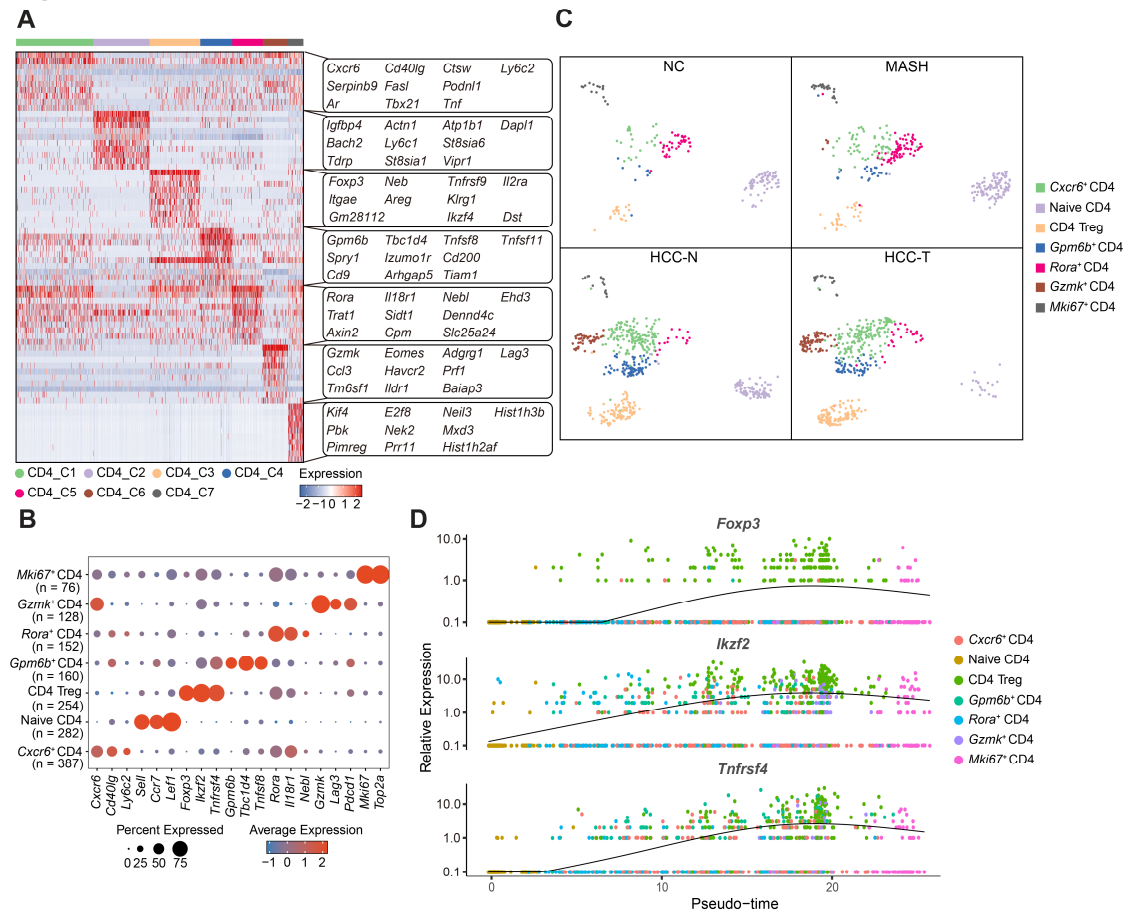
Supplementary Fig. S2. Flow cytometric gating strategy for sorting liver immune cells and the quality control of single-cell sequencing data filtering. (A) Flow cytometry was utilized to sort liver/tumor CD45⁺ cells (immunocytes) for single-cell RNA sequencing (scRNA-seq). (B, C) Violin plots were generated to display the nFeature_RNA, nCount_RNA, log₁₀GenesPerUMI, percent.mito and percent.HB metrics of the raw scRNA-seq data (B) and quality control measures data (C) for mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), as well as paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with hepatocellular carcinoma (HCC). Each dot in the scatter plot represents an individual cell. (D) Shown is an overview of gene quantitative quality control for sequencing libraries and the alignment outcomes of scRNA-seq data.

Figure S3

Supplementary Fig. S3. Marker genes and immune inflammation scores for distinct cell types. (A) Dot plot illustrated the expression patterns of marker genes among distinct cell types. (B) Box plots illustrated the immune inflammation scores for each cell type. (C) Feature plot depicted the immune inflammation scores related to (B). (D, E) Pearson's correlations across immune cells were performed between our single-cell RNA sequencing (scRNA-seq) dataset and the dataset from two combined mouse metabolic dysfunction-associated steatohepatitis (MASH)-related hepatocellular carcinoma (HCC) studies (D) or one human MASH study (E).

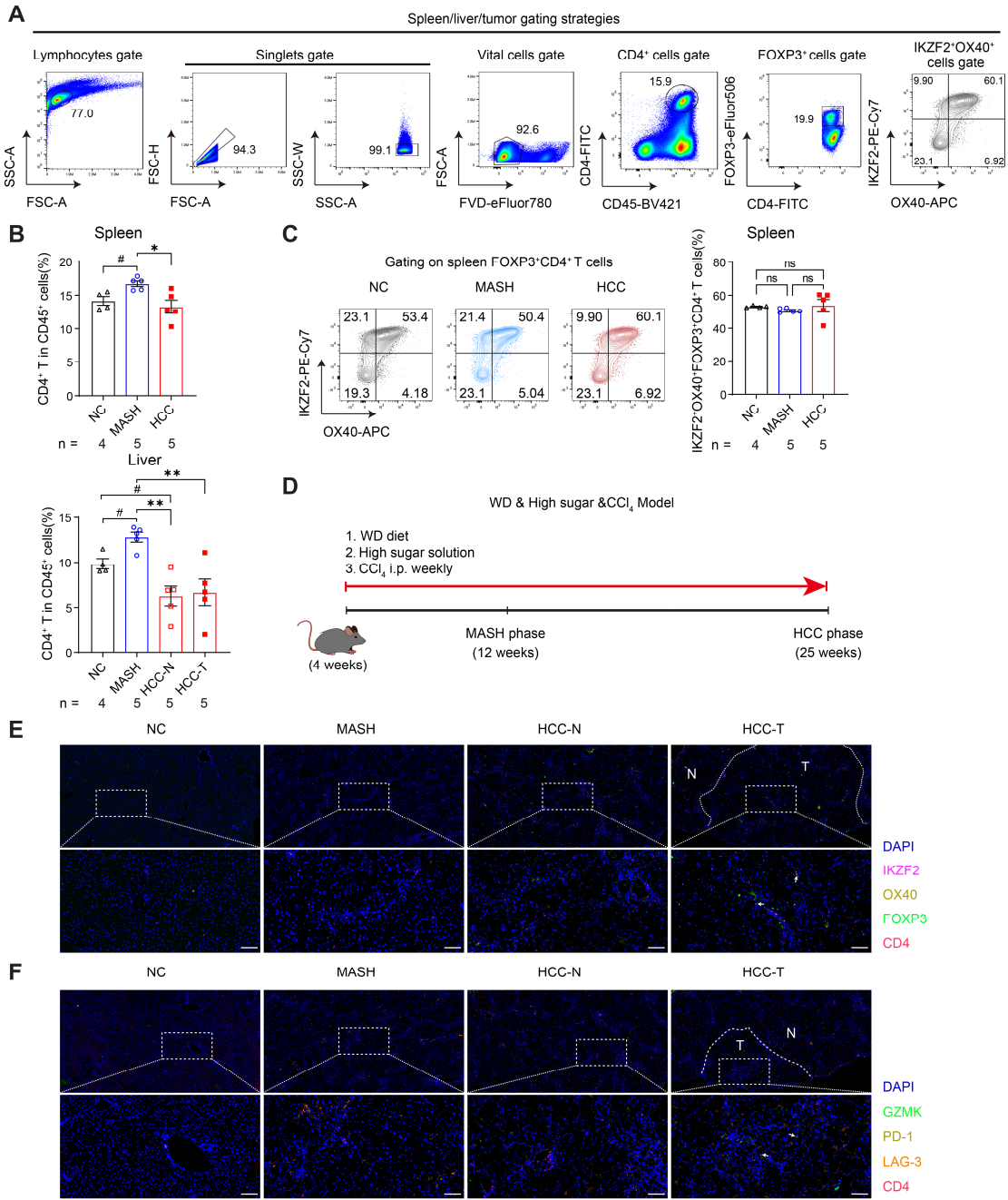
Figure S4

Supplementary Fig. S4. The expression of signature genes and the percentages for distinct T cell subtypes. (A, B) UMAP visualization of clusters (A) and cell subtypes (B) for T cells. (C) Bar chart depicted the percentages of distinct T cell subtypes. (D) UMAP visualization illustrated the expression patterns of distinct signature genes in T cells, with each point representing an individual cell. The black lines represent the spatial density of cells which expressed higher levels of indicated genes than the mean expression level.

Figure S5

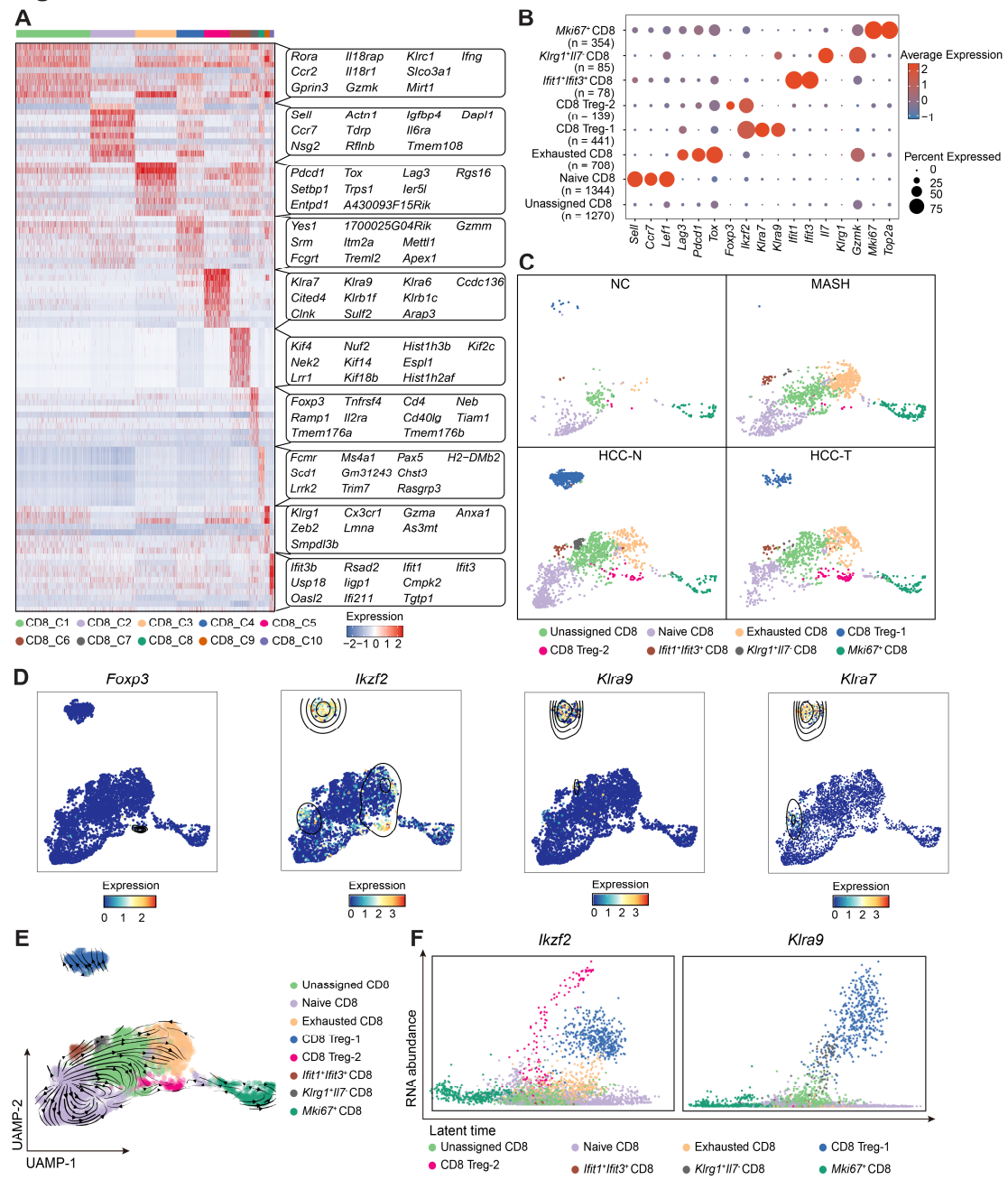
Supplementary Fig. S5. Expression patterns of signature genes or important genes for distinct CD4 T subtypes. (A) Heatmap displayed the top 10 genes for each CD4 T subcluster. (B) Dot plot illustrated the expression patterns of signature genes among distinct CD4 T subtypes. (C) UMAP visualization of distinct CD4 T subtypes among four groups, including liver tissues from mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with hepatocellular carcinoma (HCC). (D) Dot plot illustrated the expression patterns of indicated genes calculated by Monocle2.

Figure S6

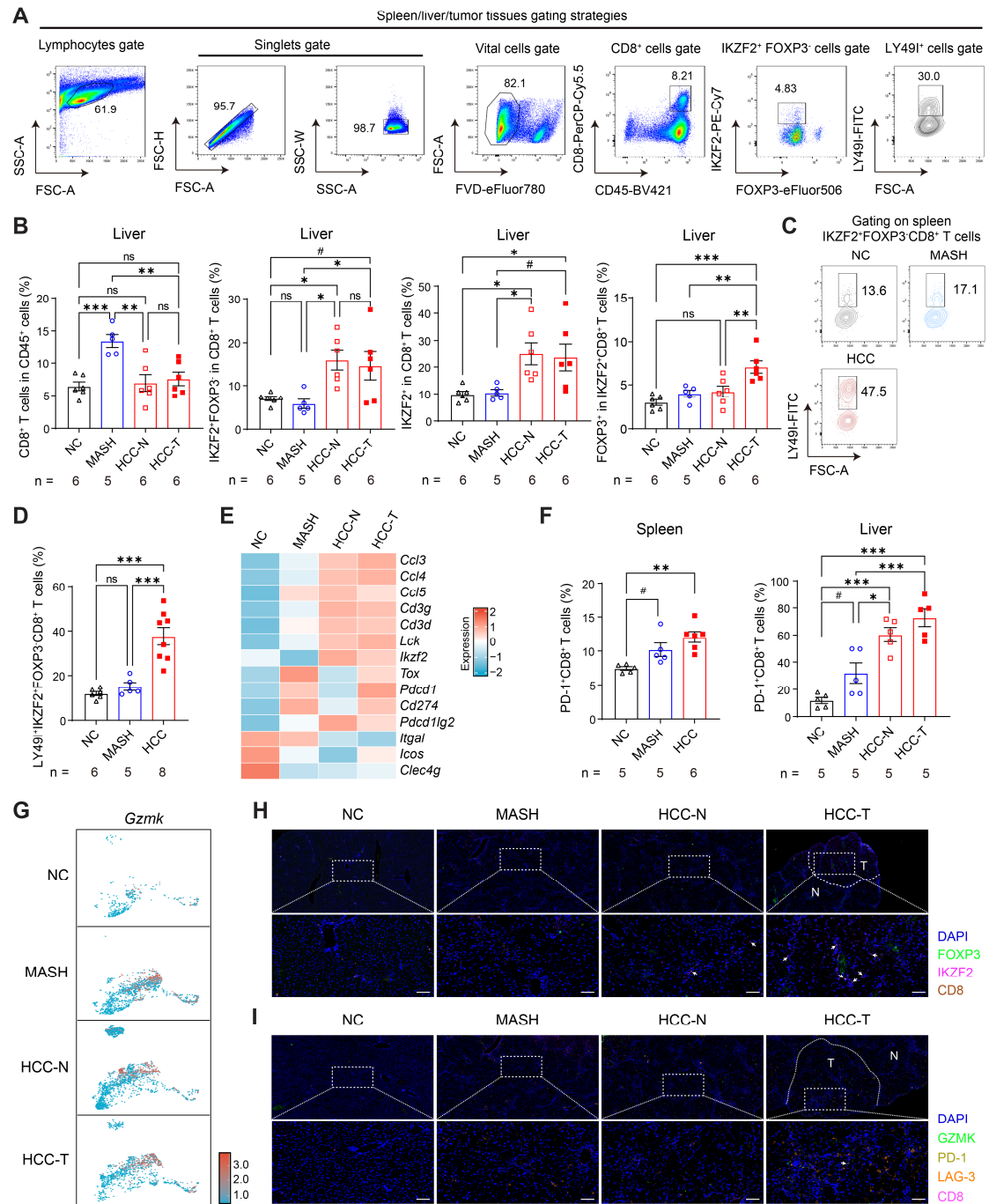


Supplementary Fig. S6. Immunosuppressive and exhausted CD4⁺ T cells were increased in MASH-related HCC. (A) The gating strategies for CD4 subtypes. Liver/tumor cell suspensions or splenocyte from mice were stained with CD45 and FVD-eF780, which was used to exclude dead cells. The sequential gating strategies are as follows: lymphocyte gate, doublets exclusion, dead cell exclusion, CD4⁺ population, FOXP3⁺ population and IKZF2⁺OX40⁺ population gate. (B) Liver CD4⁺ T cells were increased in metabolic dysfunction-associated steatohepatitis (MASH), but decreased in MASH-related hepatocellular carcinoma (HCC). The percentages of CD4⁺ T cells in the spleen (upper) or liver/tumor (lower) were analyzed in the mice with normal chow (NC) or MASH, as well as paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with HCC by using flow cytometry analysis. (C) The percentages of IKZF2⁺OX40⁺FOXP3⁺CD4⁺ T cells in the spleen were not different among different groups. The representative flow cytometry images are shown in the left panel. In the right panel, the percentages of IKZF2⁺OX40⁺FOXP3⁺CD4⁺ T cells were analyzed among different groups. Each data point represents an individual mouse in B and C, and the number of mice in each group is shown in the panels accordingly. (D) Schematic diagram of WD & High sugar solution & CCl₄ model. WD, western diet. MASH, metabolic dysfunction-associated steatohepatitis. HCC, hepatocellular carcinoma. (E, F) IKZF2⁺OX40⁺FOXP3⁺CD4⁺ and GZMK⁺LAG-3⁺PD-1⁺CD4⁺ T cells were increased in MASH-related HCC by using WD & High sugar solution & CCl₄ model. Shown are the representative multiplexed immunohistochemistry images. The white arrows show the IKZF2⁺OX40⁺FOXP3⁺CD4⁺ and GZMK⁺LAG-3⁺PD-1⁺CD4⁺ T cells in E and F, respectively. Scale bar = 100 μ m. One-way ANOVA was used to determine significance. * $P < 0.05$, ** $P < 0.01$. # All were significant using Student's t test. ns, not significant difference.

Figure S7



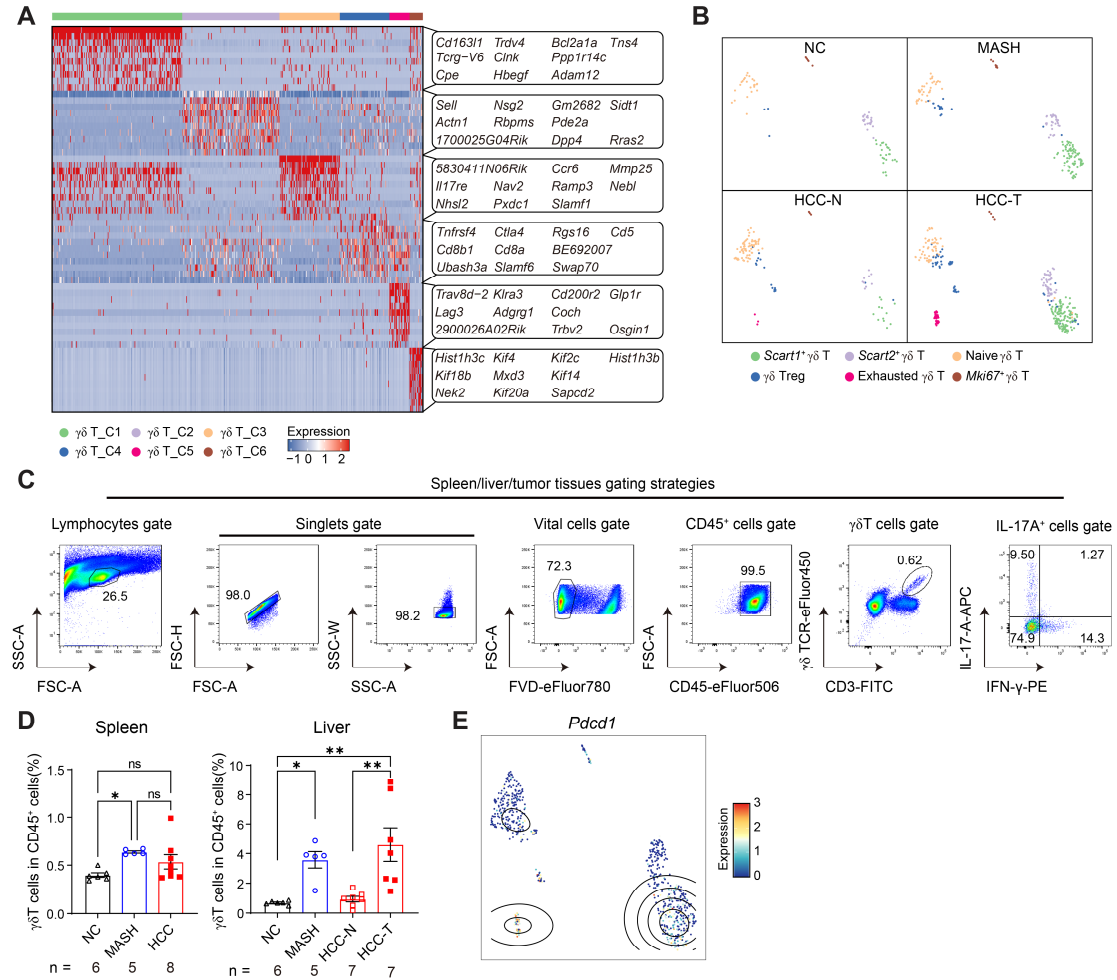
Supplementary Fig. S7. Expression patterns of signature genes or important genes for distinct CD8 T subtypes. (A) Heatmap displayed the top 10 genes for each CD8 T subcluster. (B) Dot plot illustrated the expression patterns of signature genes among distinct CD8 T subtypes. (C) UMAP visualization of distinct CD8 T subtypes among four groups, including liver tissues from mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with hepatocellular carcinoma (HCC). (D) UMAP visualization illustrated the expression patterns of indicated genes in CD8 T cells, with each point representing an individual cell. The black lines represent the spatial density of cells which expressed higher levels of indicated genes than the mean expression level. (E) RNA velocity-based cell lineage of CD8 T cells. (F) Shown are the dot plots displayed by expression levels according to RNA velocity analysis.

Figure S8

Supplementary Fig. S8. Immunosuppressive and exhausted CD8⁺ T cells were increased in MASH-related HCC. (A) The gating strategies for CD8 subtypes. Liver/tumor cell suspensions or splenocyte from mice were stained with CD45 and FVD-eF780, which was used to exclude dead cells. The sequential gating strategies are as follows: lymphocyte gate, doublets exclusion, dead cell exclusion, CD8⁺ population, IKZF2⁺FOXP3⁻ population and LY49I⁺ population gate. (B) Liver CD8⁺ T cells were increased in metabolic dysfunction-associated steatohepatitis (MASH), while the immunosuppressive CD8⁺ T cells were increased in MASH-related hepatocellular carcinoma (HCC). The percentages of liver CD8⁺, IKZF2⁺FOXP3⁻, IKZF2⁺ and FOXP3⁺ T cells in the indicated populations were analyzed in the mice with normal chow (NC) or MASH, as well as paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with HCC by using flow cytometry analysis. (C, D) The percentages of LY49I⁺IKZF2⁺FOXP3⁻CD8⁺ T cells in the spleen were increased in MASH-related HCC. The representative flow cytometry images are shown (C) and the percentages of LY49I⁺IKZF2⁺FOXP3⁻CD8⁺ T cells were analyzed among different groups (D). (E) The levels of genes related to CD8 T cell exhaustion were increased, whereas the levels of genes involved in T cell activation were decreased in the MASH-to-HCC transition according to our single-cell RNA sequencing. (F) PD-1⁺CD8⁺ T cells were increased in the spleen and liver during the transition from MASH to HCC. The percentages of PD-1⁺CD8⁺ T cells in the spleen (left) or liver (right) were analyzed among different groups by using flow cytometry analysis. (G) UMAP visualization illustrated the expression patterns of *Gzmk* in CD8 T cells among different groups, with each point representing an individual cell. (H, I) IKZF2⁺FOXP3⁻CD8⁺ and LAG-3⁺PD-1⁺GZMK⁺CD8⁺ T cells were increased in MASH-related HCC by using WD & High sugar solution & CCl₄ model. Shown are the representative multiplexed immunohistochemistry images. The white arrows show the IKZF2⁺FOXP3⁻CD8⁺ and LAG-3⁺PD-1⁺GZMK⁺CD8⁺ T cells in H and I, respectively. Scale

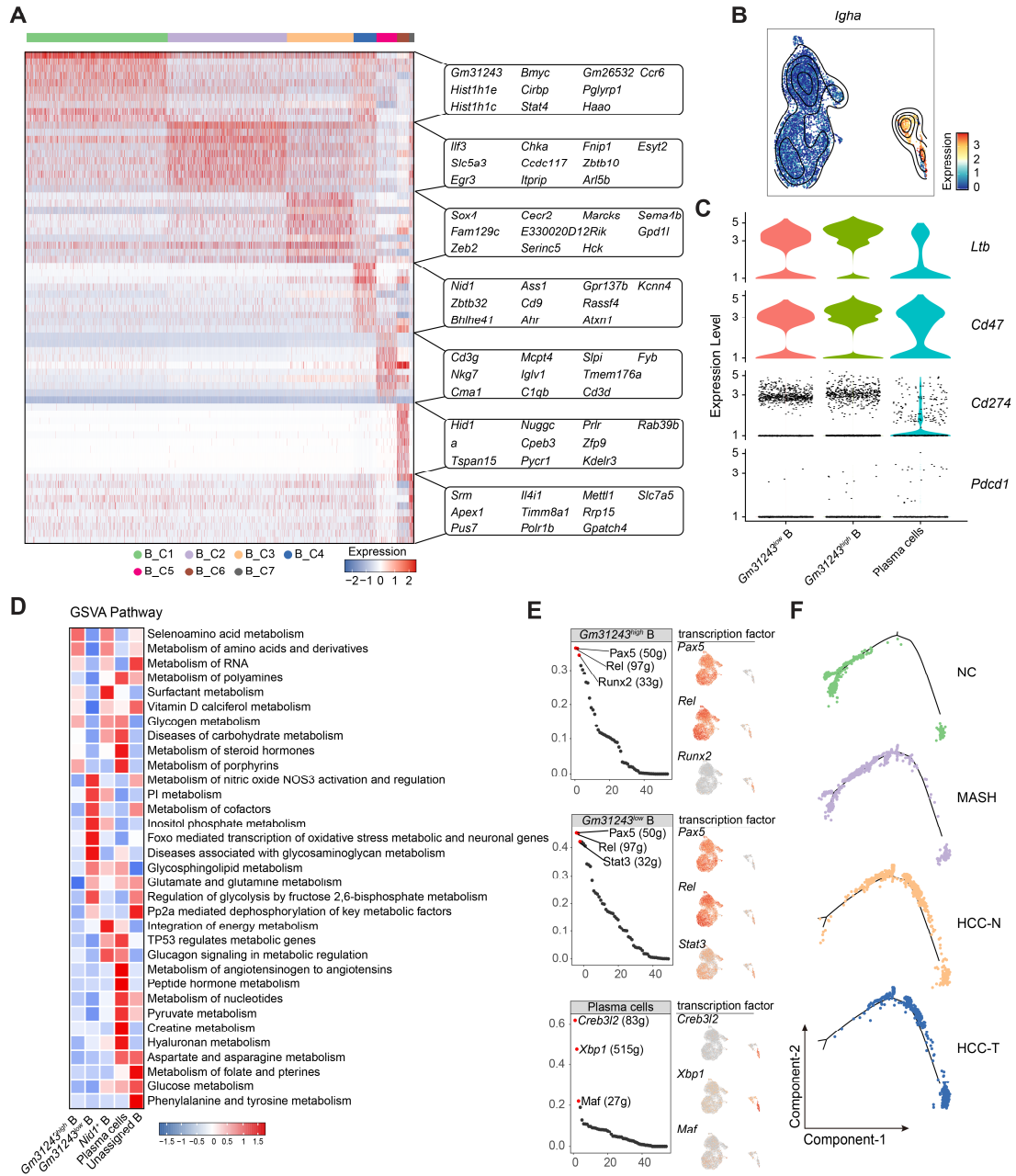
bar = 100 μ m. Each data point represents an individual mouse in B, D and F, and the number of mice in each group is shown in the panels accordingly. One-way ANOVA was used to determine significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. # All were significant using Student's t test. ns, not significant difference.

Figure S9



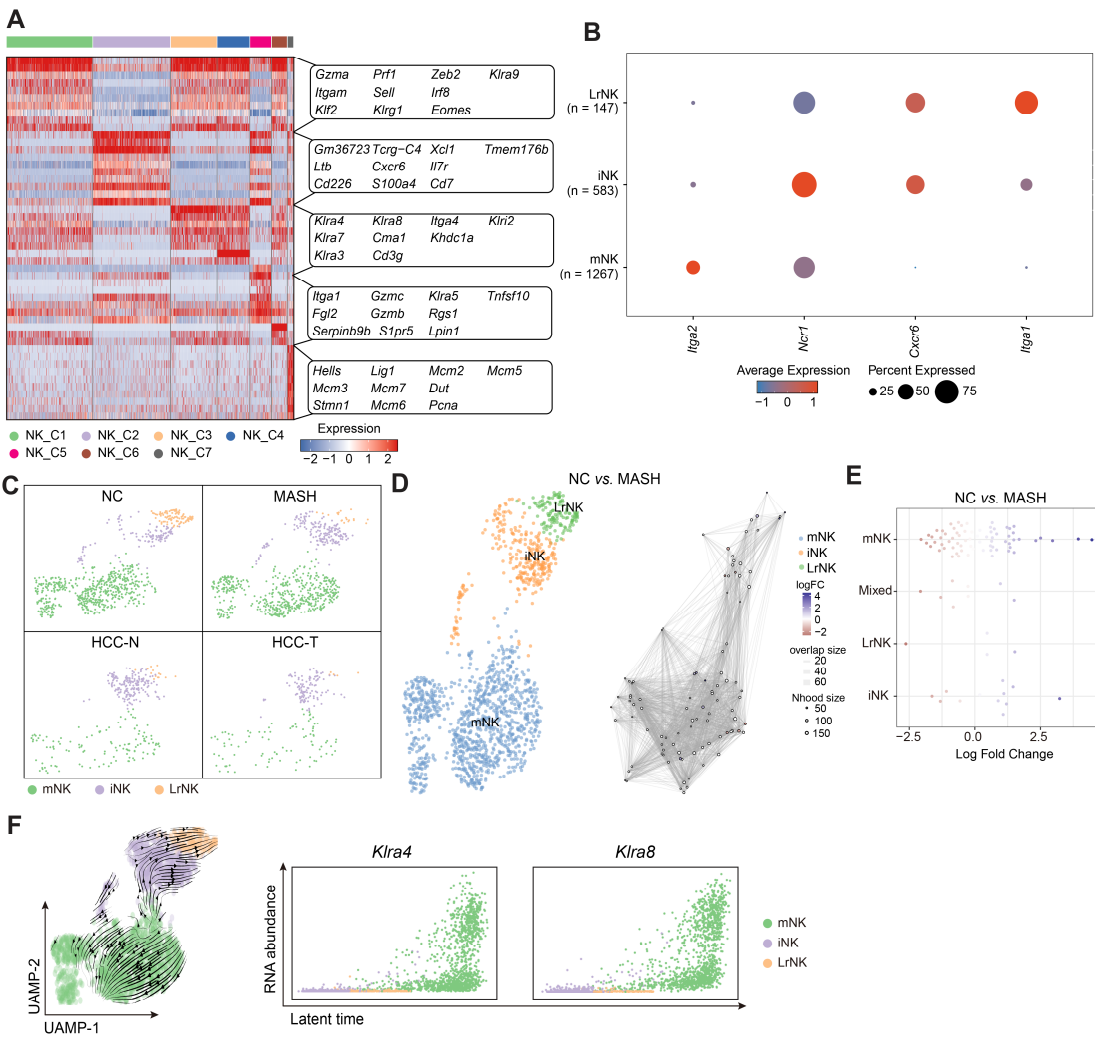
Supplementary Fig. S9. Expression patterns of signature genes, UMAP visualization and the percentages for $\gamma\delta$ T cells in the MASH-to-HCC transition. (A) Heatmap displayed the top 10 genes for each $\gamma\delta$ T subcluster. (B) UMAP visualization of distinct $\gamma\delta$ T subtypes among different groups, including liver tissues from mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with hepatocellular carcinoma (HCC). (C) The gating strategies for $\gamma\delta$ T subtypes. Liver/tumor cell suspensions or splenocyte from mice were stained with CD45 and FVD-eF780, which was used to exclude dead cells. The sequential gating strategies are as follows: lymphocyte gate, doublets exclusion, dead cell exclusion, CD45⁺ population, $\gamma\delta$ T population and IL-17A⁺ population gate. (D) Liver $\gamma\delta$ T cells were increased in MASH and MASH-related HCC. The percentages of $\gamma\delta$ T cells in the spleen (left) or liver/tumor (right) were analyzed in the mice with normal chow or MASH, as well as paired paracancerous and cancer tissues from mice with HCC by using flow cytometry analysis. Each data point represents an individual mouse, and the number of mice in each group is shown. (E) UMAP visualization illustrated the expression patterns of *Pdcd1* in $\gamma\delta$ T cells, with each point representing an individual cell. The black lines represent the spatial density of cells which expressed higher levels of *Pdcd1* than the mean expression level. One-way ANOVA was used to determine significance. * $P < 0.05$, ** $P < 0.01$. ns, not significant difference.

Figure S10

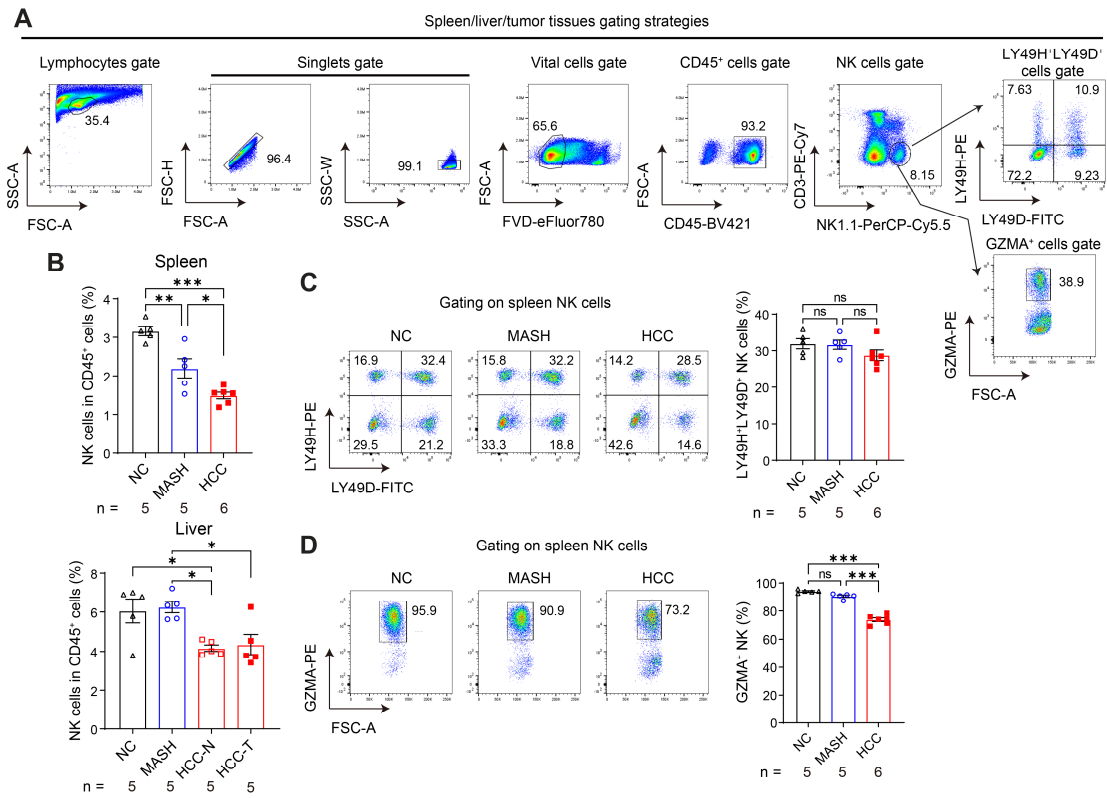


Supplementary Fig. S10. Expression patterns of signature genes, enriched pathways and regulons for B cells in the MASH-to-HCC transition. (A) Heatmap displayed the top 10 genes for each B subcluster. (B) UMAP visualization illustrated the expression patterns of *Igha* in B cells, with each point representing an individual cell. The black lines represent the spatial density of cells which expressed higher levels of *Igha* than the mean expression level. (C) Violin plot delineated the expression levels of the indicated genes in the corresponding cells, with each point representing an individual cell. (D) Gene Set Variation Analysis (GSVA) of B cell subpopulations based on the GO database. (E) Rank for regulons in B cell subsets based on regulon specificity scores. The regulon specificity scores were analyzed by single-cell regulatory network inference and clustering (SCENIC). (F) Visualization of B cell subpopulation along the differentiation trajectory. The plots were stratified by groups, including liver tissues from mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with hepatocellular carcinoma (HCC).

Figure S11

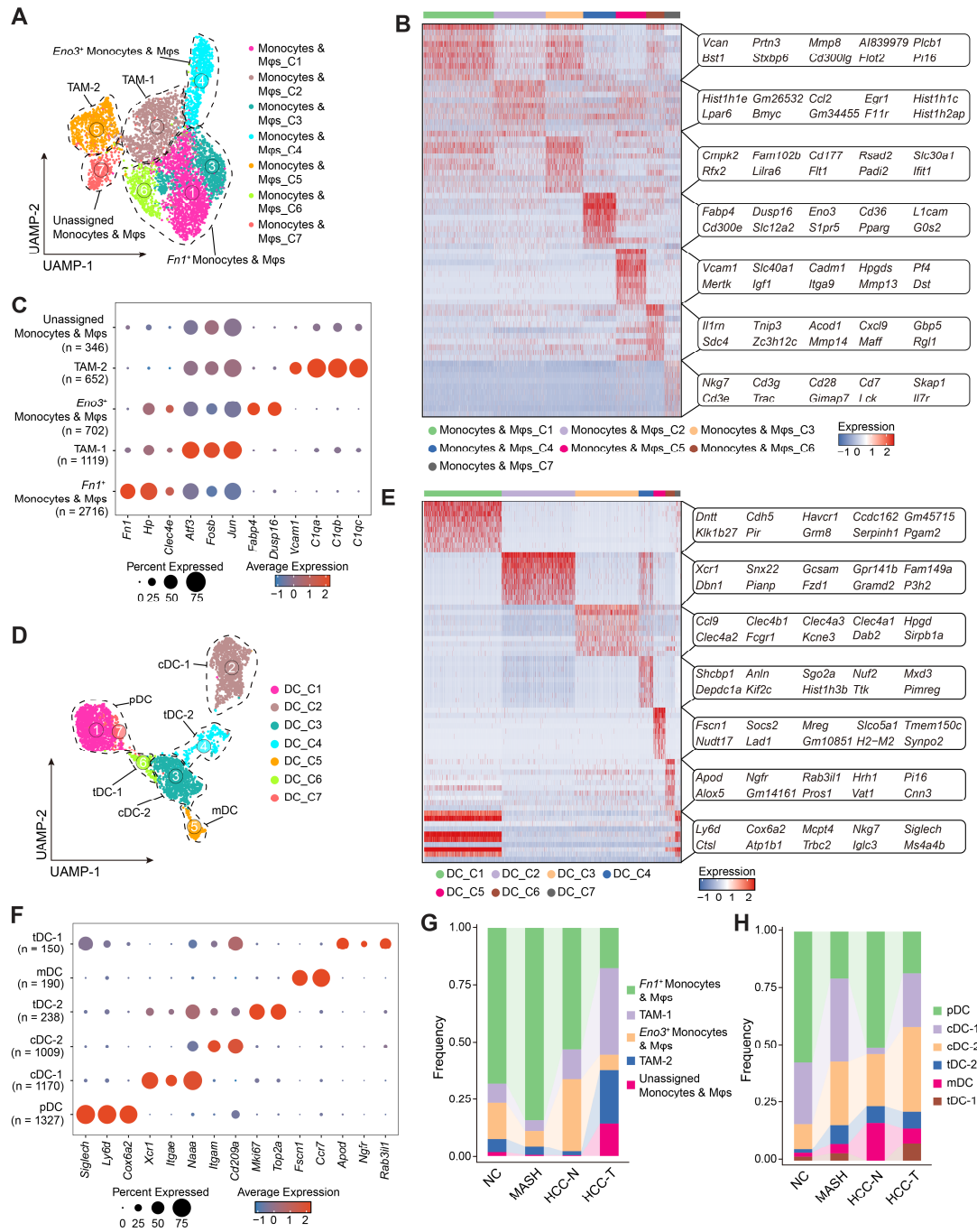


Supplementary Fig. S11. Expression patterns of signature genes or important genes for distinct NK cell subtypes. (A) Heatmap displayed the top 10 genes for each NK cell subcluster. (B) Dot plot illustrated the expression patterns of signature genes among distinct NK cell subtypes. (C) UMAP visualization of distinct NK cell subtypes among different groups, including liver tissues from mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with hepatocellular carcinoma (HCC). (D) A neighborhood graph of the results from Milo differential abundance testing between MASH and NC. Nodes represent neighborhoods, colored by their log fold change across groups. Non-differential abundance neighborhoods (FDR 10%) are colored white, and sizes correspond to the number of cells in each neighborhood. Graph edges depict the number of cells shared between neighborhoods. The layout of nodes is determined by the position of the neighborhood index cell from the UMAP. (E) Beeswarm plots illustrated the fold change of the cell abundance for NK cells between MASH and NC. (F) Shown are the RNA velocity-based cell lineage of NK cells and the dot plots displayed by expression levels according to RNA velocity analysis.

Figure S12

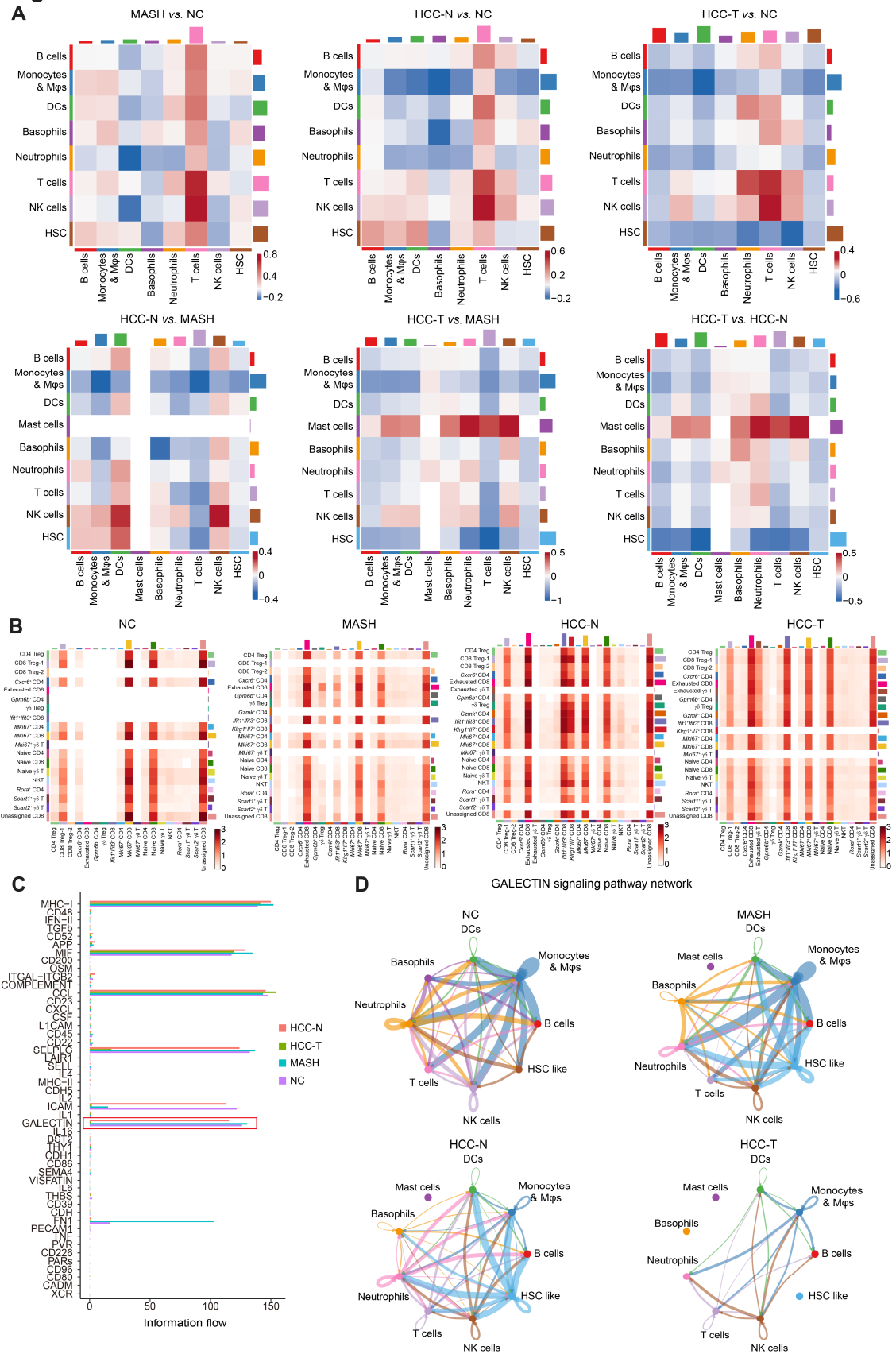
Supplementary Fig. S12. NK cells were decreased in the spleen and liver during the transition from MASH to HCC. (A) The gating strategies for NK cell subtypes. Liver/tumor cell suspensions or splenocyte from mice were stained with CD45 and FVD-eF780, which was used to exclude dead cells. The sequential gating strategies are as follows: lymphocyte gate, doublets exclusion, dead cell exclusion, CD45⁺ population, NK cell population and NK cell subtypes gate. (B) NK cells were decreased in the spleen and liver during the transition from metabolic dysfunction-associated steatohepatitis (MASH) to hepatocellular carcinoma (HCC). The percentages of NK cells in the spleen (upper) or liver/tumor (lower) were analyzed in the mice with normal chow (NC) or MASH, as well as paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with HCC by using flow cytometry analysis. (C) The percentages of LY49H⁺LY49D⁺ NK cells in the spleen were not different among different groups. (D) The percentages of GZMA⁺ NK cells in the spleen were significantly decreased in MASH-related HCC. In C and D, the representative flow cytometry images are shown in the left panel, and the percentages of indicated cells were analyzed among different groups in the right panel. Each data point represents an individual mouse in B-D, and the number of mice in each group is shown in the panels accordingly. One-way ANOVA was used to determine significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, not significant difference.

Figure S13

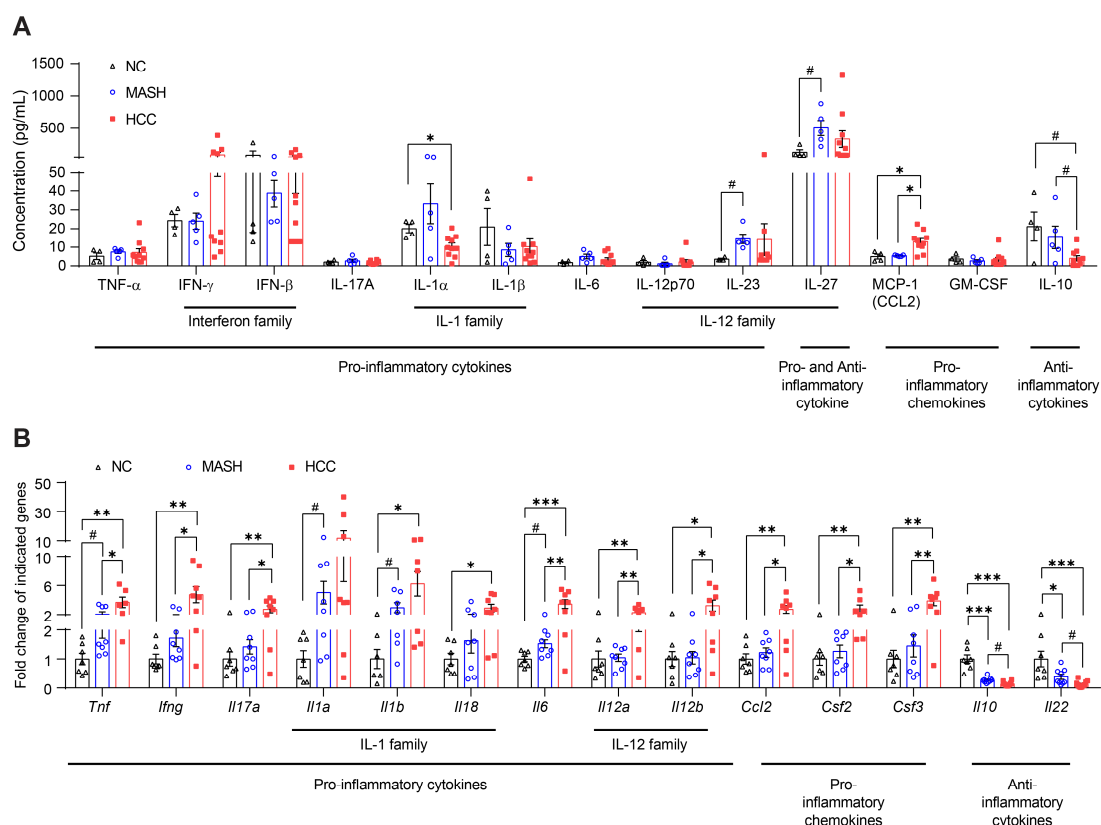


Supplementary Fig. S13. Characterization of myeloid cell subsets. (A) UMAP visualization of clusters and cell subtypes for Monocytes & Mφs. (B) Heatmap displayed the top 10 genes for each Monocytes & Mφs subcluster. (C) Dot plot illustrated the expression patterns of signature genes among distinct Monocytes & Mφs subtypes. (D) UMAP visualization of clusters and cell subtypes for DCs. (E) Heatmap displayed the top 10 genes for each DCs subcluster. (F) Dot plot illustrated the expression patterns of signature genes among distinct DCs subtypes. (G, H) Bar chart depicted the percentages of distinct Monocytes & Mφs (G) and DCs (H) subtypes among different groups, including liver tissues from mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), paired paracancerous (HCC-N) and cancer tissues (HCC-T) from mice with hepatocellular carcinoma (HCC).

Figure S14

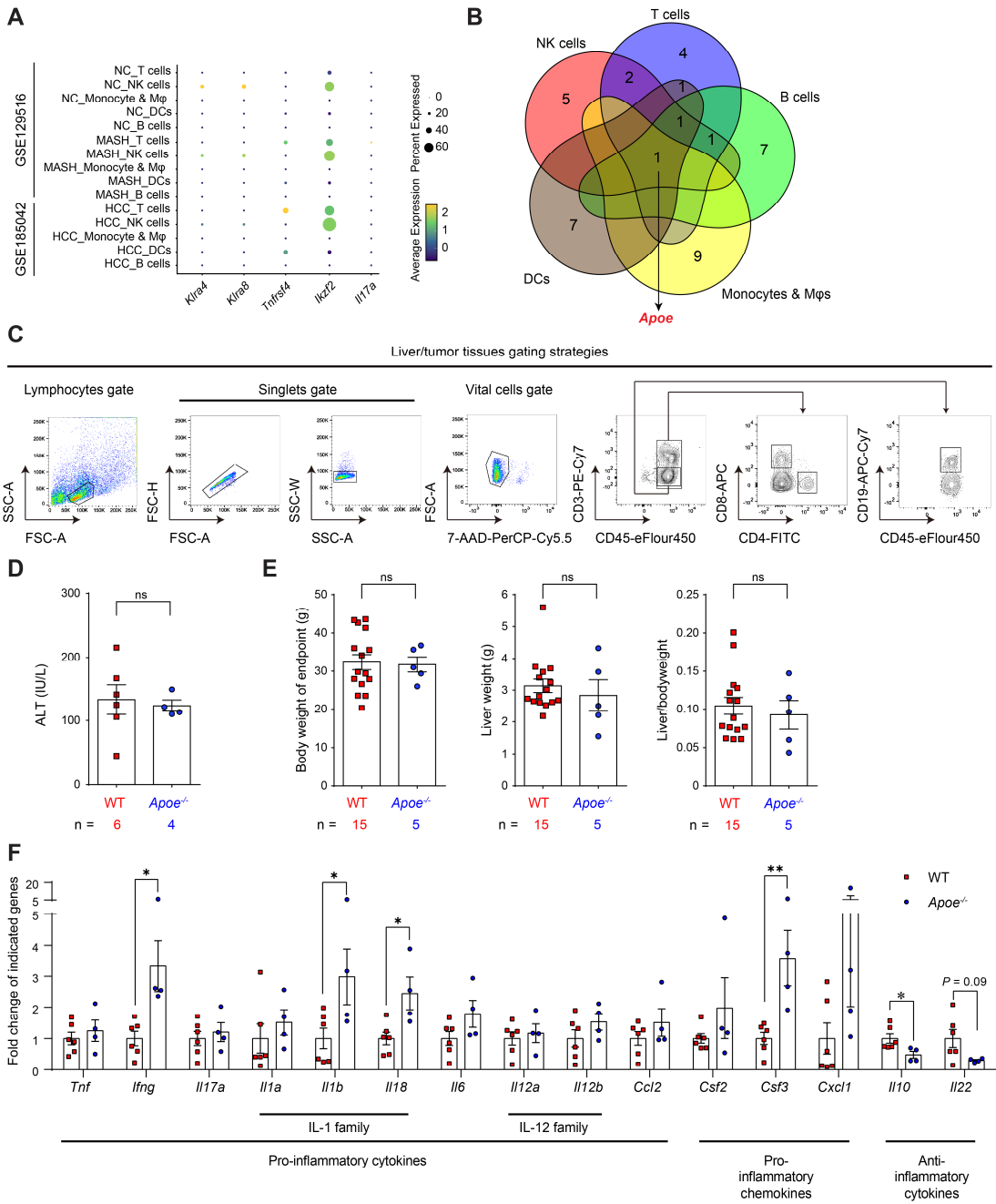


Supplementary Fig. S14. The multi-lineage immune cells interactome in the MASH-to-HCC transition. (A) Shown are the interaction strength heatmaps of distinct immune cells among different groups comparison. (B) Shown are the interaction strength heatmaps of distinct T cell subtypes among different groups. (C) Shown is the bar graph for the information flow of different signaling pathways among different groups. (D) Shown are the network diagrams representing the interaction strength among distinct cell types. The nodes denote distinct cellular entities, with the thickness of the connecting lines indicating the magnitude of interaction strength between these cell types. The color of the lines corresponds to the ligand-producing cell types. NC, normal chow. MASH, metabolic dysfunction-associated steatohepatitis. HCC-N, paracancerous tissues from mice with hepatocellular carcinoma. HCC-T, cancer tissues from mice with hepatocellular carcinoma.

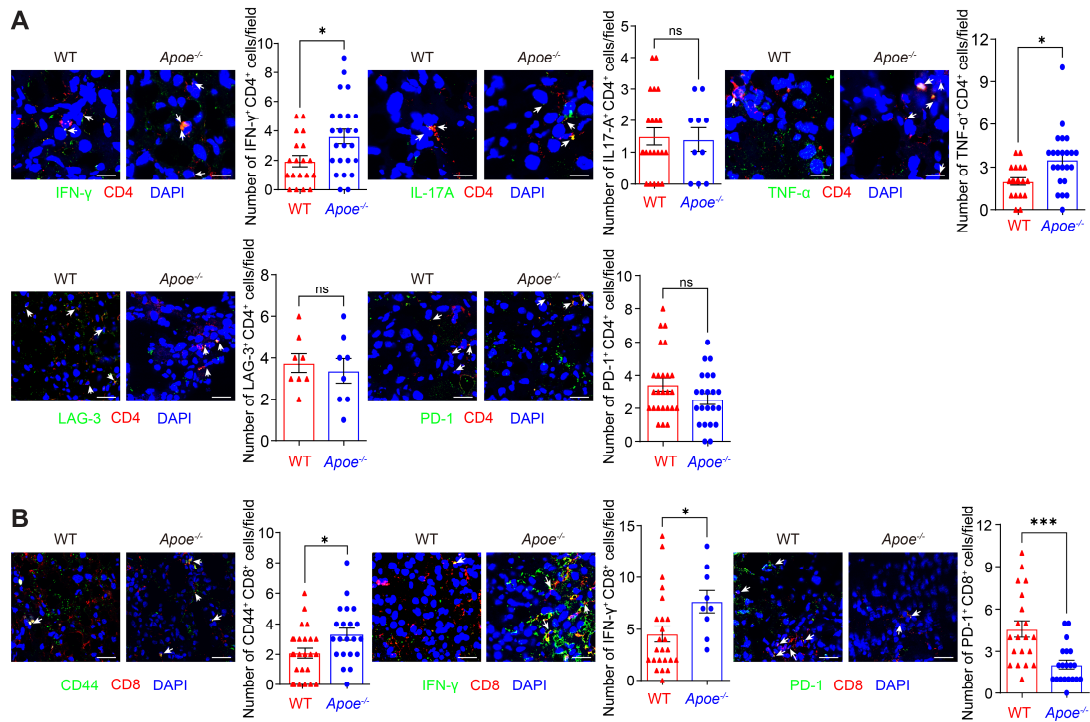
Figure S15

Supplementary Fig. S15. The levels of cytokines and chemokines in sera and hepatic tissues during MASH-driven hepatocarcinogenesis. (A) The concentrations of cytokines and chemokines in serum were quantified using the LEGENDplex Mouse Inflammation Panel (13-plex). NC, normal chow. MASH, metabolic dysfunction-associated steatohepatitis. HCC, hepatocellular carcinoma. **(B)** The mRNA levels of cytokines and chemokines were detected in the mouse liver tissues from mice at normal chow (NC) and metabolic dysfunction-associated steatohepatitis (MASH), as well as tumor tissue from mice at hepatocellular carcinoma (HCC) stages by using qRT-PCR. In A and B, each data point represents an individual mouse. One-way ANOVA was used to determine significance. # All were significant using Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

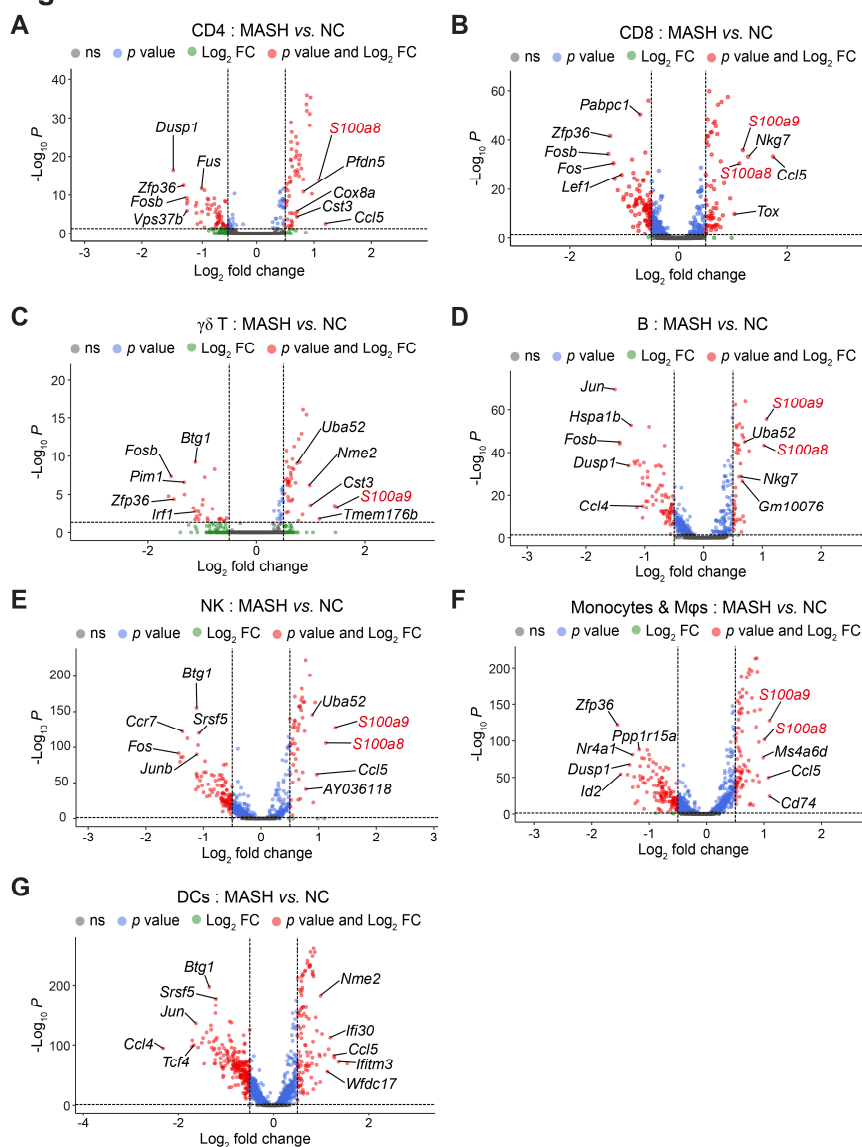
Figure S16



Supplementary Fig. S16. ApoE ablation did not affect liver damage, body weight and liver weight, but promoted a pro-inflammatory hepatic environment. (A) Shown is the dot plot for the expression pattern of indicated genes in different immune cells among different groups, including liver tissues from mice with normal chow (NC) or metabolic dysfunction-associated steatohepatitis (MASH), tumor tissues from mice with hepatocellular carcinoma (HCC). The expression levels of indicated genes and the percentages of expressed cells were obtained from two mouse MASH-related HCC studies. (B) Venn plot showing the overlapping genes that were significantly upregulated in the tumor group (HCC-T) among distinct immune cells. (C) The gating strategies for sorting CD4⁺ T, CD8⁺ T and B cells. Liver/tumor cell suspensions from mice were stained with CD45 and 7-AAD, which was used to exclude dead cells. The sequential gating strategies are as follows: lymphocyte gate, doublets exclusion, dead cell exclusion, CD45⁺ population, CD4⁺ T and CD8⁺ T populations, B cells gate. (D) Serum ALT levels were not different between *ApoE*^{-/-} mice and wild type (WT) controls at HCC stage. (E) Body weight of endpoint (left), liver weight (middle) and liver/body weight ratio (right) were not different between *ApoE*^{-/-} mice and WT controls at HCC stage. (F) The mRNA levels of cytokines and chemokines were detected in the tumor tissues from *ApoE*^{-/-} mice and WT controls. In D-F, each data point represents an individual mouse, and the number of mice in each group is shown in the panels accordingly. Student's *t* test was used to determine significance. * *P* < 0.05, ** *P* < 0.01. ns, not significant difference.

Figure S17

Supplementary Fig. S17. Hepatic tissues of *Apoe*^{-/-} mice exhibited increased effector T cells infiltration, accompanied by reduction of exhausted T cells. (A) The liver tissues from indicated mice were stained for IFN- γ , IL-17A, TNF- α , LAG-3 or PD-1 (green) and CD4 (red) by using immunofluorescence staining. **(B)** The liver tissues from indicated mice were stained for CD44, IFN- γ or PD-1 (green) and CD8 (red) by using immunofluorescence staining. Yellow signals indicate co-localization of indicated markers (white arrow). Scale bar = 25 μ m. In A and B, each data point represents an individual field. Student's *t* test was used to determine significance. * $P < 0.05$, *** $P < 0.001$. ns, not significant difference.

Figure S18

Supplementary Fig. S18. Differentially expressed genes in metabolic dysfunction-associated steatohepatitis. (A-G) Volcano plots showed the differentially expressed genes in MASH among CD4 T (A), CD8 T (B), $\gamma\delta$ T (C), B cells (D), NK cells (E), Monocytes & M ϕ s (F) and DCs (G).