Identification of a distinct NK-like hepatic T-cell population
activated by NKG2C in a TCR-independent manner

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Supplementary methods

Study samples and cell isolation

Indications for liver transplantation among patients with hepatitis B virus (HBV)-associated chronic liver disease (CLD) who were enrolled in our study included decompensated liver cirrhosis (LC) accompanied by ascites, hepatic encephalopathy, and variceal bleeding (n = 9), as well as hepatocellular carcinoma (HCC) according to Milan criteria (n = 19). Detailed information on the recipients is presented in Supplementary Table 1. Livers were perfused with Custodiol HTK (Essential Pharmaceuticals) solution during bench preparation of the liver from living donors. The first 500 ml of 1000 ml of total perfusate was discarded, and the second 500 ml was collected and filtered as described previously.1 PB mononuclear cells (PBMCs) and liver sinusoidal mononuclear cells (LSMCs) were separated by density gradient centrifugation using Lymphocyte separation medium (Corning). In some cases, liver tissues were acquired, minced, and enzymatically digested using a Tumor Dissociation Kit (Miltenyi Biotec), a gentleMACS dissociator (Miltenyi Biotec), and 70-µm cell strainer (SPL Lifesciences). Single-cell suspensions were finally obtained and cryopreserved in fetal bovine serum (Corning) containing 10% dimethyl sulfoxide (Sigma-Aldrich) until use.

CITE-seq analysis and basic quality control

Single-cell CITE-seq and TCR-seq libraries were generated using the Chromium Single Cell 5’ Library & Gel Bead Kit (PN-10000006), 5’ Feature Barcode Library Kit (PN-1000020), and V(D)J Kit, Human T Cell (PN-1000005) (10X Genomics) following the manufacturer’s instructions. Libraries were constructed and sequenced at a depth of approximately 20,000
reads per cell for gene, antibody-derived tags (ADTs), and TCR sequence expression using the Nextseq 550 or Novaseq 6000 platform (Illumina). The sequenced data were demultiplexed using the mkfastq function (Cell Ranger, 10X Genomics, v3.0.1) to generate fastq files. Demultiplexed fastq files of gene and ADT expression were aligned to the reference human genome (GRCH38; 10X Cell Ranger reference GRCh38 v3.0.0) and the barcode sequence of each ADT, and filtering, barcode counting, and UMI counting were performed to generate feature-barcode matrices using the count function (Cell Ranger, v3.0.1). The feature-barcode matrices were analyzed in the following analysis using the Seurat R package (Seurat, v3.2.2). For basic quality control, we filtered low-quality cells expressing mitochondrial genes in > 10% of their total gene expression, < 200 genes or > 3,500 genes, or < 1,500 UMI counts or > 15,000 UMI counts. We also excluded doublets that expressed multiple hashtag ADTs.

Next, standard normalization was performed for gene expression of each cell based on the total read count and identified highly variable genes (n = 4,000). To correct for a batch effect and harmonize the datasets, we identified ‘anchor’ vectors of genes using the FindIntegrationAnchors function and integrated the datasets using the IntegrateData function (Seurat, v3.2.2). The integrated samples were scaled (ScaleData function) and principal component analysis (PCA) (RunPCA function) carried out to dimensional reduction. Lastly, the cells underwent unsupervised clustering (FindClusters function, resolution = 0.5) and were visualized by uniform manifold approximation and projection (UMAP) using the top 20 principal components (PCs) (RunUMAP function) for whole-cell types. To subcluster the ‘T cells’ and ‘CD8+ T cell’ populations, dimensional reduction and visualization were performed with the top 20 PCs for ‘T cells’ (resolution = 0.5) and 6 PCs for ‘CD8+ T cells’ (resolution = 0.4) using PCA and the UMAP algorithm.
**Cell type annotation through marker gene identification in each cluster**

To identify marker genes, differential expressed genes (DEGs) in each cluster relative to the other clusters were selected on the basis of the Wilcoxon rank sum test using the FindAllMarkers function (Seurat, v3.2.2, parameter; Seurat default for whole-cell types and T cells, > 0.2 log fold change compared to the other clusters, > 0.3 min.pct [minimum fraction of test genes detected in cells of each cluster], and Bonferroni-adjusted p < 0.05). Gene set module scores were calculated using the AddModuleScore function (Seurat, v3.2.2).

**TCR-seq analysis**

Single-cell TCR sequence data were assembled and quantified following the Cell Ranger (v.3.0.1) VDJ protocol against a GRCh38 reference genome. Non-productive contigs were discarded and productive contigs assigned to each cell matching a cell barcode. To identify clonal diversity and overlap among CD8⁺ T cells, the inverse Simpson index and Morisita-Horn index were calculated using the immunarch R package (immunarch, v0.6.6).³ The Morisita-Horn index between subclusters of CD8⁺ T cells and cell counts of each subcluster of CD8⁺ T cells were expressed in a network plot using the ggnet2 function (GGally, v2.0.0).

**CITE-seq antibodies**

The following DNA-barcoded monoclonal antibodies were used for CITE-seq: anti-CD45RA-TotalSeq™-C (HI100), anti-CD45RO-TotalSeq™-C (UCHL1), anti-CD14-TotalSeq™-C (63D3), anti-CD19-TotalSeq™-C (HIB19), anti-CD56-TotalSeq™-C (5.1H11), anti-TCRα/β -

**Re-analysis of publicly available scRNA-seq data**

Publicly available scRNA-seq datasets for mononuclear cells from various organs were re-analyzed (GSE158485, GSE48452, GSE136831, GSE134809, GSE114374, E-MTAB-8007, E-MTAB-8474, E-MTAB-8476, E-MTAB-8484, and E-MTAB-8486). The datasets had been produced from 10 healthy livers, 5 cirrhotic livers, 55 healthy and pathologic (idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease) lungs, and 9 healthy intestines. Intrahepatic mononuclear cells from a single dataset (GSE158485) were
analyzed after batch effect correction with the Seurat standard integration algorithm using the IntegrateData function. UMAP was performed with the top PCA components (total mononuclear cells, dimension = 20, NK and T cells, dimension = 10) for cell clustering and visualization using the RunUMAP and FindNeighbors function.

To merge and correct the batch effect according to data sets from various organs, an ‘integrated’ data matrix was calculated using the Seurat standard integration algorithm. UMAP was performed using the top PCA components (NK and T cell, dimension = 20, CD8 T cells, dimension = 20). Next, the cells were clustered by an unsupervised clustering method (NK and T cell, resolution = 0.8, CD8 T cells, resolution = 0.6, FindClusters function). DEGs were calculated based on the Wilcoxon rank-sum test using default parameters (FindAllMarkers function).

Flow cytometry

Cryopreserved PBMCs and LSMCs were thawed and incubated with fluorochrome-conjugated antibodies for 15 minutes at room temperature. Dead cells were excluded using the Live/Dead Cell Stain Kit (Invitrogen). For intracellular staining, fixation and permeabilization was performed using the Foxp3/Transcription Factor Staining Buffer Kit (Invitrogen) for 15 minutes, and then antibodies added for another 15 minutes to label cytoplasmic proteins. Multicolor flow cytometry was performed using an LSR II instrument (BD Bioscience) and the data analyzed using FlowJo software version 10.4 (Treestar).

Immunofluorescent staining

Liver tissues were fixed with 4% PFA for 2 hours at 4°C, and then dehydrated with
30% sucrose for 24 hours at 4°C. Processed tissues were transferred into a cryomold pre-filled with OCT and cryopreserved at -80°C until use. For cryosections, OCT-embedded tissue was cut into 12-μm sections using a Cryocut Microtome (Leica). The smeared sectioned tissues on the slides were washed with PBS, permeabilized with 0.3% Triton X-100 in PBS, and blocked with PBS containing 5% fetal bovine serum (FBS; corning) for 30 minutes. Primary antibodies were incubated overnight at 4°C, washed with 0.3% Triton X-100 in PBS, and then washed twice with PBS. The following antibodies were used for immunostaining: anti-CD3 (mouse monoclonal; clone SK7; BD Bioscience), anti-CD8 (mouse monoclonal; clone RPA-T8; BD Bioscience), and ant-CD56 (mouse monoclonal; clone NCAM16.2; BD Bioscience). Nuclei were stained with DAPI (Invitrogen). Immunofluorescent images were acquired using a Nikon A1 HD25 confocal microscope (Nikon). NIS-Elements AR (Nikon) was used to process images.

In vitro stimulation of LSMCs

For TCR-mediated stimulation, thawed LSMCs were stimulated by soluble anti-CD3 (100 ng/ml; Miltenyi Biotec) and anti-CD28 (1 μg/ml; BD Biosciences) mAbs and cultured for 6 hours at 37°C. After 1 hour of stimulation, brefeldin A (BD Biosciences) and monensin (BD Biosciences) were added and the culture continued for 5 hours. For cytokine stimulation, thawed LSMCs were stimulated with recombinant human IL-12 (50 ng/ml; Peprotech) and IL-18 (50 ng/ml; R&D Systems), and IL-15 (10 ng/ml; Peprotech) for 24 hours at 37°C unless otherwise indicated. After 13 hours of stimulation, brefeldin A and monensin were added and culture continued for 11 hours. After assay, staining for flow cytometry was performed as described above. For cell proliferation assays, LSMCs were cultured in RPMI 1640 medium
(Corning) containing 10% FBS and 1% Penicillin-Streptomycin (WELGENE) with IL-15 (10 ng/ml) for 48 hours, and Ki-67 expression was analyzed using flow cytometry.

**PHA blast-stimulated functional assays**

To assess a functional capacity of T cells, we co-cultured cells with autologous PHA-stimulated cells (PHA blasts). For PHA blast generation, LSMCs were stimulated with the mitogen PHA-L (5 μg/ml; Sigma-Aldrich) in RPMI 1640 medium containing 10% FBS and 1% Penicillin-Streptomycin with recombinant human IL-2 (10 ng/ml; Peprotech). We co-cultured them with LSMCs at an E:T ratio of 1:1 for 12 hours. Anti-CD107a antibody was present throughout the assay to assess degranulation. After 1 hour of stimulation, brefeldin A and monensin were added and the culture continued for 11 hours. In blocking assays, we pre-incubated PHA blasts with 10 μg/ml isotype (IgG1κ; R&D Systems) and anti-MHC-I antibodies (clone W6/32; BioLegend) for 30 minutes at 37°C. After the assay, staining for flow cytometry was performed as described above.

**P815-redirected NKR stimulation**

For P815-redirected NKR stimulation, P815 cells (TIB-64™; ATCC®) were used as the target cells and maintained in DMEM (Corning) with 10% FBS and 1% Penicillin-Streptomycin. We pre-incubated P815 cells with 10 μg/ml isotype (IgG1κ), 10 μg/ml anti-NKG2D antibodies (clone 1D11; BD Biosciences), and 10 μg/ml anti-NKG2C (clone 134591; R&D Systems) antibodies for 30 min at 37°C. We then co-cultured the P815 cells with effector cells at an E:T ratio of 1:1 for 12 hours. After 1 hour of stimulation, brefeldin A and monensin were added and the culture continued for 11 hours. After the assay, staining for
flow cytometry was performed as described above.

**In vitro expansion of the CD56\textsuperscript{hi}CD161\textsuperscript{-} CD8\textsuperscript{+} T-cell population**

CD8\textsuperscript{+} T cells were isolated from LSMCs using anti-CD8 microbeads (Miltenyi Biotec). Isolated CD8\textsuperscript{+} T cells were stained with fluorochrome–conjugated monoclonal antibodies (CD8: clone SK1, BD Bioscience; CD56: clone NCAM16.2, BD Biosciences; CD161: clone HP-3G10, BioLegend; TCR V\textalpha7.2: clone 3C10, BioLegend; TCR \gamma\delta: clone B1, Biolegend), and CD56\textsuperscript{hi}CD161\textsuperscript{-}-depleted conventional CD8\textsuperscript{+} T cells were sorted using an Aria II cell sorter (BD Biosciences). CD56\textsuperscript{hi}CD161\textsuperscript{-}-depleted conventional CD8\textsuperscript{+} T cells were cultured with either IL-15 (10 ng/ml) or anti-CD3 (100 ng/mL) in RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin, and IL-2 (10 ng/ml; Peprotech) for 2 weeks at 37°C in a 5% CO\textsubscript{2} incubator. The culture medium was changed every 3-4 days. The frequency of CD56\textsuperscript{hi}CD161\textsuperscript{-} T cells among conventional CD8\textsuperscript{+} T cells were analyzed by flow cytometry on days 0, 2, 4, 7, 10, 12, and 14.

**Statistical analysis**

Statistical analyses were performed using Prism software version 8.4 (GraphPad, La Jolla, CA). Data did not follow a normal distribution. The Wilcoxon signed-rank test was used to compare data between two paired groups and the Mann-Whitney U test to compare data between two unpaired groups. DEGs in the CITE-seq analysis were calculated using the Wilcoxon rank-sum test. Significance was considered \( p < 0.05 \).
**Supplementary Figures**

**Fig. S1.**

(A) UMAP plot of liver sinusoid CD45$^+$ mononuclear cells colored according to cluster. (B) UMAP plot of liver sinusoid CD45$^+$ mononuclear cells reflecting the distribution of cells according to experimental batch and patient. (C) UMAP plots of liver sinusoid CD45$^+$ mononuclear cells showing normalized expression of known marker genes and ADTs.

**Fig. S1. Clustering analysis of CD45$^+$ cells among liver sinusoidal mononuclear cells according to marker gene expression.**
Fig. S2. Clustering analysis of T cells among liver sinusoidal mononuclear cells according to
marker gene expression.

(A) UMAP plot of liver sinusoid T cells colored according to cluster. (B) UMAP plot of liver sinusoid T cells reflecting the distribution of cells according to patient. (C) UMAP plots of liver sinusoid T cells showing normalized expression of known marker genes and ADTs.
Fig. S3. Clustering analysis of CD8+ T cells among liver sinusoidal mononuclear cells according to marker gene expression. (A) UMAP plot of liver sinusoid CD8+ T cells reflecting the distribution of cells according to patient. (B) UMAP plots of liver sinusoid CD8+ T cells showing normalized expression of known marker genes and ADTs.
Fig. S4. Marker ADT and gene expression of CD8+ T cells among liver sinusoidal
mononuclear cells. (A) Dot plots showing the average normalized expression of DEGs in each CD8+ T-cell subcluster. (B) Violin plots showing normalized expression of CD57, CD56 and CD161 ADT among CD8+ T-cell subclusters.
Fig. S5. Re-analysis of scRNA-seq data for mononuclear cells from healthy liver tissues. (A) UMAP projections of 10,624 intrahepatic T/NK cells from healthy donors (n = 5). (B) Dot plots showing the average normalized expression of marker genes in each immune cell cluster. (C) UMAP projection of 2,715 intrahepatic CD8+ T cells. (D) Dot plots showing the average normalized expression of marker genes and ADTs in each CD8+ T-cell cluster. (E)
Violin plots showing normalized marker gene expression among CD8$^+$ T-cell clusters. (F) Dot plots showing the average normalized expression of marker genes in each NK and CD8$^+$ T-cell cluster.
Fig. S6. Re-analysis of scRNA-seq data of mononuclear cells from various organs. (A) UMAP projections of 88,781 intra-organ T/NK cells from various organs (healthy livers, n = 10;
cirrhotic livers, n = 5; healthy and pathologic lungs, n = 55; healthy intestine, n = 9). (B) Dot plots showing the average normalized expression of marker genes in each immune cell cluster. (C) UMAP projection of 28,257 intra-organ CD8^+ T cells. (D) Dot plots showing the average normalized expression of marker genes in each CD8^+ T-cell cluster. (E) Proportion of CD56^+ CD8 T cell-cluster among conventional CD8^+ T cells from each group.
Fig. S7. Gating strategy for CD56^hi^CD161^−^CD8^+^ T cells and other CD8^+^ T-cell populations. (A) Gating strategy to observe non-MAIT TCRαβ^+^ conventional CD8^+^ T cells. (B) Gating strategy to observe CCR7^+^CD45RA^+^, CCR7^+^CD45RA^−^, CCR7^−^CD45RA^+^, and CCR7^−^CD45RA^−^ cells, and bar plots showing the proportion of each subset among non-MAIT TCRαβ^+^ CD8^+^ T cells (n = 6). (C) Gating strategy to observe T_EM, T_EMRI, CD57^+^, and CD56^hi^CD161^−^CD8^+^ T cells among non-MAIT TCRαβ^+^ CD8^+^ T cells.
**Fig. S8.**

(A) Proportion of CD56<sup>hi</sup>CD161<sup>-</sup>CD8<sup>+</sup> T cells among CD8<sup>+</sup> T cells from the LSMCs of healthy donors (n = 26), patients with HBV-CLD without HCC (n = 9), and patients with HBV-CLD with HCC (n = 19).

(B) Immunofluorescent staining of liver tissues revealing CD56<sup>hi</sup>CD8<sup>+</sup>CD3<sup>+</sup> T cells. HBV-CLD, hepatitis B virus-associated chronic liver disease.
Fig. S9. MFI of perforin, granzyme B, and granulysin in CD8+ T-cell populations after cytokine stimulation. (A) MFI of perforin, granzyme B, and granulysin in T_{EM}, T_{EMRA}, CD57+, and CD56^hiCD161-CD8+ T cells after IL-12/18 (50 ng/ml; 50 ng/ml) or (B) IL-15 stimulation (10 ng/ml) (n = 8).
Fig. S10. Expansion of the CD56^hiCD161^-CD8^+ T-cell population.

(A) Representative flow cytometry plots of CD56^+CD161^-CD8^+ T cells among CD56^+CD161^- depleted conventional liver sinusoidal CD8^+ cells after various stimulations.
Fig. S11. Comparison of liver sinusoidal and intrahepatic CD8+ T cells. (A) The proportion of T\textsubscript{EM}, T\textsubscript{EMRA}, CD57\textsuperscript{+}, and CD56\textsuperscript{hi}CD161\textsuperscript{−} cells among CD8+ T cells from LSMCs and PBMCs are plotted against the proportion of T\textsubscript{EM}, T\textsubscript{EMRA}, CD57\textsuperscript{+}, and CD56\textsuperscript{hi}CD161\textsuperscript{−} cells among CD8+ T cells from LIMCs (n = 20).
Fig. S12. Comparison of liver sinusoidal and intrahepatic NK cells. (A) The proportion of NKG2C\(^+\), NKG2A\(^+\), and KIR\(^+\) cells among NK cells from LSMCs and PBMCs are plotted against the proportion of NKG2C\(^+\), NKG2A\(^+\), and KIR\(^+\) cells among NK cells from LIMCs (n = 20).
Fig. S13. Comparison of the T<sub>RM</sub> features of CD56<sup>hi</sup>CD161<sup>−</sup>CD8<sup>+</sup> T cells from liver sinusoid
and intrahepatic cells. (A-B) Proportion and MFI of $T_{RM}$ marker-expressing cells in each CD8$^+$ T-cell subset from LSMCs (A) and LIMCs (n = 10) (B). (C) The proportion or MFI of $T_{RM}$ marker-expressing cells among CD56$^{hi}$CD161$^-$CD8$^+$ T cells from LSMCs are plotted against the proportion or MFI of $T_{RM}$ marker-expressing cells among CD56$^{hi}$CD161$^-$CD8$^+$ T cells from LIMCs (n = 10).
Fig. S14. Comparison of cytotoxic molecule expression in CD56hiCD161+CD8+ T cells from liver sinusoid and intrahepatic cells. (A-B) Proportion of cytotoxic molecule-expressing cells in each CD8+ T-cell subset from LSMCs (A) and LIMCs (n = 7) (B). (C) The proportion of...
cytotoxic molecule-expressing cells among CD56$^{hi}$CD161$^{-}$CD8$^{+}$ T cells from LSMCs are plotted against the proportion of cytotoxic molecule-expressing cells among CD56$^{hi}$CD161$^{-}$CD8$^{+}$ T cells from LIMCs (n = 7).
Fig. S15. Comparison of NKR expression in CD56\textsuperscript{hi}CD161\textsuperscript{-}CD8\textsuperscript{+} T cells from liver sinusoid and intrahepatic cells. (A–B) Proportion or MFI of NKR-expressing cells in each CD8\textsuperscript{+} T-cell subset from LSMCs (A) and LIMCs (n = 10) (B). (C) The proportion or MFI of NKR-expressing cells among CD56\textsuperscript{hi}CD161\textsuperscript{-}CD8\textsuperscript{+} T cells from LSMCs are plotted against the proportion or MFI of NKR-expressing cells among CD56\textsuperscript{hi}CD161\textsuperscript{-}CD8\textsuperscript{+} T cells from LIMCs (n = 7).
Supplementary table captions

(Tables included in separate Excel files)

Table S1. Patient clinical information

Table S2. DEGs of six major immune cell types among LSMCs

Table S3. DEGs of seven immune cell types among CD8⁺ T cells
**Supplementary references**


3. Vadim Nazarov ib, & Eugene Rumynskiy. immunomind/immunarch: 0.6.5: Basic single-cell support: Zenodo; 2020.


