Research Article
Experimental and Translational Hepatology

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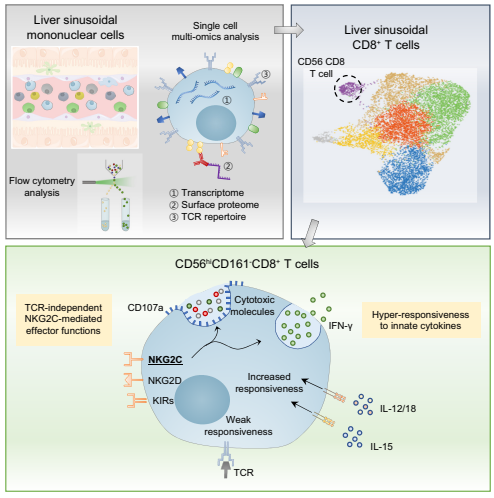
Graphical abstract



Highlights

- Single-cell analysis revealed heterogeneity among liver sinusoidal CD8$^+$ T cells.
- The CD56^{hi}CD161$^-$CD8$^+$ T-cell population expands in HBV-associated chronic liver disease.
- CD56^{hi}CD161$^-$CD8$^+$ T cells have NK-like transcriptomes and unique TCR repertoire.
- CD56^{hi}CD161$^-$CD8$^+$ T cells exert TCR-independent, NKG2C-mediated effector functions.
- CD56^{hi}CD161$^-$CD8$^+$ T cells exhibit hyper-responsiveness to IL-12/IL-18 and IL-15.

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Lay summary

The role of different immune cell populations in the liver is becoming an area of increasing interest. Herein, we identified a distinct T-cell population that had features similar to those of natural killer (NK) cells – a type of innate immune cell. This distinct population was expanded in the livers of patients with chronic liver disease and could thus have pathogenic relevance.

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

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See Editorial, pages 915–917

Background & Aims: The liver provides a unique niche of lymphocytes enriched with a large proportion of innate-like T cells. However, the heterogeneity and functional characteristics of the hepatic T-cell population remain to be fully elucidated.

Methods: We obtained liver sinusoidal mononuclear cells from the liver perfusate of healthy donors and recipients with HBV-associated chronic liver disease (CLD) during liver transplantation. We performed a CITE-seq analysis of liver sinusoidal $CD45^+$ cells in combination with T cell receptor (TCR)-seq and flow cytometry to examine the phenotypes and functions of liver sinusoidal $CD8^+$ T cells.

Results: We identified a distinct $CD56^{hi}CD161^-CD8^+$ T-cell population characterized by natural killer (NK)-related gene expression and a uniquely restricted TCR repertoire. The frequency of these cells among the liver sinusoidal $CD8^+$ T-cell population was significantly increased in patients with HBV-associated CLD. Although $CD56^{hi}CD161^-CD8^+$ T cells exhibit weak responsiveness to TCR stimulation, $CD56^{hi}CD161^-CD8^+$ T cells highly expressed various NK receptors, including CD94, killer immunoglobulin-like receptors, and NKG2C, and exerted NKG2C-mediated NK-like effector functions even in the absence of TCR stimulation. In addition, $CD56^{hi}CD161^-CD8^+$ T cells highly respond to innate cytokines, such as IL-12/18 and IL-15, in the absence of TCR stimulation. We validated the results from liver sinusoidal $CD8^+$ T cells using intrahepatic $CD8^+$ T cells obtained from liver tissues.

Conclusions: In summary, the current study found a distinct $CD56^{hi}CD161^-CD8^+$ T-cell population characterized by NK-like activation via TCR-independent NKG2C ligation. Further studies are required to elucidate the roles of liver sinusoidal $CD56^{hi}CD161^-CD8^+$ T cells in immune responses to microbial pathogens or liver immunopathology.

Lay summary: The role of different immune cell populations in the liver is becoming an area of increasing interest. Herein, we identified a distinct T-cell population that had features similar to those of natural killer (NK) cells – a type of innate immune cell. This distinct population was expanded in the livers of patients with chronic liver disease and could thus have pathogenic relevance.

Keywords: $CD8^+$ T cell; liver; NKG2C; NK cell receptor; NK-like T cell; CITE-seq.
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Introduction

The liver acts as an immune sentinel against gut-derived microbes. Antigen-rich blood from the gut passes through the liver sinusoid, the specialized vasculature of the liver.[1,2] This distinct vascular structure enables efficient immune surveillance by maximizing immune cell-pathogen interactions.[3] The liver also provides a unique niche of lymphocytes. In particular, the liver harbors a substantially higher number of innate lymphocytes, including natural killer (NK) cells and innate T cells, compared to peripheral blood (PB) and other tissues.[3–5] Innate T cells include invariant natural killer T (iNKT) cells, mucosal-associated invariant T (MAIT) cells, and γδ T cells.[6]

In the liver, conventional T-cell receptor (TCR) $\alpha\beta^+$ T cells exhibit different phenotypes and characteristics than PB TCR$\alpha\beta^+$ T cells.[5,7] In particular, the hepatic T-cell population includes more activated and terminally differentiated cells than the PB T-cell population.[8–10] In addition, compared to the PB, the liver population is enriched with $CD8^+$ T cells expressing

receptors that are typically expressed by NK cells.[11,12] For example, $CD8^+$ T cells expressing killer immunoglobulin-like receptors (KIRs) or NKG2A are more prevalent in the liver than the PB.[9,13] Moreover, the hepatic T-cell population includes a large number of $CD56^+$ cells, which characteristically express high levels of various NK cell receptors (NKRs), including NKG2D, NKp44, NKp46, and DNAM-1, and cytotoxic granule molecules.[14–18] However, the heterogeneity in the hepatic T-cell population at the single-cell level remains to be fully elucidated. In addition, the functional characteristics of each hepatic subpopulation have not been elucidated.

In recent years, the development of single-cell technology has enabled high-resolution mapping of the cellular heterogeneity, development, and dynamics of immune cells.[19,20] Novel immune cell subsets have been discovered using single-cell RNA sequencing (scRNA-seq) analysis.[21,22] More recently, single-cell multi-omics approaches have been developed to facilitate concurrent profiling of multiple molecular modalities in the same cell.[23] In particular, simultaneous profiling of proteins and RNA with cellular indexing of the transcriptomes and epitopes by sequencing (CITE-seq)[24] and TCR-sequencing allow the comprehensive and detailed evaluation of T cells.

In the present study, we performed CITE-seq analysis of liver sinusoidal $CD45^+$ cells from healthy donors and patients with HBV-associated chronic liver disease (CLD) in combination with TCR-seq. We focused on the $CD8^+$ T-cell population and identified a distinct NK-like $CD8^+TCR\alpha\beta^+$ T-cell population characterized by high expression of CD56 without CD161 expression. This $CD56^{hi}CD161^-CD8^+$ T-cell population predominantly expresses various NKRs, including CD94, KIRs, and NKG2C. We also found NK-like effector functions of $CD56^{hi}CD161^-CD8^+$ T cells that are activated by NKG2C in a TCR-independent manner. In addition, we validated the results from liver sinusoidal $CD8^+$ T cells in intrahepatic $CD8^+$ T cells obtained from liver tissues.

Materials and methods

Study samples and cell isolation

This research was reviewed and approved by the institutional review board of Severance Hospital (Seoul, Republic of Korea; 4-2014-0261 and 4-2018-0777) and conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from all study participants. PB and liver perfusates were obtained from healthy living donors and recipients with HBV-associated CLD (HBV-CLD) during living-donor liver transplantation.

Results

CITE-seq analysis of liver sinusoidal mononuclear cells

Liver sinusoidal mononuclear cells (LSMCs) were collected from healthy donors (n = 4) and patients with HBV-CLD (n = 5) (Table S1). Using the Chromium system (10x Genomics), we performed CITE-seq analysis of sorted $CD45^+$ cells in combination with TCR-seq (Fig. 1A).[23,24] We analyzed a total of 41,372 cells after filtering dead cells and doublets and performing batch correction. We subjected the cells to the uniform manifold approximation and projection (UMAP) algorithm based on highly variable genes using the Seurat package and identified 16 different clusters unbiased by patient or experimental batch (Fig. S1A,B).[25] These clusters were assigned to 6 major immune cell types (Fig. 1B) according to the expression of marker genes and cell surface proteins stained by DNA-barcoded antibodies, antibody-derived tags (ADTs): monocytes, dendritic cells, NK cells, T cells, B cells, and cycling cells (Fig. 1C, Fig. S1C, and Table S2). We subclustered the T-cell population into 10 clusters according to highly variable genes (Fig. S2A-C) and further subclustered the naïve T-cell population into naïve $CD4^+$ and $CD8^+$ T-cell clusters according to ADT expression (Fig. 1D). Total $CD8^+$ T cells were identified (Fig. 1E,F) and subjected to further analysis.

Identification of heterogenous populations among liver sinusoidal $CD8^+$ T cells

Among the 10,101 $CD8^+$ T cells, 7 different subclusters were identified after projection to the UMAP algorithm (Fig. 2A, Fig. S3A). We calculated cluster-specific differentially expressed genes and ADTs (Fig. 2B, Fig. S3B, 4A, and Table S3). 'Naïve CD8' and 'CD127 CD8' subclusters commonly exhibited high expression of *CCR7* and *IL7R*, which encode CD127, though 'naïve CD8' exhibited upregulation of *SELL* and *TCF7* and 'CD127 CD8' upregulation of *ANXA1* and *CDKN1A* (Fig. 2C,D). 'EM CD8_1' and 'EM CD8_2' exhibited features of effector memory T (T_{EM}) cells. However, 'EM CD8_1' was characterized by upregulation of *GZMK, CXCR4, CXCR3*, and *PDCD1*, whereas 'EM CD8_2' was characterized by upregulation of *JUN, FOS, CD69, IFNG, TNF, CCL3*, and *CCL4*. Interestingly, 'EMRA CD8', 'CD57 CD8', and 'CD56 CD8' subclusters exhibited high expression of genes related to cytotoxicity and NK cells, such as *GZMB, GNLY, NKG7, NCR3, TYROBP, KLRC3*, and various KIR genes. Among them, 'EMRA CD8' typically expressed CD45RA ADT without CCR7 ADT expression, which is compatible with $CD45RA^+$ effector memory T (T_{EMRA}) cells known as terminally differentiated T_{EM} cells.[26,27] 'CD57 CD8' expressed *B3GAT1*, which encodes a glucuronyltransferase that generates CD57 antigens. The expression of CD57 protein in 'CD57 CD8' was confirmed by CD57 ADT expression (Fig. S4B). $CD57^+CD8^+$ T cells are known as replicative senescent T cells.[28] 'CD56 CD8' was identified as a unique population characterized by upregulation of CD56 protein, *IFITM3, IFITM2, XCL1*, and *ZNF683*, in addition to *GNLY, TYROBP, KLRC3*, and various KIR genes. When we compared the proportions of each $CD8^+$ T-cell cluster between healthy donors and patients with HBV-CLD, we found that 'CD56 CD8' tended to be increased in HBV-CLD, though the difference from healthy donors was not significant ($p = 0.06$; Fig. 2E).

We examined the clonal relationships among 7 $CD8^+$ T-cell clusters by analyzing the TCR repertoire overlap, which was calculated by the Morisita's overlap index using the immunarch package.[29] As expected, the TCR sequences of 'naïve CD8' did not overlap with those of the other $CD8^+$ T-cell clusters (Fig. 2F). Among non-naive $CD8^+$ T-cell clusters, 'CD56 CD8' had a unique TCR repertoire that minimally overlapped with the other clusters, whereas the other non-naïve $CD8^+$ T-cell clusters showed considerable levels of TCR overlap with each other. When we calculated the inverse Simpson index representing the TCR diversity in each cluster, the TCR diversity of 'CD56 CD8' was significantly restricted compared to the other clusters (Fig. 2G). Collectively, the CITE-seq and TCR-seq analyses of LSMCs identified a distinct $CD56^+CD8^+$ T-cell population with a unique, restricted TCR repertoire.

We further analyzed whether the $CD56^+CD8^+$ T-cell population is present in various organs by re-analyzing publicly available scRNA-seq data. First, we re-analyzed a total of 10,624 cells from healthy liver tissues[30] (n = 5) and obtained 7 clusters (Fig. S5A,B). In sub-clustering analysis of the CD8 T-cell cluster, 3 sub-clusters were obtained: 'CD8 T cell_1', 'CD8 T cell_2', and 'CD8 T cell_3' (Fig. S5C). Among these sub-clusters, 'CD8 T cell_2'

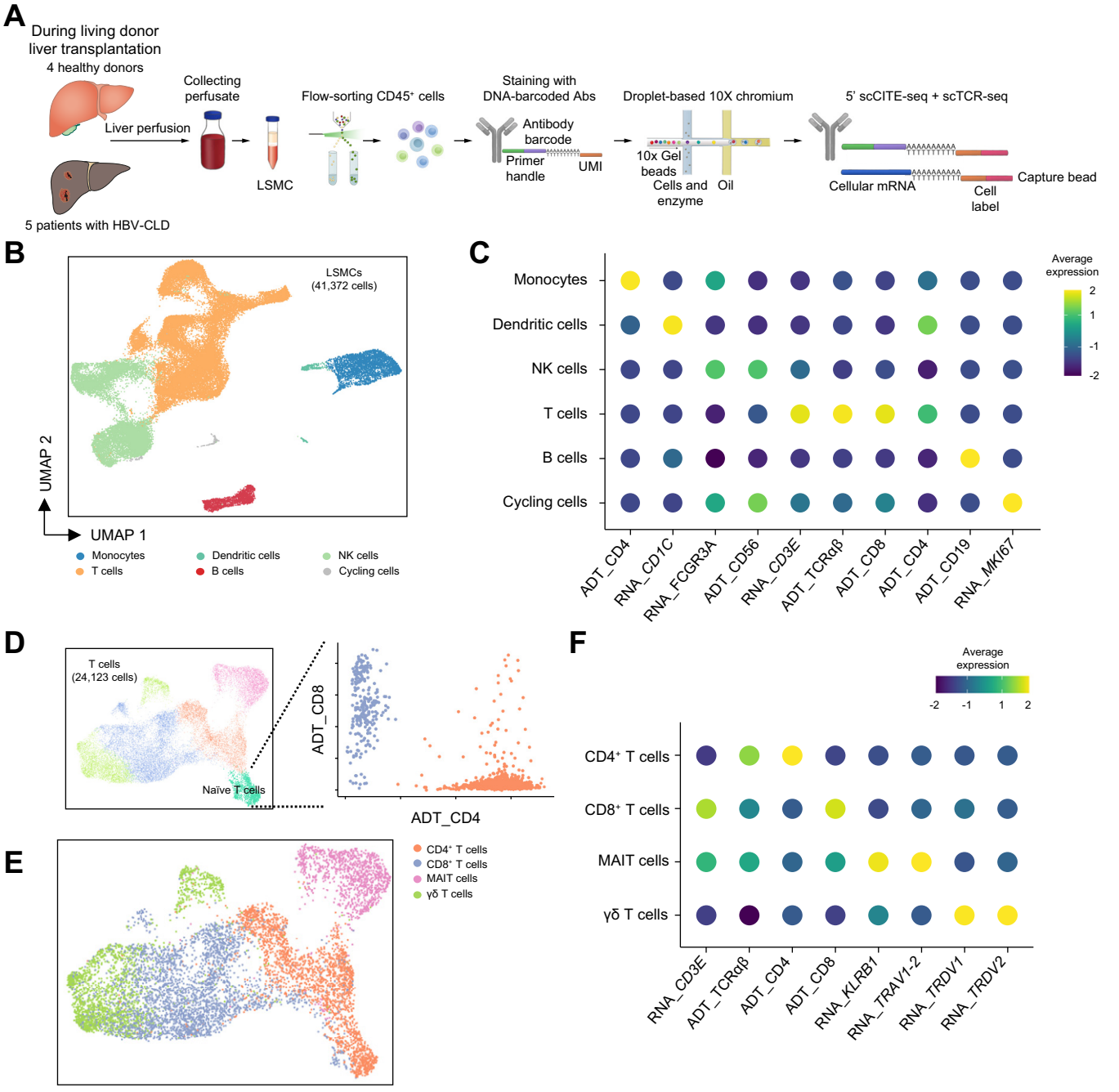

Fig. 1. Multi-omics analysis of liver sinusoidal mononuclear cells from healthy donors and patients with HBV-CLD. (A) Summary of the experimental design. (B) UMAP projections of 41,372 liver sinusoid CD45$^+$ cells from healthy donors (n = 4) and patients with HBV-CLD (n = 5) colored by immune cell type. (C) Dot plots showing the average normalized expression of marker genes and ADTs in each immune cell cluster. (D) UMAP projection of 24,123 liver sinusoid T cells and scatter plots showing the normalized expression of CD4 and CD8 ADTs in naïve T cells. (E) UMAP projection of liver sinusoid T cells colored by cell type. (F) Dot plots showing the average normalized expression of marker genes and ADTs in each T-cell subcluster. ADT, antibody-derived tag; HBV-CLD, HBV associated chronic liver disease; UMAP, uniform manifold approximation and projection.

exhibited high expression of *NCAM1, KLRC2, TYROBP, IFITM3*, and genes encoding diverse KIRs (Fig. S5D-F), indicating that the 'CD8 T cell_2' sub-cluster resembles CD56$^+$CD8$^+$ T cells. We also re-analyzed publicly available scRNA-seq data from various organs, including healthy[30,31] (n = 10) and cirrhotic[31] (n = 5) livers, healthy and pathologic (idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease) lungs[32] (n = 55), and healthy intestines[33,34] (n = 9). Ten clusters were obtained from a total of 88,781 NK and T cells (Fig. S6A,B). Further analysis of the CD8 T-cell cluster revealed 4 sub-clusters: 'CD56$^+$CD8 T cell', 'effector CD8 T cell', 'memory CD8 T cell', and 'HSP$^+$CD8 T cell' (Fig. S6C). Among these sub-clusters, 'CD56$^+$CD8 T cell' exhibited high expression of *NCAM1, KLRC2, TYROBP, GNLY,* and genes encoding diverse KIRs, as well as low expression of *KLRB1* (Fig. S6D), indicating that the 'CD56$^+$CD8 T cell' sub-cluster overlaps with the CD56$^+$CD8$^+$ T cells that were identified in our

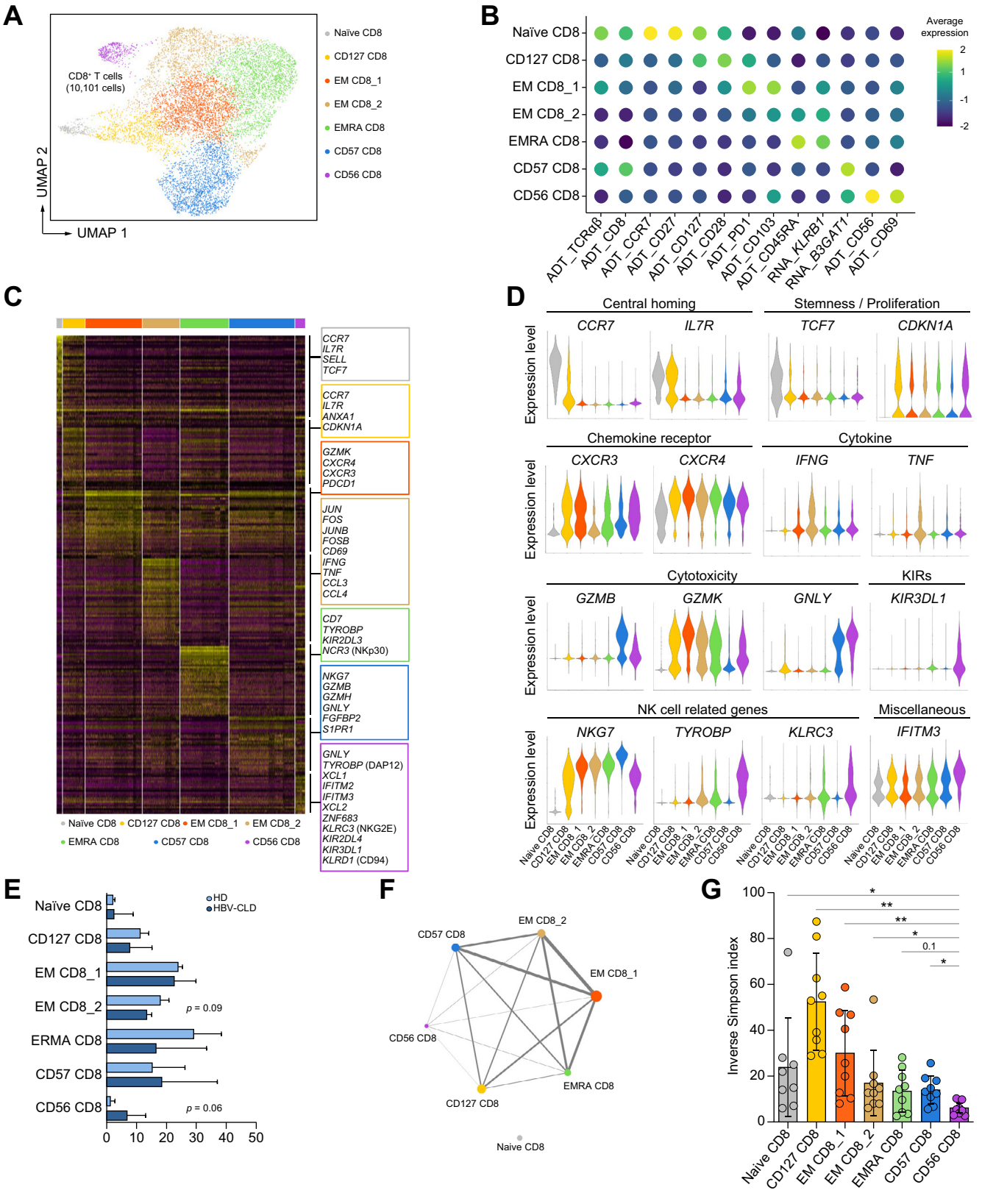

Fig. 2. Immune landscape of liver sinusoidal CD8⁺ T cells. (A) UMAP projection of 10,101 liver sinusoid CD8⁺ T cells colored by cell type information. (B) Dot plots showing the average normalized expression of marker genes and ADTs in each CD8⁺ T-cell subcluster. (C) Heatmap of cluster-specific DEGs for each CD8⁺ T-cell cluster. (D) Violin plots showing normalized marker gene expression among CD8⁺ T-cell clusters. (E) Bar plots showing the proportion of each CD8⁺ T-cell cluster in healthy donors (n = 4) and patients with HBV-CLD (n = 5). (F) Network plot representing the TCR repertoire overlapping among CD8⁺ T-cell clusters as calculated by Morisita's overlap index. The thickness of the line reflects the degree of TCR repertoire overlap between each cluster. (G) Bar plots showing TCR diversity calculated by the inverse Simpson index for each CD8⁺ T-cell cluster. *p <0.05, **p <0.01 according to Mann-Whitney U test. ADT, antibody-derived tag; DEGs, differentially expressed genes; HBV-CLD, HBV associated chronic liver disease; TCR, T-cell receptor; UMAP, uniform manifold approximation and projection.

CITE-seq analysis. The 'CD56$^+$CD8 T cell' sub-cluster was present in various healthy and pathologic organs (Fig. S6E).

Distinct immunophenotype of CD56^{hi}CD161$^-$CD8$^+$ T cells

As described above, we identified the 'CD56 CD8' cluster expressing CD56 protein and harboring a uniquely restricted TCR repertoire among liver sinusoidal CD8$^+$ T cells. The 'CD56 CD8' cluster is reminiscent of CD56$^+$CD8$^+$ T cells that were known previously as liver-enriched T cells with an NK-like feature.[14,15] Because the molecular characteristics and functions of CD56$^+$CD8$^+$ T cells have not been fully addressed, we focused on the 'CD56 CD8' cluster. We re-analyzed ADT expression among 7 CD8$^+$ T-cell clusters and found that CD56^{hi}CD161$^-$ cells were present exclusively in the 'CD56 CD8' cluster, indicating that a CD56^{hi}CD161$^-$ phenotype better defined the 'CD56 CD8' cluster (Fig. 3A).

We confirmed the results from the scRNA-seq analysis by performing flow cytometry analysis of LSMCs. We excluded γδ T cells by including anti-TCRγδ antibodies in dump staining and further excluded MAIT cells by staining them with anti-TCR Vα7.2 antibodies (Fig. S7A). More than 95% of non-MAIT TCRαβ$^+$ conventional liver sinusoidal CD8$^+$ T cells were non-naïve (non-CCR7$^+$CD45RA$^+$) memory cells (Fig. S7B). Among them, CD56^{hi}CD161$^-$, CD57$^+$, T_{EM}, and T_{EMRA} cells were well-recognized by flow cytometry (Fig. 3A and Fig. S7C).

Among non-MAIT TCRαβ$^+$ liver sinusoidal CD8$^+$ T cells, CD56^{hi}CD161$^-$CD8$^+$ T cells were the smallest population with a relative frequency of 0.5-15.4%, whereas CD57$^+$ and T_{EM} cells were relatively large populations (Fig. 3B). However, the relative frequency of CD56^{hi}CD161$^-$ cells among non-MAIT TCRαβ$^+$ CD8$^+$ T cells was significantly higher in the liver sinusoid than the PB (Fig. 3C). The relative frequency of CD56^{hi}CD161$^-$ cells among liver sinusoidal non-MAIT TCRαβ$^+$ CD8$^+$ T cells was significantly higher in patients with HBV-CLD than in healthy donors (Fig. 3D). When patients with HBV-CLD were further classified based on whether they had decompensated cirrhosis or hepatocellular carcinoma, the relative frequency of CD56^{hi}CD161$^-$ cells was significantly higher in patients with hepatocellular carcinoma than in healthy donors (Fig. S8A). We also analyzed the frequency of CD56^{hi}CD161$^-$CD8$^+$ T cells among intrahepatic CD8$^+$ T cells obtained from enzymatically digested liver tissues. The frequency of CD56^{hi}CD161$^-$CD8$^+$ T cells tended to be higher among intrahepatic CD8$^+$ T cells from patients with HBV-CLD than those from healthy donors, though the difference was not significant (p = 0.09; Fig. 3E). In liver tissues from patients with HBV-CLD, CD56$^+$CD8$^+$CD3$^+$ T cells could be detected by immunofluorescent staining (Fig. S8B).

In the analysis of markers for differentiation, almost all liver sinusoidal CD56^{hi}CD161$^-$CD8$^+$ T cells exhibited a CCR7$^-$CD45RA$^-$ effector phenotype (Fig. 3F). We also analyzed the expression of markers for tissue residency in CD56^{hi}CD161$^-$ cells compared T_{EM}, T_{EMRA}, and CD57$^+$ cells among liver sinusoidal CD8$^+$ T cells. CD56^{hi}CD161$^-$ cells had a higher frequency of CD69$^+$CD103$^+$ tissue-resident memory T (T_{RM}) cells than T_{EMRA} and CD57$^+$ cells, but not T_{EM} cells (Fig. 3G). The frequency of CD69$^+$CD103$^-$ T_{RM}-like cells, which are known to be regulated by HIF-2α(10), was highest in CD56^{hi}CD161$^-$ cells. In addition, the expression of other markers for T_{RM} cells, including CXCR6, CD49a, and CD11a, was significantly higher in CD56^{hi}CD161$^-$ cells than T_{EM}, T_{EMRA}, and CD57$^+$ cells despite an insignificant difference in the frequency of CXCR6$^+$ cells between CD56^{hi}CD161$^-$ and T_{EM} cells. These results indicate that CD56^{hi}CD161$^-$ cells predominantly exhibit T_{RM}-like features in the liver sinusoid.

Next, we examined the expression of cytotoxic molecules (Fig. 3H). The frequency of perforin$^+$ cells was significantly higher in CD56^{hi}CD161$^-$ cells than in T_{EM} and T_{EMRA} cells, but it was significantly lower in CD56^{hi}CD161$^-$ cells than in CD57$^+$ cells (Fig. 3I). Similarly, the frequency of granzyme B$^+$ cells was significantly higher in CD56^{hi}CD161$^-$ cells than in T_{EM} and T_{EMRA} cells but was not different between CD56^{hi}CD161$^-$ and CD57$^+$ cells. The frequency of granulysin$^+$ cells was highest in CD56^{hi}CD161$^-$ cells. In addition, we examined the expression level of Nur77, which is known to be upregulated exclusively by TCR signals.[35] We examined this expression without any *ex vivo* stimulation. CD56^{hi}CD161$^-$ cells exhibited higher expression of Nur77 than other conventional CD8$^+$ T cells (Fig. 3J), indicating that CD56^{hi}CD161$^-$ cells were exposed to a considerable level of TCR signals in liver sinusoidal environments. However, CD56^{hi}CD161$^-$ cells exhibited weak effector functions in terms of the production of IFN-γ and TNF and cytotoxic degranulation activity – measured by CD107a staining compared to other CD8$^+$ T-cell populations in intracellular cytokine staining assays following *ex vivo* anti-CD3/CD28 stimulation, which mimics TCR stimulation (Fig. 3K). In summary, CD56^{hi}CD161$^-$ cells express high levels of cytotoxic molecules with evidence of TCR signals. However, they have reduced effector functions upon *ex vivo* TCR stimulation.

NKG2C-mediated, TCR-independent cytotoxic activity of CD56^{hi}CD161$^-$CD8$^+$ T cells

As TCR-stimulated effector functions were attenuated in CD56^{hi}CD161$^-$CD8$^+$ cells, we examined the NK-like phenotypes and functions of CD56^{hi}CD161$^-$ cells compared to T_{EM}, T_{EMRA}, and CD57$^+$ cells among liver sinusoidal CD8$^+$ T cells. We examined the expression of various NK-activating receptors, such as NKG2D and NKG2C, NK-inhibitory receptors, such as NKG2A and KIRs, and CD94, a dimerizing partner for the NKG2 receptor family (Fig. 4A). The mean fluorescent intensity (MFI) of NKG2D was highest in CD56^{hi}CD161$^-$ cells (Fig. 4B). In addition, the frequencies of NKG2C$^+$, CD94$^+$, NKG2A$^+$, and KIR$^+$ cells were highest in CD56^{hi}CD161$^-$ cells, indicating that CD56^{hi}CD161$^-$ cells exhibit the most NK-like phenotype among liver sinusoidal CD8$^+$ T cells.

We analyzed the NK-like transcriptomic feature among liver sinusoidal CD8$^+$ T cells. To this end, we obtained a gene set from liver sinusoidal NK cells (Table S2) and examined whether it is enriched in liver sinusoidal CD8$^+$ T cells by calculating the NK gene set module scores. We found that the NK gene set module score was highest in CD56^{hi}CD161$^-$CD8$^+$ T-cell population (Fig. 4C). Next, we analyzed the gene set related to NK cell-mediated cytotoxicity. The NK cell-mediated cytotoxicity gene set module score was also highest in CD56^{hi}CD161$^-$ cells, confirming that CD56^{hi}CD161$^-$ cells exhibit the most NK-like transcriptomic features among liver sinusoidal conventional CD8$^+$ T cells.

Next, we investigated the effector functions of CD56^{hi}CD161$^-$ cells compared to T_{EM}, T_{EMRA}, and CD57$^+$ cells among liver sinusoidal CD8$^+$ T cells without *ex vivo* TCR stimulation. We co-cultured LSMCs with autologous phytohemagglutinin (PHA) blasts and found that the frequencies of IFN-γ$^+$ cells were significantly increased among T_{EM}, CD57$^+$, and CD56^{hi}CD161$^-$ cells. The frequency of IFN-γ$^+$ cells was highest in CD56^{hi}CD161$^-$ cells, but we found no significant difference in the frequency of

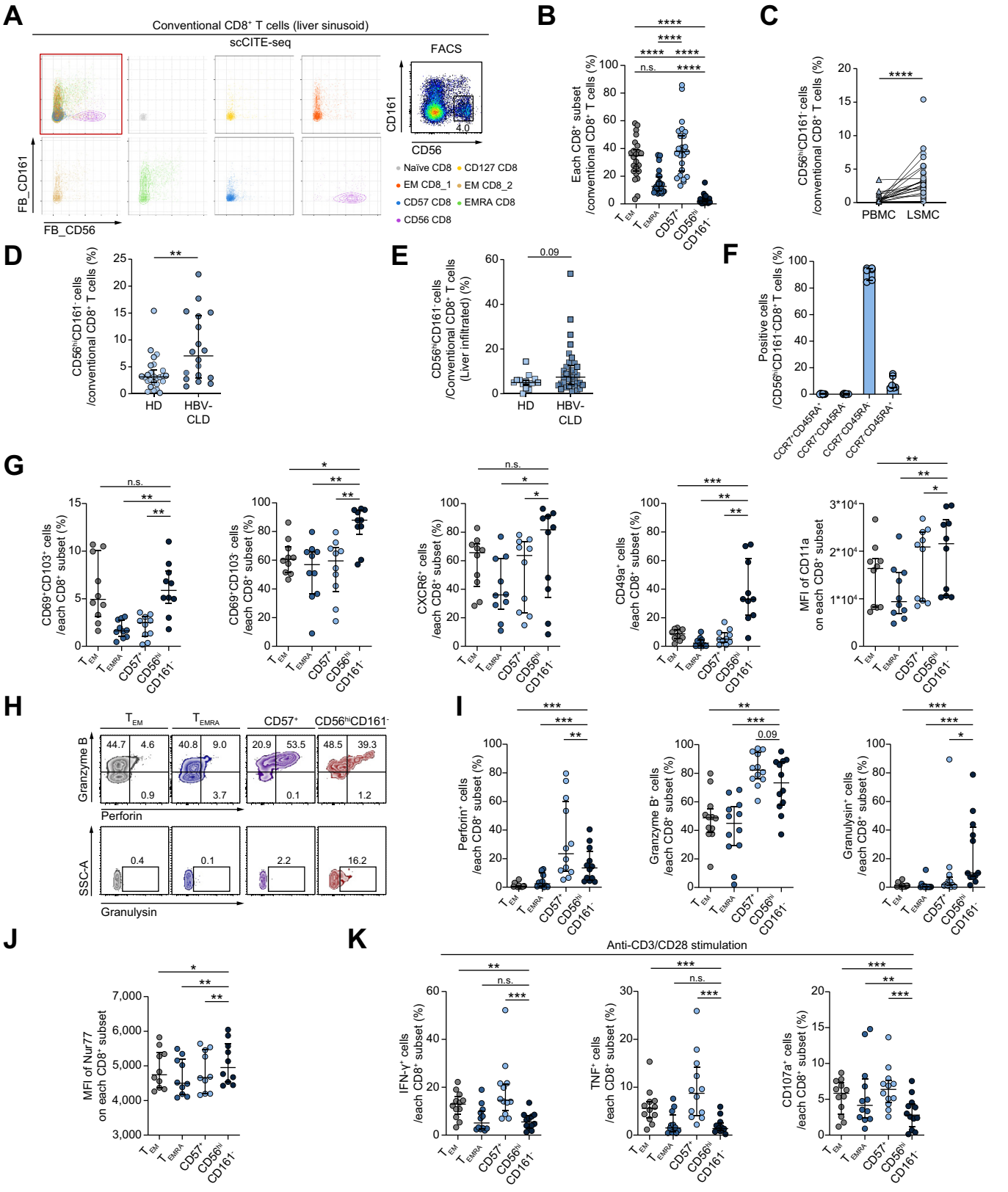

Fig. 3. *Ex vivo* immunophenotyping of liver sinusoidal CD8⁺ T cells. (A) Density plots of ADT expression in each cluster in scCITE-seq and representative flow cytometry plots of CD56⁺CD161⁻CD8⁺ T cells among non-MAIT TCRαβ⁺ liver sinusoidal CD8⁺ T cells from healthy donors. (B) Proportion of each CD8⁺ T-cell subset among CD8⁺ T cells from healthy donor LSMCs (n = 26). (C) Proportion of CD56⁺CD161⁻ T cells among CD8⁺ T cells from healthy donor PBMCs (n = 19) and LSMCs (n = 35). (D) Proportion of CD56⁺CD161⁻ T cells among CD8⁺ T cells from the LSMCs of healthy donors (n = 21) and patients with HBV-CLD (n = 28). (E) Proportion of CD56⁺CD161⁻ T cells among CD8⁺ T cells from the LIMCs of healthy donors (n = 14) and patients with HBV-CLD (n = 33). (F) Proportion of each subset among

IFN-γ^+ cells between CD56^{hi}CD161$^-$ and T_{EM} cells (Fig. 4D). The co-culture with PHA blasts significantly increased the frequencies of CD107a$^+$ cells in T_{EM} and CD56^{hi}CD161$^-$ cells. Among conventional CD8$^+$ T cells, the frequency was highest in CD56^{hi}CD161$^-$ cells. We examined the effect of antibodies specific to MHC class I (MHC-I) and found that anti-MHC-I blocking antibodies did not decrease, but rather increased, the frequency of CD107a$^+$ cells among liver sinusoidal CD56^{hi}CD161$^-$CD8$^+$ T cells (Fig. 4E). This result indicates that the PHA blast-induced cytotoxic degranulating activity of CD56^{hi}CD161$^-$CD8$^+$ T cells does not require MHC-I/TCR interaction and may be suppressed by MHC-I/KIR interaction, which was abrogated by anti-MHC-I blocking antibodies. Finally, we elucidated which receptors other than TCR can directly stimulate the effector functions of CD56^{hi}CD161$^-$CD8$^+$ T cells. To this end, we performed P815-redirected assays using antibodies specific to NK-activating receptors. The frequencies of IFN-γ^+ and CD107a$^+$ cells were significantly increased by the addition of anti-NKG2D antibodies among CD57$^+$ and CD56^{hi}CD161$^-$ cells, and they were highest in CD56^{hi}CD161$^-$ cells, though we found no significant difference in the frequency of IFN-γ^+ cells between CD56^{hi}CD161$^-$ and T_{EM} cells (Fig. 4F). Anti-NKG2C stimulation resulted in more robust effector functions in CD56^{hi}CD161$^-$ cells. The frequencies of IFN-γ^+ and CD107a$^+$ cells were significantly increased by the addition of anti-NKG2C antibodies among CD57$^+$ and CD56^{hi}CD161$^-$ cells. Among liver sinusoidal conventional CD8$^+$ T cells, the frequencies were highest in CD56^{hi}CD161$^-$ cells.

In summary, liver sinusoidal CD56^{hi}CD161$^-$CD8$^+$ T cells resemble NK cells in terms of their phenotypes and transcriptomic features. Moreover, they exert NK-like effector functions triggered by NK-activating receptors, such as NKG2C and NKG2D, in a TCR-independent manner.

Increased cytokine responsiveness of CD56^{hi}CD161$^-$CD8$^+$ T cells

As CD56^{hi}CD161$^-$CD8$^+$ cells exhibit TCR-independent, NKG2C-mediated effector functions, we examined another aspect of NK-like effector functions stimulated by innate cytokines, such as IL-12, IL-18, and IL-15,[36] in the absence of TCR stimulation. We examined the expression of cytokine receptors first. The expression of CD212 (IL-12Rβ) was significantly higher in CD56^{hi}CD161$^-$ cells than in T_{EMRA} and CD57$^+$ cells, but it was not different between CD56^{hi}CD161$^-$ and T_{EM} cells (Fig. 5A). The expression of CD218α (IL-18α) and CD122 (IL-2/IL-15Rβ) was highest in CD56^{hi}CD161$^-$ cells. When we examined the expression of PLZF, a transcription factor that contributes to the maintenance of the innate-like property of T cells,[37] it was highest in CD56^{hi}CD161$^-$ cells.

We compared the cytokine-induced effector functions of CD56^{hi}CD161$^-$ cells to those of T_{EM}, T_{EMRA}, and CD57$^+$ cells among liver sinusoidal CD8$^+$ T cells. When we stimulated LSMCs with IL-12/IL-18, the frequencies of IFN-γ^+, perforin$^+$, granzyme B$^+$, and granulysin$^+$ cells were highest in CD56^{hi}CD161$^-$ cells, but we found no difference in the frequency of granzyme B$^+$ cells between CD56^{hi}CD161$^-$ and CD57$^+$ cells (Fig. 5B). These results were recapitulated when the MFIs of perforin, granzyme B, and granulysin were analyzed (Fig. S9A). Upon IL-15 stimulation, the frequencies of IFN-γ^+, perforin$^+$, granzyme B$^+$, and granulysin$^+$ cells were highest in CD56^{hi}CD161$^-$ cells, but we found no difference in the frequency of IFN-γ^+ cells between CD56^{hi}CD161$^-$ and T_{EM} cells (Fig. 5C). These results were reproduced when we analyzed the MFIs of perforin, granzyme B, and granulysin (Fig. S9B). We also examined the expression of Ki-67, a cell proliferation marker, following IL-15 stimulation and found that the percentage of Ki-67$^+$ cells was highest in CD56^{hi}CD161$^-$ cells, indicating that IL-15 preferentially stimulates proliferation of CD56^{hi}CD161$^-$ cells (Fig. 5D). Furthermore, we examined whether IL-15 can expand the CD56^{hi}CD161$^-$CD8$^+$ T-cell population. We obtained CD56^{hi}CD161$^-$ cell-depleted conventional CD8$^+$ T cells from LSMCs and cultured them in the presence of IL-15 or anti-CD3 for 14 days (Fig. S10A). IL-15 stimulation significantly increased the percentage of CD56^{hi}CD161$^-$ cells among CD8$^+$ T cells, but anti-CD3 did not (Fig. 5E). Taken together, the results show that CD56^{hi}CD161$^-$CD8$^+$ T cells exhibit not only high expression of receptors for IL-12, IL-18, and IL-15, but also high levels of responsiveness to these innate cytokines.

Comparison of liver sinusoidal and intrahepatic CD8$^+$ T cells

Finally, we investigated whether LSMCs reflect the intrahepatic immune environment. To this end, we obtained LSMCs and intrahepatic liver infiltrating mononuclear cells (LIMCs) from liver perfusates and enzymatically digested liver tissues, respectively, from identical individuals (n = 20) with or without HBV-CLD and compared the cellular compositions and phenotypes between LSMCs and LIMCs. First, we focused on subsets of non-MAIT TCRαβ$^+$ conventional CD8$^+$ T cells as gated in Fig. S7. The percentages of T_{EM}, T_{EMRA}, CD57$^+$, and CD56^{hi}CD161$^-$ cells among liver sinusoidal CD8$^+$ T cells significantly correlated with the percentages among intrahepatic CD8$^+$ T cells (Fig. S11). We also examined subsets of CD3$^-$CD56$^+$ NK cells. The percentages of memory-like NKG2C$^+$ NK cells, NKG2A$^+$ NK cells, and KIR$^+$ NK cells among liver sinusoidal NK cells significantly correlated with the percentages among intrahepatic NK cells (Fig. S12). In further analysis, we analyzed the tissue-resident phenotypes of T_{EM}, T_{EMRA}, CD57$^+$, and CD56^{hi}CD161$^-$ cells between liver sinusoidal and intrahepatic CD8$^+$ T cells. The percentages of CD69$^+$CD103$^-$ T_{RM}-like cells, CXCR6$^+$ cells, and CD49a$^+$ cells and MFI of CD11a among liver sinusoidal CD56^{hi}CD161$^-$ CD8$^+$ T cells significantly correlated with the percentages and MFI among intrahepatic CD56^{hi}CD161$^-$ CD8$^+$ T cells (Fig. S13).

Next, we compared the expression of cytotoxic molecules between liver sinusoidal and intrahepatic CD56^{hi}CD161$^-$CD8$^+$ T cells. The percentages of perforin$^+$ cells, granzyme B$^+$ cells, and granulysin$^+$ cells among liver sinusoidal CD56^{hi}CD161$^-$CD8$^+$ T

CD56$^+$CD161$^-$CD8$^+$ T cells from the LSMCs (n = 6). (G) Proportion and MFI of T_{RM} marker-expressing cells in each CD8$^+$ T-cell subset (n = 10). (H) Representative flow cytometry plots for perforin, granzyme B, or granulysin-expressing cells among each CD8$^+$ T-cell subset. (I) Proportion of perforin$^+$, granzyme B$^+$, or granulysin$^+$ cells in each CD8$^+$ T-cell subset (n = 12). (J) MFI of Nur77 in each CD8$^+$ T-cell subset (n = 10). (K) Proportion of IFN-γ^+, TNF$^+$, or CD107a$^+$ cells after anti-CD3/CD28 stimulation (100 ng/ml; 1 μg/ml) in each CD8$^+$ T-cell subset (n = 12). *p <0.05, ** p <0.01, ***p <0.001, ****p <0.0001, according to a Wilcoxon signed-rank test for paired groups and Mann-Whitney U test to for unpaired groups. ADT, antibody-derived tag; HBV-CLD, HBV associated chronic liver disease; LSMCs, liver sinusoidal mononuclear cells; LIMCs, liver infiltrating mononuclear cells; MAIT, mucosal-associated invariant T; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; scCITE-seq, single-cell cellular indexing of the transcriptomes and epitopes by sequencing.

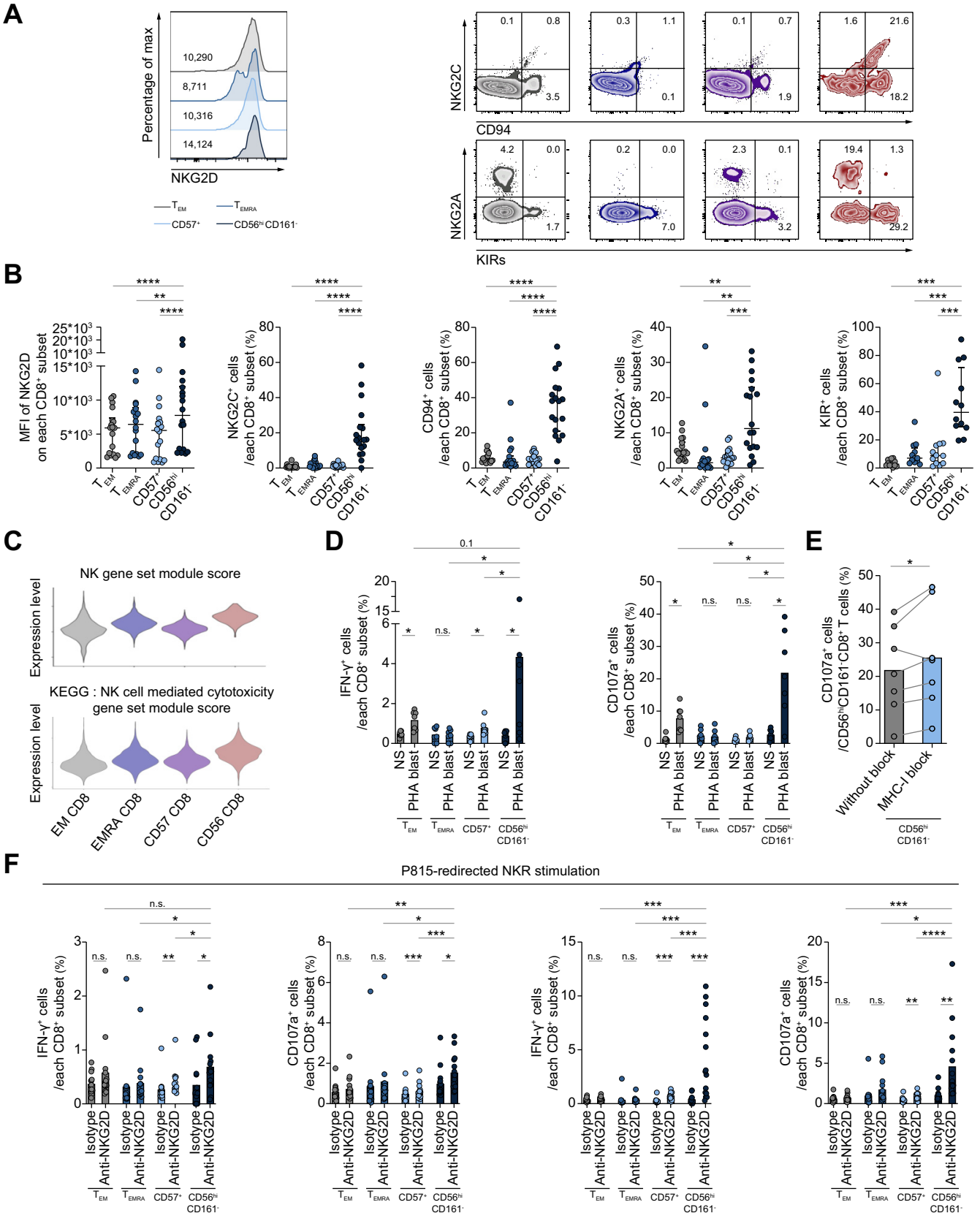

Fig. 4. NK-like phenotypes and functions of liver sinusoidal CD8⁺ T cells. (A) Representative flow cytometry plots of cells expressing NKG2D, NKG2C, CD94, NKG2A, and KIRs among each CD8⁺ T-cell subset. (B) MFI and proportion of NKG2D (n = 18), NKG2C⁺ (n = 18), CD94⁺ (n = 18), NKG2A⁺ (n = 18), and KIR⁺ (n = 12) cells in each CD8⁺ T-cell subset. (C) Violin plots showing the module scores for gene sets related to NK cells in each CD8⁺ T-cell cluster. EM CD8 includes naïve CD8,

cells significantly correlated with the percentages among intrahepatic CD56^{hi}CD161^{-}CD8^{+} T cells (Fig. S14). We also compared the expression of various NRKs between liver sinusoidal and intrahepatic CD56^{hi}CD161^{-}CD8^{+} T cells. The MFI of NKG2D and percentages of NKG2C^{+}, CD94^{+}, and KIR^{+} cells among liver sinusoidal CD56^{hi}CD161^{-}CD8^{+} T cells significantly correlated with the MFI and percentages among intrahepatic CD56^{hi}CD161^{-}CD8^{+} T cells (Fig. S15). Collectively, these data demonstrate that LSMCs reflect the intrahepatic immune environment.

Discussion

In the present study, we examined liver sinusoidal CD56^{hi}CD161^{-}CD8^{+} T cells in comparison to other liver sinusoidal non-MAIT TCR$\alpha\beta^{+}$ conventional CD8^{+} T-cell populations, including T_{EM}, T_{EMRA}, and CD57^{+} cells. CD56^{hi}CD161^{-}CD8^{+} T cells were characterized by NK-related gene expression and a uniquely restricted TCR repertoire. In addition, CD56^{hi}CD161^{-}CD8^{+} T cells highly responded to innate cytokines, such as IL-12/18 and IL-15, though they exhibited weak responsiveness to TCR stimulation. Importantly, CD56^{hi}CD161^{-}CD8^{+} T cells exerted NKG2C-mediated NK-like effector functions in a TCR-independent manner. Herein, we report that CD56^{hi}CD161^{-}CD8^{+} T cells are a unique NK-like T-cell population with NKG2C-dependent, TCR-independent activation, and are particularly enriched in the liver sinusoid. Furthermore, we demonstrated that LSMCs reflect the intrahepatic immune environment by performing a comparative analysis of liver sinusoidal and intrahepatic CD8^{+} T cells.

CD56^{+} T cells have previously been reported as 'natural T cells' or 'NK-like T cells' that are enriched in the liver.[14,15,17] CD56^{+} T cells are known to exhibit a potent capacity for T helper 1 cytokine production and TCR-independent cytotoxicity following stimulation with mitogen and IL-2.[14] In addition, CD56^{+} T cells express high levels of NKRs, including NKG2D, NKp44, NKp46, and DNAM-1.[18] CD56^{+} T cells are known to inhibit the replication of HCV in hepatocytes,[38] and the frequency of CD56^{+} T cells is increased in PB from patients with cytomegalovirus infection compared to healthy donors.[39] However, the mechanisms by which CD56^{+} T cells are functionally activated remain to be elucidated. In the current study, though we did not examine whether the liver sinusoidal CD56^{hi}CD161^{-}CD8^{+} T-cell population is identical to the previously known CD56^{+} T cells, we demonstrated that CD56^{hi}CD161^{-}CD8^{+} T cells respond to TCR-independent stimulation, including innate cytokines and NKG2C ligation.

NKG2C is a member of the C-type lectin NKG2/CD94 receptor family that binds to HLA-E and transduces activation signals.[40] NKG2C-expressing CD8^{+} T cells lyse target cells more efficiently when co-stimulated by anti-CD3 and anti-CD94 antibodies.[41] In addition, NKG2C-expressing CD8^{+} T cells have been shown to exert TCR-independent cytotoxicity in various diseases, including celiac disease, Stevens-Johnson syndrome, and toxic epidermal necrolysis.[42,43] Furthermore, NKG2C-expressing CD8^{+} T cells mediate antimicrobial activity against intracellular bacteria in both a TCR-dependent and -independent manner.[44] NKG2C recognizes cell surface HLA-E that binds peptides from leader sequences of classical MHC I proteins[45,46] or pathogen-derived peptides.[47] Given that HLA-E expression is upregulated by various cellular stresses,[48,49] NKG2C-expressing cells, including CD56^{hi}CD161^{-}CD8^{+} T cells, may play a role in immune surveillance to eliminate stressed cells by monitoring the upregulation of HLA-E.

In the present study, we used innate cytokines, such as IL-12/IL-18 and IL-15, to stimulate CD8^{+} T cells without TCR stimulation and found that CD56^{hi}CD161^{-}CD8^{+} T cells highly respond to these cytokines. IL-18, a member of the IL-1 family, is known to be a potent cytokine inducing TCR-independent activation of memory CD8^{+} T cells, and IL-12 acts synergistically with IL-18.[50–52] IL-15 belongs to a group of common γ-chain cytokines and has been known to activate memory CD8^{+} T cells even without antigenic stimulation.[53] Although IL-12/IL-18 and IL-15 cause TCR-independent activation, each has different functional effects on memory CD8^{+} T cells. IL-15 stimulates memory CD8^{+} T cells to upregulate the expression of activating NKRs, such as NKG2D and NKp30, and exert NK-like cytotoxicity,[54–56] whereas IL-12/IL-18 stimulates memory CD8^{+} T cells to produce IFN-γ.

Recently, virtual memory T (T_{VM}) cells, a new type of CD8^{+} T-cell subset, were identified in mice.[57] T_{VM} cells have hyper-responsiveness to IL-12/IL-18 and IL-15, with IL-12/IL-18 eliciting IFN-γ production and IL-15 driving T_{VM} cell proliferation and TCR-independent NK-like cytotoxicity.[13,57,58] T_{VM} cells exhibit a memory phenotype without antigenic experience[59] and have been reported to have a high affinity for self-antigens. T_{VM} cells have high basal levels of TCR signals that are thought to be driven by self-antigens. Notably, liver sinusoidal CD56^{hi}CD161^{-}CD8^{+} T cells exhibit high expression of Nur77, which is known to be upregulated exclusively by TCR signals,[35] demonstrating that CD56^{hi}CD161^{-}CD8^{+} T cells continuously receive a considerable level of TCR signaling in the liver sinusoidal environment. Taken together, these results suggest that CD56^{hi}CD161^{-}CD8^{+} T cells resemble T_{VM} cells in terms of high basal levels of TCR signals and hyper-responsiveness to innate cytokines.

In the current study, we found that the relative frequency of liver sinusoidal CD56^{hi}CD161^{-}CD8^{+} T cells is increased in patients with HBV-CLD. In addition, the frequency of CD56^{hi}CD161^{-} cells among intrahepatic CD8^{+} T cells obtained from enzymatically digested liver tissues tended to be increased in patients with HBV-CLD. Although a role of CD56^{hi}CD161^{-}CD8^{+} T cells in immune responses against HBV has not been elucidated, we propose a mechanism by which the frequency of CD56^{hi}CD161^{-}CD8^{+} T cells increases in patients with HBV-CLD. Given that IL-15-induced proliferation capacity among liver sinusoidal conventional CD8^{+} T cells was highest in CD56^{hi}CD161^{-}CD8^{+} T cells, the CD56^{hi}CD161^{-}CD8^{+} T-cell population may be expanded through persistent stimulation by the IL-15 produced by chronic viral replication[60–62] and liver injury/regeneration.[63,64]

In summary, we identified CD56^{hi}CD161^{-}CD8^{+} T cells as unique NK-like CD8^{+} T cells that are particularly enriched in the liver sinusoid using single-cell multi-omics and flow cytometric analyses. These cells have a uniquely restricted TCR repertoire

CD127 CD8, EM CD8_1, and EM CD8_2 subclusters. (D) Proportion of IFN-γ^{+} or CD107a^{+} cells after 12 hours of PHA blast co-culture stimulation in each CD8^{+} T-cell subset (n = 7). (E) Proportion of CD107a^{+} cells after 12 hours of combined stimulation with PHA blast and antagonistic MHC-I blocking antibody (n = 7). (F) Proportion of IFN-γ^{+} or CD107a^{+} cells after 12 hours of P815-redirected NKR stimulation with anti-NKG2D or NKG2C antibodies (n = 15). *p <0.05, ** p <0.01, ***p <0.001, ****p <0.0001, according to a Wilcoxon signed-rank test for paired groups and Mann-Whitney U test to for unpaired groups. MFI, mean fluorescence intensity; PHA, phytohemagglutinin.

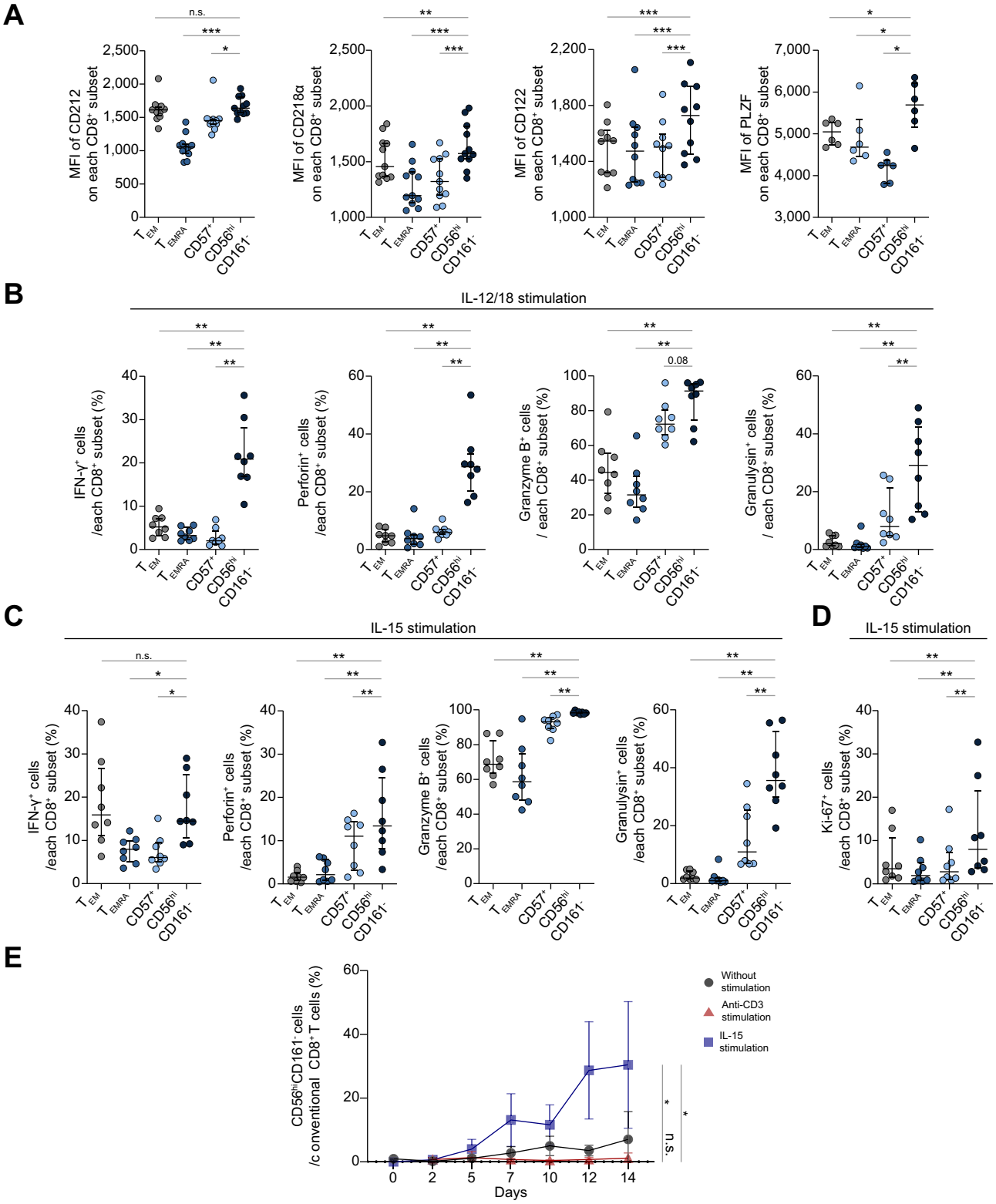

Fig. 5. Cytokine-responsiveness of liver sinusoidal CD8⁺ T cells. (A) MFI of CD122 (n = 10), CD218α (n = 10), CD122 (n = 10), and PLZF (n = 6) in each CD8⁺ T-cell subset. (B) Proportion of IFN-γ⁺, perforin⁺, granzyme B⁺, or granulysin⁺ cells after 12 hours of IL-12/18 (50 ng/ml; 50 ng/ml) or (C) IL-15 (10 ng/ml) stimulation in each CD8⁺ T-cell subset (n = 8). (D) Proportion of Ki-67⁺ cells after 48 hours of IL-15 (10 ng/ml) stimulation in each CD8⁺ T-cell subset (n = 8). *p <0.05, ** p <0.01, ***p <0.001, according to a Wilcoxon signed-rank test for paired groups and Mann-Whitney U test to for unpaired groups. (E) Proportion of CD56^{hi}C61^{-} cells among CD56^{hi}CD161^{-} cell-depleted conventional CD8⁺ T cells after stimulation with IL-15 (10 ng/ml) or anti-CD3 (100 ng/ml) according to time course (n = 6). MFI, mean fluorescence intensity.

and exhibit hyper-responsiveness to innate cytokines. Importantly, liver sinusoidal $CD56^{hi}CD161^{-}CD8^{+}$ T cells exert NK-like cytotoxic activity mediated by NKG2C ligation even without TCR stimulation. Further studies are required to elucidate the roles of liver sinusoidal $CD56^{hi}CD161^{-}CD8^{+}$ T cells in immune responses to microbial pathogens or immunopathology in infected and injured livers.

Abbreviations

ADT, antibody-derived tag; CITE-seq, cellular indexing of the transcriptomes and epitopes by sequencing; CLD, chronic liver disease; HBV-CLD, HBV-associated CLD; iNKT, invariant natural killer T; KIR, killer immunoglobulin-like receptor; LSMC, liver sinusoidal mononuclear cell; LIMC, liver infiltrating mononuclear cell; MAIT, mucosal-associated invariant T; MFI, mean fluorescence intensity; MHC-I, MHC class I; NKR, NK cell receptor; PB, peripheral blood; PHA, phytohemagglutinin; PHA blast, PHA-stimulated cell; scRNA-seq, single-cell RNA sequencing; TCR, T-cell receptor; T_{EM}, effector memory T; T_{EMRA}, $CD45RA^{+}$ effector memory T; T_{RM}, tissue resident memory T; T_{VM}, virtual memory T; UMAP, uniform manifold approximation and projection.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualization: J.-Y.K., M.-S.R., S.J.,C., and E.-C.S. Methodology: J.-Y.K., M.-S.R., S.J. C., H.S.L., J.W.H., H.N. and D.-U. K. Resources: J.G.L., M.S.K., J.Y.P. and D.J.J. Writing: J.-Y.K., and E.-C.S. Review and editing: J.-Y.K., M.-S.R., and E.-C.S. Supervision: S.-H.P., J.Y.P., D.J.J., and E.-C.S. Funding: S.-H.P. and E.-C.S.

Data availability statement

The RNA-seq data has been deposited in the GEO database under the primary accession code GSE200173. For further details regarding the materials and methods used, please refer to the supplementary information and CTAT table.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.05.020.

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Author names in bold designate shared co-first authorship

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