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Authors

Hudspeth, Kelly
Donadon, Matteo
Cimino, Matteo
et al.

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Human liver-resident CD56^{bright}/CD16^{neg} NK cells are retained within hepatic sinusoids via the engagement of CCR5 and CXCR6 pathways

Kelly Hudspeth¹, Matteo Donadon², Matteo Cimino², Elena Pontarini¹, Paolo Tentorio¹, Max Preti¹, Michelle Hong³, Antonio Bertoletti³, Silvio Biccato⁴, Pietro Invernizzi^{5,6}, Enrico Lugli¹, Guido Torzilli², M. Eric Gershwin⁶, and Domenico Mavilio^{1,7}

¹Unit of Clinical and Experimental Immunology, Humanitas Clinical and Research Center, 20089 Rozzano, Italy

²Department of Hepatobiliary & General Surgery, Humanitas Clinical and Research Center, 20089 Rozzano, Milan, Italy

³Viral Hepatitis Laboratory, Singapore Institute for Clinical Sciences, Agency of Science; Technology and Research (A*STAR), 169587, Singapore

⁴Department of Life Sciences, Center for Genome Research, University of Modena and Reggio Emilia, 41100, Modena, Italy

⁵Liver Unit and Center for Autoimmune Liver Diseases, Humanitas Clinical and Research Center, 20089 Rozzano, Italy

⁶Division of Rheumatology, Allergy, and Clinical Immunology, University of California Davis, Davis, CA 95616, CA, USA

⁷Department of Medical Biotechnologies and Translational Medicine, University of Milan, 20089 Rozzano, Italy

Abstract

Corresponding authors: Correspondence to: Domenico Mavilio, MD, Ph.D., Unit of Clinical and Experimental Immunology, Department of Medical Biotechnologies and Translational Medicine, University of Milan School of Medicine, Humanitas Clinical and Research Center, Via Alessandro Manzoni, 113, Rozzano (Milan), Italy; Phone: +39-02.8224.5157; FAX: +39-02.8224.5191; domenico.mavilio@unimi.it or M. Eric Gershwin, M.D., Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis School of Medicine, 451 Health Sciences Drive, Suite 6510, Davis, CA 95616; telephone: 530-752-2884; fax: 530-752-4669; megershwin@ucdavis.edu.

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Rationale—The liver-specific natural killer (NK) cell population is critical for local innate immune responses, but the mechanisms that lead to their selective homing and the definition of their functional relevance remain enigmatic.

Objectives—We took advantage of the availability of healthy human liver to rigorously define the mechanisms regulating the homing of NK cells to liver and the repertoire of receptors that distinguish liver-resident NK (lr-NK) cells from circulating counterparts.

Findings—Nearly 50% of the entire liver NK cell population is composed of functionally relevant CD56^{bright} lr-NK cells that localize within hepatic sinusoids. Further, CD56^{bright} lr-NK cells express CD69, CCR5 and CXCR6 and this unique repertoire of chemokine receptors is functionally critical as it determines selective migration in response to the chemotactic stimuli exerted by CCL3, CCL5 and CXCL16. In addition, hepatic sinusoids express CCL3^{pos} Kupffer cells, CXCL16^{pos} endothelial cells and CCL5^{pos} T and NK lymphocytes. The selective presence of these chemokines in sinusoidal spaces creates a tissue niche for lr-CD56^{bright} NK cells that constitutively express CCR5 and CXCR6. CD56^{bright} lr-NK cells co-exist with CD56^{dim} conventional NK (c-NK) cells that are, interestingly, transcriptionally and phenotypically similar to their peripheral circulating counterparts. Indeed, CD56^{dim} c-NK cells lack expression of CD69, CCR5, and CXCR6 but express selectins, integrins and CX₃CR1.

Conclusion—Our findings disclosing the phenotypic and functional differences between lr-NK cells and c-NK cells are critical to distinguish liver-specific innate immune responses. Hence, any therapeutic attempts at modifying the large population of CD56^{bright} lr-NK cells will require modification of hepatic CCR5 and CXCR6.

Keywords

Liver immunology; Homing of hepatic NK cells; Chemokine Receptors

Introduction

Natural killer (NK) cells are important effectors of the innate immune system that can lyse tumor-transformed or virus-infected cells in the absence of prior antigen sensitization. NK cells are also endowed with immune-regulatory functions at tissue sites of inflammation through the establishment of cellular interactions and the production of pro-inflammatory cytokines including IFN- γ , TNF- α , CCL3 (Mip1- α), CCL4 (Mip1- α and CCL5 (RANTES) [1, 2]. Human peripheral blood NK (PB-NK) cells are divided into two functionally distinct subsets characterized by a different distribution of CD56 and CD16 surface markers. CD56^{bright}/CD16^{neg-low} (CD56^{bright}) NK cells account for 5–15% of all PB-NK cells and, while poorly cytotoxic, can produce large amounts of cytokines. CD56^{dim}/CD16^{pos} (CD56^{dim}) NK cells represent the majority of PB-NK cells (up to 95%) and serve primarily as cytotoxic effectors [3]. In order to spare autologous cells from cytotoxicity and ensure tolerance to self, NK cells receive inhibitory signals from a large family of inhibitory NK cell receptors (iNKR), that include Killer cell immunoglobulin-like receptors (KIRs) and C-type lectins recognizing specific alleles of self MHC-class-I molecules (MHC-I). NK cell effector-functions are generally induced by the engagement of another family of activating NK cell receptors (aNKR) that binds their ligands expressed on stressed, infected or tumor-

transformed target cells that either lack or have a decreased expression of self-MHC-I [1, 4–6].

NK cells can account up to 50% of the total lymphocyte population in the human liver, which is in contrast to their lower frequency in blood (5–15%) or other peripheral tissues such as lymph nodes. Hence, great efforts have been placed over the recent years to understand the role of hepatic NK cells in the pathogenesis of liver disorders including fibrosis, viral infections, tumors and autoimmune diseases [7–9]. In this regard, a distinct subset of hepatic NK cells endowed with adaptive immune properties and exhibiting antigen-specific recall responses to viruses and haptens has been recently described in mice [10, 11]. This memory-like response is exerted by a unique subset of CD49a^{pos}/DX5^{neg} liver-resident NK (lr-NK) cells that are phenotypically and functionally distinct from peripheral blood CD49a^{neg}/DX5^{pos} conventional NK (c-NK) cells circulating throughout spleen and liver. Likewise, it has been reported that NK cells in human liver are different from their circulating counterparts [7, 12–16], thus suggesting that a unique lr-NK cell subset also exists in the human liver under homeostatic conditions. However, the overall frequency and the precise phenotype of human lr-NK cells are still being debated, while the distribution and the homing mechanisms regulating their retention in the liver are unknown. The present study characterizes CD56^{bright} lr-NK cells selectively located within hepatic sinusoids and accounting for half of the entire hepatic NK cell population. We demonstrate that CD56^{bright} lr-NK cells are phenotypically and transcriptionally distinct from PB-CD56^{bright} NK cells and constitutively express high levels of markers associated with tissue residency including CD69, CCR5 and CXCR6. This unique phenotype is functionally relevant as CD56^{bright} lr-NK cells migrate in response to CCL3, CCL5 and CXCL16, the CCR5 and CXCR6 ligands highly expressed within liver sinusoids on Kupffer cells, T and NK lymphocytes as well as endothelial cells.

Material and Methods

Human Donors

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors obtained in accordance with clinical protocols approved by the Institutional Review Board of Desio Hospital, Milan, Italy. Liver specimens were obtained from patients undergoing liver resection to remove liver metastases of colorectal carcinoma. Fragments of hepatic tissues used for our experiments were macroscopically and microscopically free of any diseases and considered healthy, as assessed by the Unit of Pathology of the Humanitas Research Hospital, Milan, Italy (Supplemental Figure 1). Specimens not meeting these criteria were excluded from our study. Liver specimens were obtained in accordance with clinical protocols approved by the Institutional Review Board (IRB) of Istituto Clinico Humanitas, Milan, Italy. Liver perfusates were obtained from Singapore Institute for Clinical Sciences as a part of the scientific collaboration with Dr. Antonio Bertoletti. The Gleneagles Hospital Ethics Committee, Singapore, Singapore approved the study and each patient gave written informed consent.

Cell preparation

PBMCs were obtained by Ficoll-Hypaque density gradient centrifugation (GE Healthcare Biosciences), as previously described [17, 18]. Liver mononuclear cells (LMNCs) were isolated by digestion of fresh liver samples using 2mg/ml of collagenase D (Roche) in HEPES Buffered Saline for 45 minutes followed by a brief mechanical digestion using the GentleMACS Dissociator (Miltenyi). Cells were layered over a 70%/30% discontinuous Percoll (GE-Healthcare) gradient. Cells between the 70%/30% layer contained LMNCs. Perfusate mononuclear cells from were isolated from healthy healthy liver before transplant using Ficoll-Paque PREMIUM (GE-Healthcare), as previously described [19].

Flow cytometry and in vitro functional assays

For multicolor flow cytofluorimetric analysis, PBMCs, LMNCs and PMNCs were stained with the following conjugated mAb as previously described: CD56-PE-Cy5 and NKp46-PE (Beckman Coulter, clone BAB281), CD16-PE-Cy7, CD19-APCH7, IFN- γ -PE, DNAM-1 PE, CD49e-PE, CD11c-PE, CCR5-PE, CD69-PE, CCR4-PE and CXCR3-PE (BD-Pharmigen), CD45-PB, CD62L-PE-Cy7, CCL5-AF647, CD161-PerCpCy5.5, CCR3-PE, CXCR2-PE and CXCR4-PerCpCy5.5 (Biolegend), CCR7-FITC and CXCR6-PE (R&D System), CX₃CR1 PE (MBL). Aqua LIVE/DEAD (Life Technologies) was used to eliminate dead cells from the analysis. Intracellular staining was performed using Cytotfix/Cytoperm (Beckton Dickinson), according to the manufacturers instructions. The gating strategy used to select NK cells from both PBMC and LMNC is depicted in Supplemental Figure 1. For measurement of IFN- γ production, whole PBMC and LMNC were stimulated with 18 hours with 20ng/ml rhIL-12 (R&D Systems) and 200U/ml rhIL-2 (Peprotech). For the final 4 hours, GolgiStop was added (Beckton Dickenson). Flow cytometry data was acquired using an LSR Fortessa (Beckton Dickinson) and data was analyzed using FlowJo Software (Tree Star).

Immunohistochemistry

Paraffin-embedded liver specimens were assessed for chemokine expression using the following mAbs: Anti-CCL3 (Mip1- α anti-CCL4 (Mip1- α (R&D) and anti-CCL5 (RANTES) (Abeam). Antigen retrieval was performed for 5 minutes at 125°C and for 3 minutes at 90°C in a pressure cooker using Diva Decloaker antigen retrieval solution (Biocare Medical). Endogenous peroxidase activity was blocked with Peroxidase I (Biocare Medical) for 5 minutes and non-specific proteins were blocked for 15 min with Background Sniper (Biocare Medical). The primary antibody was incubated for 1 hour at room temperature. For CCL5, MACH 4 HRP Polymer was used as the secondary antibody (Biocare Medical). For CCL3 and CCL4, Goat-on-Rodent HRP Polymer (Biocare Medical) was used as the secondary antibody. For identification of NK cells, a mAb anti-NKp46 (R&D Systems) was used. Samples were prepared by flash-freezing pieces of fresh liver in isopentane-cooled dry ice in OCT (Tissue-Tek). Sections were fixed in freshly prepared 4% paraformaldehyde and incubated with H₂O₂ in methanol. Blocking of non-specific proteins was performed using 0.04% horse serum followed by overnight incubation with the primary antibody at 4C. MACH 4 HRP Polymer was used as the secondary antibody. (Biocare Medical). All antigen detection was performed using 3,3-diaminobenzidine (DAB

chromogen) and counterstaining of nuclei was performed using hemotoxylin. Images were obtained using optical microscopy (Olympus BX53).

Immunofluorescence

For visualization of CCR5^{POS} NK cells in liver sinusoids, samples of fresh liver were fixed with fresh 2% PFA for 2 hours, washed in PBS for 4 hours and then embedded in 30% sucrose overnight. Samples were then frozen in OCT. Sections were incubated with primary mAbs against NKp46 (R&D Systems), CCR5 (BD) and Lyve-1 (Abcam) to mark hepatic sinusoids for 1 hour at room temperature, washed and then incubated with the following secondary antibodies raised in donkey for 30 minutes: anti-mouse 488, anti-goat 647, anti-rabbit 594 (Life Technologies). Nuclei were counterstained with DAPI at a concentration of 1:50000 in distilled water. For visualization of CCL3^{POS}/CD68^{POS} cells, slides of paraffin-embedded healthy liver were exposed to ultraviolet light for 48 hours to remove background staining. Staining was performed as described above for immunohistochemical staining using anti-CCL3. For identification of Kupffer cells, anti CD68 (Dako) was used. After incubation with primary antibodies, the following secondary antibodies raised in donkey for 30 minutes: anti-mouse 488 and anti-goat 647 (Life Technologies). Nuclei were counterstained with DAPI at a concentration of 1:50000 in distilled water. Imaging was obtained by confocal microscopy (Olympus, Tokyo, Japan).

Chemotaxis assay

CD56^{POS} cells were isolated from LMNC using CD56 positive selection kit (Stem Cell), and serum starved for 2 hours. Cells were then placed in serum-free medium in the upper well of transwell chambers (Costar, 5 µm insert, 24 well plate). Recombinant chemokines CCL3, CCL4, CCL5, CXCL16 and fractalkine (R&D Systems) were placed in the lower chamber. After 2 hours of migration at 37°C, the cells in the lower compartment were harvested, stained using antibodies specific for NK cells and analyzed by flow cytometry. Due to low recovery of purified NK cells from the liver, optimal concentrations of chemokines were determined by performing a titration of 4 concentrations on peripheral blood NK cells. From these data, 2 concentrations (1nM and 100nM) were selected based on the percentage of NK cells migrated. Each assay using hepatic NK cells was performed using these two concentrations and data displayed represents the chemokine concentration that gave the best results. The optimal concentrations for each chemokine were the following: 1nM for CCL3, CCL4, CCL5 and CXCL16, 100nM for fractalkine. The frequency of migration of hepatic NK cell subsets was determined as previously described [20]. Briefly, the percentage of hepatic NK cell populations was determined by dividing the number of cells migrated by the total number of cells in the input control well. The number of cells migrated was calculated by subtracting the background (from negative control) from the number of cells migrated to chemokine. Cells placed in the lower portion of the trans-well chamber only were defined as input control. Negative control is defined as the frequency of cells present in upper portion of the chamber in the absence of chemotactic stimulus in the lower portion of the chamber. Each chemokine concentration was performed in duplicate while the input control and negative control were performed in 4 different wells each. Each chemokine concentration was performed in duplicate while the input control and negative control were performed in 4 different wells each.

Gene expression

NK cells from liver perfusates and peripheral blood were FACS-sorted on the basis of their expression of CD56 and CD16 into the two subsets of CD56^{bright}/CD16^{neg} and CD56^{dim}/CD16^{pos} NK cell subsets (FACS Aria III, BD). All cells were sorted using a BD FACS Aria. Lysates from a minimum of 10,000 cells were analyzed using the preassembled nCounter GX Human Immunology Kit and the nCounter system (NanoString Technologies, Seattle, WA), a cut-off of 2 times the mean of the negative controls supplied in the kit was used to discriminate against nonspecific probe binding (noise). Samples were then normalized based on the geometric means of both the supplied positive controls and the panel of housekeeping genes, as recommended by the manufacturer. All data analyses were performed in R (version 3.0.2) using Bioconductor libraries (BioC 2.13) and R statistical packages on log² expression values. Global unsupervised clustering was performed using the function *hclust* of R *stats* package with Pearson correlation as distance metric and average agglomeration method. Gene expression heatmaps were generated using the software dChip (<http://www.hsph.harvard.edu/cli/complab/dchip/>) after row-wise standardization of the expression values. To assess cluster-specific reproducibility, we calculated p-values for sample clusters via the multiscale bootstrap resampling method coded in the R *pvclust* package [21]. Then, p-values were computed for all clusters of the original data as the frequency that any cluster appears in the bootstrap replicates (Bootstrap Probability).

Statistical analysis

Statistical calculations were performed using the Student's t test. Details of each calculation appear in the figure legends.

Results

CD56^{bright} hepatic NK cells are enriched at high frequencies in the healthy human liver

Similar to their circulating counterparts, human hepatic NK cells can be distinguished into two CD56^{pos}/CD16^{neg} and CD56^{pos}/CD16^{pos} cell subsets under homeostatic conditions [3, 19]. However, the frequency of CD56^{pos}/CD16^{neg} hepatic NK cells is significantly higher compared to that of CD56^{pos}/CD16^{neg} PB-NK cells in matched donors [7, 22] (Figures 1 A and 1C). CD56^{pos}/CD16^{neg} PB-NK cells are conventionally defined as CD56^{bright} NK cells due to the higher mean fluorescence intensity (MFI) of CD56 compared to that of CD56^{pos}/CD16^{pos} PB-NK lymphocytes. Indeed, this latter population is defined as CD56^{dim} NK cells. In freshly purified liver mononuclear cells (LMNCs) the MFI of CD56 on CD16^{neg} NK cells is significantly lower compared to that of their circulating counterparts and is similar to that of CD16^{pos} NK cells from both peripheral blood mononuclear cells (PBMCs) and LMNCs (Figures 1A, 1B and 1D). In this regard, it has been demonstrated that collagenase, the enzyme conventionally used to disrupt liver tissue for isolating LMNCs, induces a decrease in the surface expression of CD56 on NK cells [23]. To assess whether the lower MFI of CD56 on CD56^{pos}/CD16^{neg} hepatic NK is indeed an artifact associated with the use of collagenase, we analyzed the degree of CD56 expression on NK cells from liver perfusate (perf-NK cells). This biological specimen is conventionally obtained by flushing the donor's healthy organ before transplantation with the cold University of Wisconsin solution, which lacks enzymes capable of cleaving or lowering the cellular

expression of surface molecules [24]. We found that the subset distribution of perf-NK cells within perfusate mononuclear cells (PMNCs) recapitulates the one observed in LMNCs, as the frequency of CD56^{pos}/CD16^{neg} NK cells was similar in both specimens (Figures 1A and 1E). These results are in line with previous data showing that PMNCs flushed out from hepatic sinusoids share with LMNCs a similar lymphocyte distribution [24, 25]. Moreover, we observed that the MFI of CD56 on CD56^{pos}/CD16^{neg} perf-NK cells is significantly higher compared to that of their LMNC counterparts and similar to that of CD56^{bright} PB-NK cells (Figures 1B and 1D). Taken together, these results reveal that the degree of CD56 expression on CD56^{pos}/CD16^{neg} hepatic NK cells is indeed lowered by the enzymatic process of liver digestion. Therefore and in line with the nomenclature used for their circulating counterparts, CD56^{pos}/CD16^{neg} hepatic NK cells will be referred to as CD56^{bright} NK cells henceforth.

CD56^{bright} hepatic NK cells display unique transcriptional and phenotypic profiles

We then analyzed the gene expression profile of sorted PB- and perf-NK cell subsets by using the Nanostring technology. As mentioned previously, perf-NK cells were analyzed as a surrogate of hepatic NK cells since PMNCs recapitulate the overall lymphocyte distribution present in LMNCs under homeostatic conditions [24, 25]. An unsupervised cluster analysis performed on the entire dataset revealed striking differences between CD56^{bright} PB- and perf-NK cell subsets that display different transcriptional profiles and separate into two main clades, thus suggesting that they represent two distinct NK cell populations. In contrast, the genetic profiles of CD56^{dim} PB- and perf-NK cell subsets clustered together into a single clade, hence indicating a high similarity between these two populations (Figure 2A).

A more in-depth analysis of the 441 genes contained in the Nanostring dataset identified 50 genes as being significantly differentially regulated between CD56^{bright} PB- and perf-NK cells (Supplemental tables 1 and 2). Among these genes, *ccr7* and *sell* mRNA copies were decreased by respectively 32 and 10.85 fold in CD56^{bright} perf-NK cells compared to their circulating counterparts. These two mRNAs encode for the surface receptors CCR7 and L-selectin, two important homing receptors known to be constitutively present on CD56^{bright} PB-NK cells and regulating their migration to secondary lymphoid tissues (SLT) [26]. In line with their gene expression, we observed by flow cytometry that CD56^{bright} hepatic NK cells lack the expression of CCR7 and L-selectin which, in contrast, are constitutively present at high levels on CD56^{bright} PB-NK cells from matching donors (Figures 2B and 2C). Moreover, we found significantly lower levels of the mRNAs encoding for α_5 (*itga5*) and α_x (*itgax*) integrins on CD56^{bright} perf-NK cells compared to CD56^{bright} PB-NK cells. The same distribution pattern of these two integrins was also confirmed by flow cytometry on the surface of CD56^{bright} hepatic and PB-NK cells from the same donors. Finally, we found that both the transcript level and surface expression of CD161 (*klrb1*) is significantly higher on CD56^{bright} hepatic NK cells compared to CD56^{bright} PB-NK cells (Figures 2B and 2C). These findings are in line with the reported high frequency of CD161-expressing lymphocytes in the human liver [19]. The phenotypic differences between CD56^{bright} hepatic and PB-NK cells were not induced by the use of collagenase, as the phenotype of

CD56^{bright} perf-NK cells is identical to that of CD56^{bright} hepatic NK cells (Supplemental Figure 2).

In contrast, perf- and PB-NK CD56^{dim} cells were found to share a similar transcriptional profile (Supplemental Table 3). This homology in gene expression is corroborated by the identical surface expression of CCR7, L-selectin, α_5 and α_x integrins on CD56^{dim} NK cell subsets from both LMNCs and PBMCs (data not shown). Instead, we found that these homing receptors are differently expressed on CD56^{bright} hepatic NK cells compared to CD56^{bright} PB-NK cells (Figures 2B and 2C). Among all aNKRs and iNKRs regulating the effector-functions of NK cells, we observed that only the expression of DNAM-1 was significantly decreased on CD56^{bright} hepatic NK cells compared to their circulating counterparts (Figures 2B and C), while the repertoire of all remaining NKRs was nearly identical on both CD56^{bright} and CD56^{dim} hepatic NK cell subsets compared to their circulating counterparts (data not shown). We also measured the expression of Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) that is constitutively expressed on a subset of murine hepatic NK cells [27]. In line with previous reports with human hepatic NK cells [7, 13], we did not find any detectable levels of TRAIL on both CD56^{bright} and CD56^{dim} H hepatic NK cell subsets (data not shown).

CD56^{bright} and CD56^{dim} hepatic NK cells express a different repertoire of chemokine receptors and markers associated with tissue-residency

We then assessed whether CD56^{bright} NK cells in liver also display a distinct homing pattern by analyzing on both hepatic and PB-NK cells from matching donors the expression of an extensive panel of chemokine receptors known to regulate the migration of circulating lymphocytes to peripheral tissues [26, 28]. First, we found that low transcript levels and surface expressions of CCR4, CCR5, CCR7, CXCR3 and CXCR6 characterize CD56^{dim} hepatic and PB-NK cells. Moreover, both these subsets express similar high constitutive amounts of CCR3, CXCR2 and CX₃CR1 that, in contrast, were found not to be present in CD56^{bright} hepatic and PB-NK cells (Figure 3 and Supplemental Table 1). Interestingly, it has been previously reported that freshly purified PB-CD56^{dim} NK cells are characterized by the high expression of CX₃CR1 [3, 26], thus indicating that CX₃CR1^{pos}/CD56^{dim} hepatic NK cells likely derives from their circulating counterparts flowing in liver parenchyma.

While CD56^{dim} hepatic and PB- NK cells share a similar repertoire of chemokine receptors, CD56^{bright} hepatic NK cells possess a unique pattern of homing receptors that differs from that of CD56^{bright} PB-NK cells (Figure 3 and Supplemental Table 1). Indeed, CXCR6 is highly expressed only on the surface of hepatic CD56^{bright} NK cells. This was also confirmed at transcriptional level as CD56^{bright} perf-NK cells showed a nearly 18-fold increase of mRNA encoding for CXCR6 compared to CD56^{bright} PB-NK cells. We also found that CD56^{bright} hepatic NK cells, but not their circulating counterparts, express high surface levels of CCR5, a chemokine receptor present on lymphocytes in non-lymphoid tissues under homeostatic conditions [29, 30]. These latter results were not confirmed at the mRNA level, likely indicating the presence of a post-transcriptional mechanism associated with the high levels of CCR5 selectively on CD56^{bright} hepatic NK cells.

The high amounts of chemokine receptors associated with tissue residency and the lack of adhesion molecules as well as chemokine receptors involved in the homing of circulating immune cells to peripheral tissues prompted us to postulate that CD56^{bright} hepatic NK cells might represent a subset of liver-resident lymphocytes in humans. To confirm this hypothesis, we analyzed the surface expression of CD69, a molecule known to characterize tissue-resident lymphocytes [31]. We found that almost all CD56^{bright} hepatic NK cells constitutively express CD69, while CD56^{bright} PB-NK as well as CD56^{dim} hepatic NK cells and CD56^{dim} PB-NK cells lack the expression of this tissue-residency marker (Figure 3). We also analyzed on CD56^{dim} hepatic NK cells the repertoire of KIRs, which are known to distinguish CD56^{dim} from CD56^{bright} PB-NK cells [3]. We observed that both transcript levels and surface expressions of KIRs on CD56^{dim} hepatic NK cells are nearly identical to those of their circulating counterpart (Supplemental Table 1 and data not shown), thus further indicating that CD69^{neg}/CD56^{dim} hepatic NK cells are not tissue-resident but retain the homing features and phenotype of CD56^{dim} PB-NK. Indeed, CD56^{dim} perf- and PB-NK cell subsets share a highly similar transcriptional profile as assessed by the Nanostring dataset showing that only 7 out of 441 genes are differentially expressed between these two populations (Supplemental Table 3).

CD56^{bright} hepatic NK cells are functional and migrate to CCR5 and CXCR6 ligands

Similar to CD56^{bright} PB-NK cells [3], we found that CD56^{bright} hepatic NK cells are able to produce large quantities of IFN- γ following stimulation with IL-2 and IL-12. This is in contrast with what we observed for CD56^{dim} hepatic NK cells, as their degree of IFN- γ production was significantly lower compared to that of CD56^{bright} hepatic NK and equivalent to that of CD56^{dim} PB-NK (Figure 4A). These results indicate that CD56^{bright} and CD56^{dim} hepatic NK cell subsets mirror the same dichotomy in immune-regulatory functions compared to their circulating counterparts.

We then proceeded to determine if the constitutive expression of CXCR6 and CCR5 on CD56^{bright} hepatic NK cells is associated with the ability of this subset to migrate to their natural ligands (Figure 4B). By using an *in vitro* chemotaxis assay, we found that CCL3 and CCL5, but not CCL4, induced a significantly higher migration of CCR5^{pos}/CD56^{bright} hepatic NK cells compared to that of CCR5^{neg}/CD56^{dim} hepatic NK cells that, as expected, did not respond to CCR5 ligands. Similarly, CXCL16 was able to induce the migration of CXCR6^{pos}/CD56^{bright} but not CXCR6^{neg}/CD56^{dim} hepatic NK cells. We then measured the chemotactic function of CX₃CR1, the chemokine receptor constitutively expressed on CD56^{dim} but not on CD56^{bright} hepatic NK cells. As expected, its ligand fractalkine induced the chemotaxis of CX₃CR1^{pos}/CD56^{dim} hepatic NK cells but not of CX₃CR1^{neg}/CD56^{bright} hepatic NK cells. Hence, the different repertoires of chemokine receptors on CD56^{bright} and CD56^{dim} hepatic NK cell subsets are functionally relevant and also associated with distinct migratory patterns of these two populations.

CCL3, CCL5 and CXCL16 are highly expressed in the sinusoids of the healthy liver

We then assessed by immunohistochemistry the expression and distribution of CCR5 and CXCR6 ligands in healthy human liver. We found that CCL3 is expressed within hepatic sinusoids on the surface of cells characterized by an amoeboid shape and adherent to

neighboring cells. These irregular morphologic features are known to characterize Kupffer cells, which are the liver macrophages residing within the hepatic sinusoids [32]. To confirm that these CCL3^{POS} cells are indeed Kupffer cells, we labeled by confocal microscopy CD68, a marker constitutively expressed on liver-resident macrophages [33, 34]. Our data showed that CCL3 co-localizes with CD68 on irregular shaped cells within hepatic sinusoids, thereby confirming that they are indeed Kupffer cells (Figure 5A and 5B).

Regarding the other two CCR5 ligands, we found that the frequency of CCL4^{POS} cells in the liver is very low and significantly smaller compared to that of CCL3^{POS} cells. In contrast, CCL5^{POS} appeared to also be localized on cells within hepatic sinusoids at a frequency similar to that of CCL3^{POS} Kupffer cells. However, CCL5^{POS} cells were small in size, circular in shape and displayed a large nucleus surrounded by little cytoplasm. These features resemble the morphology of a lymphocyte rather than a macrophage. To confirm this and to identify the lymphocyte subset expressing CCL5, we performed flow cytometry on freshly purified LMNCs. Our results showed that CCL5 is indeed expressed on CD3^{POS} hepatic T cells and CD3^{neg}/CD56^{POS} hepatic NK cells, but not on CD19^{POS} hepatic B cells (Figures 5C, 5D and 5E). We also found a similar constitutive expression and distribution of CCL5 in liver perfusates (data not shown), thus confirming that this chemokine is located within hepatic sinusoids.

As for CXCL16, it has been previously reported that liver sinusoidal endothelial cells constitutively express this chemokine under homeostatic conditions [35].

CD56^{bright} hepatic NK cells are localized within the hepatic sinusoids

We then analyzed whether the high frequency of CCL3, CCL5 and CXCL16 in hepatic sinusoids is associated with the retention of CD56^{bright} hepatic NK cells expressing both CCR5 and CXCR6 in liver sinusoidal spaces. Given that Nkp46 is constitutively present on both CD56^{bright} and CD56^{dim} hepatic NK cell subsets and is also widely used to identify NK cells in tissues [36], we determined by immunohistochemistry the expression of this natural cytotoxicity receptor (NCR) in sections of healthy liver. Our results showed that Nkp46^{POS} NK cells are preferentially localized within liver sinusoids, while their frequency is very low in portal spaces (Figures 6A, 6B and 6C).

To assess if Nkp46^{POS} hepatic NK cells found in hepatic sinusoids under homeostatic conditions belong to the CD56^{bright} hepatic NK cell subset, we performed confocal microscopy on frozen sections of healthy liver using an anti-CCR5 monoclonal antibody (mAb) in combination with another mAb specific for Lyve-1, a marker specific for liver sinusoidal endothelial cells delineating sinusoidal spaces [37]. We found that the co-localization of Nkp46 with CCR5 on hepatic NK cells is confined within the areas defined by Lyve-1, thus demonstrating that the subset of CCR5^{POS}/CD56^{bright} hepatic NK cells is contained within sinusoidal spaces (Figure 6D).

Finally, we analyzed by flow cytometry the expression of chemokine receptors on perf-NK cells. Our results showed that, similar to what we observed in liver specimens, liver perfusates contain both CCR5^{POS}/CD56^{bright} and CX₃CR1^{POS}/CD56^{dim} NK cells (Supplemental Figure 3), thus further confirming that that CD56^{bright} NK cells expressing

markers associated with tissue residency are present in the healthy liver and are located in the hepatic sinusoids.

Discussion

The present study characterizes a novel subset of CD56^{bright} Ir-NK cells, whose transcriptional and phenotypic profiles differ from those of its circulating counterparts. We show that CD56^{bright} Ir-NK cells constitutively express markers of tissue-residency including CD69, CCR5 and CXCR6. The unique repertoire of chemokine receptors on CD56^{bright} Ir-NK cells is functionally relevant as this subset selectively migrates in response to the chemotactic stimuli given by CCR5 (i.e. CCL3 and CCL5) and CXCR6 (i.e. CXCL16) ligands. Under homeostatic conditions, CD56^{bright} Ir-NK cells are preferentially located within hepatic sinusoids containing CCL3^{pos} Kupffer cells as well as CCL5^{pos} T and NK lymphocytes. Moreover, it has been reported that hepatic sinusoidal endothelial cells also express high constitutive levels of CXCL16 [35]. Hence, the abundance of these chemokines in liver sinusoidal spaces creates a unique tissue niche hosting CD56^{bright} Ir-NK cells constitutively expressing CCR5 and CXCR6.

The key roles of these two chemokine receptors in regulating the homeostasis of CD56^{bright} Ir-NK cells are further supported by previous studies performed in murine models. Indeed, CCR5-deficient mice have a drastic reduction of NK cell frequency [38], while CXCR6 regulates the survival, the trafficking and the effector-function of murine hepatic NK cells [11]. Our experimental evidence demonstrates, for the first time, that the engagement of these two chemokine receptors is required to ensure the constitutive presence of a large subset of Ir-NK cells in human hepatic sinusoids.

CD56^{bright} Ir-NK cells coexist with another subset of CD56^{dim} c-NK cells that, instead, are transcriptionally and phenotypically similar to their circulating counterparts. In particular, CD56^{dim} c-NK cells are characterized by a CD69^{neg}/CCR5^{neg}/CXCR6^{neg} phenotype and express adhesion molecules as well as functionally relevant chemokine receptors such as CX₃CR1 associated with the homing of circulating immune cells to peripheral tissues. These results indicate that CD56^{dim} c-NK cells are capable of circulating throughout the liver without being retained in hepatic sinusoids. Notably, it has been reported that, similar to human CD56^{dim} c-NK cells, CX₃CR1 is also expressed on murine NK cells able to circulate in the periphery but not on liver-resident NK cells [10]. In line with previous studies [39, 40], we also observed that fractalkine is not present within hepatic sinusoids but is preferentially expressed by bile ducts under homeostatic conditions (data not shown). Nevertheless, we found very few NK cells in the portal areas surrounding the bile ducts of healthy liver. In this regard, it has been reported that the frequency of NK cells increases during the course of autoimmune hepatic disorders such as primary biliary sclerosis. This phenomenon is associated with the establishment of a pathologic inflammatory milieu at injured liver sites inducing a higher expression of fractalkine and several adhesion molecules such as ICAM-1 and VCAM-1 on biliary epithelial cells [40, 41]. We also show that CD56^{dim} c-NK cells constitutively express CCR3, CXCR2, and CXCR4, but the potential binding of these chemokine receptors with their ligands in a redundant chemokine network does not appear to be relevant in the homing of this subset in liver sinusoids under

homeostatic conditions. Indeed, many studies have shown the lack of expression of the ligands to CXCR4 (CXCL12), CXCR2 (CXCL1 and CXCL8) and CCR3 (CCL7, CCL15, CCL28) in hepatic sinusoids of healthy human liver [15, 22, 42–45].

A unique subset of Ir-NK cells expressing markers of tissue-residency and located within hepatic sinusoids has recently been reported in mice [10, 11, 46]. These NK cells are characterized by a CD49a^{pos}/DX5^{neg} phenotype and are endowed with memory-like features in contrast to CD49a^{neg}/DX5^{pos} c-NK cells found in liver, blood, bone marrow and spleen. The direct translation of this knowledge gained in mice into the human setting is precluded by the fact that DX5 is not conserved in humans and CD56 does not define murine NK cells. Nonetheless, a population of human CD56^{pos}/CD49a^{pos} hepatic NK cells displaying some similarities with the murine CD49a^{pos}/DX5^{neg} Ir-NK cells has been recently described [16]. In this study, human CD56^{pos}/CD49a^{pos} hepatic NK cells were detected at low frequencies (mean of 2.3 % of the entire hepatic NK cell population) in the healthy liver of only a fraction (41 %) of all donors tested. Our experimental evidence indicates that this subset of CD49a^{pos}/DX5^{neg} hepatic NK cells represents only a small fraction of the large human subset of Ir-NK cells. In fact, we show here that human CD69^{pos}/CD56^{bright} Ir-NK cells expressing constitutively high levels of CXCR6 and CCR5 (means 48,5 % and 56,5%, respectively) account for half of the entire hepatic NK cell population (mean of 47.8 %) and are consistently found in all donors tested. Furthermore, we show here that the frequencies of both CD56^{bright} and CD56^{dim} NK cells in liver perfusates differ from those normally detected in peripheral blood PBMCs [3], but are similar to those observed in liver [7, 47]. Indeed, draining immune cells from liver before transplantation flushes out from hepatic sinusoids high frequencies of CD69^{pos}/CD56^{bright} Ir-NK normally retained within this anatomic compartment and not circulating in the blood. On the other side, CD69^{neg}/CD56^{bright} PB-NK cells do not express CCR5 and CXCR6 but were positive for CCR7 and L-selectin, a phenotype classically associated with the homing of circulating NK cells to secondary lymphoid tissues [26]. These data demonstrate that CD56^{bright} NK cells in blood and liver represent two distinct populations present in different anatomic compartments. The fact that the liver harbors a large population of tissue-resident CD56^{bright} NK cell is not unique to this organ, as high frequencies of unique CD56^{bright} NK cell subsets have been found in lymph nodes, decidua and intestinal mucosa [3, 48, 49]. These NK cells generally display a phenotype and function specific to the organ of occupancy, while they are either absent or present at very low frequencies in peripheral blood [50–52]. Similar to their counterparts in other tissues, the existence of Ir-NK cells certainly plays a key role in the immune-surveillance against tumor transformation and pathogens. Moreover, it is conceivable to hypothesize an important contribution exerted by CD56^{bright} Ir-NK in setting a threshold for immunologic tolerance in the liver given the large numbers of foreign antigens drained daily from the gut. Indeed, NK cell dysfunctions have been reported to be greatly involved in the pathogenesis of autoimmune liver disease [7].

Our data showing the enrichment of CD56^{bright} Ir-NK cells in the hepatic sinusoids alongside the abundant Kupffer cell population reveals that the sinusoidal spaces represent an important tissue niche where macrophages and NK cells interact. Indeed, many lines of evidence clearly demonstrated over the past decade that NK cells are able to engage bi-directional interplays with other members of innate immunity, such as dendritic cells (DCs),

macrophages and neutrophils. The final outcome of these synergic interactions mediated by both cell-to-cell contacts and soluble mediators is the coordination and optimization of both innate and adaptive immune responses [2, 53–63]. In particular, the NK cell-macrophage cross-talk is highly relevant in the context of host-pathogen interactions and tumor biology as both these innate immune cells are deeply involved in the pathogenesis of microbial infection and cancer. Disclosing the mechanisms regulating the homing and the synergic interactions between hepatic NK and Kupffer cells under homeostatic conditions is key to better understand the physiopathology of liver diseases, including viral infections, autoimmune diseases and tumor.

The characterization of Ir-NK cells has also prompted immunologists to hypothesize that certain tissues may replace the bone marrow as a site for NK cell development and/or differentiation in order to generate organ-specific NK cells [10, 64]. This idea is supported by data showing that CD56^{bright} NK cells can develop not only from CD34^{pos} hematopoietic stem cell (HSC) precursors present in the bone marrow, but also from HSCs found in the SLT, thymus, fetal liver, uterus and intestine [65–71]. Moreover, all five NK cell developmental intermediates (NKDIs) have been identified in the adult liver and it has been also reported that differentiation stages 1–3 can give rise to CD56^{bright} NK cells *in vitro*, similar to what was shown for SLT [72]. Preliminary data developed in our laboratory have confirmed that healthy human liver contains all NKDIs (data not shown). Additional studies are required to determine if these NKDIs can give rise *in vitro* to the unique subset of human CD56^{bright} Ir-NK cells we have identified and characterized in the present study. Finally, we are delighted that this manuscript will be part of an issue, which recognizes the enormous contributions of Diego and Giordina Vergani in autoimmunity. It is part of the special series of the Journal of Autoimmunity that devotes topics and issues to important figures and critical events that we believe will greatly advance improved care of patients [73–75].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

Ir-NK cells	liver resident Natural Killer cells
c-NK cells	conventional Natural Killer cells
perf-NK cells	perfusate Natural Killer cells
iNKR s	inhibitory NK cell receptors

aNKR s	activating NK cell receptors
KIR s	Killer cell immunoglobulin-like receptors
MHC-I	MHC-class-I molecules
MAIT	mucosal-associated invariant T cells
PBMC s	peripheral blood mononuclear cells
LMNC s	liver mononuclear cells
PMNC s	perfusate mononuclear cells
MFI	mean fluorescence intensity
SLT	secondary lymphoid tissues
TRAIL	Tumor necrosis factor (TNF)-related apoptosis-inducing ligand

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Highlights

1. Characterization of the mechanisms regulating the homing of NK cells to liver and the repertoire of receptors that distinguish liver-resident NK (lr-NK) cells from circulating counterparts (c.NK cells).
2. Nearly 50% of the entire liver NK cell population is composed of functionally relevant CD56^{bright} lr-NK cells that localize within hepatic sinusoids.
3. CD56^{bright} lr-NK cells express CD69, CCR5 and CXCR6 and this unique repertoire of chemokine receptors is functionally for their migration to hepatic sinusoids expressing their putative ligands.
4. The selective presence of these chemokines in sinusoidal spaces creates a tissue niche for lr-CD56^{bright} NK cells that constitutively express CCR5 and CXCR6.
5. This study releases the phenotypic and functional differences that distinguish lr-Nk cells from c-NK cells.

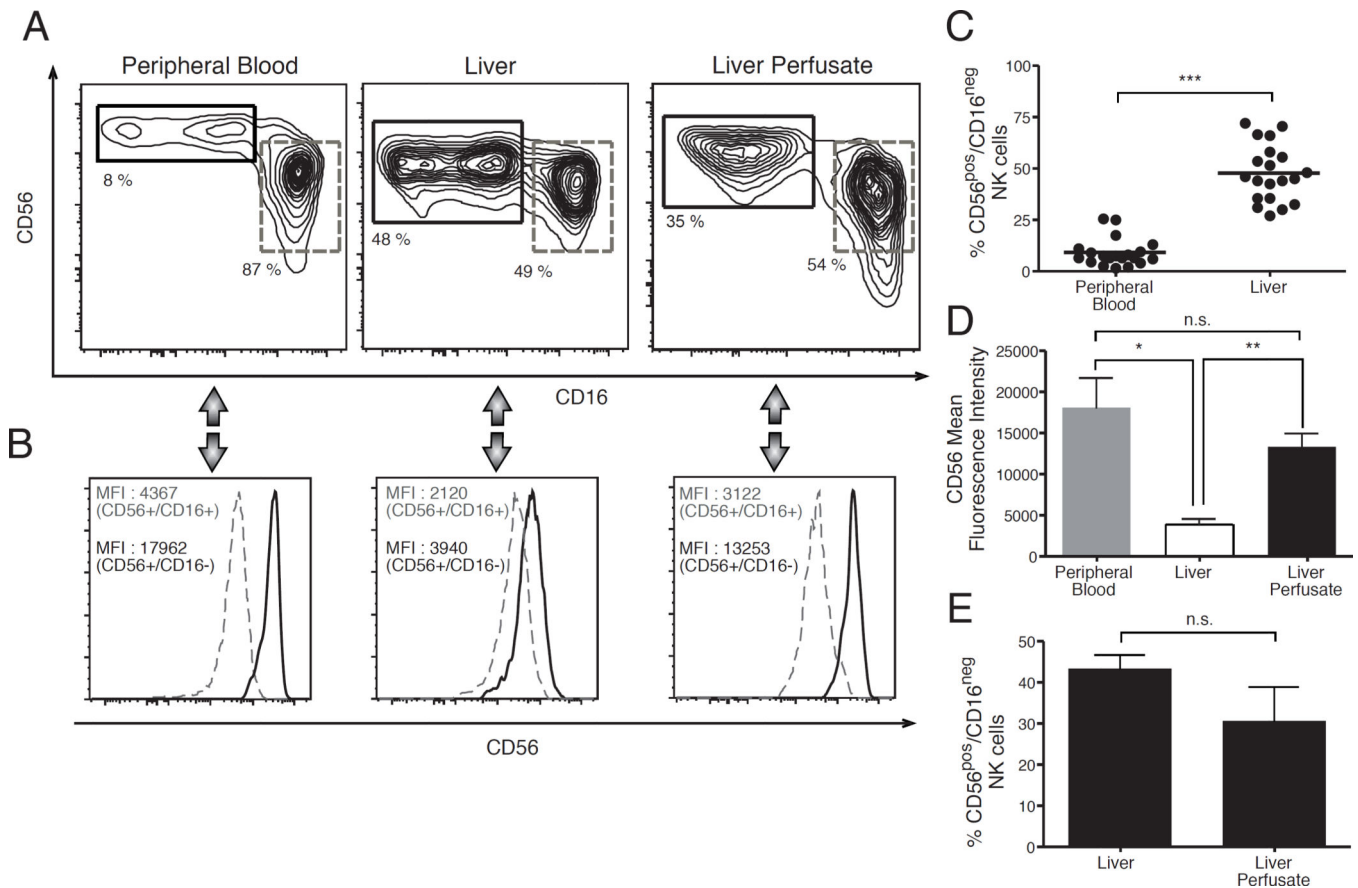


Figure 1. Distribution of NK cell subsets in peripheral blood, liver tissues and liver perfusates (A,B) Flow cytometric contour plots (A) and histogram (B) graphs from a representative example showing the phenotypic distribution of CD56 and CD16 (A) and the mean fluorescence intensity (MFI) of CD56 (B) on CD56^{pos}/CD16^{neg} (black line) and CD56^{pos}/CD16^{pos} (dashed gray line) NK cell subsets freshly purified from peripheral blood (left), healthy liver tissue (middle) and healthy liver perfusates (right). (C) Summary graphs of statistical dot plots with p values and means showing the relative frequencies of CD56^{pos}/CD16^{neg} NK cell subset within total NK cell populations purified from peripheral blood and healthy liver tissue of matching donors. Data are presented as percentages and are representative of 20 donors. (D) Summary graphs of statistical histogram bars with p values and SD showing the MFI of CD56 on CD56^{pos}/CD16^{neg} NK cells freshly purified from peripheral blood (left), healthy liver tissues (middle) and healthy liver perfusates (right). Data are representative of 3 donors. (E) Summary graphs of statistical histogram bars with p values and SD showing the relative frequencies of CD56^{pos}/CD16^{neg} NK cell subset within total NK cell populations purified from healthy liver tissues (left) and healthy liver perfusates (right). Data are presented as percentages and are representative of 3 donors. * = p < 0,05 – ** = p < 0,01 – *** = p < 0,001

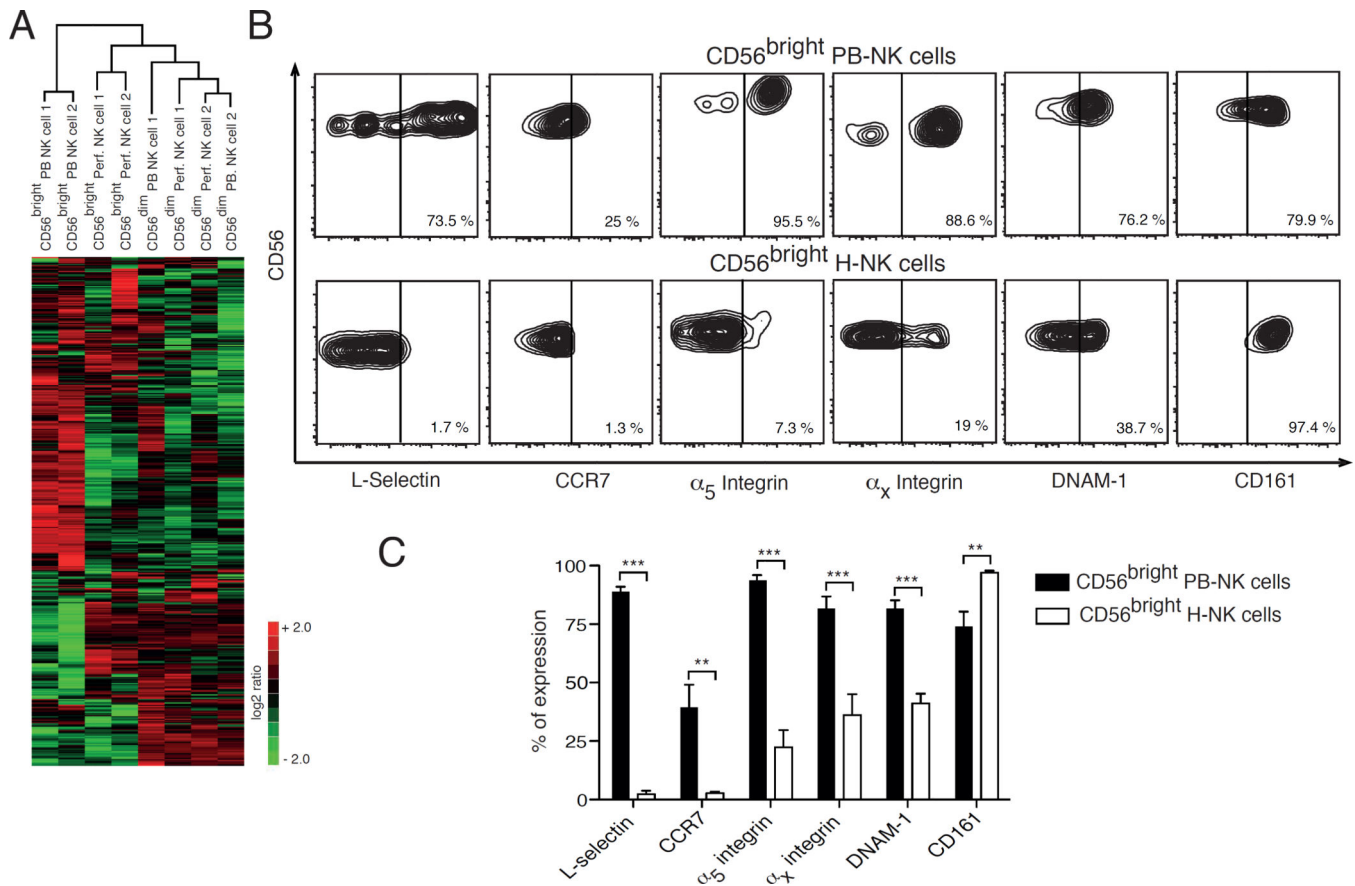


Figure 2. Transcriptional and phenotypic profiles of CD56^{bright} hepatic NK cells

(A) Heat map showing the unsupervised clustering of 441 genes differently expressed in the Nanostring dataset on CD56^{bright} NK cell subset freshly purified from perfusates (Perf.) of healthy liver compared to that of CD56^{bright} NK cell from peripheral blood (PB) and to CD56^{dim} Perf- and PB-NK cells. (B) Flow cytometric contour plots from a representative example showing the percentages of L-selectin, CCR7, α₅ integrin, α_x integrin, DNAM-1 and CD161 on CD56^{bright} NK cells from peripheral blood (PB-NK cells in the upper line) and healthy liver (H-NK cells in the lower line) of the same donor. (C) Summary graphs of statistical histogram bars with p values and SD showing the surface expression of L-selectin, CCR7, α₅ integrin, α_x integrin, DNAM-1 and CD161 on CD56^{bright} NK cells from peripheral blood (black bars) and healthy livers (white bars) of matching donors. Data are presented as percentages and are representative of 5 human donors.

** = p < 0,01 – *** = p < 0,001

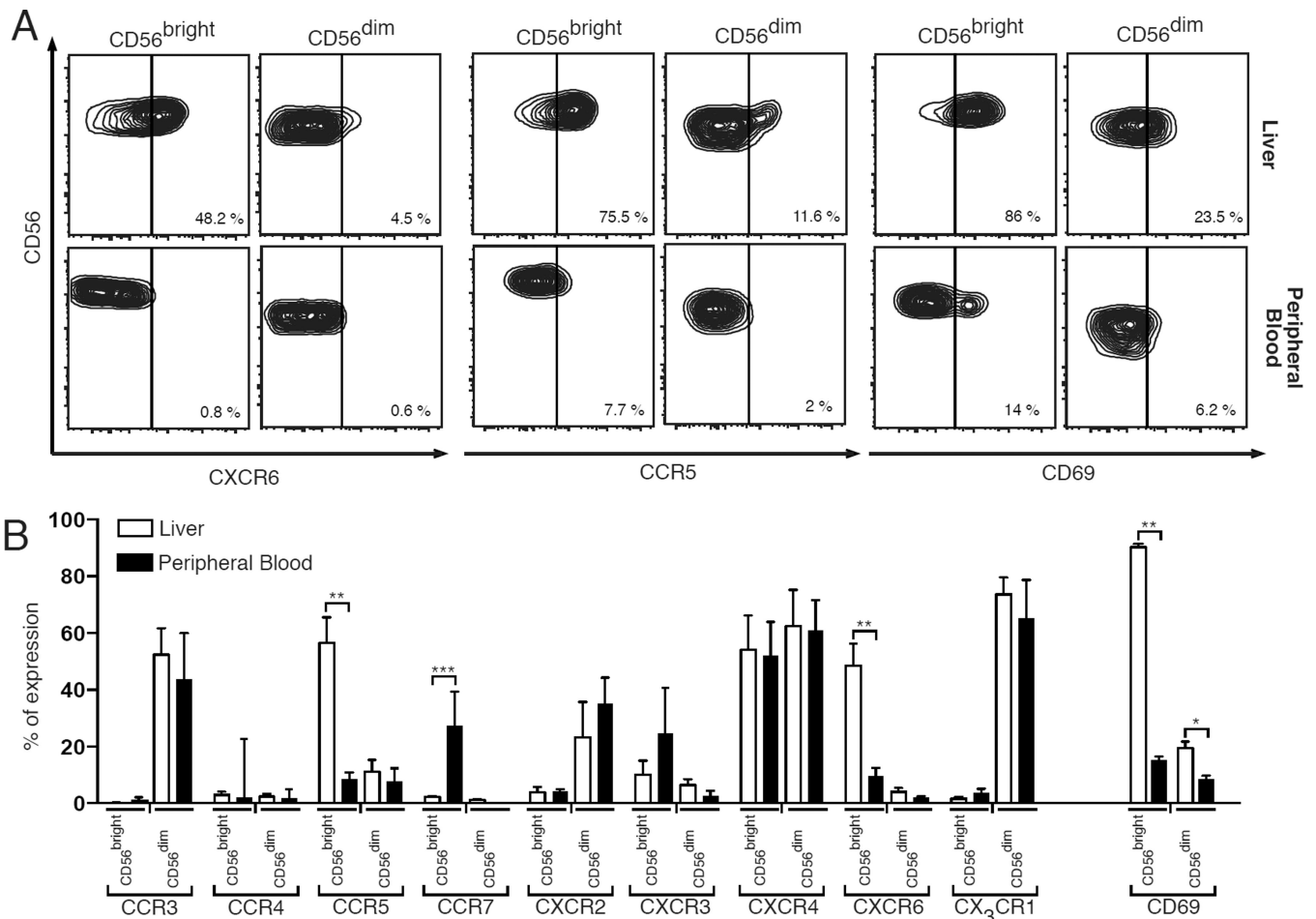


Figure 3. Repertoire of chemokine receptors and CD69 expression on hepatic NK cell subsets compared to their circulating counterparts

(A) Flow cytometric contour plots graphs showing the surface expression of the chemokine receptors CXCR6, CCR5 and CD69 on CD56^{bright} (first column) and CD56^{dim} (second column) purified from liver (upper line) and peripheral blood (lower line) of matching donors. (B) Summary graphs of statistical histogram bars with p values and SD showing the surface expression of CCR3, CCR4, CCR5, CCR7, CXCR2, CXCR3, CXCR4, CXCR6, CX₃CR1 and CD69 on CD56^{bright} and CD56^{dim} NK cells from healthy livers (white bars) and peripheral blood (black bars) of matching donors. Data are presented as percentages and are representative of 5 donors.

* = $p < 0,05$ – ** = $p < 0,01$ – *** = $p < 0,001$

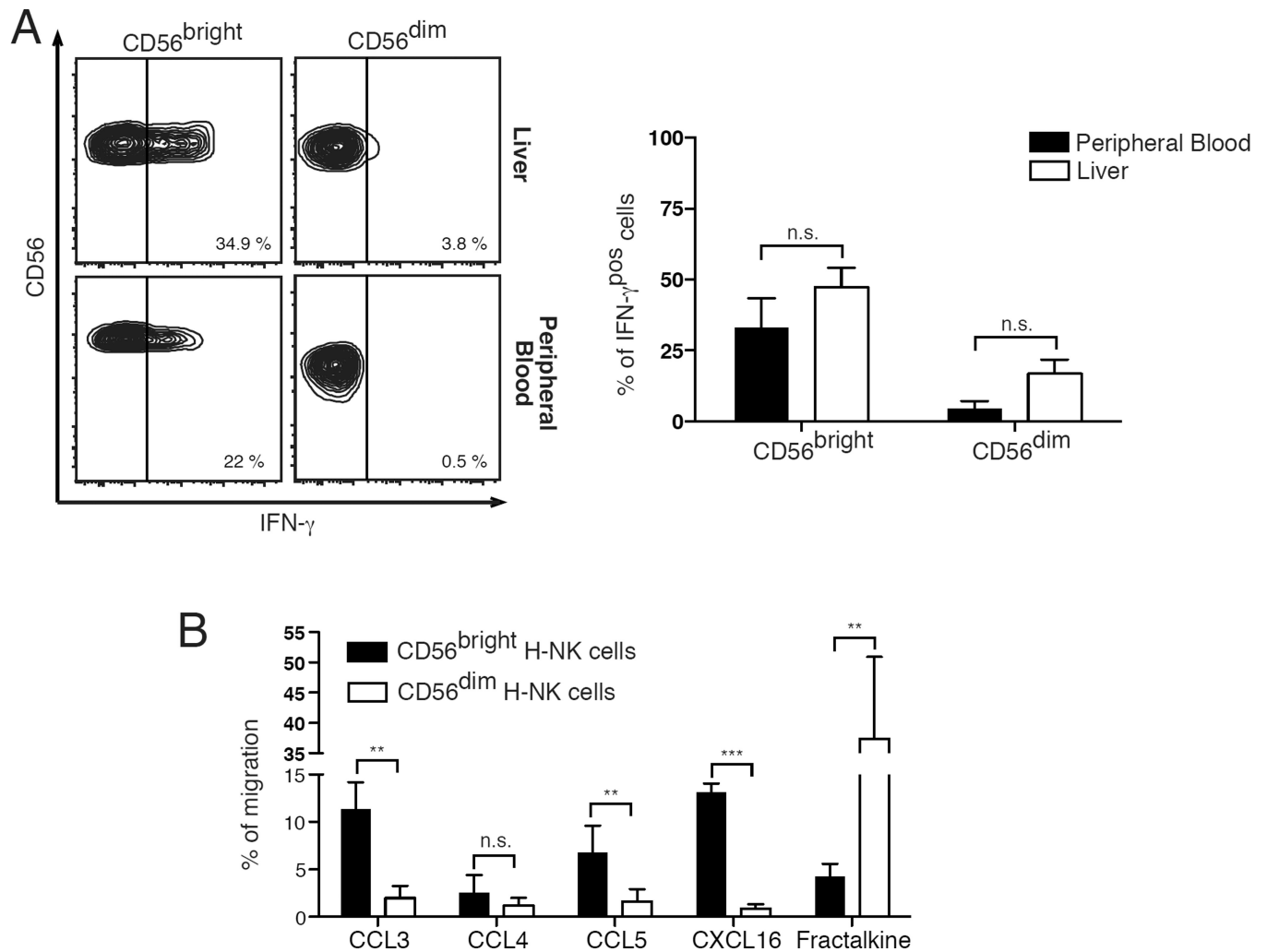


Figure 4. Functional relevance and migration patterns of hepatic NK cell subsets

(A) Flow cytometric contour plots graphs from the same representative donor showing the percentages of IFN- γ ^{pos}/CD56^{bright} (left column) and IFN- γ ^{pos}/CD56^{dim} (right column) NK cells purified from liver (upper line) and peripheral blood (lower line)

(B) Summary graphs of statistical histogram bars with p values and SD from matching donors showing the percentages of IFN- γ ^{pos}/CD56^{bright} and IFN- γ ^{pos}/CD56^{dim} NK cells purified from healthy livers (white bars) and peripheral blood (black bars). Data are presented as percentages and are representative of 6 human donors.

(C) Summary graphs of statistical histogram bars with p values and SD showing the percentages of CD56^{bright} (black bars) and CD56^{dim} (white bars) H-NK cells from matching donors migrating in response to CCL3, CCL4, CCL5, CXCL16 and fractalkine. Data are representative of 5 human donors.

* = $p < 0,05$ – ** = $p < 0,01$ – *** = $p < 0,001$

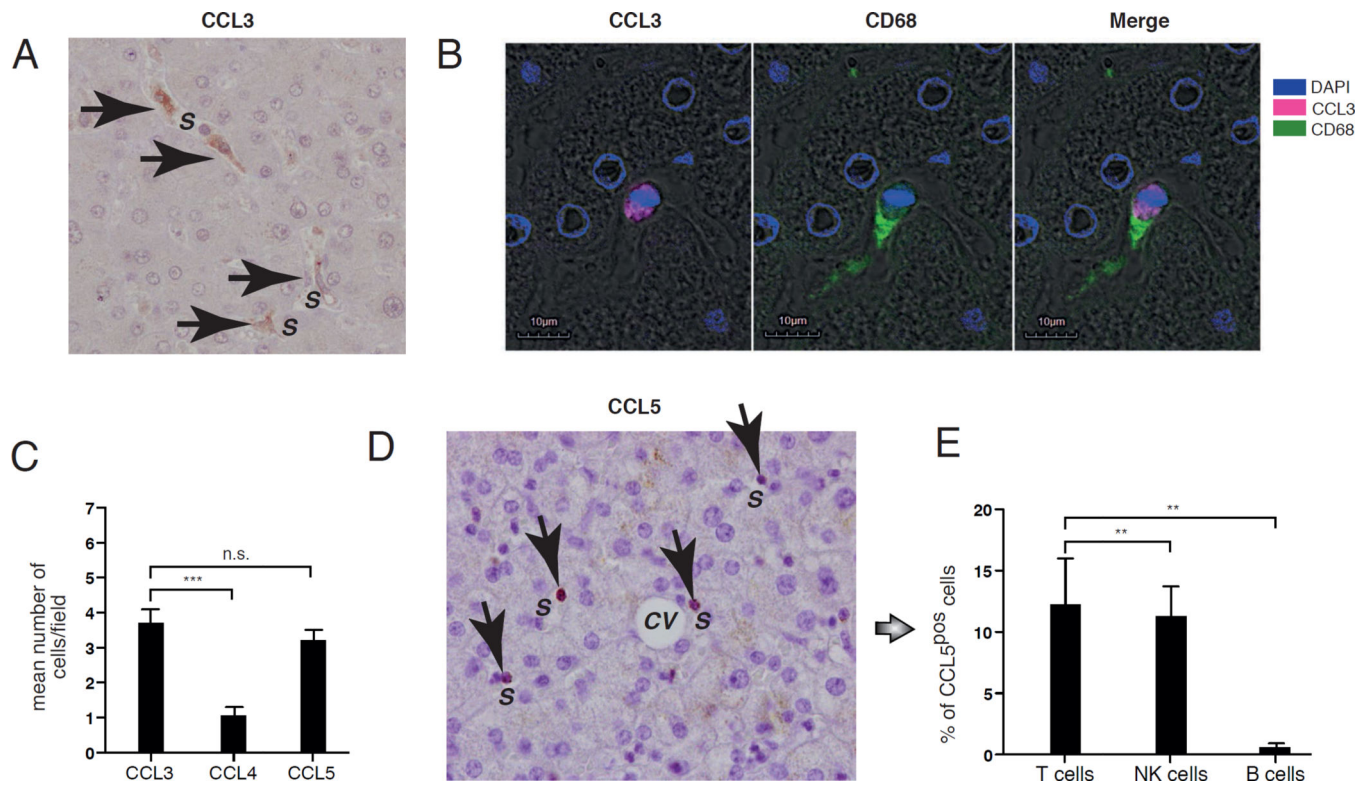


Figure 5. Distribution of CCR5 ligands in healthy human liver

(A) Representative immunohistochemistry image (magnification of 20X) showing CCL3^{POS} (brown indicated with arrows) cells in healthy liver. The hepatic sinusoids are indicated with S. (B) Representative fluorescence microscopic image showing the expression of CCL3 (purple on the left) and CD68 (green in the middle) alone or co-localized on the same cells (right). DAPI in blue labels cell nuclei. The hepatic sinusoids are indicated with S. (C) Summary graphs of statistical histogram bars with p values and SD showing the mean number for field of CCL3^{POS}, CCL4^{POS} and CCL5^{POS} cell in the in parenchyma of healthy liver determined by immunohistochemistry experiments. Cells were counted at 20X magnification and a least 10 fields per specimen were analyzed. Data are representative of 5 donors. (D) Representative immunohistochemistry image showing the CCL5^{POS} (brown indicated with arrows) cells in parenchyma of healthy liver. The hepatic sinusoids are indicated with S. (e) Summary graphs of statistical histogram bars with p values and SD showing the percentage of CCL5^{POS}/CD3^{POS} T cells, CCL5^{POS}/CD3^{neg}/CD56^{POS} NK cells and CCL5^{POS}/CD19^{POS} B cells freshly purified from healthy liver and analyzed by flow cytometry. Data are representative of 5 donors.

** = $p < 0,01$ – *** = $p < 0,001$

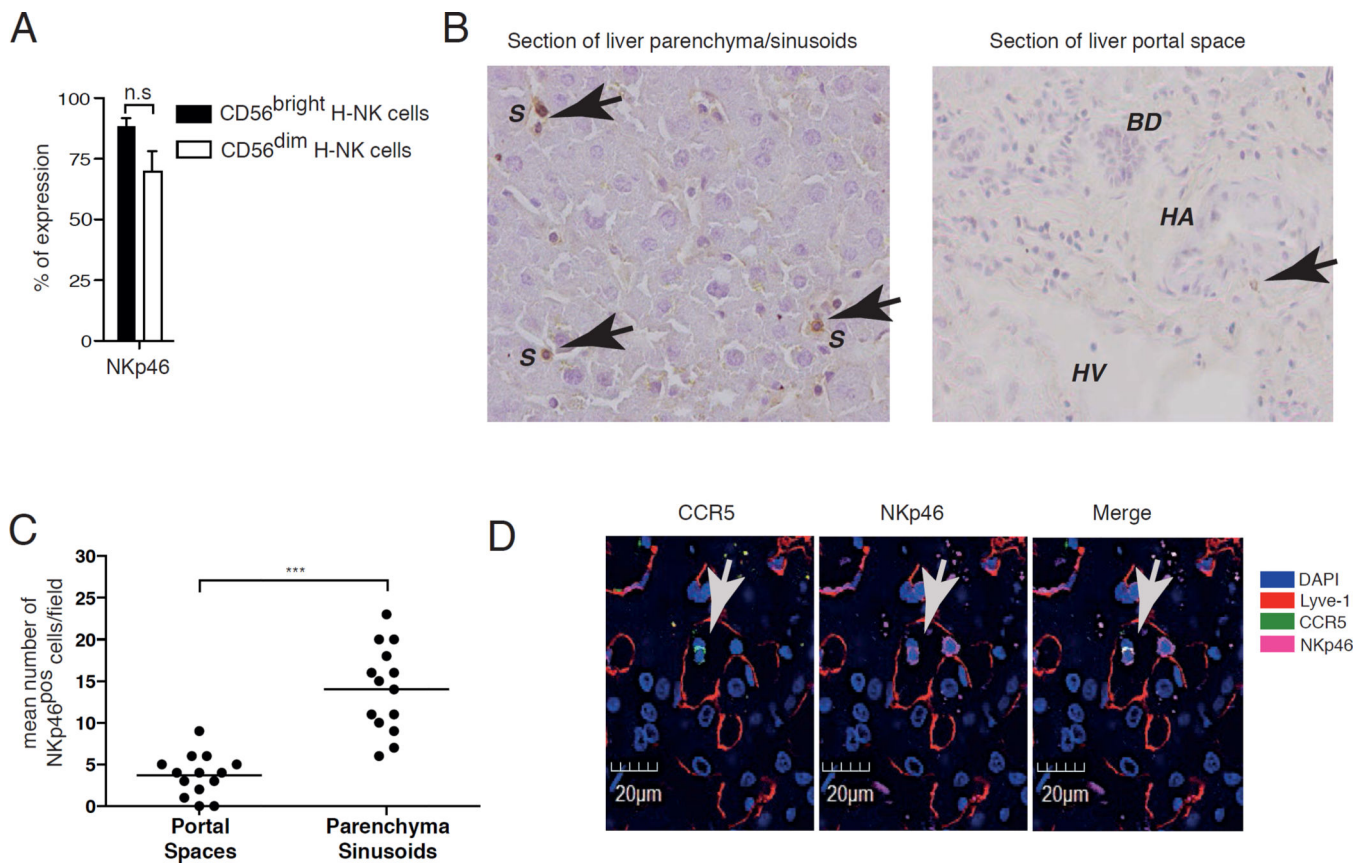


Figure 6. CD56^{bright} NK cells are preferentially located in hepatic sinusoids

(A) Summary graphs of statistical histogram bars with p values and SD showing the percentage of expression of NKp46 on CD56^{bright} and CD56^{dim} H-NK cells from healthy liver and analyzed by flow cytometry. Data are representative of 5 donors. (B) Representative immunohistochemistry image showing NKp46^{pos} H-NK cells (brown indicated with arrows) cells in sections of parenchyma (left) and portal spaces (right) of healthy liver. The hepatic sinusoids are indicated with S, bile ducts with BD, hepatic arteries with HA and hepatic veins with HV. (C) Summary graphs of statistical dot plots with means and P value showing the mean number for field of NKp46^{pos} H-NK cells in the in parenchyma of healthy liver determined in immunohistochemistry experiments. Cells were counted at 40X magnification and a least 10 fields per specimen were analyzed. Data are representative of 3 donors. (D) Representative fluorescence microscopic image showing the expression of CCR5 (green on the left) and NKp46 (green in the middle) alone or co-localized on the same cells (right) within hepatic sinusoids labeled with Lyve-1 (red). DAPI in blue labels cell nuclei.