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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

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ABSTRACT

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 functionally 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. 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. Here, we also show that 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 unique 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 CX_3CR1.

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.

List of abbreviations: lr-NK cells, liver resident Natural Killer cells; c-NK cells, conventional Natural Killer cells; perf-NK cells, perfusate Natural Killer cells; iNKRs, inhibitory NK cell receptors; aNKRs, activating NK cell receptors; KIRs, Killer cell immunoglobulin-like receptors; MHC-I, MHC-class-I molecules; MAIT, mucosal-associated invariant T cells; PBMCs, peripheral blood mononuclear cells; LMNCs, liver mononuclear cells; PMNCs, perfusate mononuclear cells; MFI, mean fluorescence intensity; SLT, secondary lymphoid tissues; TRAIL, Tumor necrosis factor (TNF)-related apoptosis-inducing ligand.

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1. 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-β)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. $CD56^{bright}/CD16^{neg\text{-}low}$ ($CD56^{bright}$) 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 (iNKRs), 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 (aNKRs) 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.

2. Material and methods

2.1. 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 Fig. 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.

2.2. 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 2 mg/ml of collagenese D (Roche) in HEPES Buffered Saline for 45 min 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 liver before transplant using Ficoll–Paque PREMIUM (GE-Healthcare), as previously described [19].

2.3. 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), CX_3CR1 PE (MBL). Aqua LIVE/DEAD (Life Technologies) was used to eliminate dead cells from the analysis. Intracellular staining was performed using Cytofix/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 Fig. 1. For measurement of IFN-γ production, whole PBMC and LMNC were stimulated with 18 h with 20 ng/ml rhIL-12 (R&D Systems) and 200 U/ml rhIL-2 (Peprotech). For the final 4 h, 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).

2.4. 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) (Abcam). Antigen retrieval was performed for 5 min at 125 °C and for 3 min at 90 °C in a pressure cooker using Diva Decloaker antigen retrieval solution (Biocare Medical). Endogenous peroxidase activity was blocked with Peroxidased I (Biocare Medical) for 5 min and non-specific proteins were blocked for 15 min with Background Sniper (Biocare Medical). The primary antibody was incubated for 1 h at room temperature. For CCL5, MACH 4 HRP Polymer was used as the secondary antibody (Biocare Medical). For CCL3 and CCL4, Goat-on-

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