Cutting Edge: Identification and Characterization of Human Intrahepatic CD49a+ NK Cells


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Although NK cells are considered innate, recent studies in mice revealed the existence of a unique lineage of hepatic CD49a⁺DX5⁻ NK cells with adaptive-like features. Development of this NK cell lineage is, in contrast to conventional NK cells, dependent on T-bet but not Eomes. In this study, we describe the identification of a T-bet⁺Eomes⁺ CD49a⁺ NK cell subset readily detectable in the human liver, but not in afferent or efferent hepatic venous or peripheral blood. Human intrahepatic CD49a⁺ NK cells express killer cell Ig-like receptor and NKG2C, indicative of having undergone clonal-like expansion, are CD56bright, and express low levels of CD16, CD57, and perforin. After stimulation, CD49a⁺ NK cells express high levels of inflammatory cytokines but degranulate poorly. CD49a⁺ NK cells retain their phenotype after expansion in vitro and in long-term in vitro cultures. These results demonstrate the presence of a likely human counterpart of mouse intrahepatic NK cells with adaptive-like features. The Journal of Immunology, 2015, 194: 2467–2471.

Natural killer cells are an essential part of the innate immune system providing rapid responses against invading pathogens and cells undergoing malignant transformation (1). NK cell responses include cytotoxicity as well as production of cytokines, for example, IFN-γ and TNF, that can fine-tune innate and adaptive immune responses (2).

Recently, several studies have shown that mouse NK cells exhibit adaptive-like features, including recall responses to allergens and viral infections (3, 4). In particular, a subset of intrahepatic NK cells with a CD49a⁺DX5⁻ phenotype has been attributed to these responses (4, 5). In the human setting, discrete subsets of expanded NK cell populations have been found in peripheral blood after infection with CMV (6, 7), hantavirus (8), and Chikungunya virus (9). Much less is known about adaptive-like features of NK cells in human peripheral tissues; specifically, it is not known whether the human liver harbors a counterpart of the mouse CD49a⁺DX5⁻ NK cell subset.

NK cells originate from the bone marrow, and several transcription factors, including E4BP4, T-bet, and Eomes, control NK cell development (2). Recently, use of knockout and reporter systems disclosed the presence of distinct NK cell lineages with separate transcription factor expression patterns in mice (10–12). Conventional mouse NK cells developing in the bone marrow are dependent on E4BP4 and the downstream targets Eomes and Id2 (10, 11). In contrast, some tissue-resident mouse NK cell populations, including CD49a⁺DX5⁻ intrahepatic NK cells, develop independently of E4BP4 and Eomes (12), but are dependent on T-bet (10). However, the transcriptional requirements for human conventional and tissue-resident NK cells remain poorly understood.

In this study, we report the existence of human tissue-resident intrahepatic CD49a⁺ NK cells. This subset of NK cells was phenotypically distinct from conventional NK (cNK) cells and non-NK innate lymphoid cells (ILCs). In contrast to intrahepatic cNK cells, the CD49a⁺ NK cells were T-bet⁺Eomes⁻ and retained this profile upon long-term expansion in vitro. Moreover, CD49a⁺ NK cells produced high levels of proinflammatory cytokines such as IFN-γ, TNF, and GM-CSF. These data support the notion of T-bet–dependent extramedullary NK cell development, as previously shown in the mouse (10), and identify a putative human counterpart to intrahepatic CD49a⁺DX5⁻ mouse NK cells.

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Abbreviations used in this article: cNK cell, conventional NK cell; ILC, innate lymphoid cell; KIR, killer cell Ig-like receptor.

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Materials and Methods

Preparation of human liver material

Human adult liver tissue was obtained either from patients undergoing surgical liver resection for removal of primary or metastatic tumors or from organ donors whose livers were not used for transplantation. Written consent was provided and the regional Ethical Review Board in Stockholm, Sweden approved the study. Of tumor-bearing livers, only nonaffected, healthy tissues were used. Immune cells were isolated using a previously described protocol (13). Portal venous blood was drawn from the portal vein after laparotomy and hepatic venous blood was drawn during wedge hepatic venous pressure measurements. Density gradient centrifugation was used to isolate leukocytes.

Flow cytometry

Abs and clones against the following proteins were used: CD3 (UCHT1, PE-Cy5, Beckman Coulter), CD7 (M-T701, Alexa Fluor 700, BioLegend, or Horizon V500, BD Biosciences), CD14 (M52E, Horizon V500, BD Biosciences), CD16 (5G8, Brilliant Violet 711 or 785, BioLegend, or VEP13, PE, Miltenyi Biotec), CD19 (HIB19, Horizon V500, BD Biosciences), CD29 (MAR4, PE-Cy5, BD Biosciences), CD34 (581, ECD, Beckman Coulter), CD45 (H130, Alexa Fluor 700, BioLegend), CD45RA (HI100, Brilliant Violet 785, BioLegend), CD49A (TS2A, PE, Alexa Fluor 647, BioLegend, or SR84, PE, BD Biosciences), CD56 (HCDC56, Brilliant Violet 711, BioLegend, or NCAM16, PE-Cy7, BD Biosciences), CD57 (TB01, purified, ebioscience), CD69 (TP1,55,3, ECD, Beckman Coulter), CD103 (Ber-Act8, PE-Cy7, BioLegend), CD107a (H4A3, FITC, BD Biosciences), CD117 (104D2D1, PE, Beckman Coulter), CD127 (R33.34, PE-Cy7, Beckman Coulter), CD161 (HP-3G10, Brilliant Violet 605, BioLegend), DNAM-1 (DX11, FITC, BD Biosciences), GM-CSF (BVDV-21C11, PE-CFS59, BD Biosciences), granzyme A (CB9, Pacific Blue, BioLegend), Granzyme B (GB11, PE-CF94, BD Biosciences), killer cell Ig-like receptor (KIR)2DL1 (I43211, allophyocyanin, R&D Systems), KIR2DL1/S1 (EB6, PE-Cy5, Beckman Coulter), KIR2DL2/S2 (CL183, PE-Cy5, Beckman Coulter), KIR2DL3 (180701, FITC, R&D Systems), KIR3DL1 (DX9, Alexa Fluor 700, BioLegend, NKG2A (Z199, custom conjugate, allophyocyanin–Alexa Fluor 750, Beckman Coulter), NKGC2 (134591, PE, R&D Systems), NKGD2 (1D11, PE-CFS59, BD Biosciences), NKp30 (p30-15, Alexa Fluor 647, BD Biosciences), NKp44 (P44-8, biotin, BioLegend), NKp46 (9E2, Brilliant Violet 421, BioLegend), perforin (d9G, FITC, eBioscience), Eomes (WF1928, FITC or e6600, eBioscience), T-bet (O4-46, CF-594, BD Biosciences), retinoic acid–related orphan receptor γt (AFFKS-9, PE, eBioscience), Helios (22F6, Pacific Blue, BioLegend), GATA-3 (TWAI, e6600, eBioscience), IFN-γ (AS.B3, Brilliant Violet 570, BioLegend), MIP-1β (D21-1351, PE, BD Biosciences), and TNF (Mab11, Pacific Blue, eBioscience). Purified NKG2C2 (134591, R&D Systems) was biotinylated using a Fluorporter Mini-biotin-X protein labeling kit (Life Technologies) and detected using Quantum Red Qdot 605 (Invitrogen). All samples were stained with Live/Dead Aqua or Green (Invitrogen) to discriminate live and dead cells. Samples were analyzed using a BD LSRFortessa flow cytometer equipped with four lasers. Data were analyzed using FlowJo 9.7.4 (Tree Star).

KIR and HLA genotyping

KIR genotyping and KIR ligand determination were performed using PCR-SSP technology with a KIR typing kit and a KIR HLA ligand kit (both Olerup SSP).

Functional NK cell assay

Mononuclear liver cells were thawed and enriched for NK cells by magnetic cell sorting using an NK cell isolation kit (Miltenyi Biotec). Cells were resuspended in complete medium and rested overnight in the presence of 5 ng/ml IL-15 (PeproTech). The cells were then stimulated with PMA (10 ng/ml) and ionomycin (500 nM) for 4 h in the presence of monensin and brefeldin A for the last 3 h of incubation. Anti-human CD107a was present throughout the assay. Finally, cells were stained and analyzed by flow cytometry, as described above.

NK cell sorting and expansion assay

Mononuclear liver cells were thawed and for some experiments CFSE labeled. Then, the cells were surface stained, followed by sorting on a FACSaria III into the following live Lin (CD3+CD56+ NK cell subsets: CD94+CD56+, CD94+CD56−, and CD94+CD56−CD16+). To avoid cross-linking of CD16, the nonagonistic anti-CD16 clone VEP13 was used. After sorting, the NK cells were cocultured with irradiated 721.221 or transfected 721.221-AEH cells (HLA-E) and HLA-C1+ C2+ feeder PBMCs in expansion medium (5% human serum and 100 ng/ml IL-15) for 3 wk. Afterward, cells were stained for flow cytometry analysis, and total cell counts were calculated using counting beads.

CMVpp65 stimulation assay

Liver mononuclear cells were thawed and distributed at a final concentration of 5 × 10⁶ cells/ml in a 96-well U-bottom plate. Cells were stimulated or not with CMVpp65 overlapping peptides (JPT Peptide Technologies, 1 μg/ml for each peptide) in the presence of brefeldin A. After 16 h of incubation, the cells were stained for extracellular receptors, permeabilized, and stained for intracellular IFN-γ. Donors were considered CMV+ when >0.1% of T cells produced IFN-γ in response to CMVpp65 overlapping peptides.

Results and Discussion

CD3+CD49a+CD56− NK cells are present in human adults

Mouse livers contain a subset of CD49a+DX5− NK cells that under certain conditions exhibit adaptive-like immune features, such as potent recall responses to viruses and allergens (4, 5). This NK cell population is virtually absent in other organs. We hypothesized that a phenotypically similar subset of cells could be present in the human liver. To test this, we investigated enzymatically prepared liver perfusates from healthy human donors and from non–tumor-affected liver sections of patients with primary and metastatic liver malignancies. Strikingly, a subset of CD3+CD49a+CD56− lymphocytes was identified in 12 of 29 livers examined. In the positive livers, this subset made up 0.11–12.7% (average 2.3%) of the total CD3+CD56− lymphocyte population (Fig. 1A). In contrast, very low frequencies of CD3+CD49a+CD56− cells were detectable in peripheral blood (Fig. 1A). Moreover, hardly any CD3+CD49a+CD56− lymphocytes were found in human first trimester fetal livers (Supplemental Fig. 1A). Taken together, we here identify a human phenotypically similar counterpart to mouse liver–resident CD49a+DX5− NK cells.

Human intrahepatic CD3+CD49a+CD56− cells are bona fide NK cells with a ‘‘T-bet’’ Eomes+ transcription factor profile

To further characterize human liver–resident CD3+CD49a+CD56− cells (hereafter referred to as CD49a+ NK cells), we first analyzed their phenotype in detail. These cells did not express CD127 (IL-7Ra) (Supplemental Fig. 1B) or the transcription factors retinoic acid–related orphan receptor γt and Helios (data not shown), indicating that CD49a+ NK cells are distinct from non–NK ILCs (14, 15). The CD49a+ NK cells also lacked expression of NKp44 and expressed only low levels of CD103 (data not shown). This suggests that the cells are different in phenotype from the population of non–NK CD3+CD56− ILC1 recently reported to reside in human tonsils (15). Instead, the CD49a+ NK cells displayed a CD3+CD7−NKp46+CD16+CD56brightCD122low phenotype, which was, except for the low expression of CD122, similar to the profile of conventional CD3+CD49a+CD56dimCD16− NK cells that represent ~50% of total intrahepatic NK cells (Fig. 1B).

The transcription factors E4BP4, T-bet, and Eomes are critical for cNK cell development and differentiation (2). However, mouse intrahepatic CD49a+DX5− NK cells develop independently of Eomes and E4BP4 but are dependent on T-bet (10, 12). Interestingly, the CD49a+ NK cells identified in human liver expressed high levels of T-bet but only low or undetectable levels of Eomes (Fig. 1C, Supplemental Fig. 1C). Hence, this profile was markedly different from that of intrahepatic Eomes−T-bet+/− cNK cells, but similar to the mouse CD49a+DX5− NK cells. Thus, based on the cell surface phenotype and transcription factor expression, our data suggest that the CD49a+ NK cell population is a distinct subset of human bona fide intrahepatic NK cells sharing features with mouse CD49a+DX5− NK cells.
The human adult liver contains CD49a+ NK cells. (A) Staining of CD56 and CD49a on freshly isolated live CD45+CD3-CD122, and CD7 on CD49a+ and CD49a- intrahepatic CD56+CD3+ cells. (B) Stainings for Nkp46, CD16, CD19, and CD7 on CD49a+ and CD49a- intrahepatic CD56+CD3+ cells. (C) Stainings for Eomes and T-bet expression on CD49a+, CD49a-CD16-, and CD49a-CD16+ intrahepatic CD56+CD3- cells.

**FIGURE 1.** The human adult liver contains CD49a+ NK cells. (A) Staining of CD56 and CD49a on freshly isolated live CD45+CD3-CD122, and CD7 on CD49a+ and CD49a- intrahepatic CD56+CD3+ cells. (B) Stainings for Nkp46, CD16, CD19, and CD7 on CD49a+ and CD49a- intrahepatic CD56+CD3+ cells. (C) Stainings for Eomes and T-bet expression on CD49a+, CD49a-CD16-, and CD49a-CD16+ intrahepatic CD56+CD3- cells.

Human intrahepatic CD49a+ NK cells express KIRs but lack CD57 and NKG2A

Next, we sought to determine the differentiation status of the intrahepatic CD49a+ NK cells. On the basis of CD11b and CD27 staining, CD49a+DX5+ mouse liver NK cells were reported to display a more immature phenotype as compared with conventional intrahepatic NK cells (5, 10). Human cNK cell differentiation is most readily assessed by the cell surface expression of CD56, NKG2A, CD57, and KIRs. In brief, more immature conventional peripheral blood NK cells are CD56brightNKG2A-CD57- and KIR-, whereas differentiation is associated with transition to CD56+lim, loss of NKG2A, acquisition of CD57, and increasing expression of KIRs (16). Similar to peripheral blood-derived NK cells, both immature and mature subsets resided within intrahepatic cNK cells (Fig. 2A, Supplemental Fig. 1D). Interestingly, almost all CD49a+ intrahepatic NK cells were CD56bright and lacked expression of CD16, CD57, and NKG2A, but >80% of them expressed KIRs (Fig. 2A, Supplemental Fig. 1D), and as such did not follow the pattern of differentiation normally seen among cNK cells.

**FIGURE 2.** Human intrahepatic CD49a+ NK cells have an immature phenotype but express high levels of self-HLA class I-binding KIRs and NKG2C.

To further characterize KIR expression on the intrahepatic CD49a+ NK cells, we genotyped donors for KIR and HLA and performed a high-resolution KIR phenotyping of the cells by flow cytometry. Intriguingly, the CD49a+ NK cells displayed an oligoclonal KIR expression pattern with at least 50% (and in some cases up to 80%) of the cells having a distinct KIR profile (Fig. 2B). In some donors, a single inhibitory KIR dominated the profile, whereas in others a specific combination of two or three inhibitory KIRs was coexpressed by the CD49a+ cells (Fig. 2B). The profile of the CD49a+ NK cells was clearly distinct from that of the CD49a+CD16- cNK cells of the same donor (Fig. 2B). Additionally, the oligoclonal KIR expression pattern of the CD49a+ NK cells was different between donors, and no clear dominance of a particular inhibitory KIR was noted. Finally, low levels of activating KIRs, such as KIR2DS1 and KIR2DS4, were coexpressed by some CD49a+ NK cells (data not shown). Taken together, the skewed KIR profile of the intrahepatic CD49a+ NK cells suggests this subset of cells to have undergone a clonal-like expansion.

In addition to the particular expression pattern of KIRs, a vast majority of the human intrahepatic CD49a+ NK cells expressed the activating receptor NKG2C (Fig. 2A, Supplemental Figs. 1D, 2D). Abundant NKG2C expression on human peripheral blood NK cells has been linked to CMV seropositivity, as well as the expression of CD57 in conjunction with a single inhibitory self-HLA class I-binding KIR (6). Additionally, expansion of NKG2C+CD57+KIR- NK cells occurs also during acute viral infections (8, 9). We did not have access to the CMV serostatus of liver donors in the present study. However, CMVpp65-specific intrahepatic CD8 T cells could be detected in three of three donors with a CD49a+ NK cell subset as well as in three of three donors without a CD49a- subset (Supplemental Fig. 1E, 1F). This suggests that there is no strict correlation between CMV seropositivity and expansion of intrahepatic CD49a+ NK cells. Furthermore, in several respects, the phenotype of the intrahepatic CD49a+ NK cell subset differed markedly from that of the expanded NKG2C+ NK cell population observed in peripheral blood following CMV infection or reactivation. This included the lack of CD16 and CD57 as described above, low expression of CD45RA, Eomes, and perforin (Fig. 3A, Supplemental Figs. 1G, 2D), as well as high expression of KIR2DL3, NKG2D, and CD69 on the intrahepatic CD49a+ NK cells (Supplemental Figs. 1H, 2D). In contrast, peripheral blood NKG2C+ NK cell expansions are CD16+CD57+ perforinhigh but express low levels of KIR3DL3, NKG2A, and CD69 (Supplemental Fig. 2D) (6, 17).
of cytokines. (A) CD49a+, CD49a− PMA/ionomycin stimulation for 4 h (proinflammatory cytokines upon stimulation).

Parabiosis experiments have revealed that mouse intrahepatic CD49a+ NK cells are largely liver-resident (5, 12). To investigate whether the human intrahepatic CD49a+ NK cells similarly showed signs of liver residency, we analyzed whether these cells could be detected in afferent or efferent venous blood of the liver. Interestingly, neither source, that is, the afferent (portal venous) or efferent (hepatic venous) blood of the liver, contained a detectable CD49a+ NK cell population (Fig. 4A), suggesting that CD49a+ NK cells are largely liver-resident.

CD49a (integrin α1) partners with CD29 (integrin β1) on the surface of cells to form an α1β1 integrin complex and binds to collagen (18). Only CD49a+ cells expressed CD29 (Supplemental Fig. 2A), indicating that these cells may form a functional receptor. Because virus-specific CD8+ T cells are kept in nonlymphoid tissues during primary immune responses in a CD49a-dependent manner (18), it is plausible that CD49a has a similar role in retaining the CD49a+ NK cells in the liver parenchyma contributing to liver residency.

**Human intrahepatic CD49a+ NK cells produce high levels of proinflammatory cytokines upon stimulation**

Having identified a new subset of intrahepatic NK cells, we next analyzed their functional properties. The CD49a+ NK cells expressed low levels of perforin and granzyme A as compared with conventional CD49a− intrahepatic NK cells but, instead, contained high levels of granzyme B (Fig. 3A, Supplemental Fig. 1G). Furthermore, CD49a+ NK cells expressed high levels of the activating receptors NKG2C, NKp30, NKG2D, and DNAM-1 (Fig. 1B, Supplemental Fig. 1H). The lack of CD16 expression and low expression of perforin and granzyme A indicated reduced cytotoxic capacity. Accordingly, upon stimulation with PMA/ionomycin, degranulation was weaker in the CD49a+ subset than in their CD49a− NK cell counterparts (Fig. 3B, 3C). Despite this poor capacity for degranulation, polyclonal stimulation led to a significantly higher production of cytokines such as IFN-γ, TNF, and GM-CSF in the CD49a+ subset as compared with CD49a− NK cells (Fig. 3B, 3C).

The capacity to produce high levels of IFN-γ, TNF, and GM-CSF, as well as the poor degranulation response and low levels of cytotoxic effector molecules, is consistent with the functional profile of mouse intrahepatic CD49a+DX5− NK cells (5, 10). A key function of NKG2C+ NK cell expansions in peripheral blood is potent Ab-dependent cellular cytotoxicity capacity (6, 7). However, the human intrahepatic CD49a+ NK cells described in the present study lack expression of CD16 and, thus, Ab-dependent cellular cytotoxicity capacity. Taken together, intrahepatic CD49a+ NK cells produce high levels of cytokines, but they degranulated poorly as compared with cNK cells.

**Human CD49a+ NK cells are largely liver-resident**

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**Human intrahepatic CD49a+ NK cells harbor a significant proliferative capacity and show a stable phenotype upon in vitro culture**

Next, we assessed the proliferative capacity of intrahepatic CD49a+ NK cells. Highly purified CD49a+, CD49a−CD16−, and CD49a−CD16+CD57− intrahepatic subsets of NK cells were stimulated for 3 wk with irradiated PBMCs as feeder cells, IL-15, and untransfected or HLA-E–transfected 721.221 cells. The CD49a+ subset expanded ∼800-fold during the 3 wk of stimulation (Fig. 4B). Additional stimulation with 721.221 cells or specifically through the NKG2C receptor via HLA-E did not further increase the proliferative response (Fig. 4B). Despite expressing low levels of CD122 (Fig. 1B), the CD49a+ cells...
expanded as efficiently as did the CD49a⁻ NK cell subsets investigated (data not shown).

Furthermore, to determine whether intrahepatic CD49a⁺ NK cells represented an immature subset with the capacity to acquire more mature characteristics, or, alternatively, possessed a stable phenotype, we performed a phenotypic analysis of the cells following 3 wk of expansion. Intriguingly, the CD49a⁺ subset had a stable phenotype, including retained low expression of Eomes, perforin, NKGA2, and CD57, and high levels of T-bet and KIR (Fig. 4C, 4D, Supplemental Fig. 2E). In contrast, conventional CD49a⁻CD16⁺ NK cells upregulated CD16, perforin, and T-bet upon proliferation (Fig. 4C, 4D). After 3 wk of culture, it was noted that sorted CD49a⁻ NK cells did upregulate CD49a. However, the level of expression was clearly lower as compared with the CD49⁺ NK cells characterized in this study (Supplemental Fig. 2B, 2C).

Stimulating the CD49a⁺ NK cells additionally through NKG2C triggering (6). These data further corroborate the phenotypic (CD57⁻CD16⁻ perforinlow Eomes⁻) and functional (high cytokine production, low cytotoxicity) data presented above, indicating that expanded intrahepatic CD49a⁺ NK cells are a stable subset distinct from previously described peripheral blood NKG2C⁺ NK cell expansions.

In conclusion, in this study we provide evidence for the existence of a previously unrecognized subset of CD49a⁺ NK cells in the human liver. These cells share phenotypic and functional traits with mouse intrahepatic tissue-resident CD49a⁺DX5⁻ NK cells possessing adaptive-like features. To some extent, these human intrahepatic CD49a⁺ NK cells resemble the peripheral blood NKG2C⁺ single KIR⁺ NK cell expansions reported in response to viruses. However, CD49a⁺ NK cells differ significantly in their transcription factor as well as effector molecule expression profiles, and in markers of differentiation. Taken together, these data support the notion of a T-bet-controlled extramedullary NK cell development in humans.

Disclosures
The authors have no financial conflicts of interest.

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