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Charakterisierung von leberresidenten CD49a+ Natürlichen Killerzellen in der humanen Leber

Publikationsdissertation

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1. ORIGINALARBEIT DER PUBLIKATIONSDISSERTATION

MARTRUS G* / KAUTZ T*, […] ALTFELD M. PROLIFERATIVE CAPACITY EXHIBITED BY HUMAN LIVER-RESIDENT CD49A+CD25+ NK CELLS.

PLOS ONE

RESEARCH ARTICLE

Proliferative capacity exhibited by human liver-resident CD49a+CD25+ NK cells

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Abstract

The recruitment and retention of Natural Killer (NK) cells in the liver are thought to play an important role during hepatotropic infections and liver cirrhosis. The aims of this study were to determine differences between liver-derived and peripheral blood-derived NK cells in the context of liver inflammation and cirrhosis. We conducted a prospective dual-center cross-sectional study in patients undergoing liver transplantation or tumor-free liver resections, in which both liver tissue and peripheral blood samples were obtained from each consenting study participants. Intrahepatic lymphocytes and PBMCs were stained, fixed and analyzed by flow cytometry. Our results showed that, within cirrhotic liver samples, intrahepatic NK cells were particularly enriched for CD49a+ NK cells when compared to tumor-free liver resection samples. CD49a+ liver-derived NK cells included populations of cells expressing CD25, CD34 and CXCR3. Moreover, CD49a+CD25+ liver-derived NK cells exhibited high proliferative capacity *in vitro* in response to low doses of IL-2. Our study identified a specific subset of CD49a+CD25+ NK cells in cirrhotic livers bearing functional features of proliferation.

Introduction

Lymphocytes in the liver consist of liver-resident cells as well as lymphocytes circulating through the liver from the portal vein and the hepatic artery [1, 2]. Liver-resident type 1 innate lymphoid cells (ILC1s), including Natural Killer (NK) cells, have been suggested to regulate liver fibrosis during chronic hepatotropic infections and chronic inflammatory processes. NK cells are classified according to their CD56 marker expression levels in $CD56^{bright}$, $CD56^{dim}$ and $CD56^{-}CD16^{+}$ NK cells and represent an enriched population within the human

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intrahepatic lymphocytes (IHLs) by constituting up to 40% of this population [3–5]. Data obtained in mouse models using parabiotic experiments have defined liver-resident NK cells as being CD49a+DX5- [6]. Furthermore, it was demonstrated that these liver-resident NK cells represent a distinct lineage from bone marrow-derived NK cells, and might have originated from hepatic progenitor cells during fetal development [7]. A number of recent studies have characterized liver-resident and tissue-resident NK cells in mouse models and humans using several surface markers, including CD49a [6, 8], DNAM-1 [9], CXCR6 [10–12], CCR5 [12], CD103 [7, 13] CD49e [14] or transcription factors including T-bet [10, 15], Eomes [10, 15] or PLZF [16]. These studies have defined liver-resident NK cells depending on specific transcription factors or integrin receptors. While our understanding of the phenotypical properties of liver-resident NK cells has been advanced by those studies, there is still some lack of knowledge about the functional capacities of liver-resident NK cells.

In our study, we have focused on several activation (CD25) and differentiation (CD34) markers, as well as integrin receptors (CD49a and CXCR3) to study the maturation and homing capacities of liver-resident NK cells. Our aim was to determine whether those markers would be especially expressed on liver-resident CD49a+ NK cells. Altogether, we demonstrate that liver-resident CD49a+ NK cells in humans with advanced liver disease include subsets of CD25+ proliferating cells responding to low doses of exogenous IL-2 as well as NK cell subsets expressing CXCR3, the receptor for IP-10, and CD34, a marker for progenitor NK cells. The results suggest that subsets of human liver-resident NK cells retain distinct functional characteristics including proliferation capacities and persistence and might contribute to liver inflammation and fibrosis.

Materials and methods

1. Study design and study population

We conducted a prospective cross-sectional study in adult patients undergoing liver transplantation at the University Medical Center Hamburg-Eppendorf (UKE). Both explant liver tissue and peripheral blood samples were obtained from each consenting study participant undergoing a transplantation procedure. All consenting adult participants (age over 18 years) undergoing liver transplantation during the study period were eligible. We furthermore obtained tumor-free liver tissue and peripheral blood samples from a cohort of individuals undergoing surgical liver resection due to hepatocellular carcinoma at the Asklepios Hospital Barmbek. Healthy liver tissue from the tumor surrounding areas was excised. All study participants provided written informed consent, according to the ethical guidelines by the Institutional Review Board of the medical faculty at the University of Hamburg that approved the study protocol. The demographics and clinically characteristics of study subjects are summarized in Tables 1 and 2.

2. Cell preparation

Intrahepatic lymphocytes (IHLs) were isolated following a hashing protocol established in our laboratory. Briefly, 10 to 20 grams (g) of liver were sliced into small pieces. Tubes containing 3 g of sliced tissue and 3 ml of RPMI+10% FBS (R10) were hashed at room temperature using the gentleMACS™ Octo Dissociator (Miltenyi Biotec, Germany). The recovered tissue was successively strained through 100μm, 70μm and 40μm Easystrainer™ filters (Greiner Bio-One GmbH). Blood samples from the same study participant obtained pre-surgery were processed by Ficoll-gradient purification to gain peripheral blood mononuclear cells (PBMCs). The recovered cells were immediately processed for flow cytometry analysis (FACS).

Table 1. Demographics and clinical characteristics from explanted liver tissue samples.

	Total number of individuals	19			
	Sex (f/m; %f/%m)	7/12; 37%, 63%			
	age (years; range)	57; 38–69			
Primary liver disease	HCV (n°) *	6			
	PSC (n°)	2			
	ALD (n°)	6			
	HCC (n°)	1			
	cholangiocarcinoma (n°)	1			
	overlap syndrome AIH/PBC or AIH/PSC (n°)	2			
	polycystic liver disease (n°)	1			
Clinical Data	CMV (neg/pos; %neg/%pos)	13/6; 68%/32%			
		Males	**Females**	**RV Males**	**RV Females**
	MELD (median /min-max)	12.15 (7–35)	19.7 (7–29)		
	INR (median /min-max)	1.26 (0.96–2.2)	1.34 (1–1.89)		
	Creatinine (mg/dl) (median /min-max)	1.18 (0.58–3.8)	1.2 (0.57–4.8)	0.6–1.3	0.5–1
	Thrombocytes (/µl (x 1000)) (median /min-max)	109 (25–201)	62 (29–210)	150–400	150–400
	Bilirubin (mg/dl) (median /min-max)	2.2 (0.3–22.9)	4.5 (0.2–22)	<1.2	<1.2

HCV: Hepatitis C Virus infection, PSC: Primary Sclerosing Cholangitis, ALD: Alcoholic Liver Disease, HCC: Hepatocellular carcinoma, AIH: Autoimmune Hepatitis, PBC: Primary Biliary Cholangitis, INR: International Normalized Ratio, MELD: Model For End-Stage Liver Disease, CMV: Cytomegalovirus status.

*5 out of HCV+ livers have developed HCC as a complication of the cirrhosis. RV: Reference Values.

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3. Antibody staining and flow cytometry

Monoclonal antibodies anti-CD56 (BUV395, clone NCAM16.2, BD Horizon™), anti-CD3 (BUV737, clone UCHT1, BD Horizon™), anti-CD25 (PE-Cy7, clone M-A251, Biolegend), anti-CD34 (PE-CF594, clone 581, Biolegend), anti-CD49a (PE, clone TS2/7, Biolegend), anti-CD16 (BV786, clone 3G8, Biolegend), anti-CD45 (BV711, clone HI30, Biolegend), anti-CXCR4 (BV605, 12G5, Biolegend), anti-CD14 (BV510, clone M5E2, Biolegend), anti-CD19 (BV510, clone HIB19, Biolegend), anti-CXCR3 (APC-Cy7, clone G025H7, Biolegend), anti-NKG2C (AF700, clone 134591, R&D Systems), anti-DNAM-1 (APC, clone 11A8, Biolegend), anti-CD57 (FITC, clone HNK-1, Biolegend) and Zombie Aqua staining (Biolegend) were used. Freshly isolated cells were washed, stained at a final volume of 100µl with PBS and incubated for 20 minutes at room temperature. Cells were subsequently washed and fixed with 4%

Table 2. Demographics and clinical characteristics from liver resection samples.

	Total number of individuals	5		
	Sex (f/m; %f/%m)	3/2; 60%, 40%		
	age (years; range)	65; 56–73		
Clinical Data	CMV (neg/pos)	1/3, 1ND		
		Males	**Females**	**Reference values**
	INR (median /min-max)	1.08 (1–1.16)	1.05 (0.95–1.22)	
	Creatinine (mg/dl) (median /min-max)	0.8 (0.7–0.9)	0.7 (0.6–1.2)	0.6–1.1
	Thrombocytes (/µl (x 1000)) (median /min-max)	205.5 (130–281)	340 (130–344)	150–370
	Bilirubin (mg/dl) (median /min-max)	0.55 (0.3–0.8)	0.7 (0.6–0.8)	<1.2

INR: International Normalized Ratio, CMV: Cytomegalovirus status. ND: non-determined

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paraformaldehyde for 20 minutes. Samples were acquired on a LSR Fortessa (BD Biosciences) and results were analyzed using FlowJo software version 10.

4. Cell sorting and proliferation assay

Freshly isolated IHLs were stained using: CD3 (PE/Dazzle 594, clone UCHT1, Biolegend), CD25 (PE-Cy7, clone M-A251, Biolegend), CD16 (BV786, clone 3G8, Biolegend), CD45 (BV711, clone HI30, Biolegend), CD56 (BV605, clone NCAM, Biolegend), CD14 (BV510, clone M5E2, Biolegend), CD19 (BV510, clone HIB19, Biolegend), CD49a (FITC, clone TS2/7, Biolegend) and Zombie Aqua (Biolegend) and, after lineage gating, 4 populations (CD49a +CD25+, CD49a+CD25-, CD49a-CD25+ and CD49a-CD25-) were sorted using a BD FACSAria™ Fusion. Subsequently proliferative capacity of the sorted cells was assessed using a CFSE proliferation assay. Briefly, cells were resuspended in PBS+2% FBS and CellTrace™ CFSE (Thermofisher Scientific) was added at a 1μM final concentration. Cells were left in the dark for 10 minutes and 1ml of FBS was added to stop the staining. After 1 minute incubation, 5ml R10 were added on top and cells were incubated for 5 extra minutes. A washing step was performed and cells were resuspended at 30,000 cells/ml in R10 + 30UI/ml of IL-2 and left in the incubator at 37˚C. CFSE fluorescence was measured using a LSR Fortessa (BD Biosciences) on day 7.

5. t-SNE analysis

The t-Distributed Stochastic Neighbor Embedding (t-SNE) [17] analysis was performed in R using the packages flowCore [18] and Rtsne [19]. For the t-SNE analysis, gated events representing living NK cells (CD56+ CD16+/-) from liver and peripheral blood from 19 patients were independently merged and randomly subsampled to 100,000 events for comparative purposes. Subsequently, liver NK cell and peripheral NK cell files were combined into one single FCS file containing 200,000 events. Fluorescence channels were then scaled according to the logicle display method [20] and t-SNE analysis was run considering the markers DNAM1, CXCR3, NKG2C, CD56, CXCR4, CD16, CD57, CD49a, CD25, CD34 and CXCR6. After analysis, the results were plotted using the R package ggplot2 [21] into merged plots of liver and PBMCs and into plots containing cells derived from only liver or only PBMCs, respectively. Additionally, liver cells were split into CD49a+ and CD49a- cells for plotting t-SNE maps.

6. Statistical methods

Percentages of cell sub-populations within the same individual were compared between PBMC and IHLs, and between CD49a+ vs CD49a- cell populations within IHLs using Wilcoxon signed rank tests. Test multiplicity was controlled by a false discovery rate (FDR) procedure accounting for dependency among statistical tests [22]. FDR-adjusted p-values <0.05 were considered statistically significant. Statistical analyses were done with SAS, version 9.3 (SAS Institute, Cary, North Carolina, USA).

Results

Unsupervised analysis revealed profound phenotypic differences between peripheral blood and intrahepatic NK cells

Human liver-resident NK cells have previously been shown to carry a distinct phenotype compared to peripheral blood NK cells using a limited number of parameters [23]. In particular, CD49a expression has been described to define liver-resident NK cells in mouse models [6, 23] as well as in human liver samples [8, 24]. We analyzed a set of surface markers, including

cytokine receptors, integrin receptors and activation markers in matched liver and blood samples from 19 individuals undergoing liver transplantation at the Department of Hepatobiliary and Transplant Surgery of the UKE and from 5 individuals undergoing surgical liver resection at the Department of General & Abdominal Surgery of the Asklepios Hospital Barmbek. The demographics and clinical characteristics of study subjects are summarized in Tables 1 and 2. All individuals undergoing liver transplantation were in advanced stages of liver disease.

The NK cell surface receptor repertoire between matched liver and peripheral blood samples was compared using the t-SNE dimensionality reduction algorithm, which provides two dimensional visualization of multiparametric single cell data [17]. To facilitate the comprehension of the final results, all 19 individuals undergoing liver transplantation were included in the same analysis. The results showed that the overall structure of peripheral blood NK (pNK) cells (Fig 1A) and intrahepatic NK (ihNK) cell (Fig 1B) repertoires differed since few plot regions were co-localizing in both groups. As expected, even the principal markers identifying NK cells, CD56 and CD16, exhibited differences. Confirming previous results, ihNK cells contained a higher proportion of cells expressing CD56 ($CD56^{bright}$ NK cells) compared to pNK cells, [12]. Furthermore, CD49a expression was almost exclusively concentrated in ihNK cells and non-existent in pNK cells, demonstrating that CD49a enables differentiation of NK cells from these two compartments (Fig 1B). When focusing on the CD49a highly dense area, ihNK cells co-expressed cytokine receptors such as CXCR4, CXCR3 and CXCR6 as well as CD25 and CD34. Peripheral NK cells in contrast contained exclusively a defined area with high density of cells expressing maturation markers such as CD57 and DNAM-1 (Fig 1A).

We next sought to quantitatively confirm the visual t-SNE analysis results by statistical approaches. Matched intrahepatic and peripheral blood NK cell populations were manually gated and the frequency of each marker was individually quantified and compared (Fig 1C and 1D). Gating strategies are shown in S1 Fig. After adjustment for test multiplicity, $CD56^{bright}$ NK cells were significantly more frequent and $CD56^{dim}$ NK cells were significantly less frequent in the intrahepatic compartment compared to the peripheral blood compartment in the liver transplant cohort (S2A Fig), confirming previous studies [12]. Within the CD56-CD16+ cells, a previously described dysfunctional NK cell subset [25–27], no differences were detected between blood and liver NK cell frequencies. Furthermore, no phenotypical differences on NK cells were observed between liver diseases such as hepatitis C virus (HCV)-liver injury, autoimmune diseases (Primary sclerosing cholangitis (PSC) and Primary Biliary Cholangitis (PBC)), alcoholic liver disease (ALD), hepatocellular carcinoma (HCC) and polycystic liver disease in the studied cohort (data not shown), potentially due to small number of cases per disease (Table 1). The same tendency for NK cell subsets distribution was observed in the tumor-free areas in liver tumor resections ($p = 0.0625$) (S2B Fig), confirming previous studies [8] and the results from the t-SNE analysis. Compared to pNK cells, ihNK cells included a significantly higher proportion of NK cells expressing CD34, and a significantly lower proportion of NK cells expressing CD57 and DNAM-1, consistent with a less differentiated phenotype (Fig 1C). In line with previous data [6, 8], we confirmed results from the t-SNE plots showing that ihNK cells contained significantly higher frequencies of CD49a+ NK cells, a molecule that is not expressed in the peripheral blood NK cell compartment. In contrast, cells positive for the chemokine receptor CXCR4 were significantly less represented among the ihNK cells. The proportion of cells expressing the same markers in liver resections showed the same tendency (Fig 1D). The liver transplant cohort, which only contained cirrhotic livers, had a statistically higher frequency of CD49a+ ihNK cells when compared to tumor-free liver resections ($p = 0.005$) (Fig 1E). Overall, these data demonstrate that ihNK cells differ significantly from pNK cells, and exhibit a less differentiated phenotype. Moreover,

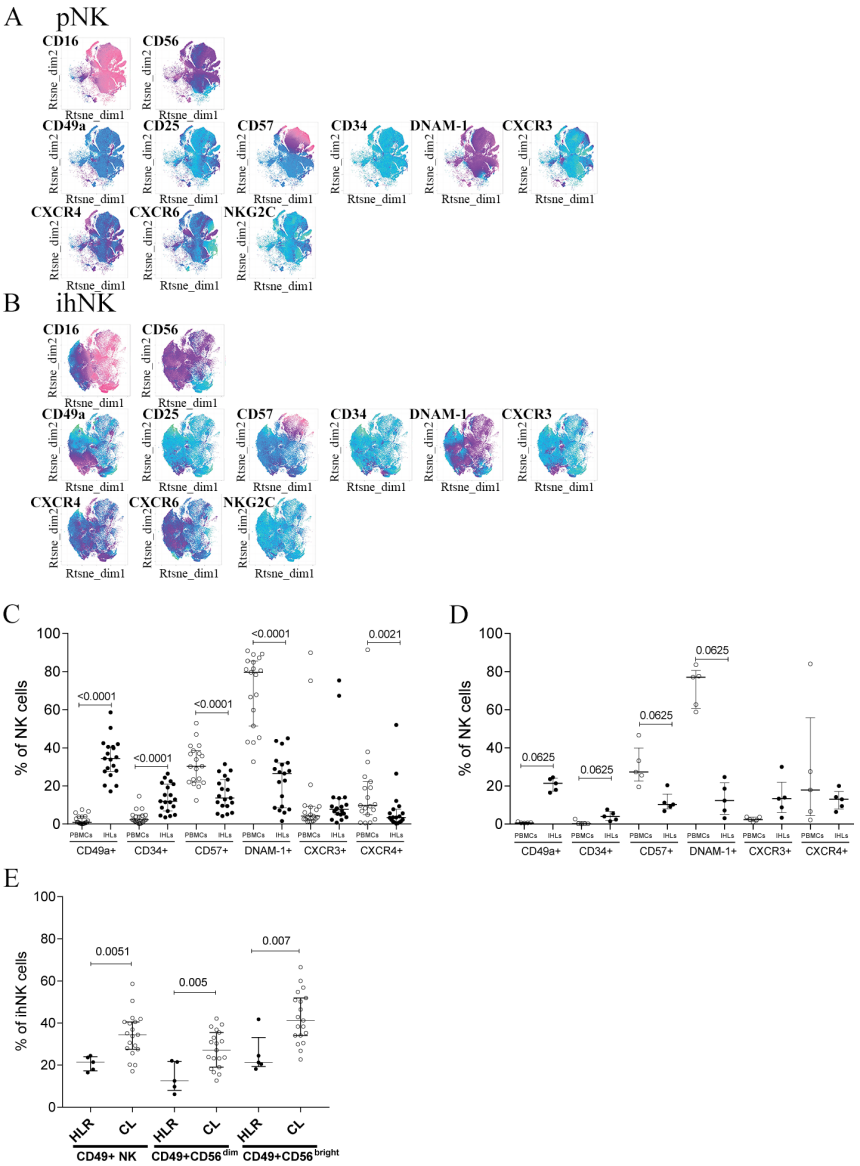

Fig 1. Immune phenotyping of combined peripheral and intrahepatic NK cells. Gated NK cells from 19 donors were concatenated and represented in t-SNE maps for the expression of chemokine receptors, activation and residency markers. **(A)** peripheral and **(B)** intrahepatic NK cells are shown. Color coding indicates the expression intensity of the surface marker, pink being higher expressed and green being lower expressed. **(C)** Proportion of NK cells derived from the liver (ihNK) and the peripheral blood (pNK) on the liver transplantation cohort expressing CD49a (pNK median (IQR): 0.9 (0.3–3.9); ihNK median (IQR): 34.4 (27.6–40.5); p<0.0001), CD34 (pNK median (IQR): 2.2 (1–4.7); ihNK median (IQR): 12 (6.8–20.9); p<0.0001), CXCR4 (pNK median (IQR): 9.8 (4.9–22.2); ihNK median (IQR): 3.4 (1.3–7.7); p = 0.0024), CD57 (pNK median (IQR): 19 (22–38.5); ihNK median (IQR): 13.7 (9.4–23.3); p<0.0001) and DNAM-1 (pNK median (IQR): 79.6 (51.5–85.6); ihNK median (IQR): 26.5 (8.5–32.1); p<0.0001) (n = 19). **(D)** Proportion of NK cells from the tumor-free liver resections expressing CD49a, CD34, CD57, DNAM-1, CXCR3 and CXCR4 within the IHLs NK cells and pNK cells (n = 5). **(E)** Frequency of CD49a+ NK cell population within the IHLs NK cells in tumor-free liver resection cohort (HLR) and the liver retransplant cohort (cirrhotic livers, CL). Data is depicted as scatter plot, with each dot corresponding to a participant. Bars indicate median and IQR. Wilcoxon signed rank tests with adjustment of p-values by false discovery rate.

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cirrhosis and inflammation were associated with higher expression levels of CD49a on ihNK cells.

Similar data were observed when comparing peripheral blood and liver CD56^{bright} and CD56^{dim} NK cells (S2C and S2D Fig). The proportion of CD49a+ cells was significantly higher within the intrahepatic CD56^{bright} and CD56^{dim} NK cell populations than in matched peripheral blood NK cells. In contrast, CXCR4+ cells were significantly more frequent within peripheral CD56^{dim} and CD56^{bright} NK cells compared to ihNK cells. Taken together, these results suggest the presence of a more immature NK cell population in inflamed livers compared to peripheral blood.

The liver-resident CD49a+ NK cell subset contains immature and pre-activated cells

CD49a (ITGA1, VLA-1) is an alpha 1 integrin binding to laminin and collagen, and has been shown to be expressed on liver-resident NK cells in mice and humans [6, 8]. We therefore investigated the phenotypical differences between intrahepatic CD49a^{+} and CD49a^{-} NK cells to further characterize this subset of liver-resident CD49a+ NK cells. CD49a+ and CD49a- NK cells showed distinct distributions within the t-SNE plots, already suggesting that these two ihNK cell populations possessed distinct phenotypical signatures (Fig 2). In particular,

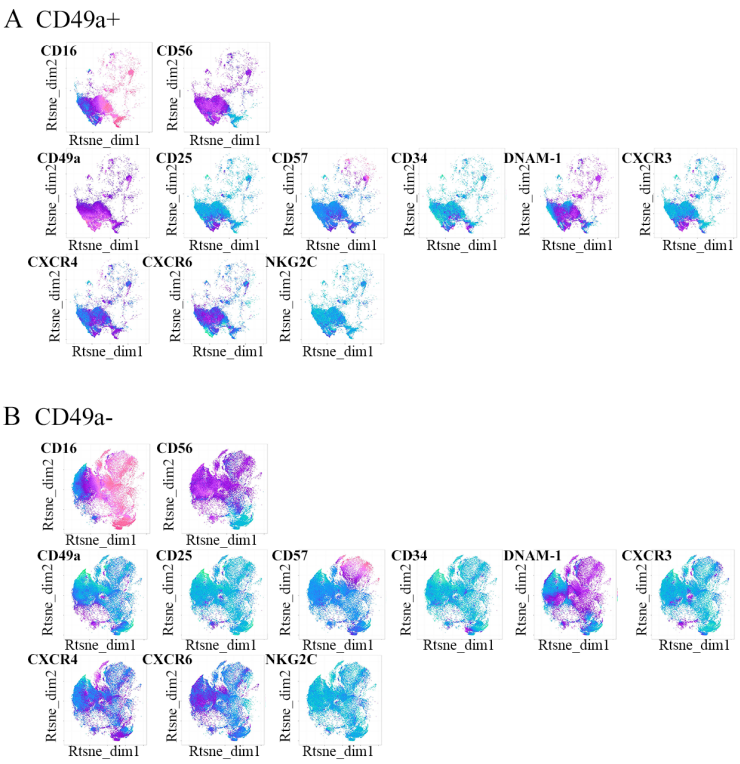

Fig 2. Unsupervised analysis of intrahepatic CD49a+ and CD49a- NK cells. (A) Gated CD49a+ and **(B)** CD49a- NK cells from 19 donors were concatenated and represented in t-SNE maps for the expression of chemokine receptors, activation and residency markers. Color coding indicates the expression intensity of the surface marker, pink being higher expressed and green being lower expressed.

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CXCR3, CXCR4, CD25 and CD34 were highly expressed in CD49a+ ihNK cells (Fig 2A), whereas CD49a- ihNK cells areas more closely resembled pNK cells (Figs 1A and 2B).

When applying manual gating and statistical testing to differentiate between CD49a+ and CD49a- NK cells in livers, we observed that, although present in $CD56^{dim}$ NK cells, CD49a+ cells were significantly more frequent within the $CD56^{bright}$ NK cell population in the liver transplant cohort ($p = 0.0001$) (Fig 3A). In the tumor-free areas of liver resections, a similar trend was observed, but did not reach statistical significance ($p = 0.185$) (Fig 3B). The respective gating strategies are shown in S3 Fig. In the liver transplant cohort, intrahepatic CD49a+ NK cells furthermore differed from CD49a- NK cells by containing significantly higher proportions of CD25+, CD34+, and CXCR3+ cells (Fig 3C). No significant differences regarding the frequency of cells positive for maturation and activation markers CD57 and DNAM-1 as well as in CXCR4 expression were observed between CD49a+ and CD49a- intrahepatic NK cells. A similar trend was observed in the tumor-free areas of liver tumor resections (Fig 3D). These significant differences in the proportion of CD34, CD25 and CXCR3 between CD49a+ and CD49a- NK cells from the liver transplant cohort were observed both within the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets (S4 Fig).

Liver-resident CD49a+CD25+ NK cells proliferate in response to low doses of IL-2

The expression of CD34, a marker associated with hematopoietic stem cells [28, 29] by liver-resident CD49a+ NK cells, indicates potential for self-renewal and an immature phenotype. The high levels of CD25 expression, the high affinity receptor for IL-2, on liver-resident CD49a+ NK cells suggests proliferative capacities of these cells in response to cytokines in the context of liver inflammation. We therefore characterized the combined expression patterns of these markers in the context of CD49a on ihNK cells using Boolean gating, which revealed 7 possible distinct NK cell populations based on these three markers after excluding the triple negative ($CD25^-CD49a^-CD34^-$) population of NK cells (Fig 4A). A pie chart representing the frequencies of each population normalized to 100% is shown to better visualize the contribution of each subset with regards to the three markers (Fig 4B). Boolean gating of the three markers confirmed that CD49a, expressed on 87% of liver-derived NK cells (excluding the triple negative population), was the main driver to differentiate ihNK cells (Fig 4B). Moreover, CD25 or CD34 were frequently co-expressed on CD49a+ NK cells (10.2% for CD49a+CD25-CD34+ cells, 6.03% for CD49a+CD25+CD34- cells), whereas single expression of CD25 or CD34 or combined expression of CD25 and CD34 in the absence of CD49a expression were less represented (9.9% for CD49a-CD34+CD25- cells, 2.4% for CD49a-CD34-CD25+ cells and 0.59% in CD49a-CD34+CD25+ cells) (Fig 4A and 4B).

To understand whether the presence of these subsets were related to high inflammation in the livers, Alanine Aminotransferase (ALT) serum levels were correlated to our flow cytometry data. ALT levels on the liver transplantation cohort positively correlated with the proportion of intrahepatic CD49a+CD25+ NK cells (Fig 4C).

CD25 is the high affinity receptor for IL-2, a cytokine overexpressed in inflammatory liver diseases [30], and showed a clear and almost exclusive expression on liver-resident CD49a+ NK cells. Since Boolean gating confirmed that CD34 and CD25 were almost mutually exclusively expressed markers, and CD49a+CD25+ ihNK cells were correlated to ALT levels, we focused on studying the functional properties of CD49a+CD25+ NK cells. CD49a+CD25+, CD49a+CD25-, CD49a-CD25+ and CD49a-CD25- NK cells were sorted from 5 livers, cell-traced with CFSE and cultured with low doses of IL-2 for 7 days (Fig 5A). Only CD49a+CD25+ cells were able to significantly proliferate in response to IL-2 when compared to other subsets

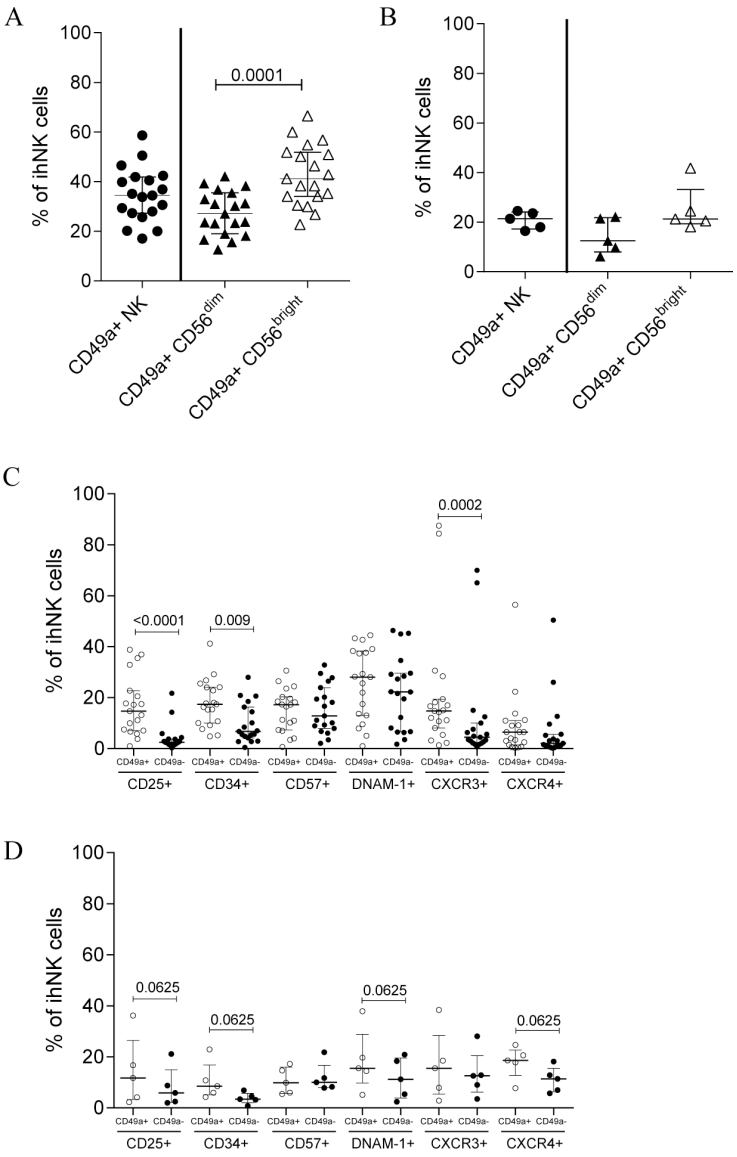

Fig 3. Immunophenotyping of intrahepatic CD49a+ and CD49a- NK cells. CD49a expression on bulk, CD56^{dim} and CD56^{bright} NK cells in the liver transplantation cohort **(A)** and in the tumor-free liver resection cohort **(B)**. Proportion of cells expressing specific markers in ihNK cells once gated on CD49a+ and CD49a- NK cells in the liver transplantation cohort **(C)** with CD25+ (CD49a+ NK cell median (IQR): 14.7 (7.1–22.7); CD49a- NK cell median (IQR): 2.5 (1.6–3.8); p<0.0001), CD34+ (CD49a+NK cell median (IQR): 17.4 (10–24.1); CD49a- NK cell median (IQR): 6.8 (4.2–16.3); p = 0.0107) and CXCR3+ (CD49a+ NK cell median (IQR): 14.8 (8.1–19.4); CD49a- NK cell median (IQR): 4.5 (2.2–10); p = 0.0002). **(D)** Proportion of cells expressing specific markers in ihNK cells once gated on CD49a+ and CD49a- in tumor-free liver resections. All data is depicted as scatter plot, with each dot corresponding to a participant. Bars indicate median and IQR. Wilcoxon signed rank tests with adjustment of p-values by false discovery rate.

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(unadjusted p = 0.009) (Fig 5B). Taken together, these results show that CD49a+CD25+ NK cells are more prevalent in inflamed livers compared to blood, and have the ability to proliferate in response to low microenvironment doses of IL-2, potentially explaining their high prevalence in inflamed liver tissues.

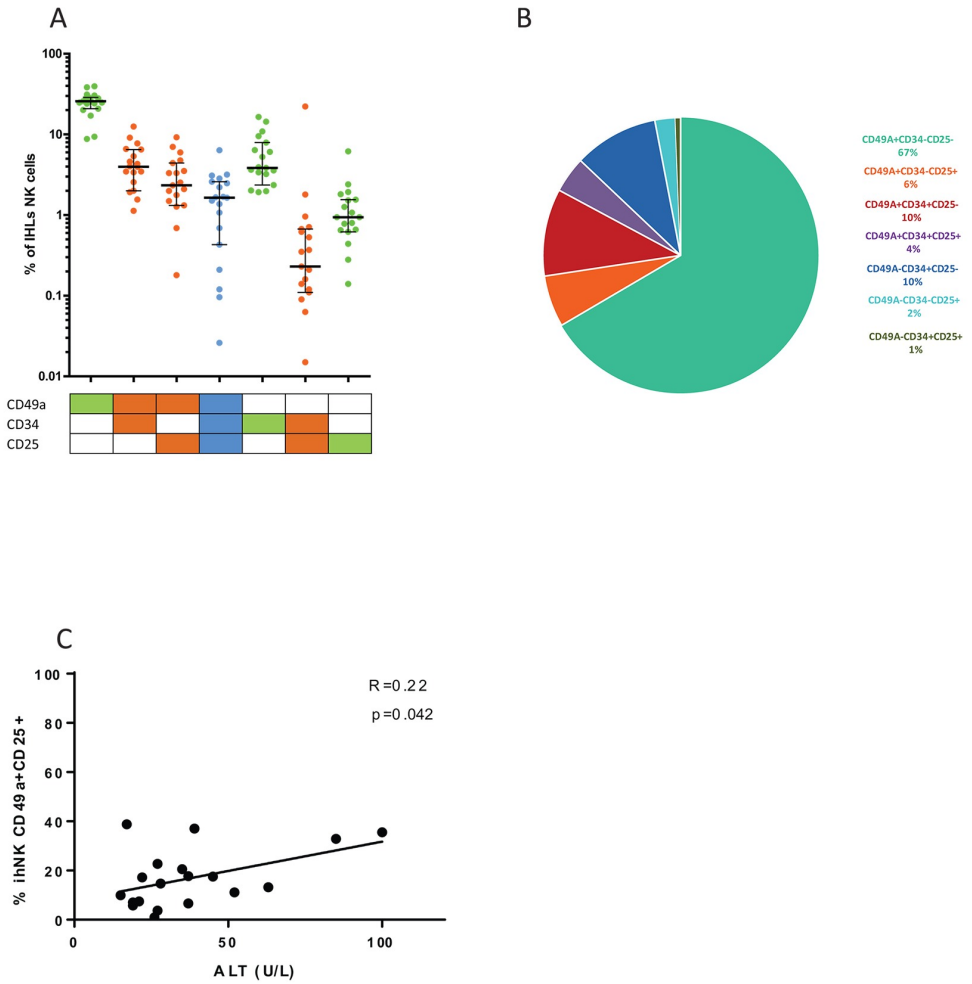

Fig 4. Relevance of CD49a+CD25+ ihNK cells. (A) Boolean gating of CD49a, CD25 and CD34 markers on ihNK cells. **(B)** Pie chart representing the frequency of each of the 7 possible combinations of the 3 markers, CD49a, CD25 and CD34. The median percentage of each population is represented. **(C)** Alanine Aminotransferase (ALT) serum levels correlation with the proportion of intrahepatic CD49a+CD25+ NK cells in the liver transplantation cohort. Data in **(A)** is depicted as scatter plot showing all individuals, the bar represents the median and the deviation is depicted as interquartile range.

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Discussion

Building evidence from mouse models and human samples has shown that liver-resident NK cells represent a specific cell population which can be identified by the expression of surface markers and transcription factors [6–12, 14, 15, 31, 32]. Specifically, it was demonstrated in mice that liver-restricted CD49a+DX5- NK cells, which also expressed high levels of CXCR6 and CD69, exhibit lower levels of CD62L and KLRG1 expression, indicating lower levels of maturation and proliferation [6, 33, 34]. Here, we compared human hepatic and peripheral NK cells and identified unique NK cell subsets in human livers which differentially expressed CD49a, CD25 and CD34 and differed in their proliferative capacity.

CD49a, an alpha-1 integrin binding to collagen and laminin, has been described in humans as a tissue-residency marker in $CD56^{bright}$ NK cells derived from liver, uterus, decidua and

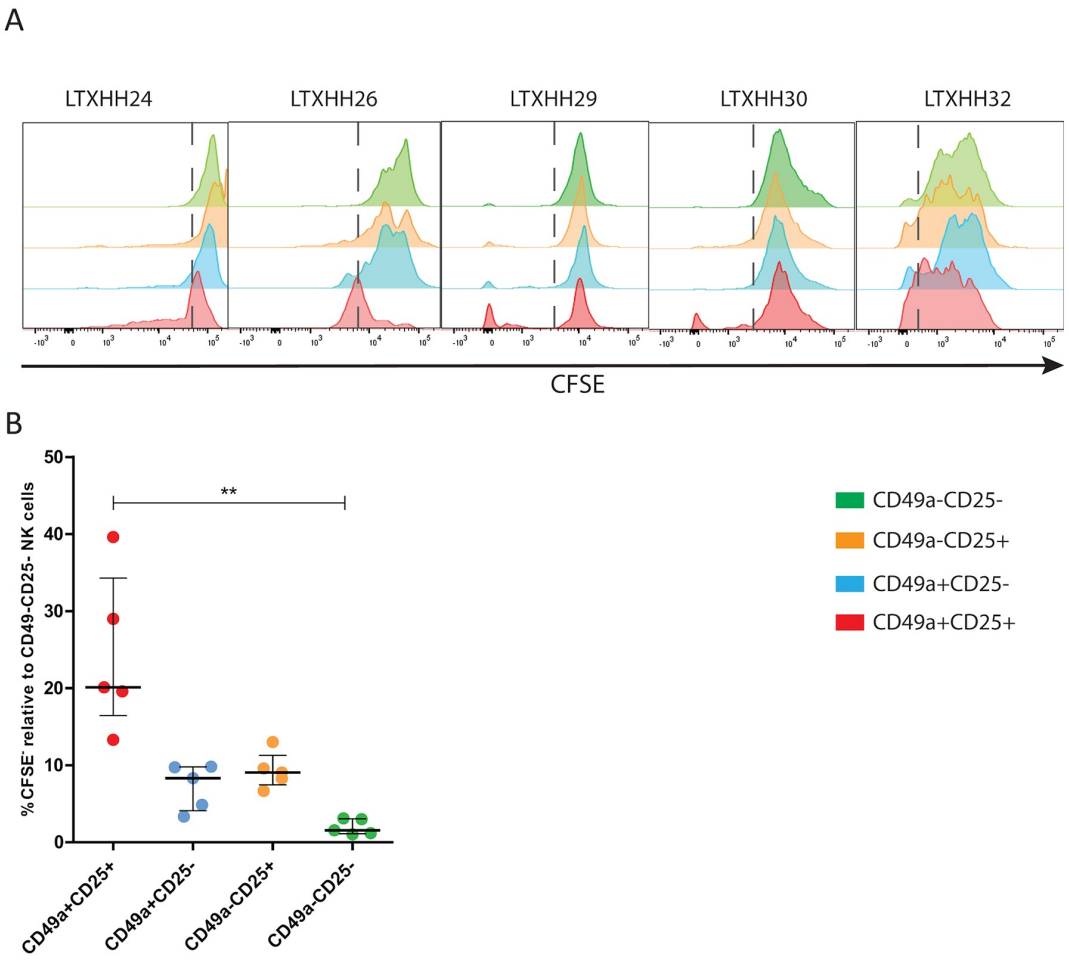

Fig 5. Functional assessment of liver-resident CD49a+ CD25 ihNK cells. (A) Representative histograms 7 days post-cell tracing with CFSE on 5 livers, LTXHH24, LTXHH26, LTXHH29, LTXHH30 and LTXHH32 (gated on live cells). Intrahepatic NK cells were sorted using a BD FACSARIA FUSION according to their expression levels of CD49a and CD25. Recovered cells were CFSE-cell stained and cultured for 7 days with low amounts of IL-2 (red: CD49a+CD25+, blue: CD49a+CD25-, orange: CD49a-CD25+, green: CD49a-CD25-). **(B)** Summary of the cell proliferation data from the four sorted populations. Percentage of CFSE+ cells was determined by setting the gate on the lower part of the CD49a-CD25- population (in green) as shown by the dashed lines. Data in **(B)** is depicted as scatter plot showing all individuals, the bar represents the median and the deviation is depicted as interquartile range.

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tonsils [6, 8, 35–37]. In line with these studies, we observed that the CD56^{bright} NK cell compartment included a high proportion of CD49a+ cells in cirrhotic explant livers. While CD56^{dim} NK cells in these livers also contained CD49a+ cells, this was to a much lesser extent. In cirrhotic livers, the proportions of CD49+ cells in liver-derived CD56^{bright} and CD56^{dim} NK cells, were higher compared to the tumor-free liver resections. This high frequency of CD49a+ NK cells in liver-derived NK cells observed in the cirrhotic livers compared to liver resections might be in part a consequence of the advanced fibrosis stages in the liver transplant samples investigated. Early studies have shown that advanced stages of liver fibrosis are associated with the accumulation of hepatic extracellular matrix (ECM) secreted by hepatic stellate cells (HSCs) [38]. ECM is rich in fibrillary collagens, which act as a ligand for CD49a [39–42] and

CD49a+ NK cells might therefore be preferentially recruited to inflamed and fibrotic livers and accumulate in these tissues.

The factors that enable CD49a+ NK cells to persist or expand in livers remain unknown. Recently, it was described that $CD56^{bright}$CD25+ decidual NK cells are trafficked towards the maternal/fetal interface in early stages of pregnancy depending on CXCR4 expression [43]. In line with those results, we observed that liver-resident CD49a+ NK cells harbored high proportions of cells expressing CD25. CD25 was not present on peripheral blood NK cells in our study, which is in line with previous studies [44]. CD49a+CD25+ NK cells exhibited a higher proliferative capacity *in vitro* compared to CD49a-CD25+, CD49a-CD25- and CD49a+CD25- NK cells when stimulated with low doses of IL-2. These data suggested that liver-resident NK cells expressing CD25 have a lower activation threshold for IL-2 stimulation when compared to non-liver-resident NK cells, potentially enabling these cells to locally expand during inflammation and cirrhotic processes. A recent study demonstrated in liver biopsies that IL-2 protein levels were significantly higher (almost 5 times) in ALD and PBC samples compared to HCV-induced cirrhotic livers and healthy liver tissue [30]. Nonetheless, and potentially due to the small amount of samples per disease, the presence of CD49a+CD25+ ihNK cells in the liver transplantation cohort was not significantly different when comparing different diseases settings. During inflammatory processes, IL-2 is synthesized in secondary lymphoid organs, such as lymph nodes, primarily by CD4+ T helper (T_H) cells, but also CD8+ T cells, NK cells and dendritic cells (DCs) contribute to its production [44–48]. In liver diseases, the production of IL-2 is increased in liver tissues as well as in serum (soluble IL-2), and is considered a biomarker for poor prognosis [49, 50]. In response to liver injury, Kupffer cells (KCs) produce IL-15, IL-12, IL-18 and TNFα to modulate the survival and activity of NK cells [51]. Additionally, overexpression of CD25 on NK cells has been described *in vitro* to be triggered by IL-15 plus IL-18 or IL-12 plus IL-18, increasing their functionality and proliferative capacity in response to picomolar concentrations of IL-2 [52]. In summary, our data suggests that CD25 expression by liver-resident CD49a+ NK cells might be a response to the inflammatory cytokines produced in inflamed livers.

Early studies described the presence of Pluripotent Stem Cells (PSCs) in liver adult mice models [53]. During human NK cell development, CD34 is expressed on NK cell precursors up to stage 2, a versatile precursor of T, DC and NK cells. In stage 3, NK cells loose CD34 expression and are already defined as committed NK cell development intermediates, as they are not able to mature into other immune cells, such as T or DC cells, *in vitro* [29, 54]. Hematopoiesis has shown to take place in the liver during the first trimester of fetal development [55, 56]. Using healthy human livers, previous studies demonstrated the presence of hematopoietic precursor cells bearing CD34 and CD45, with approximatively 37% of these CD34+ cells also expressing CD56 [56, 57]. Human hepatic NK cell progenitors were suggested to be recruited from the circulating peripheral lymphocyte population and were capable to differentiate into functional mature NK cells [58]. In line with these studies, we observed the presence of CD34+ NK cells exclusively within the CD49a+ ihNK cell population, representing around 18% of the total CD49a+ NK cell population. Using Boolean gating, we showed that the simultaneous presence of CD34 and CD25 on CD49a+ NK cells was low (1.64%), indicating that a very small population of NK cell progenitors would immediately respond to low concentrations of IL-2. All together, our results suggested two plausible hypotheses to explain the presence of CD49a+CD34+CD25- NK cells. Peripheral CD34 + NK cells might be recruited in the adult inflamed liver, and expression of CD49a in response to liver collagen retains them in the tissue to generate novel populations of NK cells. Alternatively, CD49a+CD34+CD25- NK cells might be retained in the liver tissue since fetal hematopoiesis, and might play a role in the generation of NK cell progenitors in

adult liver. Whether CD49a+CD34+CD25- NK cells originate in the liver or are recruited to it remains to be elucidated.

In the liver, CXCR3-ligands are produced by hepatic sinusoidal endothelial cells, by activated KCs and by infiltrating leukocytes in response to IFNγ and TNFα stimulation [59–63]. In chronic liver diseases, the interferon gamma inducible protein-10 (IP-10), MIG and I-TAC, ligands for CXCR3, are overexpressed in liver tissues, leading to the recruitment of effector cells towards the liver. It has been suggested that liver-homing NK cells express high levels of CXCR3 in response to IP-10 microenvironment concentrations and that $CD56^{bright}$CXCR3 + NK cells are functionally impaired and expanded in peripheral blood [64]. Our results using inflamed explant liver tissues demonstrate that CXCR3 was indeed significantly upregulated on CD49a+ liver-resident NK cells.

In conclusion, our data show that intrahepatic NK cells differ phenotypically from peripheral NK cells in individual-matched samples and that liver-resident CD49a+ NK cells additionally express exclusively CD25 or CD34. Indeed, the presence of liver-resident CD49a+CD34 + and CD49a+CD25+ NK cells demonstrate different phenotypical and functional features of liver-resident NK cells compared to peripheral blood NK cells including potential self-renewal and persistence.

Supporting information

S1 Fig. Gating strategy for the identification of ihNK (A) and pNK (B) cells. Representative contour plot for the identification of NK cells from liver samples. Lymphocytes were identified with CD45 after an initial gating on Forward (FCS-Area) and Sideward Scatter (SSC-Area) with a subsequent exclusion of doublets (FSC Width and SSC Width). NK cells were defined as CD3-CD14-CD19-CD56+CD16+/- lymphocytes. Zombie aqua was used for the exclusion of dead cells.
(TIF)

S2 Fig. Immunophenotyping of liver and peripheral NK cells. Proportion of $CD56^{dim}$, $CD56^{bright}$ and $CD16^{+}CD56^{-}$ NK cells within the intrahepatic and peripheral blood NK cells compartment in **(A)** liver transplantation cohort and **(B)** tumor-free resection cohort. (**C, D**) $CD56^{bright}$ and $CD56^{dim}$ NK cells immunophenotyping from the liver transplantation cohort with the shown markers. **(C)** On $CD56^{bright}$ NK cells, the following markers were observed: CD49a ($CD56^{bright}$ pNK median (IQR): 3.2 (1.5–6.9); $CD56^{bright}$ ihNK median (IQR): 41.2 (34.1–50.9); $p < 0.0001$), CD34 ($CD56^{bright}$ pNK median (IQR): 4.1 (1.3–6.2); $CD56^{bright}$ ihNK median (IQR): 10.8 (4.5–16.8); $p = 0.0008$), DNAM-1 ($CD56^{bright}$ pNK median (IQR): 74.8 (56.7–87.5); $CD56^{bright}$ ihNK median (IQR): 15.6 (7.6–20.8); $p<0.0001$) and CXCR4 ($CD56^{bright}$ pNK median (IQR): 17 (1.4–26.6); $CD56^{bright}$ ihNK median (IQR): 3.2 (0.6–6.7); $p = 0.0004$) when comparing $CD56^{bright}$ ihNK and pNK cells. **(D)** Similarly, on $CD56^{dim}$ NK cells, the following markers were observed: CD49a ($CD56^{dim}$ pNK median (IQR): 0.4 (0.2–2.8); $CD56^{dim}$ ihNK median (IQR): 27.1 (19–35.3); $p < 0.0001$), CD34+ cells ($CD56^{dim}$ pNK median (IQR): 1.9 (0.4–4.2); $CD56^{dim}$ ihNK median (IQR): 27.1 (5.1–14.2); $p = 0.0001$), DNAM-1+ cells ($CD56^{dim}$ pNK median (IQR): 82.2 (51.9–77.4); $CD56^{dim}$ ihNK median (IQR): 51.1 (26.1–67); $p = 0.0001$) and CXCR4+ cells ($CD56^{dim}$ pNK median (IQR): 7.8 (2.9–22); $CD56^{dim}$ ihNK median (IQR): 2.8 (1.5–5.6); $p = 0.0024$) when comparing $CD56^{dim}$ ihNK and pNK cells. Data is depicted as scatter plot, with each dot corresponding to a participant. Bars indicate median and IQR. Wilcoxon signed rank tests with adjustment of p-values by false discovery rate.
(TIF)

S3 Fig. Gating strategy of intrahepatic (A) CD49a+ and (B) CD49a- NK cells for CD25, CXCR3 and CD34 markers. Following the identification shown in S1 Fig, characterization of **(A)** CD49a+ and **(B)** CD49a- was performed. Representative contour plots are shown.
(TIF)

S4 Fig. Immunophenotyping of intrahepatic CD49a+ and CD49a+- NK cells derived from the liver transplantation cohort on $CD56^{bright}$ and $CD56^{dim}$ NK cells. (A) $CD56^{bright}$ ihNK showed the following proportions for CD25+ (CD49a+$CD56^{bright}$ NK cell median (IQR): 13.5 (7.3–26.3); CD49a- $CD56^{bright}$ NK cell median (IQR): 2.3 (1.9–7.7); $p<0.0001$), CD34+ (CD49a+ $CD56^{bright}$ NK cell median (IQR): 15.4 (8.5–22.7); CD49a-$CD56^{bright}$ NK cell median (IQR): 4.7 (3.4–14.2); $p = 0.0030$) and CXCR3+ (CD49a+ $CD56^{bright}$ NK cell median (IQR): 15.6 (11.8–29.6); CD49a- $CD56^{bright}$ NK cell median (IQR): 4.8 (3.1–14); $p = 0.0004$) in CD49a + ihNK cells when compared to CD49a- ihNK cells. **(B)** As for $CD56^{dim}$ NK cells, the data also displayed the following proportions of CD25+ (CD49a+$CD56^{dim}$ NK cell median (IQR): 12.4 (7.5–23.4); CD49a- $CD56^{dim}$ NK cell median (IQR): 2.4 (1.9–3.9); $p<0.0001$), CD34+ (CD49a +$CD56^{dim}$ NK cell median (IQR): 14.8 (9.6–23.5); CD49a- $CD56^{dim}$ NK cell median (IQR): 6 (4.2–14.7); $p = 0.0027$), and CXCR3+ (CD49a+$CD56^{dim}$ NK cell median (IQR): 7 (2.2–15.1); CD49a- $CD56^{dim}$ NK cell median (IQR): 2.4 (1.1–6.2); $p = 0.0184$) cells in the CD49a+ intrahepatic subset compared to the CD49a- intrahepatic subset. Data is depicted as scatter plot, with each dot corresponding to a participant. Bars indicate median and IQR. Wilcoxon signed rank tests with adjustment of p-values by false discovery rate.
(TIF)

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2. ZUSAMMENFASSENDE DARSTELLUNG

2.1. Einleitung

Natürliche Killer (NK)-Zellen nehmen eine essentielle Rolle in der angeborenen Abwehr gegen virusinfizierte und maligne veränderte Zellen ein. In Abhängigkeit von der Expression des Oberflächenmarkers CD56 können NK-Zellen in $CD56^{bright}$, $CD56^{dim}$ und $CD56^{neg}$ CD16+ NK-Zellen unterteilt werden (Jost and Altfeld 2013). In der Leber repräsentieren NK-Zellen mit bis zu 40% den größten Anteil der intrahepatischen Lymphozyten (IHL) (Racanelli and Rehermann 2006). Im peripheren Blut sind es hingegen zwischen 5-15% der mononukleären Zellen (PBMC) (Hudspeth, Donadon et al. 2016). In vorherigen Studien wurde beschrieben, dass NK-Zellen im Rahmen von chronisch inflammatorischen Immunreaktionen sowohl fibrosierenden Prozessen vorbeugen, als auch Leberschäden verursachen können (Dunn, Brunetto et al. 2007, Gur, Doron et al. 2012). In parabiotischen Mausmodellen konnte gezeigt werden, dass CD49a+DX5- NK-Zellen in der Leber residieren (Peng, Jiang et al. 2013). In der humanen Leber repräsentieren NK-Zellen hingegen eine sehr heterogene Population. Zum einen konnten verschiedene Oberflächenmarker, wie CD49a (Marquardt, Beziat et al. 2015), CXCR6 (Stegmann, Robertson et al. 2016), CD49e (Aw Yeang, Piersma et al. 2017), CCR5 und CD69 (Hudspeth, Donadon et al. 2016) als Residenzmarker auf humanen NK-Zellen charakterisiert werden und zum anderen wurden auch verschiedene Transkriptionsfaktorprofile für leberresidente NK-Zellen (lrNK) identifiziert (Cuff, Robertson et al. 2016, Stegmann, Robertson et al. 2016, Lunemann, Martrus et al. 2017). Jedoch sind bislang die funktionellen Eigenschaften von lrNK nicht hinreichend untersucht.

In unserer Studie haben wir die Differenzierung und das „Homing-Potential“ von lrNK näher untersucht. Dahingehend lag der Fokus auf verschiedenen Rezeptoren wie z.B. CD25 (Marker für die Aktivierung), CD34 (Marker für die Differenzierung), CXCR3 (Marker für Migration) sowie CD49a (Residenzmarker). Unser Ziel war es, aufzuzeigen, welche Marker insbesondere von CD49a+ lrNK exprimiert werden. In diesem Zusammenhang konnten wir zeigen, dass im Rahmen von fortgeschrittenen Lebererkrankungen vorrangig CD49a+CD25+ lrNK in der humanen Leber

nachzuweisen sind und diese *in vitro* Abhängigkeit von exogenen, niedrigdosierten Interleukin-2 (IL-2) ein proliferatives Potential aufweisen. Zudem koexprimierten CD49a+ lrNK sowohl CD34, einen Marker für die Differenzierung, als auch CXCR3, einen Marker für Migration. Die Ergebnisse suggerieren, dass sowohl die proliferativen Eigenschaften als auch die Persistenz der genannten NK-Zellpopulationen eine wichtige Rolle bei inflammatorischen Prozessen der Leber spielen.

2.2. Material und Methoden

Es wurde eine prospektive Querschnittstudie mit einer Kohorte aus volljährigen Patienten (im Folgenden als LTX-Kohorte bezeichnet) durchgeführt, die im Rahmen einer Lebertransplantation (LTX) am Universitätsklinikum Hamburg-Eppendorf (UKE), sowohl periphere Blutpräparate als auch explantiertes Lebergewebe zur Verfügung gestellt haben. Zudem wurden auch tumorfreie Leberproben (nach Tumorresektion bei Hepatozellulärem Karzinom) sowie periphere Blutpräparate von volljährigen Patienten aus der Asklepios-Klinik Barmbek in die Studie mit eingeschlossen. Alle Patienten haben dahingehend ihr schriftliches Einverständnis nach den vorgeschriebenen Richtlinien der Ethikkommission erteilt.

Die Blutpräparate wurden durch ein Ficoll-basierendes Dichtegradienten-zentrifugationsverfahren separiert, um die PBMC zu erhalten. Die IHL wurden aus den Leberproben durch ein in unserem Labor etabliertes Protokoll isoliert. Abschließend wurden die PBMC und IHL mit monoklonalen Antikörpern gefärbt und mittels Durchflusszytometrie (FACS) analysiert. Die Auswertung der Daten erfolgte mit FlowJo Software Version 10.

Für die Zellseparation verwendeten wir frisch isolierte IHL. Die IHL wurden mit monoklonalen Antikörpern gefärbt und anschließend an Hand der spezifischen Expressionsmuster mit dem BD FACSAria™ Fusion in 4 Subpopulationen separiert (CD49a-CD25-, CD49a-CD25+, CD49a+CD25- sowie CD49a+CD25+). Die Untersuchung des proliferativen Potentials der separierten IHL wurde mittels CFSE Proliferations-Assay etabliert. Am Tag 7 erfolgte die Messung der CFSE Fluoreszenz mit dem LSR Fortessa (BD Biosciences).

Die Visualisierung der Daten erfolgte unter anderem mit der t-SNE (T-distributed Stochastic Neighbor Embedding) Analyse. Dafür wurden Daten von ihNK und pNK von 19 Spendern, unabhängig und zufällig, zu jeweils 100.000 Events zusammengefügt. Abhängig vom Expressionsmuster konnten dann verschiedene Abbildungen mittels „R package ggplot2“ erstellt werden. Für die statistischen Methoden wurde der Wilcoxon-Vorzeichen-Rang-Test verwendet. Eine statistische Signifikanz ergab sich bei einem Falscherkennungsrate-adjustiertem p-Wert von <0.05.

2.3. Ergebnisse

2.3.1. Intrahepatische NK-Zellen (ihNK) unterscheiden sich phänotypisch von NK-Zellen aus dem peripheren Blut (pNK).

Zur Bestätigung der Hypothese, dass sich ihNK von pNK phänotypisch signifikant unterscheiden, wurden die gepaarten Leber- und Blutpräparate der Spender aus dem UKE und der Asklepios-Klinik Barmbek in Bezug auf die Expression von Zytokinrezeptoren, Integrinrezeptoren und Aktivierungsmarkern untersucht.

Die vergleichende t-SNE Analyse konnte zeigen, dass sich ihNK von pNK, bis auf vereinzelte Kolokalisationen, in Hinblick der Rezeptorexpressionen unterscheiden. Insbesondere die Expression von CD56 und CD16 war wie bereits angenommen unterschiedlich. So konnten in den Lebern der LTX-Kohorte, im Vergleich zum peripheren Blut, ein signifikant höherer Anteil an $CD56^{bright}$ NK-Zellen und ein signifikant geringerer Anteil von $CD56^{dim}$ NK-Zellen nachgewiesen werden. Dies ist vereinbar mit vorherigen Studien (Marquardt, Beziat et al. 2015, Hudspeth, Donadon et al. 2016).

Darüber hinaus bestätigte sich, dass, wie auch in anderen Studien gezeigt, CD49a ausschließlich auf NK-Zellen in der Leber exprimiert wird (Peng, Jiang et al. 2013, Marquardt, Beziat et al. 2015). Zudem zeigten sich auf den ihNK Koexpressionen von CXCR3, CXCR4, CXCR6 sowie CD25 und CD34. Die statistischen Resultate bestätigten einen signifikant höheren Anteil an CD34+ NK-Zellen sowie einen signifikant niedrigeren Anteil an CD57 und DNAM-1 exprimierenden ihNK im Vergleich

zum peripheren Blut, was mit einem niedrig differenzierten Phänotypen vereinbar wäre. Zudem war auch die Expression von CXCR4 signifikant weniger repräsentiert auf den ihNK. Interessanterweise wiesen die ihNK der LTX-Kohorte eine signifikant höhere Frequenz von CD49a+ ihNK verglichen mit den ihNK-Zellen aus den tumorfreien Leberresektaten. Des Weiteren zeigte sich eine signifikant höhere Expression von CD49a auf unreifen $CD56^{bright}$ NK-Zellen im Vergleich zu reiferen $CD56^{dim}$ NK-Zellen.

Zusammenfassend wiesen die ihNK einen eher unreifen Differenzierungsgrad auf. Insbesondere zirrhotisch veränderte Lebern sind verbunden mit einer hohen Expression von CD49a auf ihNK.

2.3.2. Leberresidente CD49a+ NK-Zellen (lrNK) offenbaren einen unreifen Phänotypen und zeigen selbsterneuernde und proliferative funktionelle Potentiale.

CD49a ist ein Alpha 1-Integrin und bildet zusammen mit CD29 (Beta 1-Integrin) den VLA-1 Komplex. Die Funktionalität des VLA-1 Komplexes spielt eine entscheidende Rolle bei der Migration und Residenz von Zellen. Insbesondere bei der Untersuchung von gewebespezifischen Markern auf NK-Zellen rückte CD49a in den vergangenen Jahren verstärkt in den Fokus. So konnte zum einen in der murinen Leber (Peng, Jiang et al. 2013) als auch in der humanen Leber (Marquardt, Beziat et al. 2015) CD49a als Residenzmarker beschrieben werden. Darüber hinaus wird dieser Marker auch von NK-Zellen in anderen humanen Organen exprimiert (Bjorkstrom, Ljunggren et al. 2016). Im peripheren Blut wiesen NK-Zellen hingegen keine Expression von CD49a auf.

In unserer Studie untersuchten wir CD49a+ und CD49a- ihNK (aus der LTX-Kohorte) im Hinblick auf verschiedene Oberflächenrezeptoren, um im weiteren Verlauf die CD49a+ ihNK bzw. lrNK detaillierter zu untersuchen. In der t-SNE Analyse ergaben sich zwei verschiedene Zellkompartimente mit unterschiedlichen Rezeptor-expressionsmustern. Insbesondere die Marker CD34, CD25, CXCR3 und CXCR4 waren auf den CD49a+ ihNK höher exprimiert als auf den CD49a- ihNK. Die signifikant

höhere Expression von CD34, CD25 und CXCR3 auf CD49a+ ihNK bestätigte sich durch unsere statistischen Analysen. Für weitere Untersuchungen fokussierten wir uns auf den Marker CD34, welcher vorrangig auf hämatopoetischen Stammzellen exprimiert wird und einen unreifen Phänotypen der Zelle repräsentiert (Freud, Yu et al. 2014) sowie auf CD25, einen hochaffinen Rezeptor für IL-2. Diese Erkenntnisse suggerieren, dass CD49a+ ihNK zum einen ein selbsterneuerndes Potential bergen (CD34) und zum anderen im Rahmen von inflammatorischen Prozessen ein hohes proliferatives Potential aufweisen (CD25).

Fortführend nutzten wir basierend auf den drei Markern (CD34, CD49a, CD25) das „Boolean gating“, um die Koexpressionsmuster auf den CD49a+ ihNK aufzuzeigen. Die Analyse konnte zeigen, dass CD49a+, bei Ausschluss der dreifach negativen Population, auf 87% der ihNK exprimiert wird. Somit kann CD49a als Signaturmarker zur Differenzierung von ihNK verstanden werden. Interessanterweise waren zudem die Marker CD34 und CD25 gehäuft koexprimiert auf CD49a+ ihNK (10,2 % vs. 6,03%). Hingegen war die Single-Expression der besagten Marker auf CD49a+ ihNK weitaus geringer (9,9% vs. 2,4%) und der Anteil der CD49a-CD34+CD25+ ihNK lag nur bei 0,59%. Zur Evaluation, ob die Anwesenheit von bestimmten NK-Zellpopulationen in der Leber abhängig ist vom Ausmaß der inflammatorischen Prozesse, korrelierten wir die Level der Alanin-Aminotransferase = ALT (als Zeichen der Leberzellschädigung) mit dem Anteil der jeweiligen NK-Zellpopulation. Final ergab sich daraus ausschließlich in der CD49a+CD25+ ihNK-Zellpopulation eine positive Korrelation.

Nach dem Erkenntnisgewinn, dass CD25+ nahezu ausschließlich auf CD49a+ ihNK vermehrt koexprimiert wird und wegen der positiven Korrelation der CD49a+CD25+ ihNK mit den ALT-Leveln, evaluierten wir die funktionellen Eigenschaften dieser NK-Zellpopulation. Die NK-Zellen wurden aus 5 Spenderlebern in Abhängigkeit ihrer Expression von CD49a und CD25 selektiert. Die sich daraus ergebenden 4 NK-Zellpopulationen (Siehe Methoden und Material) wurden mit Hilfe von CFSE markiert und 7 Tage mit niedrig dosiertem IL-2 kultiviert. Einzig die CD49a+CD25+ ihNK zeigten eine signifikant erhöhte Proliferationsrate im Vergleich zu den anderen NK-Zellpopulationen.

Daraus konnte geschlussfolgert werden, dass CD49a+CD25+ NK-Zellen, im Vergleich zum peripheren Blut, eine höhere Prävalenz in inflammatorisch veränderten Lebern aufweisen und zudem in Abhängigkeit zu einer niedrig dosierten IL-2 Exposition das Potential zur Proliferation besitzen. Dies scheint die hohe Prävalenz im Rahmen von inflammatorischen Prozessen der Leber zu erklären. Die CD49a+CD34+ ihNK hingegen stellen eher gering differenzierte Zellen mit einem hämatopoetischen Potential dar. In Hinblick auf vorangegangene Studien könnten sie auch ein Indiz für die intrahepatische Entwicklung von NK-Zellen aus NK-Progenitorzellen sein (Renoux, Zriwil et al. 2015).

2.4. Diskussion

Vorherige Studien in Maus- und humanen Modellen konnten zeigen, dass ihNK eine, im Vergleich zu pNK, heterogene Population mit spezifischen Expressionen von Oberflächenmarkern und Transkriptionsfaktoren darstellt (Peng, Jiang et al. 2013, Sojka, Plougastel-Douglas et al. 2014, Marquardt, Beziat et al. 2015, Harmon, Robinson et al. 2016, Hudspeth, Donadon et al. 2016). Insbesondere der Residenzmarker CD49a, der vor allem auf $CD56^{bright}$ NK-Zellen exprimiert wird, stellt einen der wesentlichen Marker zur Identifizierung von gewebespezifischen NK-Zellen in diversen humanen Geweben dar (Fuchs, Vermi et al. 2013, Marquardt, Beziat et al. 2015, Montaldo, Vacca et al. 2015). In Übereinstimmung mit diesen Studien konnte in unserer Studie gezeigt werden, dass CD49a auf $CD56^{bright}$ NK-Zellen in zirrhotisch veränderten Lebern höher exprimiert wird als in tumorfreien Leberresektaten. Die erhöhte Expression von CD49a in zirrhotisch veränderten Lebern mag damit begründet sein, dass im Rahmen der Leberzirrhose ein Remodeling der Extrazellulären Matrix (EZM) stattfindet (Wells 2008). Ein wesentlicher Bestandteil der EZM ist das Kollagen, welches gleichzeitig als Ligand für CD49a fungiert (MacDonald, Horton et al. 1990). CD49a+ ihNK könnten daher in inflammatorisch sowie zirrhotisch veränderte Lebern rekrutiert werden und dort residieren. Welche Einflüsse es ermöglichen, dass CD49a+ NK-Zellen in der Leber residieren, ist bislang unklar.

Tao und Li et al. berichteten in Ihrer 2015 erschienen Publikation, dass $CD56^{bright}$ CD25+ deziduale NK-Zellen in Abhängigkeit von CXCR4 in frühen Stadien der

Schwangerschaft zum uteroplazentaren Übergang migrieren können (Tao, Li et al. 2015). In unserer Studie konnten wir zeigen, dass CD49a+ lrNK eine hohe Expression von CD25 aufwiesen. Hingegen zeigte sich keine Expression von CD25 auf pNK. Zudem offenbarten ausschließlich CD49a+CD25+ lrNK ein proliferatives Potential gegenüber niedrigen Mengen von IL-2, was bedeutet, dass die Stimulationsschwelle von CD49a+CD25+ lrNK geringer ist im Vergleich zu nicht lrNK. So ist es schlussfolgernd möglich, dass CD49a+CD25+ lrNK das Potential besitzen, im Rahmen von inflammatorischen und zirrhotischen Prozessen zu proliferieren. Hauptsächlich während inflammatorischer Prozesse kommt es zu einer vermehrten Freisetzung von Interleukin 2 durch CD4+ T-Helferzellen, CD8+ T-Zellen, NK-Zellen und Dendritische Zellen (Granucci, Vizzardelli et al. 2001, Setoguchi, Hori et al. 2005, Malek, Yu et al. 2008, Boyman and Sprent 2012). Zudem können Kupffer-Zellen im Rahmen der Leberzellschädigung IL-12, IL-15, IL-18 und TNF-α produzieren und somit die Aktivität sowie das Überleben von NK-Zellen wesentlich beeinflussen (Li and Diehl 2003). Darüber hinaus konnte *in vitro* gezeigt werden, dass bestimmte Interleukin-Konstellationen, wie z.B. IL-12 und IL-18 sowie IL-15 und IL-18 voraktivierte NK-Zellen nach zusätzlicher Stimulation mit pikomolaren Konzentrationen von IL-2, eine Überexpression von CD25 auf NK-Zellen hervorrufen können und somit die Funktionalität und proliferativen Potentiale steigern (Leong, Chase et al. 2014). Schlussfolgernd kann die Expression von CD25 auf CD49a+ lrNK die Antwort auf die Zytokinproduktion in inflammatorisch veränderten Lebern darstellen.

Die NK-Zell-Differenzierung geht mit einem Verlust des hämatopoetischen Stammzellmarkers CD34 einher, sodass NK-Zellen sich nicht mehr in andere Zelllinien differenzieren können. Frühere Studien in gesunden Lebern zeigten zum einen, dass die Hämatopoese bereits im ersten Trimester der embryonalen Entwicklung in der Leber stattfindet und ca. 37% der CD34+ und CD45+ Vorläuferzellen auch den Oberflächenmarker CD56 exprimieren (Crosbie, Reynolds et al. 1999, Golden-Mason, Curry et al. 2000). Übereinstimmend mit diesen Studien konnten wir nachweisen, dass 18% der CD49a+ lrNK CD34 koexprimieren. Hingegen war die gleichzeitige Koexpression mit CD25 sehr gering (1,64%). Dies suggeriert, dass nur ein sehr kleiner Anteil CD49a+CD34+ lrNK in Abhängigkeit von niedrig dosierten IL-2 proliferieren kann.

Unsere Ergebnisse bringen zwei plausible Hypothesen für die Existenz von CD49a+CD34+ lrNK hervor. In der ersten Hypothese migrieren CD34+ NK-Zellen aus dem peripheren Blut in die adulte inflammatorisch veränderte Leber und exprimieren dort in Abhängigkeit von Kollagen CD49a, um in der adulten Leber als neue Population zu residieren. In der zweiten Hypothese residieren CD49a+CD34+ NK-Zellen bereits in der frühen embryonalen Entwicklung in der Leber und generieren im späteren Verlauf eine Art Vorläuferpopulation in der adulten Leber. Welche der beiden Hypothesen am ehesten die Bedeutung dieser Population widerspiegelt wird die Grundlage für weitere Untersuchungen sein.

Für die Investigation des Homing-Potentials von NK-Zellen, evaluierten wir die Expression von verschiedenen Chemokinrezeptoren (CXCR3, CXCR4 und CXCR6).
In vorherigen Studien zeigte sich, dass CXCR3-Liganden von sinusoidalen Endothelzellen in Abhängigkeit von der Stimulation von IFN-γ und TNF-α exprimiert werden (Helbig, Ruszkiewicz et al. 2004). Diese Liganden, wie z.B. das interferoninduzierbare Protein-10 (IP-10), sorgen für eine Migration von CXCR3+ Effektorzellen aus dem peripheren Blut in die Leber. Zudem ging aus einer früheren Studie hervor, dass $CD56^{bright}$ CXCR3+ ihNK bei fortgeschrittenen Fibrosegraden vermehrt in der Leber akkumulieren und in Bezug auf die Degranulation sowie IFN-γ Produktion eine funktionell limitierte Population darstellen (Eisenhardt, Glassner et al. 2012). In unserer Studie konnten wir in der Tat zeigen, dass CXCR3 signifikant höher exprimiert war auf CD49a+ lrNK in inflammatorisch veränderten Leberproben der LTX-Kohorte im Vergleich zu CD49a- ihNK, was suggeriert, dass CXCR3+ NK-Zellen während inflammatorischer Prozesse in der Leber eine wichtige Rolle spielen könnten, da sie das Potential besitzen, in die Leber zu migrieren und daraufhin auch dort zu residieren.

Zusammenfassend konnten unsere Ergebnisse zeigen, dass sich ihNK phänotypisch von pNK in gepaarten Proben (Blut/Leberprobe) unterscheiden und dass CD49a+ lrNK in besonderem Maße CD25 und CD34 exprimieren. Die Existenz von CD49a+CD25+ (prolifertives Potential) und CD49a+CD34+ (selbsterneuerndes Potential) lrNK zeigte zudem unterschiedliche phänotypische und funktionelle Eigenschaften von leberresidenten NK-Zellen im Vergleich zu pNK.

2.5. Zusammenfassung der Ergebnisse

In unserer Studie haben wir die Differenzierung und das „Homing-Potential“ von lrNK näher untersucht. Insbesondere wollten wir die CD49a+ lrNK phänotypisch und funktionell näher charakterisieren. Dahingehend untersuchten wir die Expressionsmuster verschiedener Oberflächenmarker, insbesondere CD56, CD16, CD25, CD34, CD57, DNAM-1, CXCR3, CXCR4 und CXCR6. Wir konnten zeigen, dass sich intrahepatische NK-Zellen von pNK phänotypisch unterscheiden. Insbesondere die Expression von CD56 und CD16 zeigte signifikante Unterschiede. So repräsentieren $CD56^{bright}$ ihNK, im Vergleich zu pNK, den größeren Anteil der NK-Zellen. Hingegen dominieren $CD56^{dim}$ NK-Zellen im peripheren Blut. Auf den ihNK konnten wir, im Vergleich zu pNK, eine signifikant höhere Expression von CD34 sowie eine signifikant geringere Expression von CD57, DNAM-1 und CXCR4 nachweisen. Zusammenfassend stellen ihNK im Vergleich zu pNK eine eher unreife NK-Zell-Population dar. Zudem bestätigte sich, dass CD49a+ ausschließlich auf ihNK und vorrangig auf den $CD56^{bright}$ NK-Zellen exprimiert wird. Darüber hinaus war der Anteil an CD49a+ lrNK in zirrhotischen Lebern signifikant höher als in tumorfreien Leberresektaten. Vergleichende Analysen von CD49a+ lrNK und CD49a- ihNK demonstrierten eine signifikant höhere Koexpression von CXCR3, CD25 und CD34 auf den CD49a+ lrNK-Zellen. Die Population der CD49a+CD25+ lrNK offenbarte *in vitro* nach Stimulation mit niedrig dosierten IL-2 ein exklusives proliferatives Potential. CD49a+ lrNK stellen somit eine Population mit proliferativen und selbsterneuernden Potentialen dar.

2.6. Result summary

In our study we want investigated the maturation and the homing-potential of lrNK cells. Our aim was to define the phenotypical and functional properties of CD49a+ lrNK. Therefore, we used a broad range of receptors, primarily CD56, CD16, CD25, CD34, CD57, DNAM-1, CXCR3, CXCR4 and CXCR6. We revealed that ihNK differ significantly in phenotypical properties from pNK. Especially the expression of CD56 and CD16 were different. $CD56^{bright}$ NK cells represented the main part of ihNK cells in contrast to peripheral blood, where $CD56^{dim}$ NK cells represented the main population. We elucidated that CD34 was significantly higher expressed compared to significant lower expressions of CD57 and DNAM-1 and CXCR4 on ihNK, in contrast to pNK. Taking the results together, ihNK might have a naïve phenotype compared with pNK. In addition, we confirmed that CD49a+ was exclusively expressed on ihNK in contrast to pNK. Interestingly the expression of CD49a+ on lrNK was significantly increased in cirrhotic inflamed livers in comparison to tumor-free liver resections. When we compared CD49a+ and CD49a- ihNK, we found a higher expression of CXCR3, CD25 and CD34 on CD49a+ lrNK. The CD49a+ lrNK revealed exclusively proliferation capacities after stimulation with low amounts of IL-2. The results implicated that CD49a+ lrNK showed unique proliferative and self-renewal properties.

3. ABKÜRZUNGSVERZEICHNIS

ALT:	Alanin-Aminotransferase
CCR:	C-C-Motiv-Chemokin-Rezeptor
CD:	Cluster of differentiation
CFSE:	Carboxyfluorescein succinimidyl ester
CXCR:	C-X-C-Motiv-Chemokin-Rezeptor
DNAM-1:	DNAX accessory molecule-1
DX5:	CD49b / alpha2-Integrin / very late antigen-2
EZM:	Extrazelluläre Matrix
FACS:	Fluorescence-activated cell sorting
IFN-γ:	Interferon-γ
IHL:	Intrahepatische Lymphozyten
ihNK:	intrahepatische Natürliche Killerzellen
IL:	Interleukin
IP-10:	Interferon-γ induziertes Protein - 10
lrNK:	leberresidente Natürliche Killerzellen
LTX:	Lebertransplantation
NK-Zellen:	Natürliche Killerzellen
PBMC:	Mononukleäre Zellen des peripheren Blutes
pNK:	Natürliche Killerzellen im peripheren Blut
TNF-α:	Tumornekrosefaktor-α
t-SNE:	T-distributed Stochastic Neighbor Embedding
UKE:	Universitätsklinikum Hamburg-Eppendorf
VLA-1:	Very late antigen-1

4. LITERATURVERZEICHNIS

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5. ERKLÄRUNG DES EIGENANTEILS

Im Zusammenhang mit meiner Promotionsarbeit habe ich im Vorfeld eine eigenständige Literaturrecherche zur Thematik durchgeführt. Zudem habe ich die Projektskizze für die Promotion eigenständig erstellt. Das Konzept dieser Publikation habe ich in Zusammenarbeit mit Herrn Prof. Dr. med. M. Altfeld und Frau Dr. rer. nat. G. Martrus in Anlehnung an die Projektskizze erarbeitet. Die Erarbeitung des Ethikantrages sowie die damit zusammenhängende Betreuung führte ich zusammen mit Herrn Prof. Dr. med. M. Altfeld durch.

Die Datenerhebung, Organisation und Integration führte ich zusammen mit Frau Dr. rer. nat. G. Martrus, Herrn Dr. rer. nat. S. Lunemann, Herrn Dr. med. univ. W. Salzberger, Frau H. Goebels, Frau Dr. rer. nat. A. Langeneckert, Herr L. Hess sowie Herrn T. Poch M. Sc. durch. Die Analysen wurden in Zusammenarbeit mit Frau Dr. rer. nat. G. Martrus, Frau L. Richert, Frau L. Glau, Frau Prof. Dr. rer. nat. Tolosa und Herrn Prof. Dr. med. M. Altfeld fertiggestellt.

Die Originalarbeit wurde von Frau Dr. rer. nat. G. Martrus, Herrn Prof. Dr. med. C. Schramm, Herrn Prof. Dr. med. K. J. Oldhafer, Frau Prof. Dr. med. M. Koch, Herrn Prof. Dr. med. B. Nashan und Herrn Prof. Dr. med. M. Altfeld erarbeitet. Die Rezension und Korrektur wurde zusätzlich durch meine Person, sowie Herrn Dr. rer. nat. S. Lunemann, Frau L. Richert, Frau L. Glau, Herrn Dr. med. univ. W. Salzberger, Frau H. Goebels, Frau Dr. rer. nat. A. Langeneckert, Herrn L. Hess, Herrn T. Poch M. Sc. sowie Frau Prof. Dr. rer. nat. E. Tolosa vorgenommen.

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7. LEBENSLAUF

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

8. EIDESSTATTLICHE VERSICHERUNG

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Tobias Kautz
