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HIGHLIGHTS

Basic

Frontline | Research Article

Tissue-resident Eomes^{hi} T-bet^{lo} CD56^{bright} NK cells with reduced proinflammatory potential are enriched in the adult human liver

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The adult human liver is enriched with natural killer (NK) cells, accounting for 30–50% of hepatic lymphocytes, which include tissue-resident hepatic NK-cell subpopulations, distinct from peripheral blood NK cells. In murine liver, a subset of liver-resident hepatic NK cells have altered expression of the two highly related T-box transcription factors, T-bet and eomesodermin (Eomes). Here, we investigate the heterogeneity of T-bet and Eomes expression in NK cells from healthy adult human liver with a view to identifying human liver-resident populations. Hepatic NK cells were isolated from donor liver perfusates and biopsies obtained during orthotopic liver transplantation ($N = 28$). Hepatic CD56^{bright} NK cells were Eomes^{hi} T-bet^{lo}, a phenotype virtually absent from peripheral blood. These NK cells express the chemokine receptor CXCR6 (chemokine (C-X-C motif) receptor 6), a marker of tissue residency, which is absent from hepatic CD56^{dim} and blood NK cells. Compared to blood populations, these hepatic CD56^{bright} NK cells have increased expression of activatory receptors (NKp44, NKp46, and NKG2D). They show reduced ability to produce IFN-γ but enhanced degranulation in response to challenge with target cells. This functionally distinct population of hepatic NK cells constitutes 20–30% of the total hepatic lymphocyte repertoire and represents a tissue-resident immune cell population adapted to the tolerogenic liver microenvironment.

Keywords: CD56^{bright} natural killer (NK) cells · Human liver · Interferon · Microenvironment · Tissue resident

See accompanying commentary by Marotel et al.

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Introduction

Natural killer (NK) cells are group 1 innate lymphoid cells, important in antiviral and tumor immunity. In human peripheral blood (PB), two subsets of NK cells are readily distinguished by their expression of CD56, a cell surface glycoprotein. CD56^{bright} NK cells are potent producers of immunoregulatory cytokines, such as interferon (IFN) γ and tumor necrosis factor (TNF) α, and are commonly attributed as the precursor population of the more mature CD56^{dim} NK cells [1, 2]. Upon maturation, CD56^{dim} NK cells gain potent cytotoxic effector functions, and produce

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less cytokines than the CD56^{bright} population [1, 2]. Studies in knockout mice have identified a number of transcription factors essential for the developmental program of NK cells, such as Nfil3, Ets-1, Id2, Id3 [3–5], as well as the two highly related T-box transcription factors, T-bet and eomesodermin (Eomes) [6, 7]. In the bone marrow (BM), T-bet expression is initially repressed, allowing the development of Eomes^{+} NK-cell precursors, with T-bet expression subsequently increased during the final stages of NK-cell maturation [6].

While conventional NK cells circulate in the PB, transiting through several organs, significant NK-cell populations also permanently reside in tissues such as the lung, gut, uterus, and liver and may develop there [8]. Among these tissue-resident NK-cell populations, the role of uterine NK cells in successful pregnancy is well described [9]; however, the functions of other heterogeneous tissue-resident NK-cell populations remain largely unknown. Within these tissue microenvironments, it is evident that the developmental, functional, and phenotypic features of NK-cell subsets are poorly characterized compared to their PB counterparts [10].

The adult human liver contains a unique and extensive lymphoid repertoire. There is a predominance of innate lymphoid cells including NK cells, NKT cells, mucosal-associated invariant T cells and γδ T cells in the liver, in addition to the presence of conventional CD4^{+} and CD8^{+} lymphocytes of the adaptive immune system [11–13]. Over 40% of the hepatic lymphoid population are NK cells, making the liver the most "NK-populated" organ in the body. These human liver NK cells are enriched in CD56^{bright} cells and display a range of cell-surface markers associated with tissue residency including CD103, CD69, and CD49a [13–15]. Hepatic NK cells play important roles in viral and tumor surveillance, as well as tissue homeostasis and immune regulation [16–19]. However, these divergent functions have yet to be attributed to distinct NK cell subsets and it is not clear whether circulating and tissue-resident NK cells are involved in distinct or overlapping immunological roles within the liver.

Murine liver resident NK cells (CD49a^{+} DX5^{-}) have been shown to differentiate independently of Eomes, in contrast to conventional NK cells [7]. These T-bet^{+} Eomes^{-} liver-resident NK cells express a broad range of cytokines and chemokines (interleukin (IL) 2, IL-4, GM-CSF, TNF, CCL3, and IFN-γ) and contribute to the immune response against intracellular pathogens such as *Francisella tularensis* [7]. Expression of the chemokine receptor chemokine (C-X-C motif) receptor 6 (CXCR6) by CD49a^{+} DX5^{-} NK cells is believed to be important for homing to and retention in the liver [20]. A comparable Eomes^{-} CD49a^{+} population of CD56^{bright} NK cells is present at low frequencies in human liver and highly express T-bet, in contrast to conventional NK cells that are Eomes^{+} T-bet^{+} [14]. The human Eomes^{-} CD49a^{+} CD56^{bright} NK-cell population was only identified in 12 of 29 livers examined, making up 0.11–12.7% (average 2.3%) of the total CD3^{-}CD56^{+} lymphocyte population [14]. Despite this low frequency of detection of Eomes^{-} CD49a^{+} NK cells in humans, almost all human hepatic CD56^{bright} NK cells express the chemokine receptor CXCR6 and the tissue-residency marker CD69, enabling them to traffic to hepatic sinusoidal spaces [15]. This indicates that other tissue-resident hepatic NK-cell populations, distinct from the previously described Eomes^{-} CD49a^{+} CD56^{bright} NK cells, exist in humans.

In the present study, we aimed to define the expression of Eomes and T-bet within healthy adult human liver NK-cell populations, with the goal of validating the tissue-resident status of human hepatic CD56^{bright} NK cells. Using LP and biopsies taken from healthy organs during liver transplant, we identified a unique Eomes^{hi} T-bet^{lo} population of CD56^{bright} NK cells in adult human liver. These Eomes^{hi} T-bet^{lo} CD56^{bright} NK cells are phenotypically distinct from PB NK-cell populations, display increased cytotoxic function, and reduced pro-inflammatory cytokine production. We propose that this functionally distinct population of hepatic NK cells is of importance in the context of tissue homeostasis, liver infection, malignancy, and transplantation.

Results

Human hepatic CD56^{bright} NK cells are characterized by increased Eomes and reduced T-bet expression

NK cells account for 51.4 ± 3% ($n = 28$) of LP lymphocytes compared to 36.5 ± 5.8% of lymphocytes from liver biopsies ($p = 0.06$, $n = 5$; using the flow cytometry gating detailed in Fig. 1A). In contrast, NK cells account for only 17.3 ± 2.3% of peripheral lymphocytes in matched blood (Fig. 1B, $n = 28$). CD56^{bright} NK cells were enriched in LP (43.8 ± 2.7%) and biopsy (53.3 ± 7.9%); while significantly fewer CD56^{bright} NK cells were detected in PB (9.4 ± 1.1%) (Fig. 1C). The ratio of CD56^{dim} CD16^{+} to CD56^{bright} CD16$^{+/-}$ NK cells in the perfusate showed high levels of interindividual variation, ranging from 0.3:1 to 4:1, and was distinct from PB where the ratio was usually greater than 10:1. The variation in CD56^{dim}CD16^{+} to CD56^{bright} CD16$^{+/-}$ hepatic NK-cell ratios was not attributable to the age or sex of the organ donor (data not shown).

A novel population of Eomes^{-} NK cells have been described in murine liver and more recently in human liver in both healthy and malignant tissue [14]. In the present study this Eomes^{-} NK-cell population was not detectable in human LP, donor biopsy or PB, although a unique population of Eomes^{hi} T-bet^{lo} NK cells was identified in liver (Fig. 2A). This population accounts for nearly 50% of perfusate NK cells (47.2 ± 3.4%), and 61.7 ± 2.8% of biopsy NK cells (Fig. 2B). Less than 2% of peripheral NK cells express this phenotype (1.8 ± 0.6%) (Fig. 2B). This phenotype is almost exclusively confined to the CD56^{bright} subset. In LP 89.5 ± 1.8% of CD56^{bright} cells are Eomes^{hi} T-bet^{lo}, while only 7.5 ± 1.5% of perfusate CD56^{dim} NK cells possesses this phenotype (Fig. 2E and F). Similarly, 87.5 ± 4% of biopsy CD56^{bright} NK cells were Eomes^{hi} Tbet^{lo}, with only 2.8 ± 1% of CD56^{dim} NK cells sharing this phenotype. Small proportions of blood CD56^{bright} and CD56^{dim} NK cells were also Eomes^{hi} T-bet^{lo} (10.4 ± 2.3% and 1.8 ± 0.5%, respectively). This altered T-bet and Eomes usage is corroborated by quantification of *EOMES* and *TBX21* mRNA

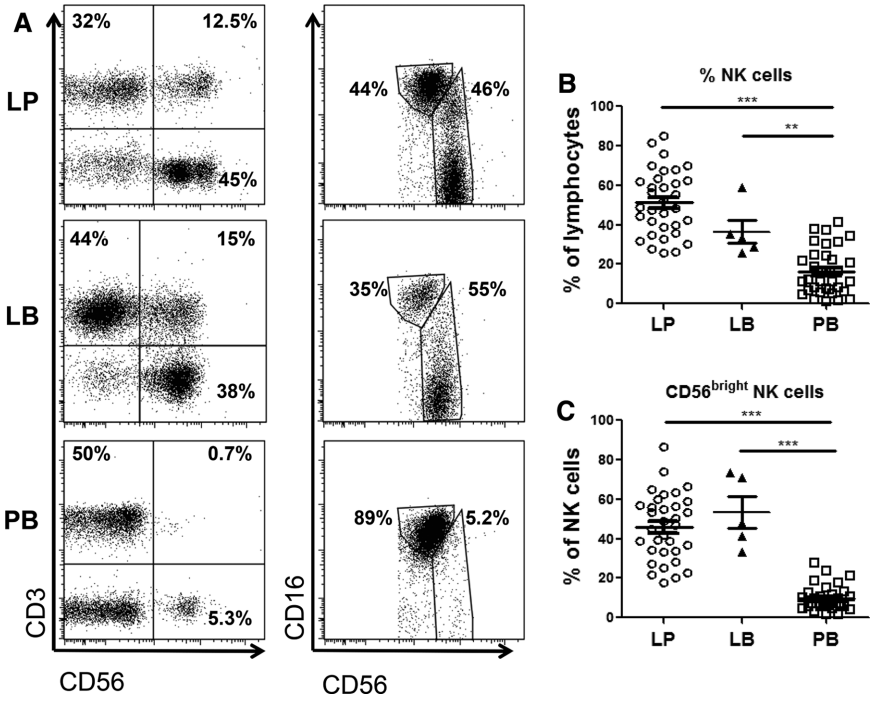

Figure 1. NK cells, in particular CD56bright NK cells, are enriched in LP compared to matched PB. (A) Representative gating strategy for NK cells from LP, matched PB, and donor LB. NK cells were identified from viable CD45+ lymphocytes as CD56+CD3−. NK-cell subsets were identified as CD56bright CD16−/+ and CD56dim CD16+. (B) Total NK cells in LP (o), DB (▲) PB (□) as a percentage of CD45+ lymphocytes. (C) CD56bright CD16−/+ NK-cell frequencies in LP (o), LB (▲) and PB (□) as a percentage of total NK cells. n = 28 LP, n = 5 LB, n = 28 PB samples. (A-C) Data are shown as mean ± SEM and are representative of 28 independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$. Data were analyzed using Kruskal–Wallis test, with Dunn's multiple comparison test.

levels in LP and PB NK cell populations. *EOMES* expression is significantly increased in perfusate NK cells compared to blood cells ($p = 0.002$), while *TBX21* expression was not significantly different ($p = 0.093$) (Fig. 2I). As the NK cell subsets isolated from LP and liver biopsy (LB) samples shared similar phenotypes, LP-derived NK cells were used in all subsequent experiments.

CXCR6 has previously been identified as a marker of tissue resident NK cells in the murine liver. The majority of hepatic CD56^{bright} NK cells express CXCR6 (68.3 ± 8.2%, Fig. 2J, $n = 8$), in contrast to the PB (3.1 ± 2%). This phenotype is not reflected in the CD56^{dim} population that do not express CXCR6 in the liver (1.33 ± 0.28%) or PB (0.6 ± 0.25%, Fig. 2K). This suggests that these hepatic CD56^{bright} NK cells may represent a human-specific tissue resident population.

Hepatic CD56^{bright} NK cells display increased expression of NCRs and NKG2D

We next analyzed NK-cell receptors to determine if hepatic CD56^{bright} NK cells display a unique profile of activatory receptors (Fig. 3). NKG2D, a C-type lectin binding receptor that recognizes stress-induced ligands such as MHC class I polypeptide associated sequence A (MIC-A), MIC-B, and ULBPs, is expressed on the majority of both hepatic and PB NK cells, but is significantly increased in hepatic NK cells with the highest expression in the CD56^{bright} subset (97.5 ± 0.9% vs. 92.6 ± 1.7%, $p = 0.001$, Fig. 3B). The expression of the activatory receptor NKG2C that recognizes human leukocyte antigen E molecules, was significantly lower in CD56^{bright} hepatic NK cells compared to their blood counterparts (8.6 ± 2.3% vs.19.1 ± 6.1%, $p = 0.0017$, Fig. 3C). Expression of the related inhibitory receptor natural-killer group 2, member A (NKG2A), was higher in CD56^{bright} hepatic NK cells compared to matched PB NK cells (13.2 ± 10.5% vs. 5.1 ± 3.8% $p = 0.02$, Fig. 3D).

Hepatic NK cells also have distinct expression of the natural cytotoxicity receptors NKp46 and NKp44. NKp46 is expressed on the majority of both peripheral and hepatic NK cells, although slightly more hepatic CD56^{bright} NK cells express this receptor (93.1 ± 3.7%) than peripheral cells (87.7 ± 2.3%, $p = 0.006$, Fig. 3E). Furthermore, the receptor is expressed at a higher concentration per cell on these cells (MFI 1271 ± 400) compared to peripheral CD56^{bright} NK cells (MFI 884 ± 513, $p = 0.03$). No differences were seen between natural cytotoxicity receptor expression on hepatic and peripheral CD56^{dim} NK cells. NKp44 is an activatory receptor that is not expressed on resting NK cells. Not surprisingly, NKp44 is low or absent on PB NK cells but the NKp44^{+} population is significantly increased in the hepatic CD56^{bright} NK cell population (8.9 ± 3.3%, $p = 0.002$, Fig. 3F) confirming the previously described activated phenotype of hepatic NK cells [13].

Hepatic NK cell populations have less granzyme B but more perforin than peripheral NK populations

To assess whether the cytotoxic potential of hepatic NK cells is altered we performed intracellular staining for granzyme B and perforin. Only 60% of hepatic CD56^{bright} NK cells express granzyme B compared to over 75% in the blood (Fig. 3H), with significantly lower expression of the molecule per cell (MFI 678 ± 306) compared to blood CD56^{bright} cells (MFI 2021 ± 1258, $P = 0.03$, Fig. 3I). In contrast, a significantly higher proportion of hepatic CD56^{bright} NK cells express perforin (92 ± 11%) compared to blood CD56^{bright} NK cells (83.7 ± 11.7%, $P = 0.02$, Fig. 3K). No significant difference was seen in the amount of

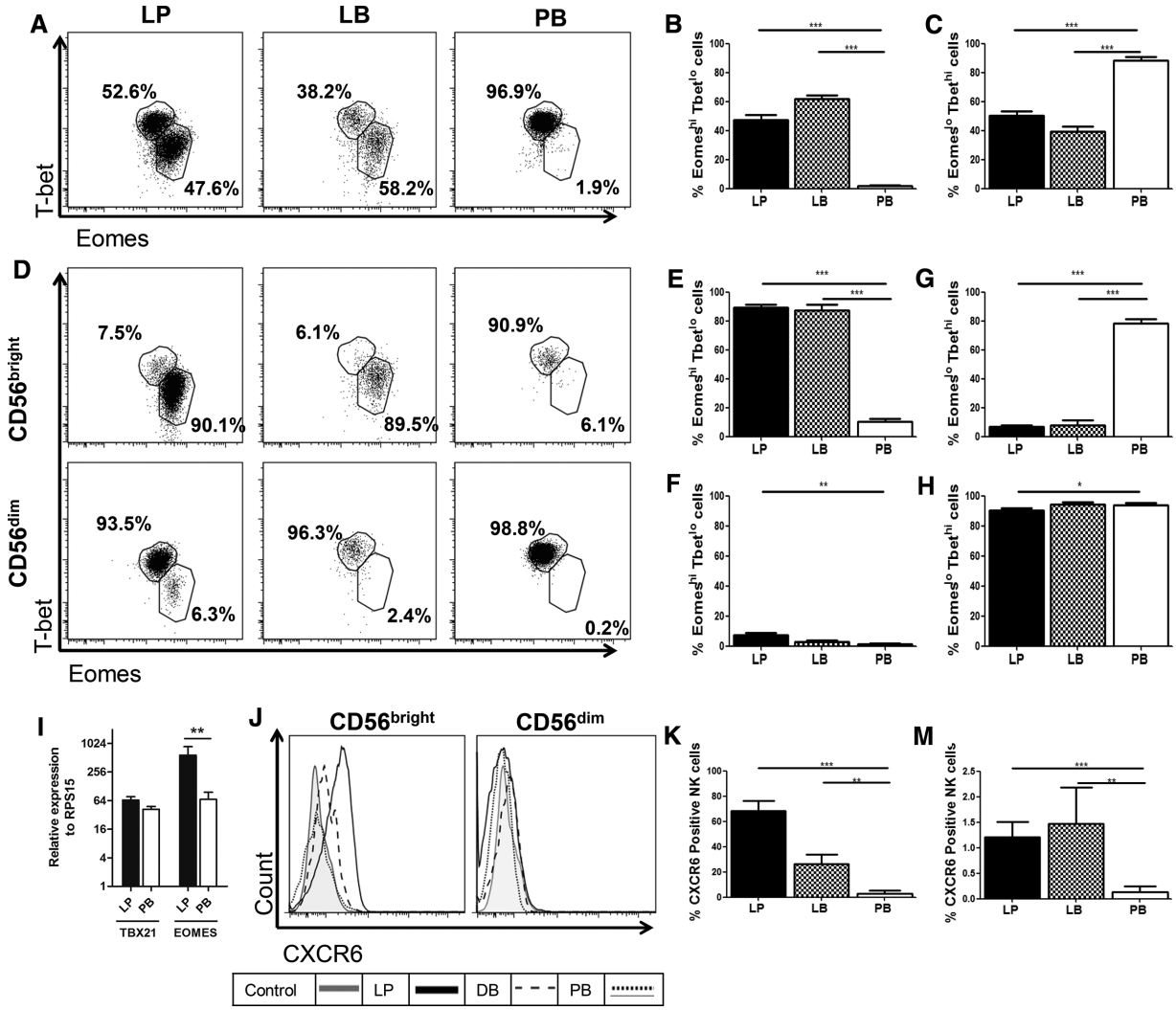

Figure 2. Hepatic CD56^{bright} NK cells have unique transcription factor usage and express markers of tissue residency. (A) Representative FACS plots of Eomes and T-bet expression in LP, matched PB, and donor LB. (B) Percentage of Eomeshi T-betlo cells in the total NK-cell population from LP, LB and PB. (C) Percentage of Eomeslo T-bethi cells in the total NK-cell population from LP, LB, and PB. (D) Representative FACS plots of Eomes and T-bet expression in CD56bright and CD56dim subsets from LP, PB, and DB. (E, F) Percentage of Eomeshi T-betlo cells in the CD56bright (E) and CD56dim (F) NK-cell populations from LP (n = 18), LB (n = 5), and PB (n = 18). (G, H) Percentage of Eomeslo T-bethi cells in the CD56bright (G) and CD56dim (H) NK-cell populations from LP (n = 18), LB (n = 5) and PB (n = 18). (I) Relative mRNA expression of TBX21 and EOMES, using RPS15 as a reference gene, in NK cells isolated from non-matched PB (n = 6) and LP (n = 6). (J) Representative histograms of CXCR6 expression in LP (solid line), LB (dashed line), and PB (dotted line) in NK-cell subsets, with FMO control (grey tint). (K, M) Percentage of CXCR6+ CD56bright (K) and CD56dim (M) NK-cell subsets in LP (n = 8), LB (n = 5), PB (n = 8). (A–M) Data are shown as mean ± SEM and are representative of 18 independent experiments.$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.All data were analyzed using Kruskal–Wallis test, Dunn's multiple comparison test, except for the mRNA expression (I), which was analyzed by Mann–Whitney *U* test.

perforin expressed per cell (LP MFI 2067 ± 787, PB MFI 2794 ± 1108, $p = 0.21$, Fig. 3L).

Hepatic NK cells demonstrate increased cytotoxicity compared to PB NK cells

To examine cytotoxic function, hepatic and PB NK cells, isolated by negative selection from donor perfusate and healthy donor blood, were incubated with MHC class I deficient K562 target cells in the presence or absence of IL-2. Hepatic NK cells display significantly higher CD107a expression compared to PB NK cells from healthy donors, with significant differences between CD56^{bright} subsets (Fig. 4). In the absence of cytokine stimulation hepatic CD56^{bright} NK cells display enhanced CD107a expression compared to PB (28.6 ± 2.5% vs. 9.3 ± 1.3%, $p = 0.011$, Fig. 4B). No significant difference was seen between hepatic and PB CD56^{dim} NK cells (13.5 ± 2.7% versus 11.9 ± 1.5%, $p = 0.8$, Fig. 4B). With the addition of IL-2, expression of CD107a increased in hepatic CD56^{bright} NK cells (53.2 ± 8.6%) and hepatic CD56^{dim} cells (38.8 ± 10.4%). No difference is seen between the proportions of IFN-γ^{+} cells between CD56^{bright} (21.4 ± 18.3%) and CD56^{dim} (24.1 ± 14.9%) within the liver after stimulation with IL-2 and target cells. This differs markedly to PB where more CD56^{bright} cells

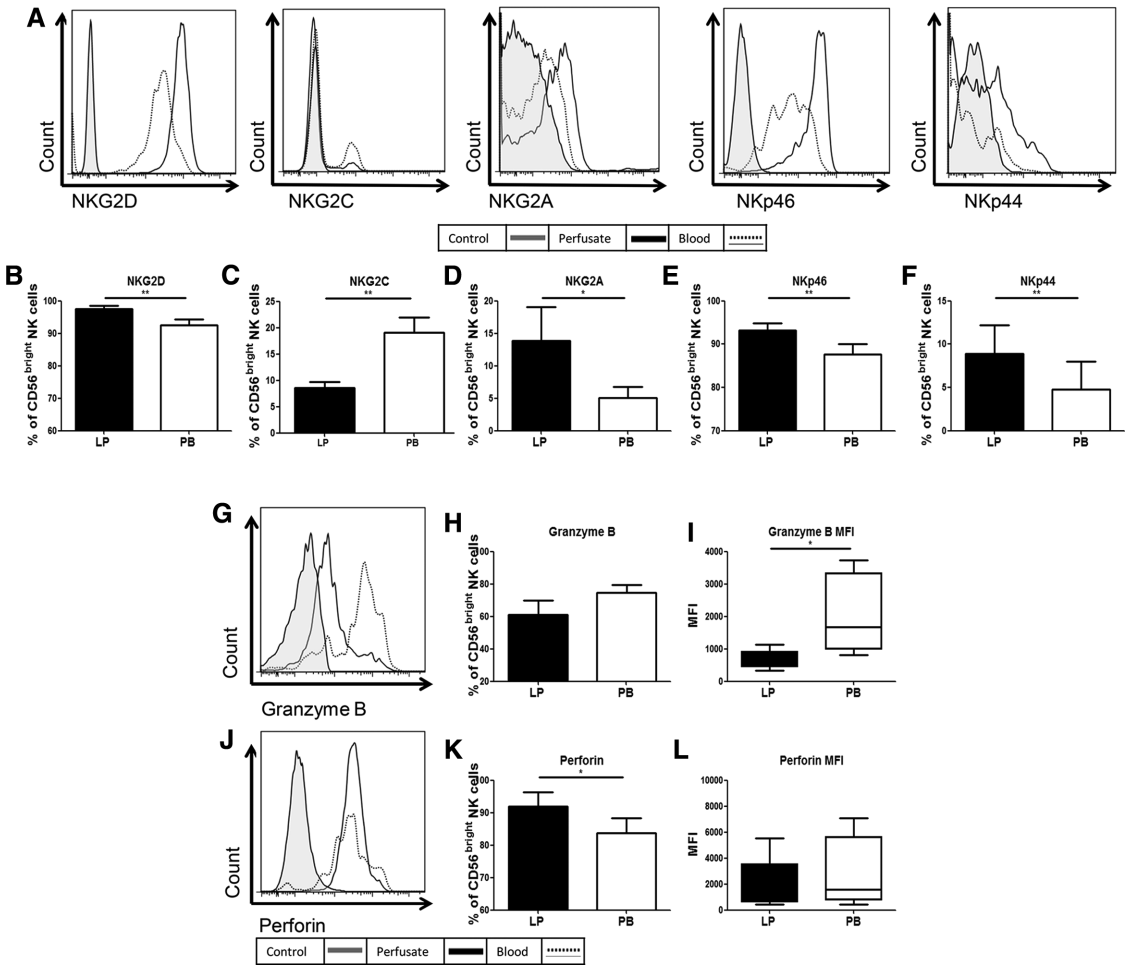

Figure 3. Expression of cytotoxic molecules and receptors in LP and PB CD56bright NK cells. (A) Representative histograms of NKG2D, NKG2C, NKG2A, NKp46, and NKp44 expression in LP (solid line) and matched PB (dotted line) CD56bright NK cells with FMO control (gray tint). Percentage of CD56bright NK cells positive for NKG2D (B), NKG2C (C), NKG2A (D), NKp46 (E), NKp44 (F), in LP (n = 17) and PB (n = 17). (A–F) Data are presented as mean ± SEM, and are representative of 17 independent experiments (G) Representative histograms of granzyme B and perforin expression in LP (solid line) and PB (dotted line) CD56bright NK cells, with FMO control (gray tint). (H) Percentage positive and (I) MFI values of granzyme B expression in LP (n = 6) and PB (n = 6) CD56bright NK cells. (J) Representative histograms of perforin expression in LP (solid line) and PB (dotted line) CD56bright NK cells, with FMO control (gray tint). (K) Percentage positive and (L) MFI values of granzyme B expression in LP (n = 6) and PB (n = 6) CD56bright NK cells. (G–L) Data are presented as mean ± SEM and are representative of six independent experiments (B–F, H–L). $^{*}p < 0.05$; $^{**}p < 0.01$; Wilcoxon signed-rank test.

express IFN-γ compared to CD56^{dim} cells (68.6 ± 7.4% versus 32.3 ± 6.4%, $p = 0.001$, Fig. 4D and E). This difference is due to a reduced ability to produce IFN-γ specifically in the hepatic CD56^{bright} NK cell population. Hepatic CD56^{bright} NK cells also produce significantly less IFN-γ in response to IL-2 stimulation alone, compared to their PB counterparts (3.2 ± 1.6% versus 21.4 ± 7.5%, $p = 0.01$, Fig. 4D). IFN-γ production by hepatic and PB CD56^{dim} cells is largely comparable.

Due to enhanced degranulation of the hepatic CD56^{bright} NK-cell population and the fact that the hepatic CD56^{bright} NK cell population is enriched for the Eomes^{hi} T-bet^{lo} phenotype, we examined the response of Eomes^{hi} T-bet^{lo} and Eomes^{lo} T-bet^{hi} subsets to target cell and cytokine stimulation. Hepatic NK cells were incubated with target cells, K562 cells, in the presence or absence of IL-2. CD56^{bright} NK cells were divided into two groups: CD56^{bright} Eomes^{hi} T-bet^{lo}, and CD56^{bright} Eomes^{lo} T-bet^{hi}. No difference was seen in CD107a expression between CD56^{bright} subsets either in the absence (26.7 ± 3% versus 22.4 ± 2.6%, $p = 0.08$, Fig. 4G) or presence of cytokine stimulation (53.7 ± 4.7% versus 50.6 ± 4.3%, $p = 0.8$, Fig. 4G). However, a significantly lower proportion of CD56^{bright} Eomes^{hi} T-bet^{lo} NK cells express IFN-γ with cytokine and target cell stimulation (15.2 ± 5.4% versus 27.8 ± 3.8%, $p = 0.01$, Fig. 4H).

Liver-conditioned media suppresses IFN-γ production by PB NK cells

Liver-conditioned media (LCM) is a rich source of liver-derived cytokines, growth factors, and chemokines (unpublished data). In order to investigate the effect of the hepatic microenvironment on NK-cell function, PB NK cells were isolated from healthy donors

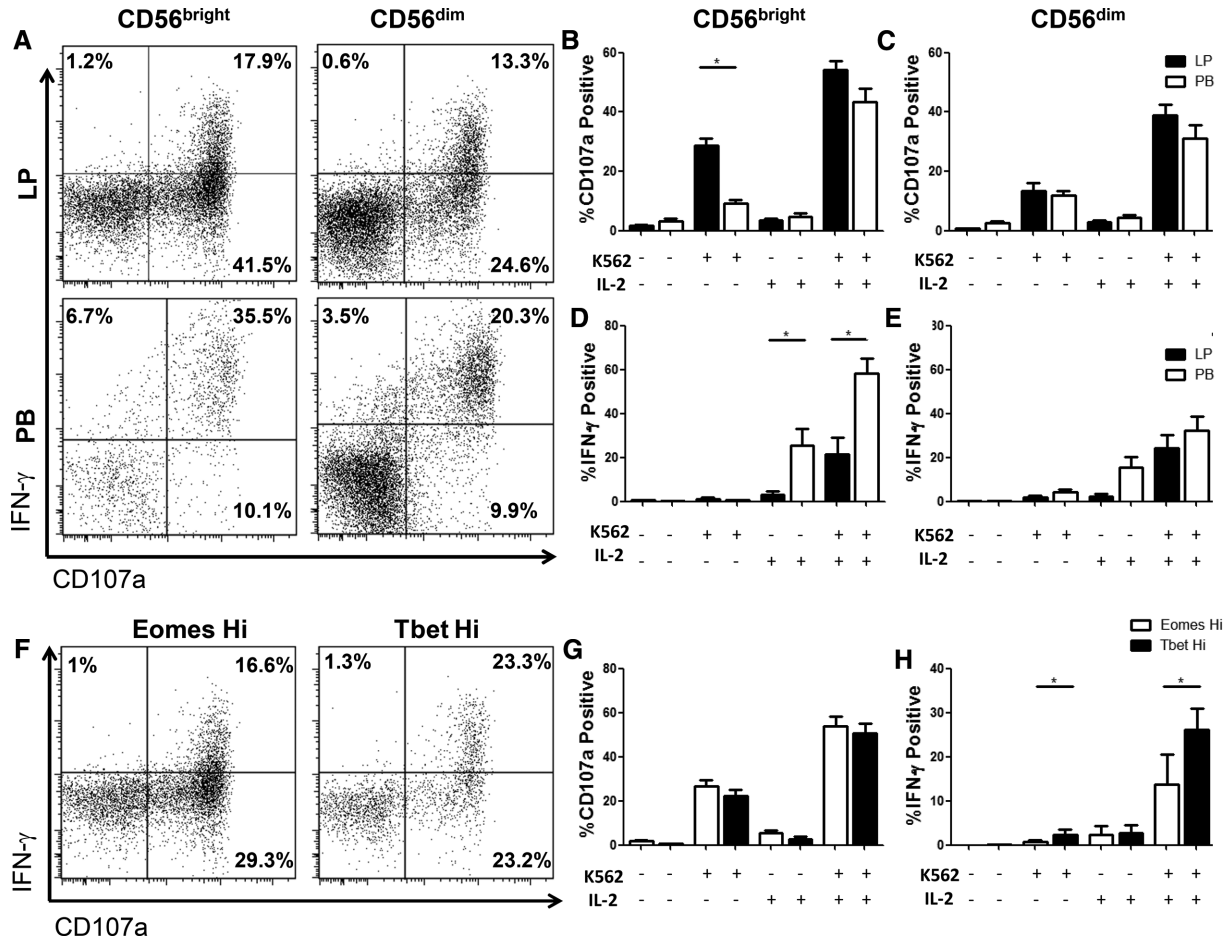

Figure 4. Comparison of degranulation and IFN-g production between LP and PB NK cells. (A) Representative FACS plots of CD107a and IFN-g staining in response to IL-2 and K562 target cell stimulation. (B–E) Percentage of CD56bright and CD56dim NK-cell subsets positive for CD107a (B, C) and IFN-g (D, E) in LP (black bars) and PB (white bars) in response to IL-2 and/or K562 target cell stimulation (LP, n = 7; PB, n = 5). (A–E) Data are presented as mean ± SEM and are representative of eight independent experiments. *p < 0.05; Mann–Whitney U test. (F) Representative FACS plots of CD107a and IFN-g staining in hepatic Eomeslo T-bethi and Eomeshi T-betlo CD56bright NK cells stimulated with IL-2 and/or K562 target cells. (G, H) Percentage of hepatic Eomeslo T-bethi (black bars) and Eomeshi T-betlo (white bars) NK cells positive for CD107a (G) and IFN-g (H) (n = 5). (F–H) Data are presented as mean ± SEM and are representative of five independent experiments. *$p < 0.05$; Wilcoxon signed-rank test.

and treated with LCM for 24 h. NK cells were subsequently stimulated with IL-2 and K562 target cells as described above. No significant difference was observed in the expression of CD107a in either CD56^{bright} (43.3 ± 6.1% vs. 34.9 ± 9.9%, p = 0.62, Fig. 5B) or CD56^{dim} (36.0 ± 10.5% vs. 23.4 ± 7.4%, p = 0.25, Fig. 5D) NK-cell populations after treatment with LCM. Interestingly, production of IFN-γ was significantly reduced in CD56^{bright} (47.6 ± 0.9% vs. 10.9 ± 4.9%, p = 0.01, Fig. 5C) and CD56^{dim} NK cells (25.7 ± 1% vs. 4.7 ± 1.4%, $p = 0.005$, Fig. 5E) suggesting that the liver microenvironment can directly suppress NK-cell proinflammatory cytokine production.

Discussion

In this study, we characterized the diverse NK repertoire of healthy human liver and have identified a novel subpopulation of hepatic NK cells with unique phenotypic features, effector function, and transcriptional regulation, similar to those recently described by Stegmann et al. [21]. As previously described, we have shown that the liver is enriched with NK cells, accounting for more than half of hepatic lymphocytes, with the CD56^{bright} subset making up over 40% of these. The Eomes^{hi} Tbet^{lo} CXCR6^{+} hepatic NK-cell population we identify represents a novel human tissue resident population. While murine models of NK-cell differentiation have proven invaluable in furthering our understanding of NK-cell biology, significant differences in the structure and immune repertoire of the liver exist between man and mouse. As such, data from murine liver may not provide a full view of the heterogeneity of human hepatic NK cells and the spectrum of tissue resident immune cells. Eomes^{hi} Tbet^{lo} hepatic NK cells have increased expression of activating receptors, such as NKG2D, NKp44, and NKp46, which recognize stress-induced ligands and viral and tumor-associated antigens. In addition, these NK cells show increased expression of the cytotoxic molecule perforin. While we have found that granzyme B expression is reduced in hepatic NK cells, it has previously been demonstrated that these cells show increased expression of granzymes A and K [13]. In line with this activated phenotype and

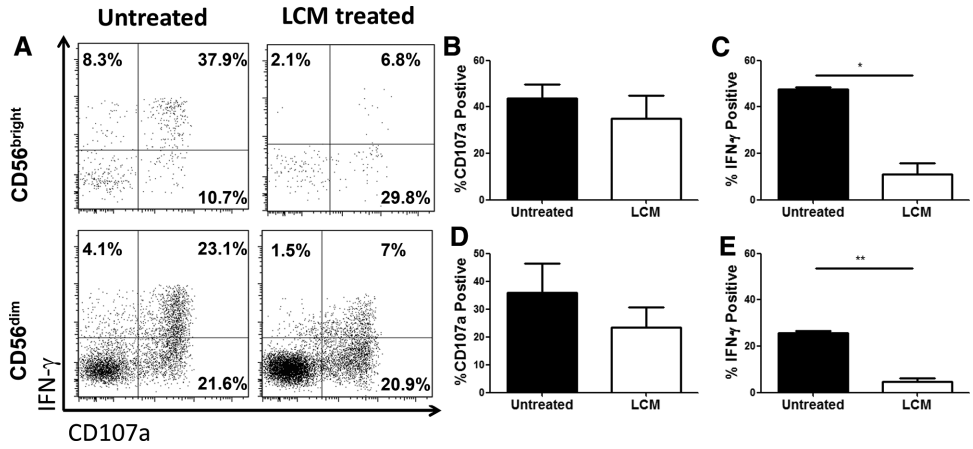

Figure 5. The LCM suppresses IFN-g production by PB NK cells. (A–E) LCM was produced by culturing portions of donor LB for 72 h in X-VIVO 15 media. Supernatants (5% v/v) from this culture were used to treat PB NK cells for 24 h. NK cells were then stimulated with IL-2 and K562 target cells. (A) Representative FACS plots of CD56bright and CD56dim NK cells treated with 5% LCM or untreated. (B, C) Percentage of CD56bright NK cells positive for CD107a (B) and IFN-g (C) in response to IL-2 and K562 target cell stimulation with/without 5% LCM. (D, E) Percentage of CD56dim NK cells positive for CD107a (D) and IFN-g (E) in response to IL-2 and K562 target cell stimulation with/without 5% LCM. (A–E) Data are presented as mean ± SEM and are representative of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; Mann–Whitney *U* Test.

increased cytotoxic molecule expression, hepatic NK cells display increased degranulation in response to target cell stimulation. In PB, the CD56^{bright} subset is normally considered poorly cytotoxic and produces large amounts of proinflammatory cytokines. In contrast, hepatic CD56^{bright} NK cells display enhanced degranulation and significantly reduced IFN-γ production, with no difference seen in the expression of IFN-γ between CD56^{bright} and CD56^{dim} hepatic NK cells. This difference between PB and hepatic populations can be attributed to the presence of the Eomes^{hi} Tbet^{lo} cells in the hepatic CD56^{bright} NK-cell population, which produce significantly less IFN-γ compared to conventional Eomes^{lo} Tbet^{hi} PB NK cells. This functional phenotype appears to be driven, as least in part, by the liver microenvironment, as treatment of PB NK cells with LCM leads to a significant reduction of IFN-γ positive NK cells following cytokine and target cell stimulation.

The origin of hepatic Eomes^{hi} T-bet^{lo} NK cells remains to be established. We have hypothesized that some hepatic NK-cell populations differentiate locally from lymphoid progenitors in the adult human liver [22, 23]. Healthy liver tissue contains a complex cytokine milieu and we have identified cytokines essential of progenitor differentiation and NK-cell activation including IL-7, IL-15, IL-12, IL-18, and IL-2 within healthy liver tissue [24–26]. As evidenced in the present study, the local microenvironment is also capable of inducing liver specific phenotypical and functional changes in BM-derived NK cells which traffic to the liver. The liver is a naturally tolerogenic environment, which maintains unique anti-inflammatory status even in the presence of immune activating dietary antigens and bacterial components. In this context, Eomes^{hi} T-bet^{lo} NK cells may provide a potent cytotoxic cellular response while minimizing wider tissue inflammation through reduced pro-inflammatory cytokine production, helping to maintain the overall tolerogenic environment in the liver.

The high interindividual variation in the relative proportions of hepatic NK-cell populations observed in this study may be of clinical significance during malignancy, viral infection, autoimmunity, and transplantation tolerance and explain inter individual variation in pathological severity of these conditions [27, 28]. The significance of NK-cell subset heterogeneity is driven by the differing immunological roles of NK-cell subpopulations. While conventional NK cells are thought of as predominantly cytotoxic and proinflammatory, emerging data suggest that NK cells can also play immunoregulatory roles in the liver, helping to maintain tissue homeostasis [16, 19, 29], and minimize immune activation during viral infection [17, 30]. Highlighting their immunoregulatory potential, hepatic Eomes^{hi} Tbet^{lo} CD56^{bright} NK cells express TRAIL, enabling these cells to regulate both virally-infected hepatocytes and activated cytotoxic T cells [21]. High levels of interindividual NK-cell subset heterogeneity may also help explain the conflicting reports on the influence of NK-cell accumulation on clinical outcomes in hepatocellular carcinoma and colorectal liver metastasis. Accumulation of NK cells has been linked with increased survival in hepatocellular carcinoma [31, 32] and significantly worse survival rates in hepatic colorectal cancer metastasis [33, 34]. The ability of hepatic NK cells to both regulate and drive liver pathology highlights the importance of defining the complexity and heterogeneity of tissue-resident immune cell populations and in particular, this abundant hepatic CD56^{bright} Eomes^{hi} Tbet^{lo} NK-cell population in the context of human liver disease.

Materials and methods

Collection of LP during orthotopic liver transplantation

Samples were collected from donor livers ($n = 28$) during orthotopic liver transplantation at St. Vincent's University Hospital. During retrieval, the donor aorta and superior mesenteric vein

were flushed with University of Wisconsin (UW) solution (Bristol-Myers Squibb, Uxbridge, UK) at the time of exsanguination. The liver was flushed again with UW solution after excision of the organ until all blood was removed and the perfusate appeared clear, at which time the liver was placed in a container with UW solution and packed on ice for transportation. Donor livers were transplanted within 12 hours. At implantation, after completion of the upper inferior vena cava anastomosis, livers were flushed with normal saline through the portal vein to wash out the UW before reperfusion. This wash-out fluid was collected from the inferior vena cava; the UW transportation solution was also collected. A matched donor blood sample was taken at the time of organ retrieval. Peripheral blood was obtained from anonymized blood donors from the Irish Blood transfusion Board (IBTS). All protocols were approved by St. Vincent's University Hospital Ethics Committee in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Isolation of hepatic mononuclear cells from LP, LB, and PB

Hepatic mononuclear cells (HMNCs) were isolated from donor LPs, as previously described [35], by filtration through 70 μm filters (BD Biosciences, Erembodegem, Belgium) followed by centrifugation at 1200 rpm for 10 min. The supernatant was aspirated and the cells resuspended in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Gibco, Wicklow, Ireland). HMNCs were separated from this suspension by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden) and residual red blood cells were removed by adding red cell lysis solution (Sigma-Aldrich, Wicklow, Ireland). HMNCs were isolated from donor liver biopsies taken at the time of transplantation by mechanical and enzymatic digestion, as previously described [12]. Matched PB mononuclear cells (PBMCs) were also isolated by density centrifugation.

Preparation of LCM

Wedge biopsies taken at the time of transplantation were used to generate tissue conditioned media. Tissue samples were measured and weighed. Tissue was then cut into sections measuring approximately 0.5 × 0.5 × 0.5cm. These were placed in a 24-well culture plate and 350 μL of X-VIVO (Lonza Biologics, Slough, UK) media was added to each well and incubated for 72 h at 37°C. Following incubation the supernatant was centrifuged to remove cell debris and stored at −20°C until use.

Phenotypic analysis of NK cells

HMNCs or PBMCs, isolated from donor perfusate or blood, were stained with fluorescently labelled monoclonal antibodies to determine the phenotypic differences between hepatic and peripheral NK cells. The following antibodies were used: CD45 (HI30) BV510, CD3 (UCHT1) BV421, CD56 (NCAM16.2) BV650, CD16 (3G8) PE-Cy7, CD16 (3G8) BV605, NKp46 (9E2) PE-Cy7 (BD Biosciences, Franklin Lakes, NJ, USA), NKp44 (P44-8) APC, NKG2D (1D11) PE (Biolegend, San Diego, CA, USA), NKG2A (# 131411) APC, NKG2C (# 134591) AF488 (R&D Systems Minneapolis, CA, USA). Intracellular staining was performed using FoxP3 staining buffer (00-5523-00, eBiosciences, San Diego, CA, USA) and the following antibodies were used: Perforin (δG9) PE-CF594, Granzyme B (GB11) AF700 (BD Biosciences), Eomes (WD1928) AF488 and T-bet (ebio4B10) PE (Biolegend). Dead cell exclusion was carried out using fixable viability stain 780 (BD Biosciences). CD107a (H4A3) APC-H7 and IFN-γ (B27) PE-Cy7 were used for functional experiments. Flow cytometric analysis was carried out using an LSR Fortessa (BD Biosciences) and data was analyzed using FlowJo (Version 7.6.5, Tree Star, Ashland, OR, USA).

Quantitative PCR of Eomes and TBX21

Total RNA was extracted from $CD3^-$ $CD56^+$ NK cells (isolated as detailed below) from fresh LPs and PB from healthy donors using TRIzol reagent (Thermo Fisher Scientific, Paisley, UK). During RNA extraction glycogen (Sigma-Aldrich) was added as an RNA carrier and reverse transcription was performed using the SuperScript VILO Master Mix (Thermo Fisher Scientific). Quantitative PCR was performed using the following primers: *RPS15* forward 5′-CGGACCAAAGCGATCTCTTC; *RPS15* reverse 5′-CGCACTGTACAGCTGCATCA; *EOMES* forward 5′-CCGACAATAACATGCAGGGC; *EOMES* reverse 5′-TGCAGTCG GGGTTGGTATTT; *TBX21* forward 5′-AACCACCTGTTGTGGTC CAA; and *TBX21* reverse 5′-ATGGGAACATCCGCCGTCC. Quantitative PCR was run using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). *RPS15* was used as a reference gene to generate normalized relative expression values for *TBX21* and *EOMES*.

Isolation of NK cells from LP and PB

$CD3^-CD56^+$ NK cells were isolated from fresh LPs and PB from healthy donors by negative selection using the NK-cell isolation kit (130–092–657; Miltenyi Biotech, Teterow, Germany), as per the manufacturers protocol. Briefly, mononuclear cells were labeled with a biotin-conjugated antibody cocktail against lineage specific targets. Anti-biotin microbeads were then added and the NK cells were separated using a magnetic cell sorting (MACS) LS column. Cells were then cultured in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. After separation NK-cell populations all had purities >90%, with an average purity of 96.9% (LP) and 95.8% (PB).

Cytoxicity assay

Degranulation of NK cells was measured by quantifying the increase of cell-surface CD107a in response to co-incubation with K562s, a MHC-deficient monocytic cell line. Briefly, freshly isolated hepatic or PB NK cells were incubated overnight at 37°C in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. K562s were cultured overnight under the same conditions. K562s were then coincubated with NK cells at an effector:target ratio of 5:1. Anti-human CD107a antibody was added to each well to assess degranulation. Samples were then incubated at 37°C for 4 h. After 1 h Golgistop (BD Biosciences) was added to each sample. Following the 4 h incubation, samples were washed with flow buffer and stained for CD56, CD3, CD45, CD16, IFN-γ and the transcription factors Eomes and T-bet. Intracellular staining was performed as above using the FoxP3 staining buffer set (eBiosicence).***

Statistical analysis

Data are presented as mean $\pm$ standard error mean (SEM). Comparison of more than two groups was performed using Kruskal–Wallis test, with Dunn's multiple comparison test. For paired comparisons, Wilcoxon signed-rank test was used. For comparison of two unmatched groups, Mann–Whitney *U* test was used. A p-value of <0.05 was considered significant.

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Abbreviations: **CXCR6**: chemokine (C-X-C motif) receptor 6 · **Eomes**: Eomesodermin · **HMNC**: hepatic mononuclear cells · **IFN-γ**: IFN gamma · **LB**: liver biopsy · **LCM**: liver-conditioned media · **MIC-A/B**: MHC class I polypeptide associated sequence A/B · **NKG2A/C/D**: natural-killer group 2, member A/C/D · **PB**: peripheral blood · **UW**: University of Wisconsin

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