Natural Killer Cell Development In the Adult Human Liver

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Master of Science Molecular Medicine

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Abstract

Natural killer (NK) cells, as a component of the innate immune system, are large granular lymphocytes, and their main effector functions are to kill virus-infected and malignant cells without prior stimulation and to activate macrophages to destroy phagocytosed microbes. In humans NK cells are identified by the expression of CD56 (NCAM) and lack of CD3, and comprise approximately 15% of all circulating lymphocytes. Apart from peripheral blood, NK cells can also be found at specific sites such as lymph nodes, tonsils, spleen and liver. These organ-specific NK cells exhibit some phenotypical and functional differences compared to blood NK cells. While the developmental pathways and sites of maturation of human B and T cells have been generally understood, only recently, studies have started unraveling the sites and pathways of NK cell development. According to these findings NK cells reach maturation through a multistep process comprising five sequential stages. Regarding the human liver, while there is evidence demonstrating the presence of hematopoietic stem cells and early progenitor cells in the adult human liver, there is no clear proof indicating whether NK cells can locally develop from hematopoietic precursors. We have previously shown that organ-specific NK cells are present in the adult human liver [1]. Here, we investigated NK cell development within the adult human liver. First, we identified all five stages of NK cell development in the adult human liver, by analyzing both liver perfusates and liver biopsy specimens. The comparison between hepatic and blood-derived NK precursors highlighted differences in their immunophenotype (expression of CD25 and CD127 on immature NK cells from liver but not from blood) as well as in the proportions of cells in various developmental stages. Upon in vitro culture, stage 2 and 3 NK precursors isolated from liver perfusates differentiated into mature NK cells exhibiting high level of cytotoxicity. To verify if hepatic NK precursors that reside within the liver can provide long-term differentiation to mature NK cells, we tested the presence of donor NK cells in blood samples and hepatic biopsies from liver transplanted patients long time after transplantation (LTX). The results indicated that only mature donor NK cells were present, while donor NK precursors could not be detected, therefore suggesting that after LTX donor NK precursors may migrate to other sites and from there generate mature NK cells. All together, our data indicate that the adult human liver is a site of physiological NK cell development. However this mechanism may be altered after LTX or in the presence of hepatic diseases, conditions that may partially influence this hematopoietic function of the liver.

Introduction

Innate immunity

Innate immunity (also called natural immunity) serves two important functions against potentially dangerous pathogens; firstly, as an early line of defence, it blocks, controls, or eliminates microbial infection in the host. Secondly, it stimulates adaptive immunity and can affect the nature of adaptive responses to make them optimally efficient against different types of microbes [2-3]. Main components of the innate immune system are: (1) the epithelial barriers, (2) leukocytes, especially phagocytes such as neutrophils and macrophages, and natural killer cells, (3) circulating effector proteins like complement and (4) non specific mediators such as IL-10, IL-12 or IFN-γ (Fig. 1). The components of the innate immunity recognize pathogen associated molecular patterns (PAMP) that are structural features of microbial pathogens. The mechanisms of innate immunity are specific to PAMPs that are common to groups of related microbes and may not distinguish fine differences between antigens. Therefore, discriminating characteristics of innate immunity are lack of specificity to the invading pathogen and early activation before adaptive immune response [4-6]. In contrast to innate immunity, the adaptive immune system is able to recognize and react to a large number of microbial and non-microbial substances. In addition, it has an extraordinary capacity to distinguish between different, even closely related, microbes and molecules. Moreover, the adaptive immune system provides immunological memory (secondary immune response) to the same antigen which are usually more rapid, larger, and often qualitatively different from the primary immune response to that antigen [4].

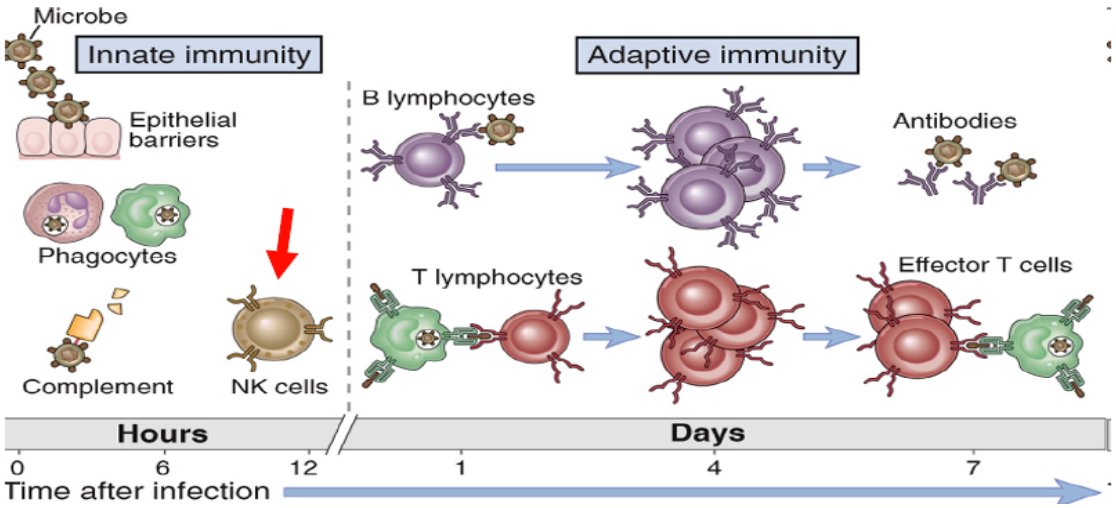

Figure 1. Innate and adaptive immunity. The mechanisms of innate immunity provide the initial defence against infection. Adaptive immune responses develop later and are determined by

activation of lymphocytes. The kinetics of the innate and adaptive immune responses are estimations and may vary in different infections [4].

Natural killer cells

Natural killer (NK) cells, as a component of the innate immune system, are large lymphocytes with numerous cytoplasmic granules [4]. The main effector functions of NK cells are to directly kill infected and malignant cells, and to activate macrophages to destroy phagocytosed microbes (Fig. 2). NK cells, like CTLs ($CD8^+$ T cells), have granules that contain lytic proteins which mediate killing of target cells. When NK cells are activated, granule exocytosis releases these proteins onto the surface of the target cells. One NK cell granule protein, called perforin, facilitates the entry of other granule proteins, called granzymes, into the cytoplasm of target cells. The granzymes are enzymes that initiate apoptosis of the target cells. By killing cells infected by viruses and intracellular bacteria, NK cells eliminate reservoirs of infection. Moreover, NK cell-derived IFN-γ serves to activate macrophages and enhances their capacity to kill phagocytosed bacteria [4-6].

NK cell activation is regulated by a balance between signals that are generated from activating receptors and inhibitory receptors. The activating signals must be blocked by inhibitory signals in order to block NK cell activation and attack of normal cells. Many of these inhibitory receptors on NK cells recognize class I MHC molecules or proteins that are structurally homologous to class I MHC molecules. The specificity of inhibitory receptors for self-class I MHC protects a normal, self cell from NK-mediated killing. Infection of the host cells, especially by some viruses, often leads to decreased expression of class I MHC molecules, and therefore the ligands for the inhibitory NK cell receptors are lost. As a result, the NK cells are released from their normal state of inhibition and thus the infected cells are killed. This specificity of the NK cell inhibitory receptors for normal class I MHC allows the innate immune system to attack virally infected cells, that might be invisible to CD8+ T cells, and malignant cells which in most of the cases have lost expression of their MHC class I molecules. This last function makes them remarkably relevant as effector cells in immune therapies for cancer [4, 6-7]. The largest family of NK inhibitory receptors is the killer immunoglobulin-like receptors (KIRs), which primarily recognizes HLA-A, -B and –C alleles. Another important NK inhibitory receptor is CD94/NKG2A, a dimer of C-type lectins recognizing the non classical MHC molecule HLA-E. The activating receptors on NK cells comprise several structurally discrete groups of molecules. CD16 is a

low-affinity IgG Fc receptor that associates with FcεRIγ and ζ proteins. Finally, natural cytotoxicity receptors, includes NKp46, NKp30, and NKp44 associates with FcεRIγ, ζ proteins and DAP12 respectively. Ligand binding to activating NK cell receptors leads to cytokine production, enhanced migration to sites of infection, and killing activity against the ligand-bearing target cells. Additionally, the expansion and activities of NK cells are also stimulated by cytokines, mainly IL-15 and IL-12, secreted by activated macrophages, and IL-2 [5-6, 8].

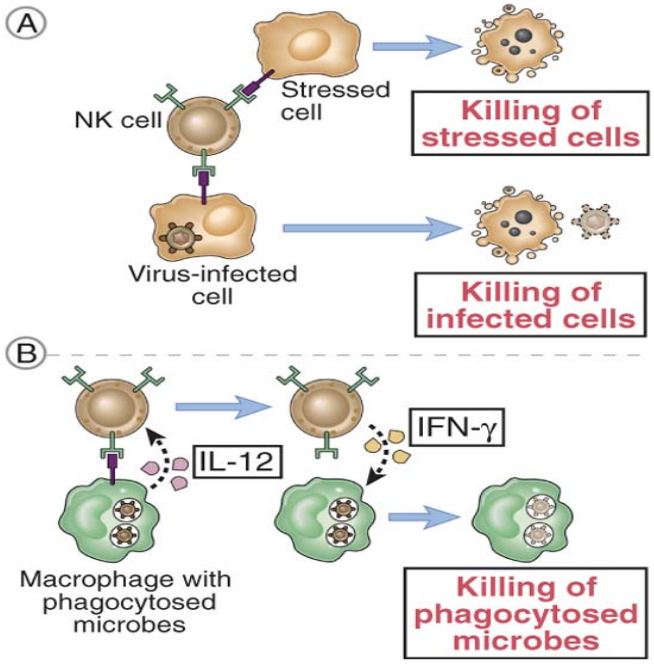

Figure 2. Role of NK cells. (A) NK cells recognize ligands on infected cells or cells undergoing other types of stress and kill the target cells. This way, NK cells eliminate reservoirs of infection as well as dysfunctional cells. (B) NK cells respond to IL-12 produced by macrophages and secrete IFN-γ, which activates the macrophages to kill phagocytosed microbes [4].

Mature human NK cells comprise ≈15% of all peripheral blood lymphocytes, and are defined phenotypically by their expression of CD56 and lack of expression of CD3 [9-10]. Almost 15 years ago, with the introduction of monoclonal antibodies (Abs) for specific NK-cell markers, two distinct populations of human NK cells were identified based on the surface expression of CD56 [11]. The majority (≈90%) of blood NK cells have a low-density expression of CD56 ($CD56^{dim}$) and express high levels of FcγRIII (CD16), whereas the remaining 10% are $CD56^{bright}$ $CD16^{dim}$ or $CD16^{-}$ (Fig. 3, 4). CD56 is an isoform of the human neural-cell adhesion molecule (NCAM) with unknown function on NK cells; CD16 mediates antibody-dependent cellular cytotoxicity (ADCC) of NK cells [12-13]. Early

functional studies of these subsets by Lanier and colleagues revealed that CD56^{bright} NK cells from blood secrete immunoregulatory cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, TNF-β and granulocyte macrophage-colony stimulating factor (GM-CSF), and they are less effective mediators of natural cytotoxicity and ADCC (antibody-dependent cell-mediated cytotoxicity**)** [14]. On the contrary, CD56^{dim} NK cells are more cytotoxic against NK-sensitive targets and produce fewer amounts of cytokines following treatment with IL-2 *in vitro* and *in vivo* [11, 15-17].

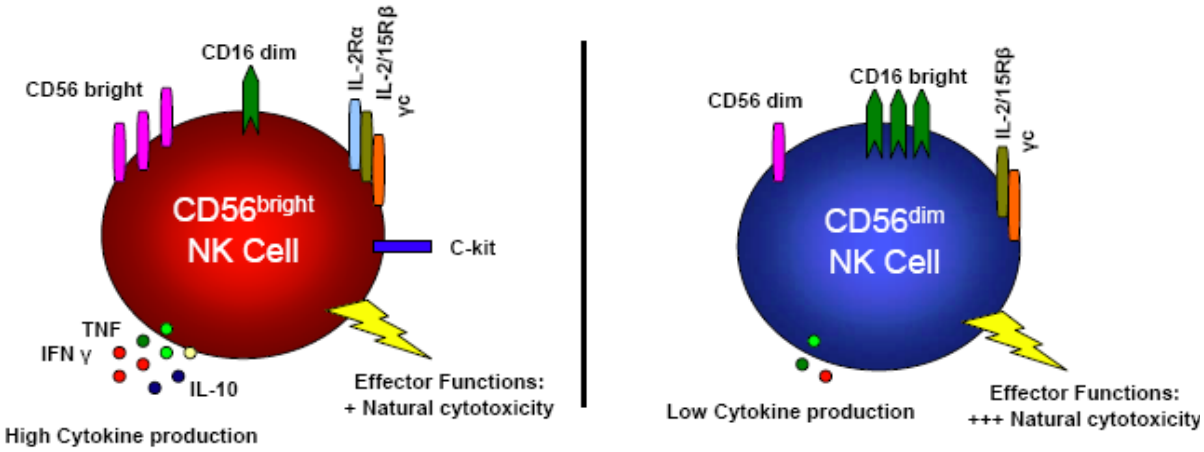

Figure 3. Human NK cell subsets. The CD56^{bright} NK cells are mainly immunoregulatory cells that produce high levels of cytokines following monokine stimulation. This subset has low expression of CD16 and low natural cytotoxicity. The CD56^{dim} NK cells are essentially cytotoxic cells and secrete low levels of cytokines in response to monokine stimulation [16].

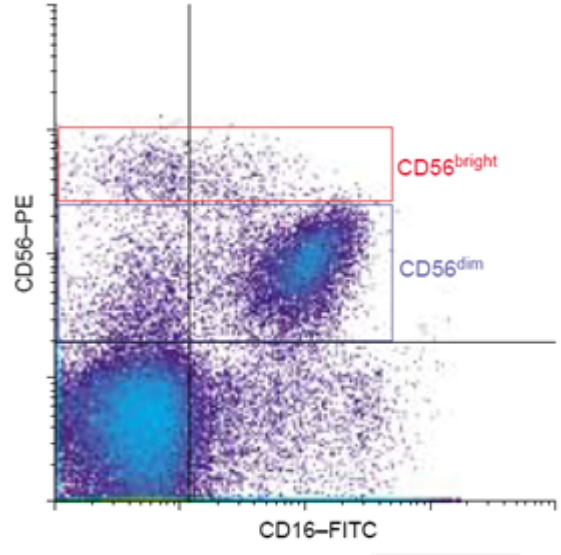

Figure 4. Flow cytometric analysis of CD56^{bright} and CD56^{dim} natural killer (NK) cells in human peripheral blood. CD56^{bright} NK cells (red box) consist of ≈10% of all blood NK cells and are

CD16^{-} or CD16^{dim}. The majority (≈90%) of NK cells is CD56^{dim} (blue box) and has high-expression of CD16 (CD16^{bright}) [15].

Hematopoiesis

The cellular components of the immune system, such as T cells, B cells, monocytes, granulocytes, macrophages, dendritic cells, and NK cells, are derived from common hematopoietic stem cells (HSCs) in the bone marrow (Fig. 5). As a first stage, hematopoietic stem cells (HSCs) differentiate into two distinct subsets: common myeloid progenitors and common lymphoid progenitors. Whereas common myeloid progenitors eventually develop into myeloid cells such as monocytes, granulocytes, macrophages, and dendritic cells, common lymphoid progenitors differentiate into B-cell precursors and common T- and NK-cell precursors (T/NKPs). Consequently, T/NKPs differentiate into NKPs and T-cell precursors. These steps are thought to take place mainly in the bone marrow, which is regarded as the most important site for primary immune cell development [18-21].

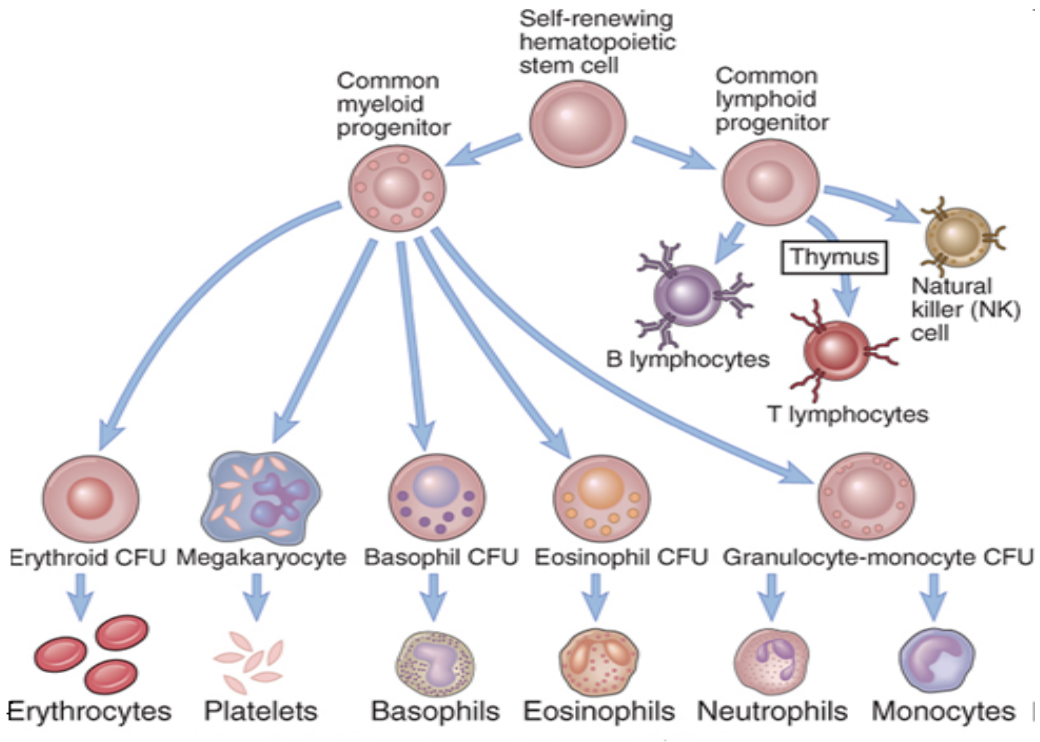

Figure 5. Hematopoiesis. The development of the different lineages of blood cells is depicted in this "hematopoietic tree" [4].

NK cell development

As mentioned above, it is known that NK cells differentiate from hematopoietic progenitor cells (HPC) that reside in the $CD34^+$ cell compartment of bone marrow (BM) [22]. Interleukin-15 (IL-15) is the main physiological cytokine present in the bone marrow and is able to induce NK cell differentiation in stroma-free cultures of human CD34+ hemotopoietic progenitors in the absence of other cytokines [23]. Although IL-2 is not a physiological cytokine for NK cell development in the bone marrow, exogenous IL-2 can produce *in vivo* NK cell expansion in a manner similar to IL-15. [16-17]. Although IL-15 induces development of $CD34^+$ cells towards functional NK cells, this occurs without significant cell expansion. Two additional bone marrow stromal factors, ligands for the receptor tyrosine kinases c-kit (KL) and flt-3, have been shown to act synergistically with IL-15 to enhance NK cell expansion in culture. This resembles the situation with other hematopoietic lineages, where c-kit ligand (KL) and flt-3 ligand (FL) act in concert with lineage-specific growth factors for expansion of cells of a specific lineage. KL and FL promote NK cell differentiation by upregulating IL-2/IL-15Rβ expression on hematopoietic progenitor cells, and these cytokines enhance NK cell precursor frequency from human hematopoietic progenitor cells on limiting dilution analysis. The IL-2/IL- $15R\beta^+$ progenitor cells are then responsive to IL-15-induced NK cell differentiation [24-25]. Therefore, human NK cell development may be divided into two stages (Fig. 6). An early phase in which early NK progenitor cells respond to stromal cell growth factors, KL and FL, and differentiate into NK precursor cells with the phenotype $CD34^{bright}$ IL-2/IL-$15R^+$ $CD56^-$. In a second phase, these precursors are responsive to IL-15 for development into mature NK cells [26]. Recently, other groups have shown that adding IL-7 and IL-21 to the *in vitro* cultures induces higher proliferation of differentiated NK cells [27-28].

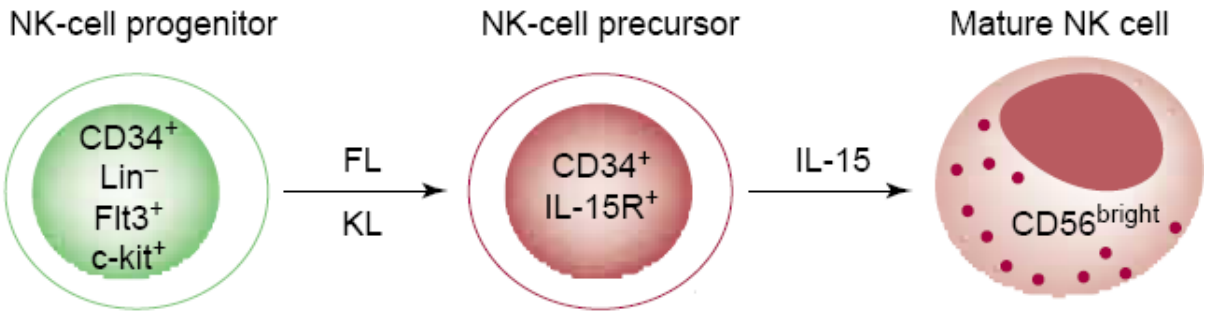

Figure 6. Human natural killer (NK) cell development. NK-cell differentiation can be divided into two main stages according to the *in vitro* models. A $CD34^+$ NK-cell progenitor, negative for lineage markers (Lin^-), which expresses the receptor tyrosine kinases flt3 and c-kit, and is responsive to flt3 ligand (FL) and/or c-kit ligand (KL), develops into a $CD34^+$ interleukin-15

receptor (IL-15R)$^{+}$ NK precursor, which is responsive to IL-15 for maturation into a functionally mature CD56^{bright} NK cell [15].

For the past three decades, it had been generally accepted that NK cell development primarily occurs within the bone marrow (BM) microenvironment [29-30]. In spite of these data, the possibility that BM-derived pre-NK cells or developmental intermediates may traffic to peripheral tissues to undergo terminal maturation has not yet been ruled out [31]. Moreover, a comprehensive and continuous pathway of NK cell development has not been identified within adult human BM. This would more definitively reveal actual differentiation *in situ*. The main reason for this lack of evidence is probably because the *in vivo* stages of human NK cell development have only recently been identified in lymph nodes and tonsils [32-33].

From the collective work of numerous investigators during the last two decades, Caligiuri and Freud proposed an inclusive sequential model of human NK cell development using the relative surface expression of CD34, CD117 and CD94 to discriminate stages of differentiation (Fig. 7).

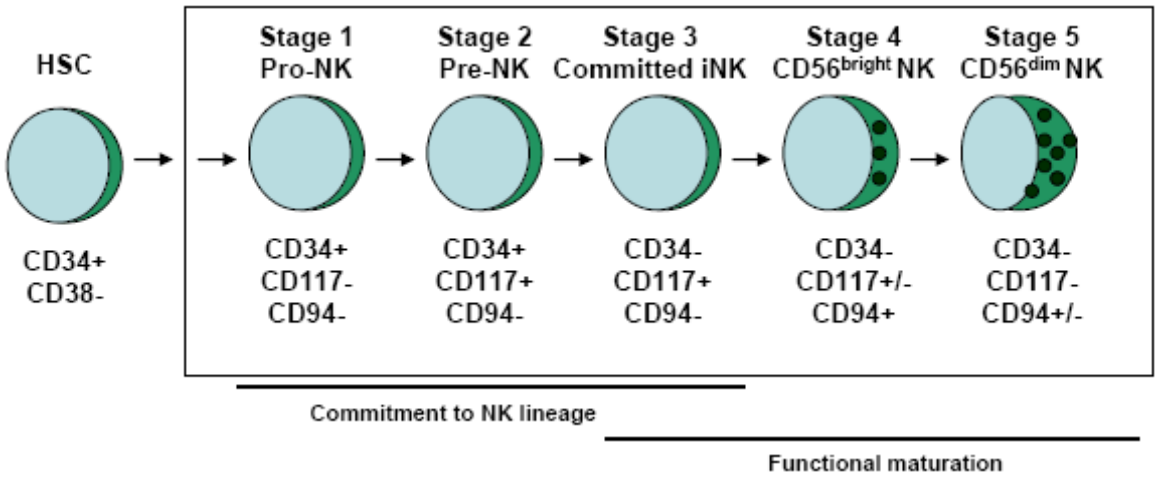

Figure 7. Proposed model of *in vivo* human NK cell development. This figure depicts the developmental stages through which human NK cells are believed to mature from BM-derived HSCs to stage 5 NK cells. The box represents developmental steps that may occur within SLT (secondary lymphoid tissues). As NK cells develop from stage 1 to stage 3, they become committed to the NK cell lineages and lose the capacity for T-cell or DC development. From stage 3 to 5, it is suggested that NK cells undergo functional maturation. Adapted from [33].

Caligiuri and Freud have shown that all aspects of terminal human NK cell differentiation, including lineage commitment, functional maturation, and KIR acquisition, occur within LNs and tonsils, and

have suggested that these processes may occur in other SLT including Peyer's patches and the white pulp of the spleen. Although the exact reason of NK cell development in the SLT is unclear, SLT may simply provide unique settings for NK cell developmental intermediates to interact with accessory cells such as DCs [32, 34]. Following these studies, Chinen and his colleagues suggested that lamina propria of human intestine can also be a possible site of NK cell development [35]. Certainly, very early steps in NK cell development, such as the generation of pro-NK cells from HSCs, likely occur within the BM. However, it is not known which developmental precursors are capable of relocating, through peripheral blood, to other tissues [33].

The Liver: an organ of innate immunity

The liver is the largest organ in the adult body weighing approximately 1,500 g and is known to be responsible for protein, carbohydrate, lipid metabolism, bile secretion and detoxification. However, after having been ignored by immunologists for years, the liver is now known to be a site of complex immune activity. The dual blood supply of the liver derives for about 80% from the gastrointestinal tract via the portal vein and for the remaining 20% consists of oxygenated blood from the systemic circulation entering through the hepatic artery. Its constant exposure to a large antigenic load that includes pathogens, bacterial particles derived from the gut flora, toxins, tumour cells and harmless dietary antigens determines the presence of a complex repertoire of immune cells capable of mediating effective surveillance and defence [36-37]. Hepatic immunity consists of innate immunological components, which rapidly detect and respond to foreign infectious agents and infected or transformed self cells, as well as adaptive immune cells like T cells. Components of innate immunity that are active in the liver include local macrophages (Kupffer cells), DCs, NK cells, NKT cells, inflammatory cytokines, the complement system, acute-phase proteins, and chemokines [38-39]. One to two million of these lymphoid cells are retrievable from 200 mg of normal human liver tissue, indicating that an average human liver of 1.5 kg contains approximately 1×10^{10} cells [40]. Immunohistochemical staining of normal liver biopsy specimens taken after extensive perfusion and washing has revealed that these lymphocytes are predominantly located around the portal tracts and can also be found spread throughout the parenchyma [40]. Up to 65% of all lymphocytes present in the normal liver consist of natural killer (NK) cells, T cells expressing γδ T-cell receptors (TCR), and T cells expressing NK molecules (NKT cells); these are found in much lower proportions in PB (13%, 2%

and 2% respectively) (Fig 8). Their dominating presence in the liver confirms that it is a site of early encounter of antigens where the immune system has a particularly important role [37, 40].

Hepatic lymphocytes can be isolated from biopsies of healthy livers as well as from liver perfusates. A liver perfusate is the fluid used to flush the preservation solution out of the liver graft after the cold storage period, right before the organ is transplanted to the recipient. We and others have found that this perfusion fluid is a rich source of hepatic immune cells and is largely representative of the hepatic pool of immune cells [41-43]. In fact, two main observations indicating the similarities between NK cells from perfusates and from liver biopsies can be reported: NK cells comprise around 30% of the lymphocytes both in perfusates and liver biopsies (while in blood they account for around 10%); secondly hepatic NK cells both from perfusates and liver biopsies have an enrichment in the $CD56^{bright}$ subset and show a number of phenotypical and functional features that discriminate them from blood NK cells [1, 44].

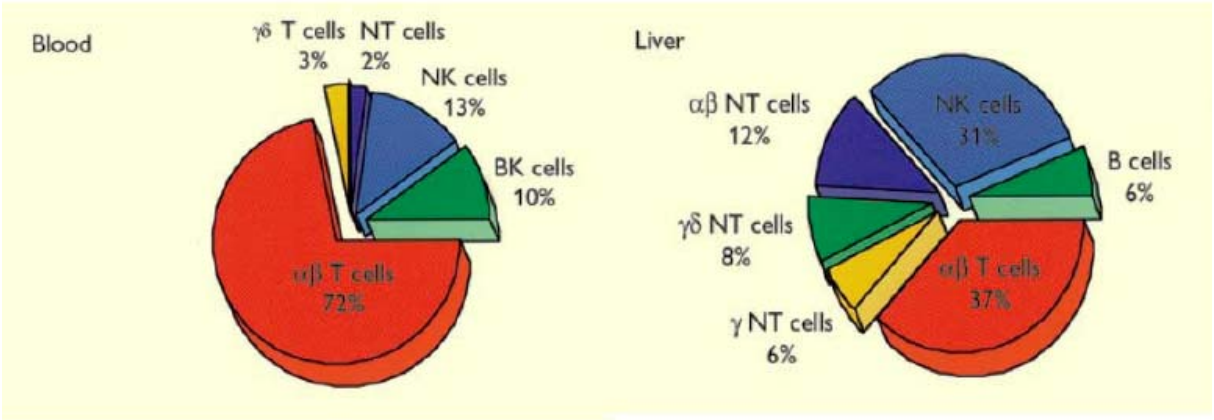

Figure 8. Distributions of lymphocyte subsets in human blood compared to adult human liver [37, 40].

NK cells in the liver

NK cells are more abundant in the liver than in any other organ [45] and human hepatic NK cells can be easily isolated from perfusate during liver transplantation (Fig. 9) [46]. $CD3^-$ $CD56^+$ hepatic NK cells account for 30–50% of intrahepatic lymphocytes compared to 5–20% in the peripheral blood and has a critical role in the immune response against hepatotrophic viruses. Their depletion seems to be a contributing factor to chronicity in hepatitis C infection [42, 47]. Hepatic NK cells are also critical antitumor agents and are thought to have a role in liver allograft rejection [48]. In the rat, NK1P + CD3− NK cells originally termed “pit cells” represent 30–50% of hepatic lymphocytes, while mouse

hepatic NK cells (DX5/integrin CD49b^{+} CD3^{-} and NK1.1^{+} CD3^{-} in some strains such as C57BL/6 mice) are found at lower frequencies (5–10% of liver lymphocytes) [49]. These species-specific differences in local populations of hepatic NK cells cause significant challenges for the successful interpretation of animal models of liver diseases and their translation to clinical practice.

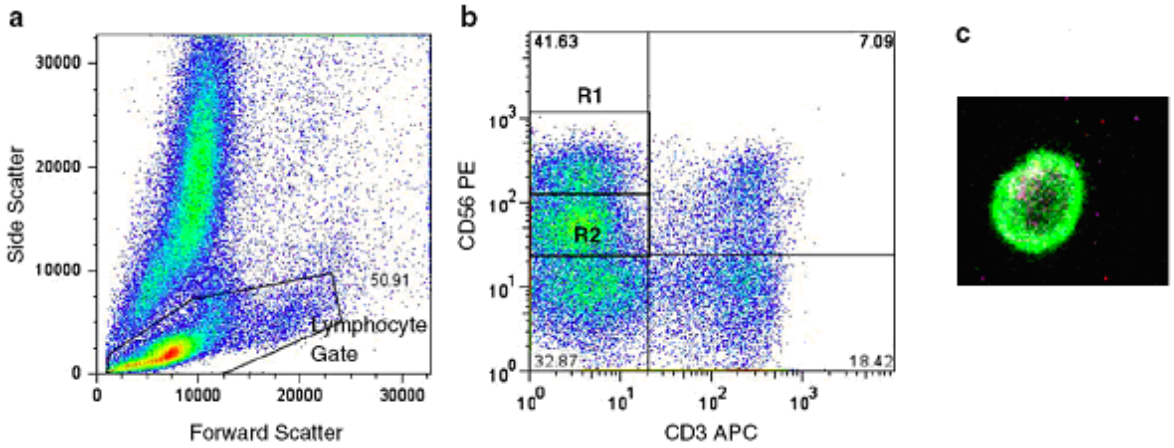

Figure 9. Hepatic NKR^{+} cells in University of Wisconsin (UW) solution flushed from transplanted liver. (a) Forward scatter vs. side scatter of hepatic mononuclear cells with the lymphogate indicated. (b) Dot plot of CD56 vs. CD3 cells and CD56^{bright} NK cells (R1) account for 35% of NK cells whereas CD56^{dim} NK cells (R2) account for 65% of NK cells. (C) Confocal microscopy image of an NK cell (CD56^{+} CD3^{-}) from a donor liver [50].

NK cell development in the liver

Besides being more frequent, hepatic NK cells also differ from blood NK cells in expression of a number of surface markers as well as in their higher cytotoxic potential [1, 14, 44]. Recently our group analyzed these differences between hepatic and circulating NK cells more in depth and suggested that hepatic NK cells may develop locally. Firstly, liver NK cells were not only more abundant but also differently distributed in their CD56^{bright} and CD56^{dim} subsets compared to blood, showing that there is a local enrichment of CD56^{bright} NK cells within the liver. Similarly, NK cells from lymph nodes are also largely CD56^{bright} [51] and develop from local precursors. In spite of the large presence of CD56^{bright} NK cells, traditionally considered non-cytotoxic and more immuno-regulatory, our recent results have shown that hepatic NK cells are more cytotoxic than NK cells from peripheral blood. In addition, our preliminary observations indicated that NK-depleted MNCs from perfusates co-cultured with allogeneic T cells induced the regeneration of a population of CD56posCD3neg cells resembling the original NK cells. Lastly observations *in vivo* have revealed that in some LTX patients donor-derived NK-like cells (CD56^{bright}CD3^{low}) are detectable in the circulation more than 1 year post LTX (unpublished data). All

together these observations suggest local development of NK cells in the adult liver, although they do not provide evidence on the origin of this local precursor. It is plausible that peripheral NK cell-precursors migrate into the liver and are there stimulated by other hepatic cells to reach a complete maturation.

Interestingly, several studies have shown that HSCs are present in the adult mouse liver and can give rise to multiple leukocyte lineages [4, 52-53]. Moreover, Crosbie et.al demonstrated the presence of hematopoietic stem cells and early progenitor cells in the adult human liver and suggested that this organ may continue to contribute to hematopoiesis after birth, and may be an important site for the differentiation of lymphohematopoietic cells involved in disease states, such as autoimmune hepatitis and graft rejection after liver transplantation [54]. However, although previous studies have shown that cytolytic NK cells can be derived in vitro following culture of $CD34^+$ cells derived from fetal liver, BM, thymus, cord blood (CB), adult blood, or secondary lymphoid tissue (SLT) [23, 55], evidence of NK-cell progenitors in adult human liver is still lacking.

Aim

The aim of this study was to examine whether the adult human liver in a physiological condition can be a possible site of local NK cell development. Using flow cytometric analysis based on the expression of CD34, CD117 and CD94, we identified 5 different stages of NK cell development in the perfusates of liver grafts. Next we sorted hepatic NK-cell precursors and studied *in vitro* the NK-cell differentiation potential. We then tested the NK cells that we differentiated in vitro for their cytotoxic potential.

Hepatic NK cell development: relevance to liver transplantation

It is generally recognized that 15-30% of liver transplant recipients become spontaneously tolerant to their graft [56]. As a result, most of recent research in this field is dedicated to unravelling the cellular basis of this phenomenon. There are indications that the presence and persistence of donor leukocytes after transplantation is relevant for tolerance induction after LTX [57]. In addition to this, it has been shown that after allogeneic bone marrow transplantation, donor-derived NK cells can eliminate graft rejection and leukemia relapse and additionally prevent Graft-versus-Host Disease (GVHD) [58-59]. If similar mechanisms would act also in LTX settings and donor NK cells would have positive effects on the outcome of LTX, the transfer of these cells could be incremented for

therapeutical use. An important prerequisite underlying this mechanism is not clarified at the moment and regards the persistence of donor cells in the recipient. Understanding for how long donor NK cells persist in the liver after transplantation and when they are completely replaced by recipient cells will help clarifying for how long their effect can be prolonged in the recipient. Trying to answer this question we tested *in vivo* the presence of donor NK cells in the peripheral blood of LTX recipients and, when possible, in their transplanted liver at different time points after transplantation.

Materials and Methods

Isolation of mononuclear cells

Perfusates were collected from human liver grafts during the back table procedure. Upon arrival at the hospital, grafts were perfused via the portal vein with 1-2 liters of University of Wisconsin (UW) preservation solution to remove residual blood from the vasculature. Immediately before transplantation, the donor livers were perfused once more with 200-500 mL of human albumin solution to flush UW preservation solution out. These latter perfusates were collected from the vena cava and, unless otherwise indicated, were used to study hepatic NK cell precursors. Mononuclear cells (MNCs) from fresh liver graft perfusates were isolated by Ficoll density gradient centrifugation (1,800 rpm for 20 min at room temperature). MNCs were harvested in the Roswell Park Memorial Institute (RPMI) medium and were counted using Burkit chambers. Cells were stained with Turk solution, which stains the nuclei of the cells, allowing the exclusion of contaminating erythrocytes from the counts. The total amount of cells was calculated according to the following formula: number of counted cells × mL of cell solution in the tube × dilution factor used to mix cells with the staining solution × 10^4. MNCs were either used directly for the experiment or stored in cryovials at -150° C. MNCs were frozen in RPMI medium supplemented with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 20% dimethyl sulfoxide (DMSO) in a slow-freeze method. In this method, DMSO is used to prevent cell death during the freezing process. Thereafter, at -150°C, the metabolism of the cells is stopped allowing samples to be conserved for long periods of time. When needed, isolated cryo-preserved MNCs were rapidly thawed in cold, filtered FBS and were counted using the Trypan blue staining. This dye selectively stains dead cells; therefore uncoloured cells can be counted as living cells, and the viability of the cells can be evaluated. The same procedure of mononuclear cell isolation here described for perfusates was performed also for peripheral blood from either healthy blood bank donors (buffy coats) or LTX patients.

Phenotypic analysis of the leukocytes obtained from perfusates has previously indicated that they well represent liver-derived leukocytes and that the contamination by lymphocytes from blood is negligible [1]. Liver graft biopsies obtained from either healthy donor livers right before transplantation or explanted livers from patients who underwent re-transplantation were collected in UW preservation

solution. First liver graft biopsies were cut into small pieces (approximately 0.5 cm^2), which were then incubated with digestion medium at 37°C at slow shaking. The digestion medium consisted of collagenase IV (0.5 mg/mL; Gibco, Breda, The Netherlands) and DNase type I (0.02 mg/mL; Roche Diagnostics,Manheim, Germany) in RPMI. While collagenase breaks the peptide bonds of collagen allowing the release of cellular components from the extracellular matrix, DNase catalyzes the hydrolytic cleavage of phosphodiester linkages in the DNA backbone and prevents the formation of clumps of dead cells. Next, hepatocytes were precipitated in washing medium containing RPMI and 10% FBS by collagenase, and discarded. MNCs from fresh liver graft biopsies were then isolated by Ficoll density gradient centrifugation. MNCs were harvested in RPMI and counted using Turk staining.

Flow cytometry

Flow cytometry is a technique that allows measurement of multiple physical characteristics of cells simultaneously, as they flow in a fluid stream through a beam of light. When particles pass through the laser intercept, they scatter laser light. There are two kinds of light scattering that allow discrimination of major leukocyte populations: forward scattered light (FSC) and side scattered light (SSC).

Cell surface antigens can be detected by use of monoclonal antibodies (mAb) labeled with various fluorochromes. These florescent molecules can be then detected in different channels by the FACS (Fluorescence-activated cell sorting) machine and visualized in dot plots or histograms. By analyzing the combined expression of these surface markers we could define the intermediate stages of NK cell development. In addition, specialized flow cytometers can sort cells into different fractions based on light scatter and fluorescence profiles.

MNCs obtained from liver graft perfusates, liver graft biopsies or PBMCs obtained from healthy individuals or LTX patients were stained with a combination of monoclonal antibodies (mAb) to assess the immunophenotypic characteristics of the cells. All antibodies and their matched isotype controls used in our experiments are listed in Table. 1. Isotype controls are used to correct the measurement for aspecific binding. Optimal dilutions of all mAbs used were established in preliminary experiments. A FACSCanto II flowcytometer was used for cell analysis, and a FACSAria machine was used for cell sorting (both from BD Biosciences, San Jose, CA). Flow cytometric data were analyzed with BD FACSDiva flow cytometry software, version 6.1.1 (BD Biosciences).

Table 1. Antibodies used for flow cytometric analysis.

Target Molecule	Conjugated fluorochrome	Clone	Company	Remarks
CD3	FITC	UCHT1	Beckman Coulter	T cell marker
	Amcyan	SK7	BD Biosciences	
CD14	FITC	MOP-9	BD Biosciences	monocyte marker
CD15	FITC	Leu-M1	BD Biosciences	Granulocyte marker
CD16	Pacific Blue	3G8	BD Biosciences	Fc-γ receptor III
CD25	Pacific Blue	BC96	BioLegend	IL-2Rα
CD34	APC	8G12	BD Biosciences	HSC marker
CD56	FITC	NCAM16.2	BD Biosciences	NK marker
	PE	MY31	BD Biosciences	
	APC	N901 (NKH-1)	Beckman Coulter	
CD94	PE	HP-3B1	Beckman Coulter	NK marker
CD107a	PE	H4A3	BD Biosciences	Degranulation marker
CD117(c-kit)	PE-Cy7	104D2D1	Beckman Coulter	HSC marker
CD127	APC-Cy7	RDR5	eBiosiences	IL-7R
CD158a	APC	EB6B	Beckman Coulter	KIR2DL1/DS1
CD158b	APC	6L-183	Beckman Coulter	KIR2DL2/DL3
HLA-A2	FITC	BB7.2	BD Biosciences	MHC-I marker
7AAD	–	–	BD Biosiences	Dead cell marker
IgG2a	PE	MOPC-173	Beckman Coulter	Isotype control for CD94
IgG1	APC	P3	eBiosciences	Isotype control for CD34
	PE-Cy7	X40	BD Biosiences	Isotype control for CD117
	PE	X40	BD Biosiences	Isotype control for CD107a

Purfication of NK precursors from perfusate

NK precursors were first pre-enriched from fresh liver perfusate MNCs by negative selection, using CD3 (T cell specific marker), CD14 (myeloid cell specific marker), CD15 and CD56 (NK cell specific

marker) microbeads (Miltenyi Biotech) according to the manufacturer's protocol. The entire cell suspension was then loaded onto a magnetic cell sorting (MACS) LS column that was placed in a magnetic field and retained only the antibody-conjugated cells. The unlabelled cells run through and this cell fraction is depleted of Lin^+ cells. All the reagents used for the isolation were diluted in calculated proportions of PBS supplemented with 2 mM EDTA and 2.5% BSA. Subsequently, the resulting cell suspension was used for cell sorting of pro, pre and immature NK cells.

Maturation of NK cells from their precursors (pro-NK) is described as a process spanning through five sequential stages [33], each one with specific immunophenotypical characteristics. Based on this model we were able to purify cells from all these phases of development from the enriched fraction by cell sorting. Stages were sorted according to expression of the following markers:

Stage 1 pro-NK cells: Lin^- $CD34^+$ $CD117^-$ $CD94^-$

Stage 2 pre-NK cells: Lin^- $CD34^+$ $CD117^+$ $CD94^-$

Stage 3 iNK cells: Lin^- $CD34^-$ $CD117^+$ $CD94^-$

Stage 4 NK bright cells: $CD56^{high}$ $CD34^-$ $CD117^-$ $CD94^+$ $CD16^-$

Stage 5 NK dim cells: $CD56^{low}$ $CD34^-$ $CD117^-$ $CD94^+$ $CD16^+$

In vitro differentiation of NK cells

Purified NK precursors were seeded into 96-well round-bottomed plates. Unless otherwise indicated, each NK precursor culture was initiated with 1×10^4 cells in 200 µl of complete medium consisting of 1640 RPMI with L-Glutamine, 10% heat-inactivated human AB serum, 10% FBS, penicillin (100 U/mL) and streptomycin (100 U/mL; both from Gibco BRL Life Technologies, Breda, The Netherlands) (PS) in the presence of the following cytokines: stem cell factor (SCF) (20ng/ml), FMS-like tyrosine kinase (FLT3-L) (20ng/ml), interleukin-7 (IL-7) (20ng/ml), IL-15 (20ng/ml) and IL-21 (20ng/ml) (all from Peprotech). Half of the culture medium was replaced every 3–4 days**.** After 25 days cells were harvested with culture medium (RPMI supplemented with 10% FBS and 1% PS) and were used for functional assays or FACS analysis.

The choice of using this combination of factors in the culture medium was based on previous studies showing how progression through the two main phases of NK cell development is dependent on various stimulating factors [16]. During the first phase early NK progenitor cells respond to stromal cell growth factors KL (c-kit ligand) and FL (flt-3 ligand) and develop into NK cell precursors with the phenotype $CD34^{bright}$ IL-2/IL-15R+ CD56-. Subsequently these precursors are responsive to IL-15 for maturation into NK cells. In addition, the presence of IL-7 and IL-21 can induce proliferation of the cultured cells.

Functional analyses of in vitro-derived NK cells

Cytotoxicity assays were performed to determine the cytotoxic activity of NK cells after *in vitro* culture. Flow cytometry was used for a combined assessment of degranulation and cytotoxicity. The first was quantified by measuring the surface expression of CD107a, a molecule detectable on cells during degranulation, and the latter was assessed by carboxyfluorescein succinimidyl ester (CFSE)-labeling of the targets and quantification of cell death by using 7AAD. 7AAD is a fluorescent compound that penetrates only cells with a compromised membrane and can then intercalate to their DNA allowing detection of dead cells. CFSE is a fluorescent dye which can be used to stain cells and monitor their proliferation, both *in vitro* and *in vivo*, due to the progressive halving of CFSE fluorescence within daughter cells following each division. K562 target cells are a human erythroleukemic cell line lacking major histocompatibility complex (MHC) class I expression required to inhibit NK activity. K562 cells were maintained in basic culture medium (RPMI, 10% FBS and 1% PS) at 37°C and were split every 3-4 days. In our assays, K562 cells were labeled with CFSE (10 nM; Molecular Probes, Eugene, OR) for 10 minutes at 37°C and then washed with FBS to block the uptake of CFSE. K562 cells were then cultured overnight at 37˚C in culture medium. The following day, K562 cells were collected, counted and coincubated with NK cells in an effector:target ratio of 2:1 and 5:1. CD107a-PE mAb, or alternatively IgG1-PE mAb, was added at the beginning of the culture to the wells to determine the proportion of degranulating NK cells. After 4 hours of incubation at 37˚C, the cells were washed once with PBS and then stained with CD56, CD16 and CD3 mAb. The proportion of viable K562 cells was determined by using 7AAD; to quantify their absolute number, a fixed amount of beads (CaliBRITE unlabeled beads; BD Biosciences, San Jose, CA) was added to each sample and measured by flow

cytometry. For each well, the absolute number of living K562 cells was calculated as a proportion to the number of beads; the percentage of specific cytotoxicity was then estimated as follows:

$$\left[1-\left(\frac{Number\ of\ K562\ in\ coculture\ with\ NK\ cells}{Number\ of\ K562\ in\ monoculture}\right)\right]\times 100$$

Two or three replicates of each sample for each condition were measured in every assay.

Purification of NK cells from perfusates

$CD3^-$ $CD56^+$ NK cells were isolated from fresh liver perfusate MNCs by negative selection, using the NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Briefly, non-NK cells were first labeled with a cocktail of biotin-conjugated antibodies against lineage-specific markers, and subsequently with anti-biotin MicroBeads. In addition to this, during NK cell isolation from perfusate MNCs, CD15 MicroBeads (Miltenyi Biotec) were added for complete granulocyte depletion. The entire cell suspension was then loaded onto a magnetic cell sorting (MACS) LD column that was placed in a magnetic field and retained only the antibody-conjugated cells and the flow-through, containing purified NK cells, was collected into a new tube. All the reagents used for the isolation were diluted in calculated proportions of MACS buffer. MACS buffer consisted of phosphate-buffered saline (PBS) (from BioWhittaker, Lonza, Belgium), 2 mM ethylenediamine tetraacetic acid (EDTA) and 2.5% bovine serum albumin (BSA) (both from Sigma, St Louis, MO). EDTA is used as a chelating agent that binds to calcium and prevents joining of cadherins between cells avoiding, this way, clumping of the cells. BSA is used as a nutrient for cells during the isolation. Purity of isolated NK cells was estimated by flow cytometry using CD3-AmCyan and CD56-APC mAbs and viability was determined by using 7AAD. The average purity of the enriched NK cell population was 98% ± 0.5%, whereas the average viability was 97% ± 1 %. Isolated NK cells were used for mRNA isolation and PCR analysis.

Quantitative PCR

Total mRNA was extracted from either the sorted NK precursors (5×10^4 cells per stage), which were pooled from several perfusate samples, or the purified mature NK cells (5×10^5) using a NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer's instructions. The kit has been used for small amounts of cells ($\leq 5 \times 10^5$). The isolated RNA was treated with rDNase to remove any

contaminating genomic DNA according to the manufacture's instruction. Absence of contaminating genomic DNA and protein as well as concentration of extracted RNA was ascertained using Nanodrop (Thermo). Complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis kit (Bio-rad) according to the manufacturer's instruction. The RNA was mixed with iScript reaction mix containing dNTPs, random hexamers, and reverse transriptase. According to the protocol, primers were allowed to anneal for 5 minutes at 25° C. Then cDNA synthesis occurred at 42° C for 30 minutes, followed by inactivation at 85° C for 5 minutes. The synthesized cDNA was held at 4° C in the cycler and then stored at -20° C. Gene specific primers to amplify the coding sequence of candidate genes were designed using Oligo 6.2 software, and were ordered via (http://medgen.ugent.be/rtprimerdb/). Using dilution series of cDNA the efficiency of the primers was calculated in positive control cells. All primers used in our experiments are listed in Table. 2. Quantitative-PCR was performed using SYBR-Green according to the manufacturer's instruction (Quantace) in duplicate. SYBR-Green binds double-stranded DNA and, as a consequence, can be detected using a fluorescent detector. After each amplification step the sample temperature is increased, dsDNA melts and releases SYBR Green, resulting in a decrease in fluorescence (melt curve). As a result, it is possible to plot the negative rate of fluorescence changes vs. temp (ΔdI/dT) to determine the amplicon size and thereby judge primer specificity. In qPCR reactions the samples containing more templates take less time to reach a threshold concentration of PCR product, while those containing fewer templates take longer. The Ct value, which represents the PCR cycle at which the fluorescence exceeds the background, is a measure of the input nucleic acid. For qPCR the following conditions were used: initial denaturation for 10 minutes at 95° C, following 40 cycles of 10 sec denaturation at 95° C, 10 sec annealing at 58° C, and 15 sec elongation at 72° C. Data were analyzed with the Bio-rad IQ-5 to establish the CT value for each sample. Experimental CT values were used to calculate gene-specific copy numbers from a standard curve, and the gene-specific copy numbers were normalized to the housekeeping gene *GAPDH* (glyceraldehyde phosphate dehydrogenase) copy numbers from the same samples. Relative expression was calculated according to $2^{-\Delta CT}$ method.

Table 2. Primers used for qPCR analysis.

Gene of interest	Primer orientation	Primer sequence
E4BP4	Forward	5'-CGCCCCTTTCTTTCTC-3'
	Reverse	5'-GGGCCTCCTTCGTTATC-3'
Id2	Forward	5'-CTCGCATCC CACTATTGT-3'
	Reverse	5'-TGCTTTGCTGTCATTTGA-3'
IL-17	Forward	5'-GAAGGCAGGAATCACAATC-3'
	Reverse	5'-GCCTCCCAGATCACAGA-3'
IL-22	Forward	5'-CCCATCAGCTCCCACTGC-3'
	Reverse	5'-GGCACCACCTCCTGCATATA-3'
RORC	Forward	5'-CCCGTCAGCAGAACTG-3'
	Reverse	5'-AGCCCCAAGGTGTAGG-3'
IFN-γ	Forward	5'-CCAGGACCCATATGTAAAAG-3'
	Reverse	5'-TGGCTCTGCATTATTTTTC-3'
GAPDH	Forward	5'-TGCACCACCAACTGCTTAGC-3'
	Reverse	5'-GGCATGGACTGTGGTCATGAG-3'

Results

Identification of NK precursors within adult human liver

To determine whether adult human liver is a site of NK development, we used the model proposed by Caligiuri and Freud [33] to identify human NK precursors within the liver. By flow cytometric analysis on MNCs from liver graft perfusates, we identified all the five described stages of the NK cell developmental pathway (Fig. 10). Generally stage 1, 2 and 3 NK cell precursors were present in low frequency among the total intrahepatic lymphocytes (Fig. 16A) and fresh samples clearly contained higher numbers than frozen ones (Fig. 11). We therefore preferred to measure NK precursor stages in the fresh perfusates right after collection without freezing. Consequently, the NK precursor frequency was higher and the cells could be identified as a better distinct cloud in fresh samples.

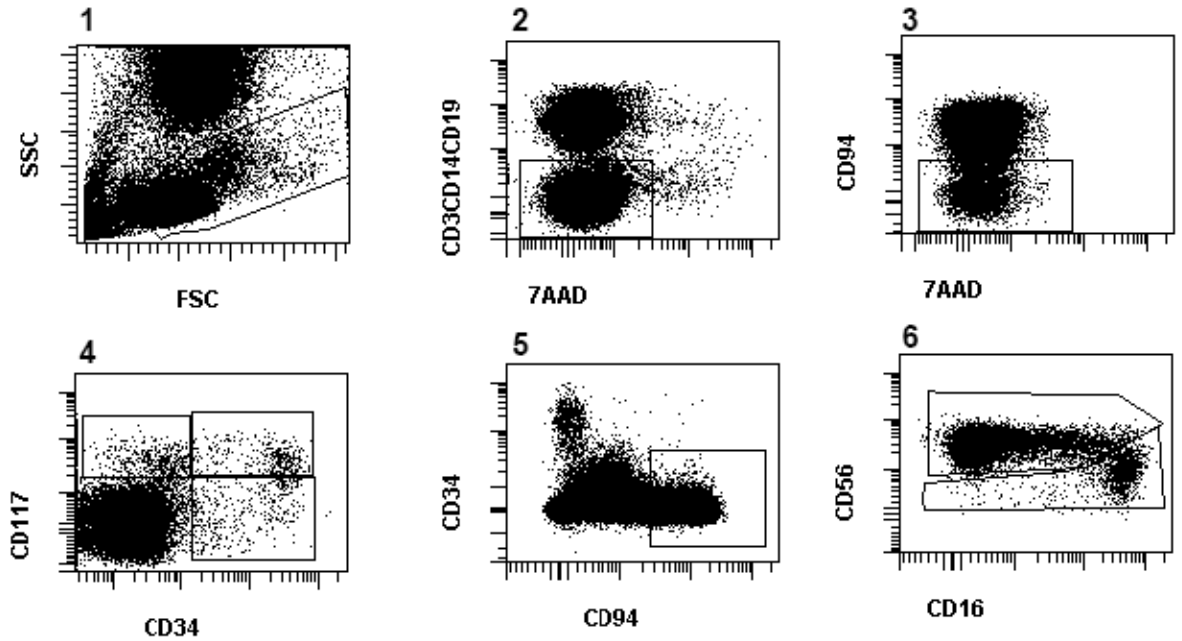

Figure 10. Sequential gating technique leading to the identification of human NK precursors in liver graft perfusate. MNC were first gated based on their side scatter (SSC) and forward scatter (FSC) profile (1). Dead cells were excluded by 7AAD gating (2). Committed lin$^+$ intrahepatic lymphocytes were excluded by CD3, CD14 and CD19 gating (2). CD94$^+$ cells were excluded (3). CD34$^+$ CD117$^-$ stage1, CD34$^+$ CD117$^+$ stage 2 and CD34$^-$ CD117$^+$ stage 3 NK precursors were gated (4). For the detection of mature NK cells, living cells were first gated as CD94$^+$ CD34$^-$ CD117$^{+/-}$ (5). Secondly NK cells were gated on the basis of their CD56 expression: CD56^{bright} (upper gate) and CD56^{dim} (lower gate) (6). One representative experiment of five is shown.

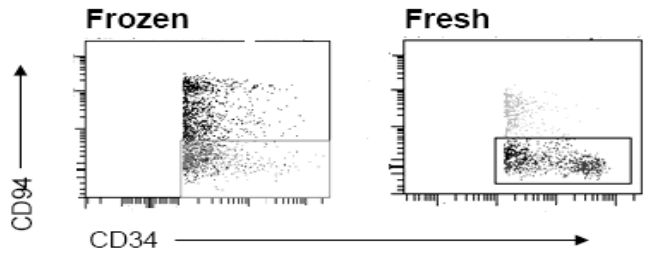

Figure 11. Comparison of gating stage 1 NK cells precursors in frozen and fresh liver graft perfusate samples. Perfusates were obtained from perfusion of human liver grafts and MNCs were isolated as indicated before. Half of the cells obtained were used freshly to detect NK precursors, while the other half was stored at -150˚C and used after 2-3 months to detect NK precursors. One representative experiment of three is shown.

To confirm that the NK cells precursors detected in liver graft perfusates represented a real population contained within the liver, we performed the same flow cytometric analysis on MNCs from liver graft biopsies. This analysis confirmed the presence of all five described stages of the NK cell developmental pathway in liver biopsies. However, we measured lower numbers of total lymphocytes in the biopsy samples (Fig. 16B), and most of them were $7AAD^+$ dead cells. As a result, we could not discriminate the 3 intermediate stages from each other properly (Fig. 12) due to lack of isotype controls and lower numbers of the cells in each stage.

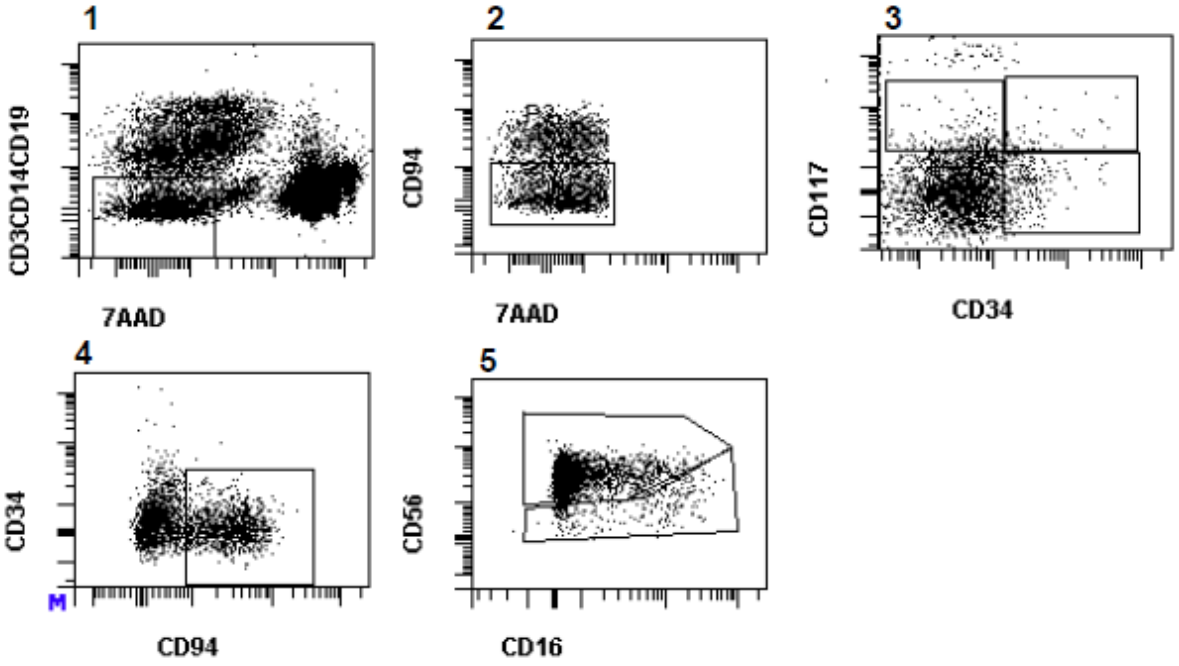

Figure 12. Identification of NK precursors in liver graft biopsies. After lymphocyte gating on FSC versus SSC plot (not shown), dead cells were excluded by 7AAD gating and committed

lin$^+$ intrahepatic lymphocytes were excluded by CD3, CD14 and CD19 gating (1). CD94$^+$ cells were excluded (2). CD34$^+$ CD117$^-$ stage1, CD34$^+$ CD117$^+$ stage 2 and CD34$^-$ CD117$^+$ stage 3 NK precursors were gated (3). For the detection of mature NK cells, living cells were first gated as CD94$^+$ CD34$^-$ CD117$^{+/-}$ (4). Secondly, NK cells were gated on the basis of their CD56 expression: CD56^{bright} (upper gate) and CD56^{dim} (lower gate) (5). One representative experiment of three is shown.

Previous studies have shown that NK precursors are present in peripheral blood. However, their proportion and immunophenotypical characteristics in peripheral blood remain largely unclear. To assess the immunophenotypical differences between hepatic and circulating NK precursors, we analyzed in PB the surface expression of the same markers considered for MNCs in perfusates and biopsies (Fig. 13). As expected most of the mature NK cells in blood belonged to the CD56^{dim} subset, in contrast with hepatic mature NK cells which mainly belonged to the CD56^{bright} subtype. The number of circulating NK precursors in each stage of development was more similar to the distribution observed for liver biopsy samples than for liver perfusates (Fig. 16C). Although the same general immunophenotypical characteristics were conserved between hepatic and circulating precursors, a clear difference was observed when analyzing the expression of CD25 (IL-2Rα) and CD127 (IL-7Rα). While stage 3 and stage 4 cells from peripheral blood did not express CD25 (IL-2Rα) and CD127 (IL-7Rα), cells at the same developmental stage did express these surface markers in liver perfusates and biopsies (Fig. 14).

Additionally, the Caligiuri group [60] has recently reported how CD56^{dim} NK cells may develop from CD56^{bright} NK cells and that CD94 surface density identifies a functional intermediate between the two human NK cell subsets in peripheral blood. Therefore, CD94^{high} CD56^{dim} was defined as the intermediate stage between CD94^{high} CD56^{bright} NK cells and CD94^{low} CD56^{dim} ones. By performing the same flow cytometric analysis as the one described in the paper, we compared peripheral blood with perfusate of liver grafts. Similarly to the proportions described by Caligiuri and his colleagues, we observed that in peripheral blood CD94^{high} CD56^{bright} NK cells accounted for 12.2 ± 8 %, while the CD94^{high} CD56^{dim} intermediate stage accounted for 35.7 ± 11.5 % and CD94^{low} CD56^{dim} NK cells accounted for 45.2 ± 15% of total NK cells. However, in perfusate of liver grafts they accounted for 49.5 ± 13 %, 18.3 ± 6.4 % and 29.1 ± 9.7 % respectively (Fig. 15).

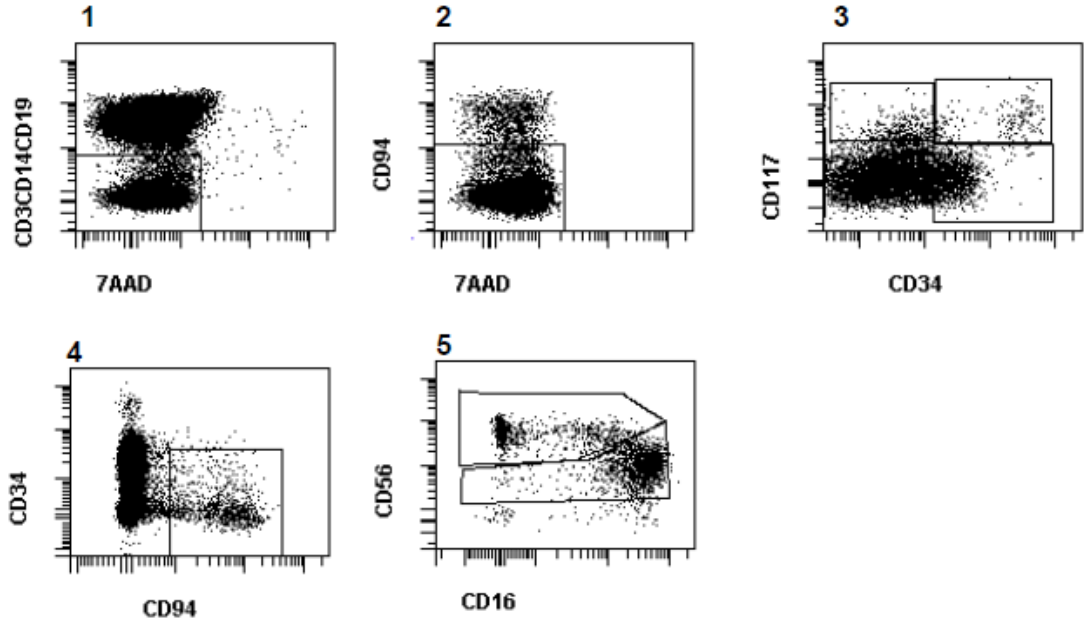

Figure 13. Identification of NK precursors in peripheral blood of healthy donors. After lymphocyte gating on FSC versus SSC plot (not shown), residual erythrocytes, debris, doublets and dead cells were excluded by 7AAD gating (1). Committed lin+ intrahepatic lymphocytes were excluded by CD3, CD14 and CD19 gating (1). CD94+ cells were excluded (2). CD34+ CD117- stage1, CD34+ CD117+ stage 2 and CD34- CD117+ stage 3 NK precursors were gated (3). For the detection of mature NK cells, living cells were first gated as CD94+ CD34- CD117+/- (4). Secondly, NK cells were gated on the basis of their CD56 expression: CD56bright (upper gate) and CD56dim (lower gate) (5). One representative experiment of three is shown.

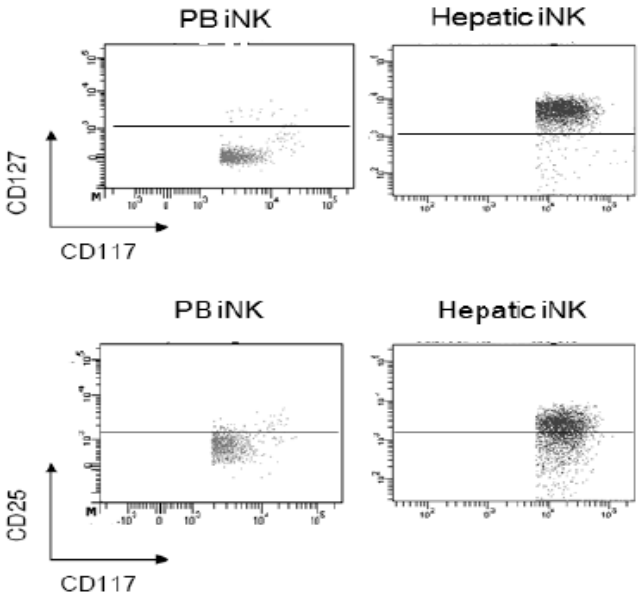

Figure 14. Differences between Peripheral Blood and hepatic iNK cells. (A) Stage 3 iNK cells were gated from MNCs of perfusates of liver grafts (hepatic iNK) or from PBMC of healthy

donors (PB iNK). CD127 and CD25 expression were then analyzed separately on these populations. One representative experiment of three is shown.

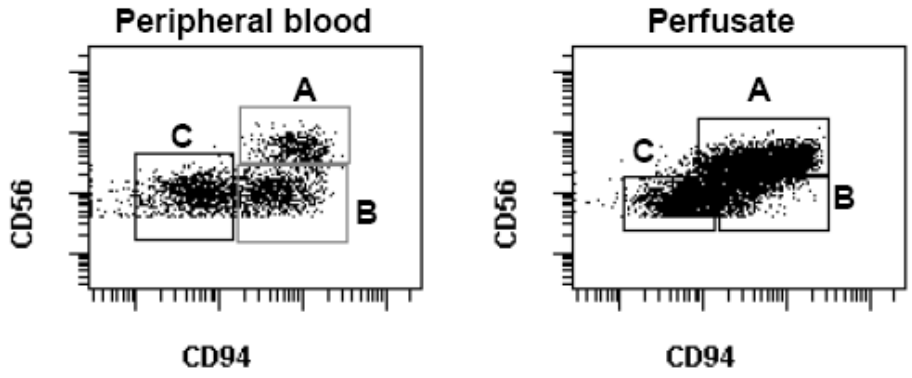

Figure 15. Comparison of the frequency of the CD94^{high} CD56^{dim} intermediate stage in peripheral blood and perfusate. Mononuclear cells isolated from peripheral blood and perfusate of liver grafts were stained with anti-CD3, anti-CD56 and anti-CD94 mAb. CD56^{+} CD3^{-} NK cells were then gated for quantification of CD94 surface density. Next, CD94^{high} CD56^{bright} NK cells (A), CD94^{high} CD56^{dim} intermediate stage (B) and CD94^{low} CD56^{dim} NK cell (C) were gated. The dot plots are representative of 5 independent experiments in perfusate and 3 independent experiments in blood.

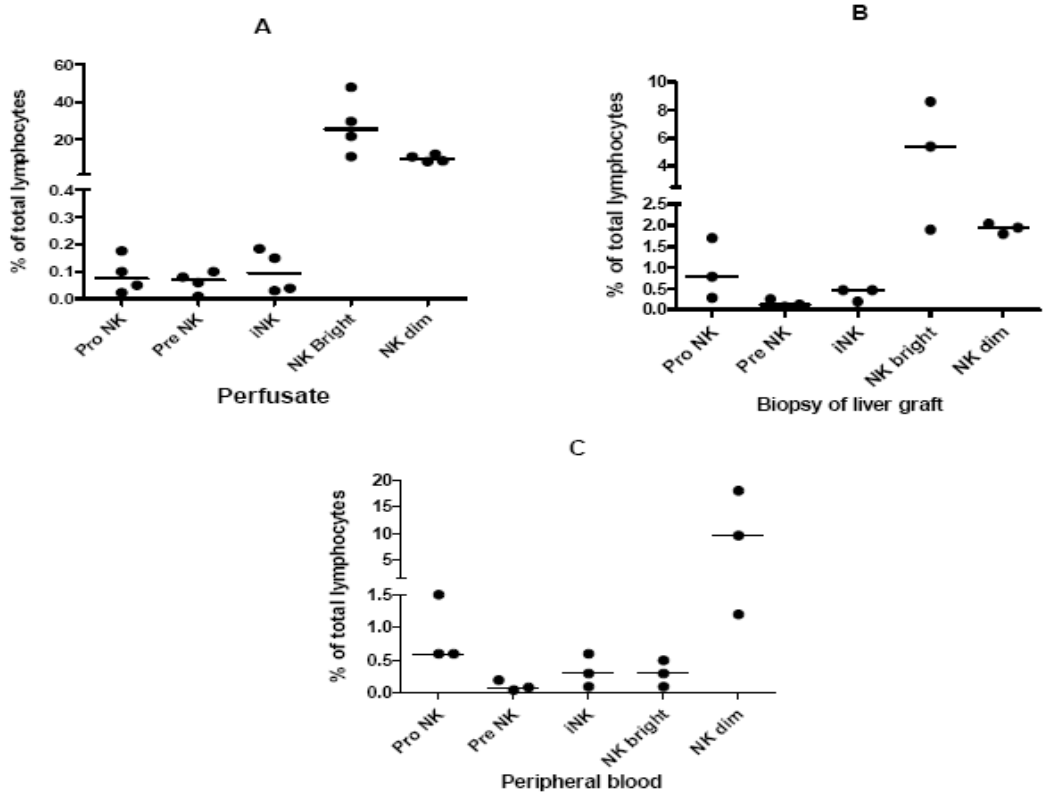

Figure 16. Distribution of NK precursors in liver perfusates, liver biopsies and peripheral blood. All the analysis here shown are performed using the same combination of mAb to define each

stage of NK cell development. Dots represent measurements of different samples from liver graft perfusates (A), liver graft biopsies (B) and peripheral blood of healthy individuals (C) horizontal lines indicate median values.

In vitro development of hepatic NK precursors

To assess the potential of hepatic NK precursors to differentiate into $CD3^-$ $CD56^+$ NK cells, we sorted stage 1, 2 and 3 cells from fresh liver graft perfusate (Fig. 17) and cultured them for approximately 4 weeks as described in the materials and methods section. While stage 2 and stage 3 hepatic precursors differentiated into mature NK cells after 25 days of culture (Fig. 18 and 19), stage 1 cells could not survive for more than 7 days in these culture conditions.

As shown in Fig. 17, right after sorting the purity of isolated stage 2 and stage 3 precursors, which then developed into mature NK cells, was higher than 95%. By contrast, the purity of sorted stage 1 cells was around 50%. After 25 days of culture the sorted stage 2 and 3 cells differentiated into $CD3^-$ $CD14^-$ $CD19^-$ $CD34^-$ $CD117^+$ iNK cells and within this population small fraction is $CD56^-$ iNK cells, while most of the cells expressed the NK cell marker CD56 (Fig. 18B and 19B). Nevertheless, among the cultured cells a small fraction expressed $CD3^+$ and was negative for CD56 (Fig. 18C and 19C). Moreover, we did not detect any $CD14^+$ cells or $CD19^+$ B cells among the differentiated cells (not shown).

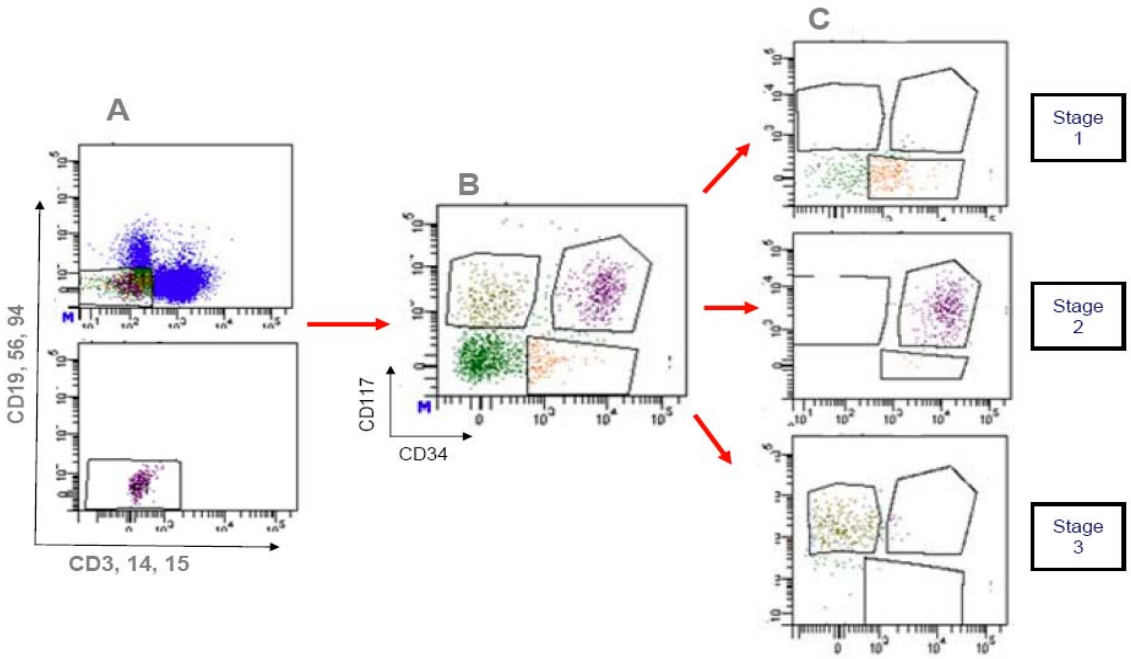

Figure 17. Sorting of hepatic stage 1-3 precursors from perfusates of liver grafts. (A) Lymphocytes were first gated on basis of FSC versus SSC. Lineage positive committed cells

were first excluded by magnetic bead isolation and then by lin marker sorting to a purity > 99% (A). From lineage negative cells, stage 1-3 cells were sorted based on expression of CD34 and CD117 (B) reaching a purity up to > 97% for stage 2 and 3 and up to 50% for stage 1 (C). Data are shown as a representative of 5 independent experiments.

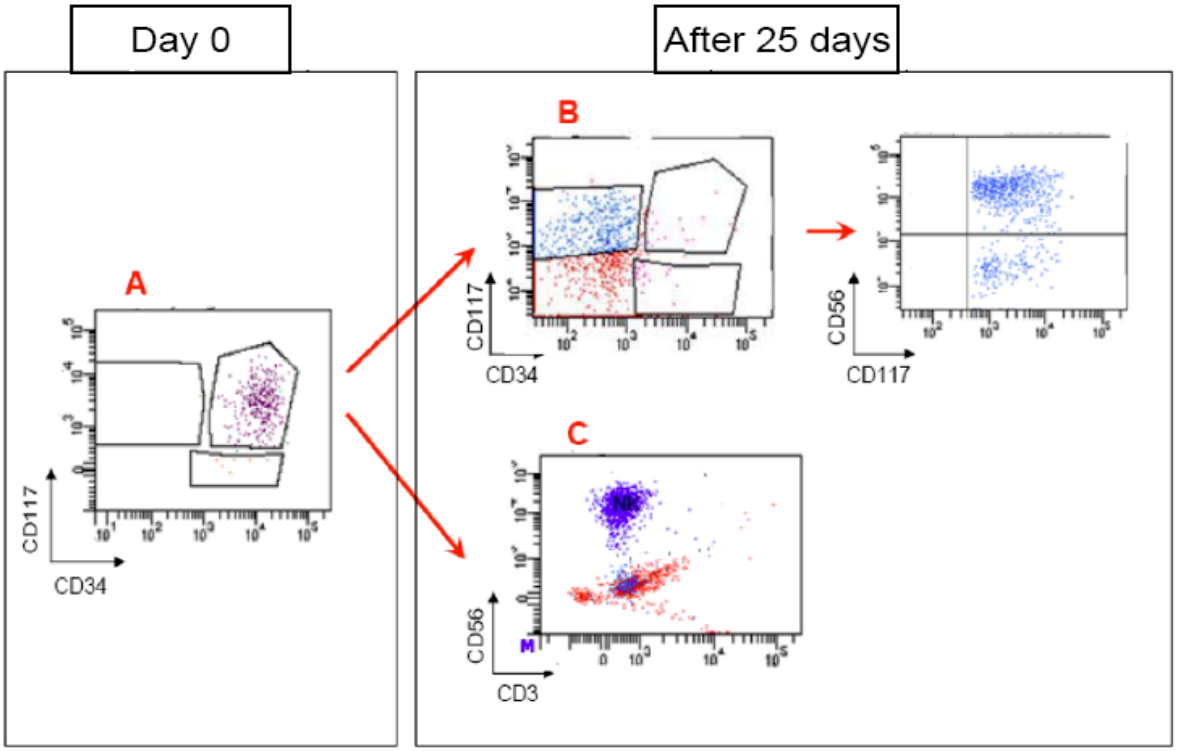

Figure 18. Differentiation of stage 2 hepatic pre-NK cells into CD3- CD56+ mature NK cells. (A) Sorted stage 2 cells were cultured for 4 weeks in the conditions previously described. Cultures were initiated with 10^4 starting cells. After 25 days, cells were analyzed by flow cytometry for expression of stage 3 immature NK cell markers (B) and mature NK cell markers(C). Lymphocytes are first gated in the FSC versus SSC plot, then Lin+ and dead cells are excluded. Phenotypical analysis of stage 2 cells before and after 25 days of culture are here shown as representative of 5 independent experiments.

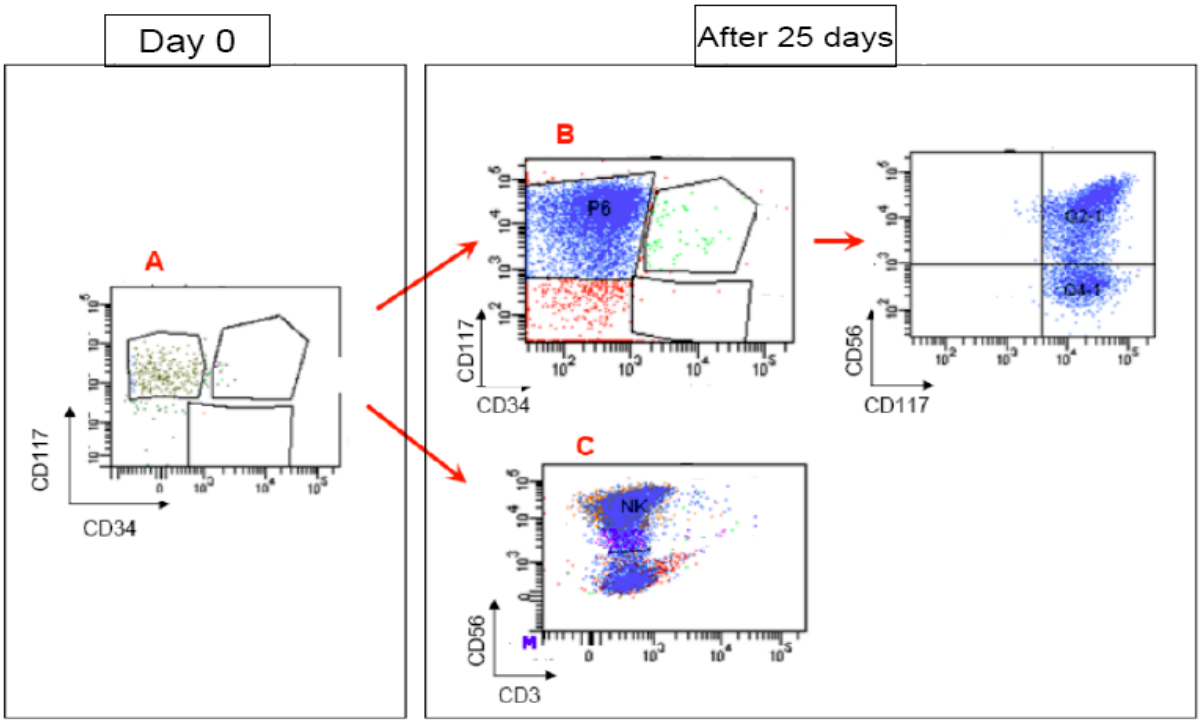

Figure 19. Differentiation of stage3 hepatic iNK cells into CD3- CD56+ NK cells. (A) Sorted stage 3 cells were cultured for 4 weeks in the conditions previously described. Cultures were initiated with 10^4 starting cells. After 25 days cells were analyzed by flow cytometry for expression of stage 3 immature NK cell markers (B) and mature NK cell markers(C). Lymphocytes are first gated in the FSC versus SSC plot, then Lin+ and dead cells are excluded. Phenotypical analysis of stage 2 cells before and after 25 days of culture are here shown as representative of 5 independent experiments.

Functional analysis of in vitro differentiated NK cells

To determine the functionality of the $CD3^-$ $CD56^+$ NK cells differentiated *in vitro* from hepatic stage 2 and 3 NK precursors, we performed cytotoxicity and degranulation assays against the target cell line K562. When surface expression of CD107a was quantified as a direct measurement of degranulation activity, in vitro differentiated NK cells coincubated with K562 targets showed a higher response than NK cells cultured alone (spontaneous degranulation) (Fig. 20A-C). Stage 3 derived NK cells showed slightly higher degranulation activity than stage 2 derived NK cells (Fig. 20D). Moreover, degranulation activity of NK cells had only a small variation by increasing the E: T ratio from 2:1 to 5:1 (not shown).

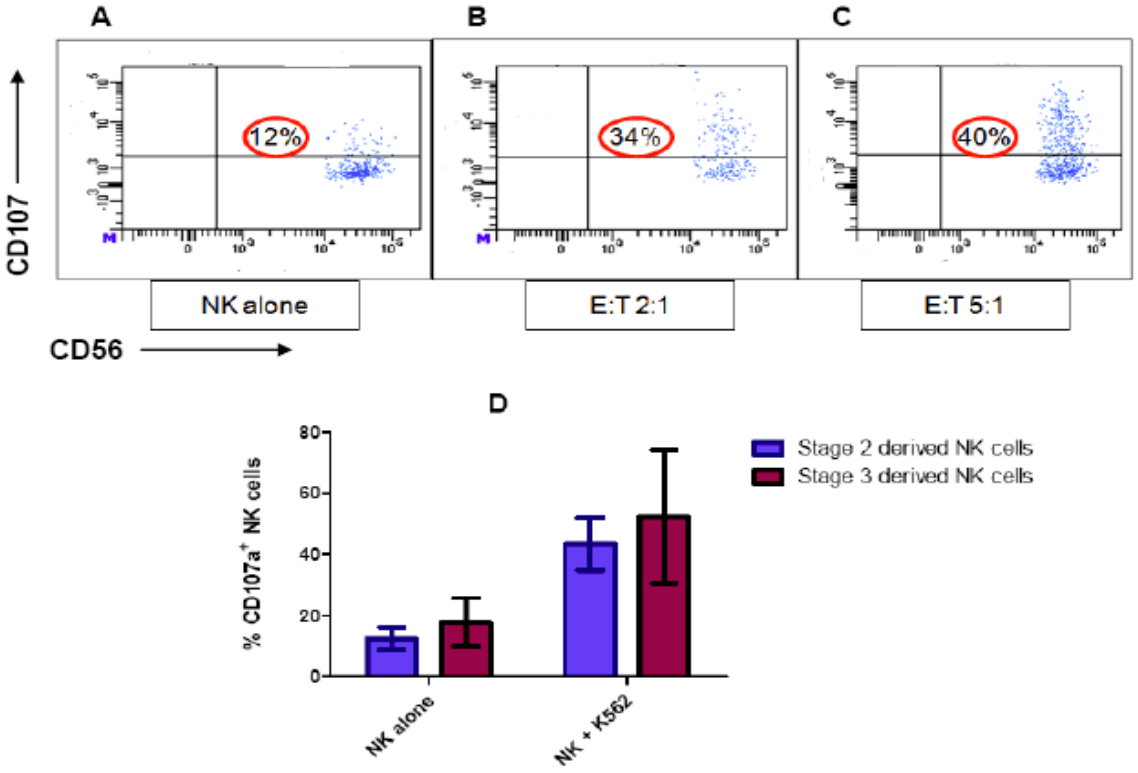

Figure 20. Degranulation assays on *in vitro* differentiated NK cells derived from hepatic stage 2 and 3 NK precursors. In vitro differentiated NK cells were coincubated with K562 cells in an effector: target (E:T) ratio of 2:1 and 5:1. Representative examples of dot plots used for this quantification are here reported: in vitro differentiated NK cells in single culture (A), in vitro differentiated NK cells coincubated with K562 targets in two different E:T ratios (B, C). Numbers in the plots indicate percentages of cells in the quadrants. After 4 hours of coincubation, degranulation of the effector NK cells was quantified by detection of CD107a. Data are shown as the average of both E:T ratio and as means ± SD of 5 independent experiments (D).

To asses the cytotoxic capacity of *in vitro* derived NK cells, numbers of CFSE-labelled K562 were quantified by flow cytometry after incubation in the presence or absence of the effector NK cells (Fig. 21A-B). The *in vitro* differentiated NK cells showed a high capacity to kill their targets. The average percentage of cytotoxicity of stage 2-derived NK cells was 80% ± 10 % in a 2:1 E:T ratio, and 82% ± 8 % in a 5:1 E:T ratio. Similarly, stage 3-derived NK cells killed 60% ± 20 % of their targets in a 2:1 E:T ratio and 80% ± 8 % in a 5:1 E:T ratio (Fig. 21C). In vitro derived NK cells were also tested for the expression of KIRs. Two groups of KIRs were measured on stage 2 and stage 3-derived NK cells: CD158a (KIR2DL1/DS1) and CD158b (KIR2DL2/DL3). However, the results were negative and neither stage 2 nor stage 3-derived NK cells expressed these KIRs (not shown).

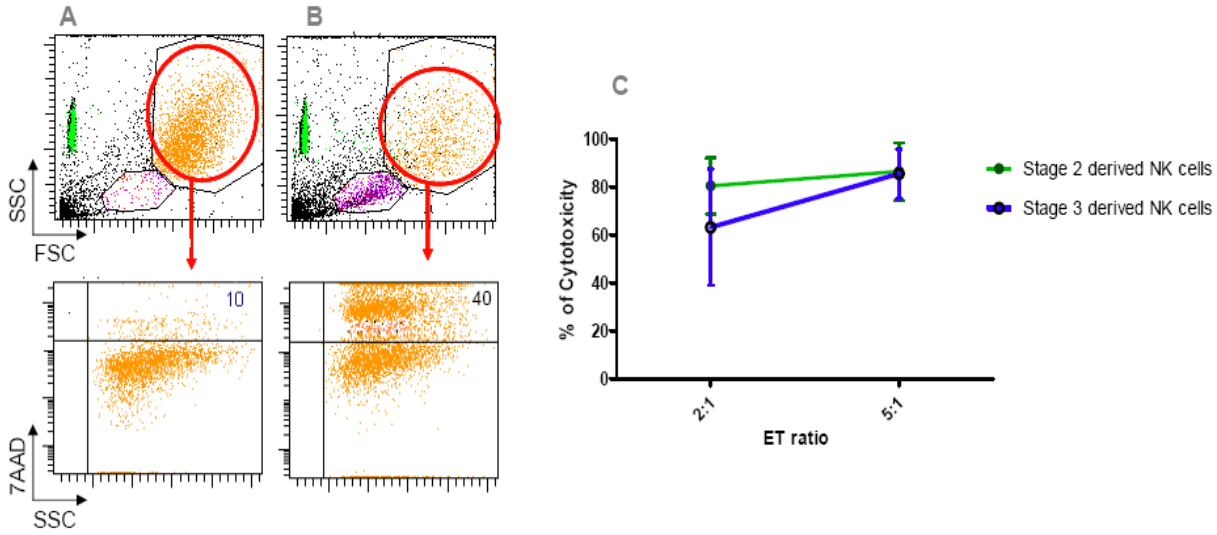

Figure 21. Cytotoxicity assays on *in vitro* differentiated NK cells derived from hepatic stage 2 and 3 NK precursors. In vitro differentiated NK cells were coincubated with K562 cells in an effector: target (E:T) ratio of 2:1 and 5:1. Representative examples of dot plots used for this quantification are here reported: K562 in single culture (A), *in vitro* differentiated NK cells in coculture with K562 targets (B). Numbers in the plots indicate percentages of cells in the quadrants. Example of typical forward scatter and side scatter plots obtained at the end of the 4-hour coincubation are here shown. Beads used for normalization are coloured in green, NK cells (absent in A) are coloured in purple, and K562 cells are coloured in orange. K562 target cells are then plotted against 7AAD to discriminate between living and dead cells. (C) By normalization with beads, the absolute numbers of living K562 after incubation were obtained, and specific cytotoxicity was calculated. Here shown are average ± SD of percentages of K562 cells killed by NK cells in 5 independent experiments.

Quantitative PCR analysis of NK developmental stages

To further support our data, we started analyzing by qPCR the mRNA expression levels of some transcription factors relevant for NK development. It has been reported that the transcription factors *ID2* together with *E4BP4* are essential for NK cell differentiation [61]. We here report our preliminary data from one single experiment. We observed that *ID2* mRNA was expressed in almost all populations of NK cell precursors and was highly expressed in mature NK cells. By contrast, in case of *E4BP4* mRNA expression was found high in mature NK cells, pro- and pre-NK progenitors, but unexpectedly low in iNK cells. In addition, as expected, mature NK cells expressed mRNA for *IFN-γ*, whereas NK cell precursors did not (Fig. 22A).

Cupedo et al. [62] have shown that in tonsils and lymph nodes, the majority of stage 3 iNK cells are $CD127^+$ $RORC^+$ lymphoid tissue inducer (LTi) cells and they express *IL-22* mRNA (similar to NK22 population proposed by Cella et.al [63]), while blood iNK cells are negative for these markers and are not LTi cells. By flow cytometric analysis, we observed that hepatic iNK cells are unique in this manner since they are mostly $CD127^+$ $RORC^-$ (Fig. 22B). However, by qPCR analysis we could detect *RORC* and *IL-22* mRNA expression in all stages, and especially in stage 3 iNK cells (Fig. 22A). Furthermore, IL-17 mRNA was not expressed in any of the four stages in the liver (not shown).

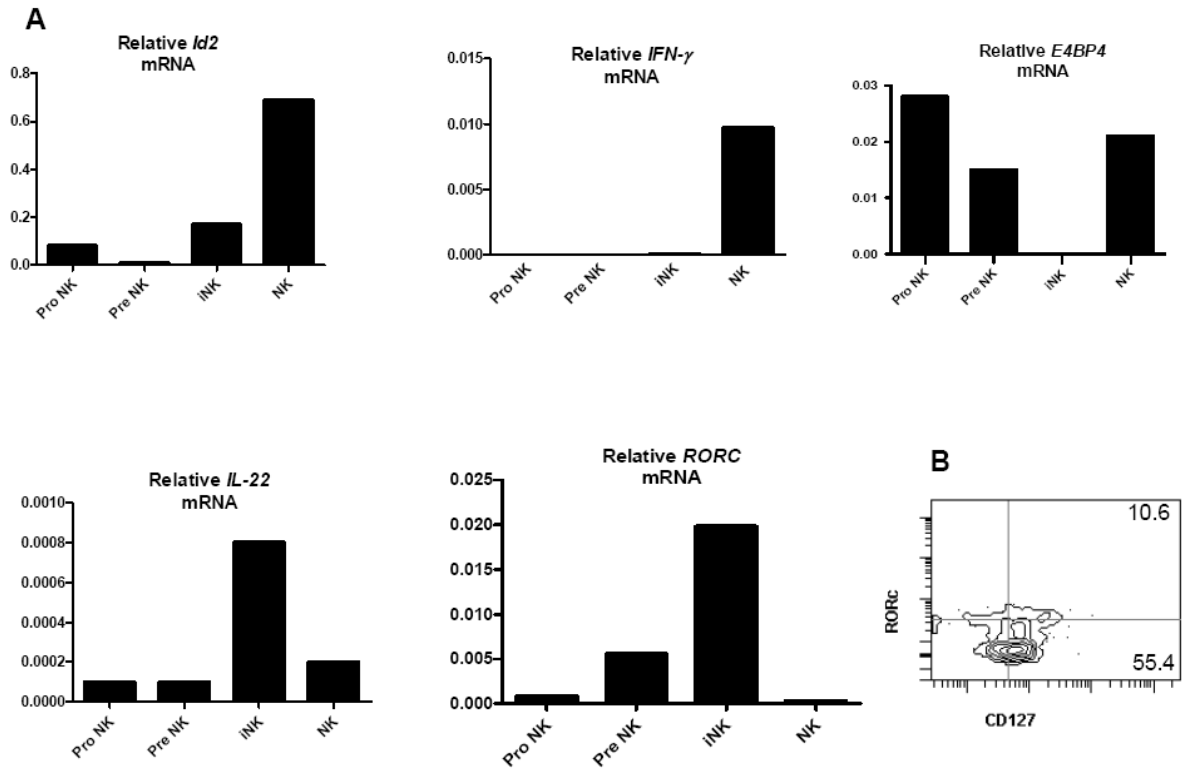

Figure. 22. Quantitative PCR analysis of hepatic NK precursors and mature NK cells. (A) Hepatic Pro-, Pre- and iNK cells were sorted from perfusates of liver grafts, while mature NK cells were purified by magnetic beads from perfusate of liver grafts. Quantitative PCR analysis of *Id2*, *IFN-γ*, *E4BP4*, *IL-22* and *RORC* mRNA was performed in various populations (horizontal axis) and normalized to *GAPDH* expression. Preliminary data of one experiment are here shown. (B) Intracellular staining of MNCs from perfusates was performed by flow cytometry to analyze *RORC* expression by hepatic iNK cells. The values indicate average number of cells in the quadrants from 2 independent experiments.

Persistence of donor immune cells in the LTX recipient

To investigate whether the local NK precursors in the liver are really able to renew the NK cell population, we analyzed presence of donor-derived NK precursors and NK cells in the liver long time after transplantation. Donor-derived NK cells were detected in the recipient by means of HLA-A2 or HLA-Bw4 disparities. When HLA-A2- recipients were transplanted with a liver from an HLA-A2+ donor, or vice versa, donor derived cells could be easily distinguished by flow cytometry by use of an HLA-A2 mAb. The same analysis could be performed in case of HLA-Bw4 disparities. By flow cytometric analysis, we therefore tested the presence of the five stages of NK development in peripheral blood of patients long time after LTX (Table. 3). In addition, after selecting the LTX cases with the proper HLA mismatches, for patients that underwent re-transplantation we obtained biopsies from the first grafted liver. From these specimens we isolated hepatic lymphocytes to detect donor NK cells precursors still resident in the graft (Table. 4). As shown in tables 3 and 4, although we could not detect donor-derived stage 1-3 NK precursors neither in the circulation of recipients nor in the explanted liver grafts, we could identify small fractions of mature NK cells of donor origin in both sites long time after LTX. Similarly to NK cells, we could identify leukocytes expressing other lineage markers, including $CD3^+$ $CD56^-$ T cells, $CD3^+$ $CD56^+$ NKT cells, and not well-characterized $CD16^+$ or $CD14^+$ cells of donor origin. The percentages of donor-derived cells were then calculated as $\left[\frac{number\ of\ donor\ within\ the\ specific\ lymphocyte\ subset}{total\ number\ of\ donor\ and\ recipient\ within\ the\ lymphocytes\ subset}\right]\times 100$. On the other hand, we identified recipient NK precursors in the donor liver of all patients even within 7 days after transplantation. Eventually, all types of mature immune cells with recipient origin were also detectable in the donor liver.

Table 3. Percentage of total and donor-derive immune cells in the peripheral blood of recipient after LTX.

% of total lymphocytes (% of donor-derived within each subset)					
	Patient 1 (10 Months)	Patient 2 (15 Months)	Patient 3 (17 Months)	Patient 4 (14 Months)	Patient 5 (15 Months)
Pro-NK	0.3 (0)	0.2 (0)	0.5 (0)	0.2 (0)	0.2 (0)
Pre-NK	0.05 (0)	0.09 (0)	0.07 (0)	0.03 (0)	0.02 (0)
iNK	0.01 (0)	0.05 (0)	0.06 (0)	0.01 (0)	0.03 (0)
NK	7.8 (0)	14.1 (0.07)	11.3 (0)	10.5 (0)	6.6 (0)
CD3+CD56+	3.2 (0)	7.2 (4.6)	5.8 (0)	8.3 (0)	1.6 (0)
CD3+CD56–	9.7 (0.01)	12.1 (0.08)	15.5 (0.08)	8.1 (0)	3.3 (0)
CD16+	16.6 (7.6)	10.9 (1.8)	13.1 (2.3)	6.2 (0)	5.2 (0.1)

Table 4. Percentage of total and donor-derived immune cells in the explanted donor liver.

% of total lymphocytes (% of donor-derived within each subset)				
	Patient 1 (2 Years)	Patient 2 (2 Years)	Patient 3 (1 Year)	Patient 4 (7 Days)
Pro-NK	0.2 (0)	0.1 (0)	0.1 (0)	0.4 (0)
Pre-NK	0.05 (0)	0.01 (0)	0.04 (0)	0.07 (0)
iNK	0.1 (0)	0.1 (0)	0.1 (0)	0.3 (0)
NK	7.9 (1.7)	32.2 (4.8)	42.8 (1.7)	10.1 (6.8)
CD3+ CD56+	5.1 (3.7)	6.6 (16.3)	5.7 (1.6)	7.5 (6.8)
CD3+ CD56 –	10.5 (16)	8.8 (18.6)	22.8 (0.8)	12.5 (2.1)
CD14+	7.2 (37)	22.2 (46)	7.8 (27.5)	15.4 (63.3)
CD16+	13.5 (20)	6.1 (0.6)	10.4 (0.6)	5.2 (3)

Discussion

While the in vivo developmental pathways for human B and T lymphocytes as well as their sites of development have been generally understood, the pathway of development of human NK cells has only recently been defined [33]. While the bone marrow is considered to be the main site of development for NK cells, alternative sites of NK cell development have recently been identified in the adult human body. NK cells can develop locally in the intestine [35] and SLT [34] including, lymph nodes, tonsils, and spleen. These sites all contain organ-specific NK cells with some phenotypical differences compared to blood NK cells [51].

Regarding the human liver, while there are evidences demonstrating the presence of hematopoietic stem cells and early progenitor cells in the adult human liver [52-54], there is no evidence indicating whether NK cells can locally develop from hematopoietic precursors. We have previously shown that organ-specific NK cells are present in the adult human liver [1]. In this present study, we have identified in the liver all the five stages of the NK developmental pathway and we have highlighted some phenotypical features that distinguish these cells from blood NK precursors. These hepatic NK precursors were able to differentiate into mature NK cells with high level of cytotoxicity *in vitro*. As a result, we propose that the adult human liver is a possible site of local NK cell development. Supporting this concept, we assessed the presence of donor-derived NK cells long time after liver transplantation in the peripheral blood of the recipient as well as in the grafted liver. The finding that donor-derived mature NK cells were detected suggested that donor NK cell development is partially maintained in the recipient after liver transplantation.

In the current study we hypothesized that if hepatic NK cells have these unique features [1] and at the same time HSC are present in the liver [54], NK cells may develop locally within the adult human liver. To investigate our hypothesis, we identified NK cell precursors based on the developmental model that, as previously mentioned, was recently proposed for the maturation of NK cells. In addition, we detected mRNA expression of Id2 and E4bp4, which are known as important transcription factors during NK cell development. Notably, E4bp4 transcription factor has until now been only described in mouse NK cell development [61]. Our preliminary data indicate that Id2 and E4bp4 are both acting

during hepatic NK cell development and their activity seems to be complementary. These data seem to be concordant with previous studies, which have shown that E4bp4 is required at the earliest stage of NK cell development and can induce Id2 expression, crucial in later stages of NK maturation [61, 64-65]. Moreover, we showed that mRNA of IFN-γ was present in mature NK cells, while it was absent in NK cell precursors. Additionally NK cell precursors as well as mature NK cells did not express IL-17 and only low levels of IL-22 mRNA, supporting the hypothesis that they are committed NK cell precursors [66]. The low levels of IL-22 expression were most probably derived from the small fraction of $CD127^+$ $RORC^+$ LTi cells within the hepatic iNK population (Fig. 22B). all together, our preliminary molecular analysis seems to support the hypothesis that hepatic NK cell precursors can develop in mature and functional NK cells. However, our qPCR results are only preliminary and need further confirmation.

We identified some differences in the number of NK precursors in perfusates and liver biopsies. In biopsy specimens, it appears that stage 1 and 3 cells were present in larger numbers than in perfusates, while stage 4 and 5 cells in biopsies were less abundant than in perfusate (Fig. 16). A possible explanation may derive from the fact that the measured total lymphocytes in the biopsy samples were by far less than in perfusate. As a result, the amount of cells in each stage was too low to discriminate them from each other. In addition to this, stage 2 NK precursor cells in liver biopsies were hardly detectable in biopsy specimens due to absence of the $CD34^+$ $CD117^+$ population, whereas the same cells were clearly present in liver perfusates (Fig.12). To exclude that the use of collagenase IV, used in the protocol to release cells from the extracellular matrix, could have affected the detection of the surface markers, we tested the effect of the enzyme on the expression of CD117 and CD34. We analyzed the expression of both CD117 and CD34 in both collagenase-treated, and non-collagenase treated lymphocytes taken from human BM and observed no difference in their expression (not shown).

Since we identified the NK precursors not only in perfusates and biopsies of liver grafts but also in peripheral blood, we studied whether the precursors found in liver tissue differed from those in blood, or were derived from contaminating blood. By looking more specifically at the phenotype of hepatic and blood-derived NK cell precursors, we observed some additional differences. Our data indicate that

the majority of stage 3 iNK cells and small fraction of stage 4 mNK cells in perfusates and biopsies of liver grafts express CD25 (IL-2Rα) and CD127(IL-7Rα), whereas circulating counterparts do not express these same markers. This difference was mainly observed for stage 3 iNK cells and small number of stage 4 NK cells, while the other stages of NK cell development pathway never expressed CD25 and CD127. It means that expression of CD25 and CD127 on the iNK precursor cells is up-regulated in the liver. Most probably, due to the secretion of IL-2 and IL-7 in the liver environment, iNK cell respond to these cytokines by up-regulation of CD25 and CD127. Doherty et. al has shown that IL-2 is secreted by hepatic T cells and NKT cells upon CD3 cross linking or PMA and ionomycin stimulation [44]. Moreover, IL-7 can be produced by murine liver parenchymal hepatocytes [67]. Other authors have shown that the majority of stage 3 iNK precursor cells in tonsils express CD127 and RORC, and show lymphoid tissue inducer activity [66]. Therefore, we analyzed the expression of RORC by qPCR and, for stage 3 hepatic iNK cells, also intracellularly by flow cytometry. Surprisingly, the majority of hepatic iNK cells did not express RORC intracellularly (Figure 13B) but had higher RORC mRNA levels than all the other developmental stages (Figure 22A). We believe that the potential LTi activity of stage 3 iNK cells within the liver should be investigated further.

It has been suggested that $CD56^{dim}$ NK cells may develop from $CD56^{bright}$ in peripheral blood [60]. Recently a new study found that the surface density of CD94 identifies an intermediary stage of development between CD56bright and CD56dim NK cells (Fig. 15). The authors have shown that in peripheral blood CD56bright CD94high NK cells develop through the intermediate stage CD56dim CD94high before completing their maturation into CD56dim CD94low. To determine whether this progression is shared by hepatic NK cells, we analyzed the presence of all three NK subsets in both blood and liver (Fig. 15). Our data indicate that in liver the intermediate CD56dim CD94high subset has a lower frequency compared to the same subset in blood, suggesting that differentiation of $CD56^{dim}$ NK cells from $CD56^{bright}$ NK cells proceeds relatively slowly within the liver, which may account for the relatively high numbers of CD56 bright NK cells in hepatic tissue.

During identification of NK precursor cells in perfusates of liver grafts, we observed that fresh samples contain higher numbers of NK precursor cell than frozen samples. We then analyzed the effect of the process of freezing and thawing on the frequency of NK precursors more in detail. By comparing fresh

and frozen samples from the same specimen, we detected a reduction of the number of NK precursors due to the freezing step. This observation is suggesting that HSCs are more sensitive to the process of freezing, and perhaps to the toxicity of DMSO, than mature cells.

While stage 2 and stage 3 hepatic precursors differentiated into mature NK cells after 25 days of culture (Fig. 18 and 19), stage 1 cells could not survive for more than 7 days in these culture conditions. A possible explanation for this failed maturation of stage 1 NK precursors may be the lack of feeder cells in the culture, as previous literature reported an essential role of a supporting layer of OP9 feeder cells for the growth of only stage 1 cells [32]. In addition, the low purity of stage 1 sorted cells (50%) may also have influence on the decreased survival of these cells. It has been previously shown that there are two populations of $CD34^+$ cells: $CD34^{high}$ and $CD34^{dim}$. In contrast to stage 1 and 2 precursors in peripheral blood, the majority of $CD34^+$ cells in human LN express this HSC marker only dimly. Current knowledge indicates that $CD34^{high}$ cells migrate from BM to LN and there develop into $CD34^{dim}$. Herewith, within lymph nodes, it has been shown that only a specific subset of NK cell precursors with dim expression of CD34 differentiated into $CD56^{bright}$ NK cells in vitro [34]. Similar to human LN, we showed that the majority of $CD34^+$ cells in the liver are also CD34dim.

After 25 days of culture of the sorted stage 2 and stage 3 cells in the presence of FCS, FL, IL-7, IL-21 and IL-15, we observed that the majority of cells were CD3-CD14-CD19-CD117+CD56+ NK cells. Moreover, we observed that in our culture conditions the cells did not differentiate in other cell lineages (B cells or DCs). The largest number of cells we obtained at the end of the culture belonged to the $CD56^{bright}$ NK subset, while a limited number of $CD3^{low}$ (T-like cells) could also be observed. Likewise, other authors have shown that in vitro differentiation of NK cells under similar culture condition can uniquely induce NK cell development [68]. Overall our results show that the culture conditions, also in the absence of feeder cells, are largely efficient in inducing differentiation of stage 2 and sage 3 precursors into mature NK cells, suggesting that hepatic NK precursor cells have a high potential to differentiate specifically into NK cells.

Mature NK cells derived from cultures of either stage 2 or stage 3 hepatic NK precursors exhibited high levels of degranulation and cytotoxicity, indicating that these precursors can give rise to a

population of functional NK cells in terms of cytotoxicity. However, these in vitro differentiated NK cells did not express KIRs. This observation is in accordance with previous data indicating that in vitro-differentiated NK cells have no KIR expressions, although capable of cytokine production [27, 34] and cytotolytic activity towards their targets [27, 34]. Since the absence of KIR expression seems to be mostly due to the lack of an "education step" and not to a failure in developing complete functionality, these in vitro-derived NK cells are often called "pseudo-mature" or "immature NK cells" [27, 68]. One single study has shown that KIR expression was induced by the presence of IL-21 in the culture medium only after 35-45 days of culture, while at 25 days, the time that we selected to end our cultures, KIRs were still not present [69]. Besides the lack of KIRs we also observed low level of CD16 expression on in vitro-derived NK cells after 25 days of culture. Combined together these data may suggest that CD56bright NK cells are the subset that is preferentially "stored" in liver, LN and tonsils. In case of infection, these $CD56^{bright}$ NK cells may be released into the bloodstream and differentiate into $CD56^{dim}$ NK cells to react against pathogens. Subsequently, a portion of these newly differentiated $CD56^{dim}$ NK cells may return to other peripheral organs.

Further indication of the existence of local NK cell progenitors in the liver can derive by analysis in liver transplantation settings. Previous research from our group has shown that upon liver transplantation variable numbers of donor leukocytes are systematically transferred into the recipient [1]. Despite these previous findings, indicating that donor NK cells are detectable in the recipient circulation for approximately two weeks [1], no data was provided with regard to the presence of donor NK cells long after LTX in peripheral blood of the recipient or within the grafted organ. Proving that donor NK cells are present in the recipient long time after LTX will not only provide additional evidence of the presence of a local hepatic precursor but can also have a strong impact on studies aiming at understanding the dynamics of tolerance in LTX settings. Since it is generally recognized that the liver represents a tolerogenic organ and the mechanisms responsible for this relative tolerogenicity are only poorly understood, advances in this field can provide useful insights.

Therefore we here analyzed the presence of donor NK cells, or their precursors, in the circulation of recipients long time after LTX. Additionally, when re-transplantations were performed in our center we obtained a biopsy of the explanted liver and detected the amount of donor lymphocytes still present in

the grafted organ. Our current results indicate that stage 1 to 3 NK precursors were below the detection limit of flow cytometry in both recipient circulation and explanted donor livers. Conversely, we could identify small fractions of mature NK cells of donor origin in all the explanted livers we analyzed, even in one case in which biopsies were obtained two years after the primary transplantation. Previous studies have shown that the half-life of circulating NK cells is 7-10 days [30, 70]. However, a recent study has detected memory NK cells in a viral-infectious mouse models up to 30-50 days post adoptive transfer [71]. As a result, it is controversial to clarify nature of donor-derived NK cells in the recipients based on the current knowledge.

We also identified donor mNK cells in the circulation of a recipient 15 months after primary LTX. Apart from NK cells, we identified a number of other leukocytes of donor origin: $CD3^+$ $CD56^-$ T cells, $CD3^+$ $CD56^+$ NKT cells, and a group of cells characterized by either expression of CD16 or $CD14^+$ or both markers. These observations support the hypothesis that the human adult liver is linked to hematopoiesis of several lineages of immune cells, and renewing of donor-derived lymphocytes is endured in the recipient long time after LTX. However, due to the absence of NK precursors in the donor liver, this organ may partially decrease its donor NK-generating activity after LTX. One hypothesis to explain this is that NK precursors may migrate to the BM and\or SLTs of the recipients, and there differentiate into mature NK cells. Thereafter, due to expression of specific chemokine receptors, they may be able to return to the donor liver and contribute to the mechanism of tolerance induction for a long time after LTX. On the other hand, the observation that recipient NK precursors and mNK cells are present in the donor liver even within 7 days after LTX may suggest that donor-derived NK precursor cells are rapidly replaced with bone-marrow and\or SLT derived NK precursors of recipient. Nonetheless, further investigation is needed, firstly, to precisely determine the type of cells expressing $CD14^+$ and\or $CD16^+$ cells, and secondly, to reveal the clinical relevance of the presence of donor lymphocytes after LTX in relation to tolerance.

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In summary, we have identified for the first time NK precursors in the human adult liver. We also have shown that these cells, upon in vitro culture, can differentiate into mature NK cells with high cytotoxic properties. To conclude, we propose that the human adult liver can be a site of NK cell development, as it was shown for SLT, mainly LN and tonsil. However, further evidence can be provided by

assessment of in vivo NK cell development in an appropriate experimental animal liver transplantation model. Furthermore, additional research is needed to determine whether different types of liver diseases can have effects on the presence or properties of hepatic NK precursors.

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List of Abbreviations

BM	bone marrow
CLP	common lymphoid progenitor
FBS	foetal bovine serum
Flt3L	fms-like tyrosine kinase 3 ligand
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
Id	inhibitor of DNA binding
IFN-γ	interferon-gamma
IL	interleukin
iNK cell	immature natural Killer cell
KIR	killer cell immunoglobulin-like receptor
Lin	lineage markers
LN	lymph node
LTi cell	lymphoid tissue–inducer cell
LTX	liver transplantation
mAb	monoclonal antibody
MHC	major histocompatibility complex
MNC	mononuclear cells
mNK cell	mature Natural Killer cell
PB	peripheral blood
PBS	phosphate buffered saline
Pre-NK cell	precursor Natural Killer cell
Pro-NK cell	progenitor Natural Killer cell
qPCR	quantitative polymerase chain reaction
SLT	secondary lymphoid tissues
SCF	stem cell factor
T/NKPs	common T and natural killer cell precursors
UW solution	university of Wisconsin solution

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