Universitätsklinikum Hamburg Eppendorf

Institut für Immunologie
Prof. Dr. Marcus Altfeld

Metabolics of Tissue resident NK cells

Dissertation

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Vorgelegt von
Dr. med. univ. Wilhelm Friedrich Salzberger
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Prüfungsausschuss, 2. Gutachter/in: Prof. Dr. Adam Grundhoff

1. Synopsis

1.1. Introduction

1.1.1. Natural killer cells and their differentiation

Natural killer (NK) cells were first described in 1975 as effector cells able to target and kill tumor cells after scientists observed a natural reactivity against tumor cells without prior sensitization in samples from both cancer patients as well as in animal models [1,2]. Subsequently, NK cells were characterized as large granular cells belonging to the innate immune system with the ability to efficiently lyse virus-infected and malignantly transformed cells without prior sensitization [3,4]. Today NK cells are known to be part of the lymphoid lineage and account for about 10% of all lymphocytes in the peripheral blood. NK cells are derived from hematopoietic progenitor cells, although the exact location of all steps of their development is not yet fully understood [3–7]. One proposed model for NK cell development is the differentiation from bone marrow derived hematopoietic progenitor cells [5], but recent studies suggest additional sites of NK cell development, such as the liver and thymus, where NK cell precursor cells have been described [6,8–11] . In the bone marrow, hematopoietic progenitor cells can differentiate into common lymphoid progenitor cells, which then further differentiate into pre-natural killer cell precursors, which in turn can further differentiate into natural killer cell precursors and then into NK cells [5,11]. Studies on the development of NK cells in other compartments such as the liver suggest that NK cell precursors can migrate into tissues and differentiate into fully mature NK cells inside these tissues [7,8,10,12,13]. The existence of a pool of stem cells in certain tissues with the potential to differentiate into tissue-resident NK cells has also been discussed [14], but so far the existence could not be proven.

Critical factors for NK cell development are two transcription factors (TFs), Eomes and T-Bet. Both are TFs critical for the human developmental process in general, but also for immune cell development [15–17]. Due to their sequence similarity, they were originally thought to have redundant roles. T-Bet and Eomes are the only known TFs of their family, the T-Box family and have been described as master regulators of human T cell development. They are expressed in the majority of all murine and human NK cells [17–19]. While it is difficult to study their role during maturation and differentiation of human NK cells, extensive studies on their role in murine NK cell function have been performed. While Eomes deficient mice die during

early embryonic development, deletion of Eomes in immune cells leads to a dramatic decrease of numbers of NK cells in peripheral blood and spleen, NK cell numbers in liver, bone marrow and lymph nodes were only slightly decreased [16,19,20]. On the other hand, it was found that T-Bet deficient mice had higher numbers of NK cells in the bone marrow, but lower numbers of NK cells in peripheral blood and tissues [16,17,19,20]. Deletion of both Eomes and T-Bet lead to a complete lack of mature NK cells in all examined compartments [16,19]. These experiments suggest that T-Bet and Eomes expression are critical in the maturation and differentiation of NK cells. But other studies have reported on a population of NK cells lacking Eomes expression in the murine liver, which have subsequently been classified as a population of class-1 innate lymphoid cells (ILC1) separate from peripheral blood NK cells [16]. Their development is dependant on IL-15 and T-Bet and is restricted in the bone marrow due to repression of T-Bet in the bone marrow, which favors the differentiation of Eomes+ NK cells [19,20]. Another TF involved in the development and maintenance of a fully functional immune system is the B lymphocyte-induced maturation protein 1 (Blimp-1) [21–23]. Blimp-1 is a critical factor in the development of B-cells and in maintaining T cell homeostasis [21]. Its expression is induced by IL-2 and in turn it negatively downregulates IL-2 production [22,24]. Homologue of Blimp-1 in T cells (Hobit), a TF with a high sequence homology to Blimp-1, was found to be specifically upregulated in both natural killer T cells (NKT cells) and peripheral blood CD56dim NK cells [23,25]. Hobit is involved in several of NK and NKT cell effector functions including the production of murine granzyme B and murine and human IFN-γ, functions NKT cells share with NK cells [23,25]. A recent study in mice identified Blimp-1 and Hobit as central regulators in tissue-residency and tissue retention for a number of both adaptive and innate lymphocyte subsets [25,26,26,27]. But while some of the functions of these transcription factors have been elucidated, much conflicting data between murine models and studies on human material has been reported, underlining the importance of further study on this topic.

Mature NK cells are large granular cells which lack the expression of several classic lymphoid lineage markers, such as CD3, CD4, CD14 and CD19, but express CD45, as well as CD56 and/or the low affinity Fc receptor CD16 (FcγRIII) [28,29]. NK cells can be further divided into two subsets, $CD56^{bright}CD16^{neg}$ (CD56bright) and $CD56^{dim}$ $CD16^{+}$ (CD56dim) NK cells. CD56bright NK cells are usually considered to be the

precursors of CD56dim NK cells and lack the expression of the late differentiation marker CD57 [4,29]. While CD56bright NK cells are capable of lysing virus infected or malignantly transformed cells, their main function seems to be the modulation of the immune response via the secretion of cytokines such as interferon-gamma (IFN-γ), tumor-necrosis factor alpha (TNF-α), granulocyte macrophage colony-stimulating factor, IL-10 and IL-13 [2,5,30]. CD56dim NK cells on the other hand are considered to represent a later maturation stage and express higher amounts of CD57 and other markers of differentiation such as killer cell immunoglobulin-like receptors (KIRs) [4,5,31,32]. These CD56dim NK cells represent the main NK cell population in peripheral blood and account for more than 90% of all peripheral blood NK cells. Their main function has been characterized as cytotoxic effectors. They can lyse their target cells via secretion of granzyme B and perforin. They can also recruit other cells of the immune cells via secretion of cytokines, such as IFN-γ, although to a lower degree than CD56bright NK cells [2,4,5].

NK cells can recognize their targets via a wide array of activating and inhibitory receptors. The most diverse group of these receptors are the KIRs, which bind specifically to matching major histocompability complex (MHC)-class I molecules [3,4,31,32]. These activating and inhibitory KIR receptors differ mostly in their cytoplasmatic tail. In the case of activating receptors, the KIR possesses a short cytoplasmatic tail, which contains an immunoreceptor tyrosine based activation motif. In the case of inhibitory KIRs, the KIR possesses a long cytoplasmatic tail, which contains an immunoreceptor tyrosine-based inhibitory motif [32–34]. Additionally, NK cells can recognize their targets via binding of antibody-coated cells by CD16 and via other receptors such as the NKG and the NKP family [2,4,5,32,35]. While ligands for many of these receptors have been identified, ligands for other NK cell receptors are still unknown and the full range of their function is still unknown. Many of these receptors are critical in the recognition and killing of external and internal pathogens threatening the organism, but in recent years evidence on additional roles of NK cells and several of their receptors in tissues has been discovered.

NK cells can also be affected by a range of cytokines, such as type I interferons, IL-2, IL-12, IL-15 and IL-18 [32]. IL-2 mediates its effects on NK cells via the high affinity IL-2 receptor, which is constituted of alpha (CD25), beta and gamma chains. In the

resting state, most NK cells express low levels of CD25. IL-2 has a stimulatory effect on T cells and NK cells, driving proliferation and activation and can enhance NK cell function during viral infections [4,30,31]. IL-15 is produced by a variety of cells, such as activated dendritic cells, macrophages, monocytes and stromal cells. IL-15 signaling is critical for NK cell development and homeostasis. It has been found to "prime" NK cells, increasing NK cell activation after a secondary stimulus such as IL-2 [5,24,30,36]. IL-12 is produced mostly by antigen presenting cells like dendritic cells, macrophages and monocytes. It has been shown to be involved in maturation of NK cells in mice, promoting the more differentiated $CD56^{dim}$ $CD16^{-}$ phenotype [5,30,32,36]. IL-18 was originally defined as an IFN-γ inducing factor. IL-18 can be produced by dendritic cells, epithelial cells macrophages, neutrophilic granulocytes and several other cell types. It is involved in inducing the production of IFN-γ in NK cells during a range of situations and can upregulate CD25 expression when combined with other cytokines [3,30]. It has been reported that treatment of murine NK cells with IL-12, IL-15 and IL-18 could induce a special NK cell population, cytokine-induced memory-like NK cells. Another recent study found that treatment of human NK cells with IL-12 and IL-15 lead to metabolic changes in peripheral blood NK cells which went along with an increase of IFN-γ production [37].

In recent years, evidence that NK cells might not be purely innate immune cells, but also possess some characteristics of adaptive immune cells has arisen. Evidence on immunological memory against haptens in mice deficient for *Recombination activating gene 1* (*Rag1)* was reported [38,39]. As *Rag1* is a gene critical for development of B- and T cells, these mice are completely deficient of classical immunological memory. Still, evidence for an immunological memory reaction after prior sensitisation was observed [18,38,39]. Following studies revealed that after murine cytomegalovirus (MCMV) infection, a certain population of NK cells was expanded. These NK cells were binding specifically to the MCMV glycoprotein m157 via the murine NK cell receptor Ly49a. Furthermore, these NK cells conferred increased resistance to MCMV infection after adoptive transfer to mice lacking Ly49a or mice lacking expression of Ly49a [40,41]. In humans a similar population of NKG2C, an activating NK cell receptor, expressing NK cells was found to be expanded after human cytomegalovirus (HCMV) infection. These cells were found to be capable of enhanced IFNγ transcriptional activity when NKG2C was engaged, an

effect attributed to epigenetic remodeling of the gene encoding for IFNγ [42,43]. NKG2C has been found to bind HLA-E on HCMV infected cells, most likely due to an HLA-E presented viral peptide [44]. These NKG2C expressing NK cells are capable of rapid expansion following HCMV reactivation [39,41,45]. The lack of the gene encoding for NKG2C leads to an impaired immune response to HCMV infection [46]. NK cells exhibiting adaptive immune system characteristics have been called memory-like NK cells. Evidence on other viral infections that can induce development or expansion of memory-like NK cells in non-human primates and humans include simian immune deficiency virus (SIV), influenza and Epstein-Barr-Virus (EBV) has recently been found [47,48].

NK cell activation is most commonly identified via flow cytometric measurement of CD69 and CD107a expression on NK cells. CD69 is most commonly associated with activation and proliferation after stimulation in lymphocytes [49]. More recently, it has also been associated with tissue residency in a variety of organs [50–52]. It is a C-type lectin, whose expression is inversely correlated with the sphingosine-1-phosphate receptor 1, thus inhibiting the cells emigration from tissues.
Increased CD69 expression can be found on NK cells shortly after a variety of stimuli [49]. Thus, identification of tissue residency in NK cell stimulation studies is commonly not done via measurement of CD69 expression, but via expression of other tissue-residency markers. CD107a or lysosomal-associated membrane protein-1 is significantly upregulated on NK cells after stimulation and correlates strongly with NK cell-mediated lysing and cytokine secretion [53].

Additional roles of NK cells, such as killing of macrophages, immature dendritic cells and activated T cells in certain circumstances, but also promotion of maturation of dendritic cells and priming of CD4+ T-helper cells via secretion of cytokines have been described, indicating that NK cells are involved in regulating immune responses and preventing autoimmunity [4,5]. An involvement of NK cells in tissue regeneration and homeostasis has been reported while other studies have reported that tissue-resident NK cells might harbor a population of memory-like NK cells, suggesting an even wider range of potential functions for NK cells, especially in different tissues [8,10,12,13,13,54,55].

1.1.2. Tissue resident NK cells

Historically, most studies on human NK cells have been performed on NK cells from peripheral blood, while studies on murine NK cells have focused on splenic NK cells. But in recent years, it has become clear that tissue-resident NK cells can differ dramatically from their peripheral blood counterparts [10,13]. But while peripheral blood can easily be taken from healthy individuals, access to organ samples is much more difficult and in general limited to diagnostic or therapeutic interventions. Thus, most studies on tissue-resident NK cells rely on samples taken from organs affected by pathological conditions, which are accessed either during diagnostics, after excision or postmortem. This is likely to affect the results. In order to compensate for this, studies in mice and non-human primates are also being performed, although human NK cells differ from murine and primate NK cells in many characteristics. Tissue-resident NK cells have been found in many different organs, including bone marrow, intestine, kidney, liver, lung, lymph nodes, skin, spleen, thymus and uterus [6,12,13,50,55–58]. These tissue-resident NK cells share many characteristics with NK cells from peripheral blood. But they also differ in many characteristics. The most obvious difference between peripheral blood and tissue-resident NK cells is the different distribution of the CD56bright and CD56dim subsets. While CD56dim NK cells amount for about 90% of all NK cells in peripheral blood, in tissues CD56bright NK cells can amount a much higher percentage of the NK cell population, depending on the tissue [7,12]. This has been discussed to either indicate that CD56bright NK cells, which are generally considered to be precursors of CD56dim NK cells, are located mostly in tissues and leave the tissues to differentiate into CD56dim NK cells, thus acting as a reservoir for peripheral blood NK cells, or that tissue-resident NK cells represent a distinct population of NK cells with a development separate from those from bone marrow and secondary lymphoid tissues [7,14]. This second theory has been bolstered by the recent finding of precursor stages of NK cells residing in the liver, lung, uterus and intestine [14,19,59]. It is currently unknown if these tissue NK cell precursors differ from NK cell precursors and differentiate specifically into tissue-resident NK cells.

One defining characteristic of tissue-resident NK cells is the expression of a number of surface proteins, which are functionally involved in tissue retention and are now used to identify tissue-resident NK cells. These include CD49a (or alpha1integrin),

CXCR6, CD69, CD103 (alphaE integrin), DNAM-1, CCR5, CD49e and the transcription factors T-Bet, Eomesodermin (Eomes) and promyelocytic leukemia zinc finger protein (PLZF) [20,51,59–63]. CD69 is a transmembrane C-type lectin that is often used to measure NK cell activation on peripheral blood NK cells. But it has been shown to also inhibit S1P1, which binds sphingosine-1-phospate (S1P). S1P is most concentrated in peripheral blood and promotes egress from tissues into peripheral blood for lymphocytes, including NK cells, expressing S1P1 [52,64]. When CD69 is expressed, this egress is inhibited and the lymphocytes are retained inside the tissue [50,50,52]. CD69 is expressed on a high percentage of NK cells from the liver, uterus, lymph nodes, spleen and intestine and is one of the most commonly used surface proteins to characterize tissue-resident NK cells [7,10,51]. CD49a is an alpha1beta1 integrin predominantly expressed in vascular and visceral smooth muscle. It is, among others, expressed by fibroblasts, bone marrow mesenchymal stem cells and hepatic stellate cells. It can bind to several collagen types, as well as a number of other factors [60,63,64]. Its expression in fibroblast lineages can be upregulated by TNF-α and INF-γ, while IL-12 and IL-15 were found to induce its expression in peripheral blood NK cells, along with CXCR6 expression [36]. A recent study revealed a population of CD49a expressing NK cells in the human liver that expressed T-Bet, but not Eomes [20,60]. CXCR6 is another surface protein expressed on many lymphocytes that is used as a marker for tissue residency, which binds the membrane-bound chemokine CXCL16, its only known ligand [65]. CXCL16 has been found to be highly expressed on endothelial cells, but also in a number of tissues [66]. While its main site of expression are endothelial cells, it can exist in a soluble form, allowing it to function as an adhesion molecule as well as a chemotactic molecule [66]. Its expression has been linked to migration of immune cells into various tissues leading to both benign as well as detrimental effects such as tumor suppression, tumor infiltration and inflammation [67–70]. CXCR6 expression has been used to identify tissue-resident NK cells in a variety of organs both in mice and in humans, including liver and spleen [13,51,71]. A subset of CXCR6 expressing murine memory-like NK cells was described, which was found mainly in the liver [38].

In recent years, the role of NK cells in many organs has been examined. It has become evident that NK cells in tissues are not limited to the roles that are attributed to peripheral blood NK cells, that of cytotoxic effectors and modulating the immune

response via secretion of cytokines. Murine studies have revealed a distinct population of thymic-resident NK cells, although studies on humans are still lacking in this regard [9,13]. NK cells can also be found in peripheral lymph nodes, although they are rare there and their purpose in this tissue is not yet clear [8]. Tissue resident NK cells have also been found and studied in other organs, where at least some of their functions, often critical for the function of organ in question, have been elucidated. In the uterus the immune system can come into close contact with foreign organisms, the unborn child, which only shares part of the MHC of the host. Thus, mechanisms for preventing the host immune system from attacking the unborn child are needed. Recent studies have focused on a special NK cell population found only in the uterus, decidual or uterine NK cells. These uterine NK cells were found to be weaker when it came to killing cancer cell lines than their peripheral blood counterparts and seem to spare trophoblasts, the fetal cells they usually come into contact with. Evidence suggests that uterine NK cells are involved in the remodeling placenta and the ingrowth of spiral arteries from the fetus's trophoblasts, a process critical for assuring sufficient blood flow and thus a key factor in a successful pregnancy [58]. Other organs have also been shown to harbor significant tissue-resident NK cell populations, such as the liver and the spleen, two other immunologically privileged organs.

1.1.3. Liver-residency

The liver is an organ with a high exposure to foreign antigens with the portal vein delivering blood from the intestine directly to the liver, thus exposing the liver to antigens from nutrients, environmental toxins and the microbiota from the gut. But in spite of this constant supply of antigens, no severe immune activation takes place in healthy livers [72]. On the other hand, during diseases overshooting immune activation in the liver can cause severe local and systemic damage as seen during autoimmune diseases and viral infections such as hepatic viral infections [73]. But again, this damage can often, at least in part, be reversed due to potent regenerative capabilities of the liver [74,75]. This suggests potent mechanisms for immunotolerance in the liver to prevent the high number of lymphocytes inside the liver from inducing potentially damaging inflammation while also maintaining control of foreign antigens. It has been suggested that a healthy human liver harbors about 10^10 intrahepatic lymphocytes. NK cells represent 20-40% of all intra-hepatic lymphocytes (in contrast to about 10% in peripheral blood) [12,56,72,75,76]. CD56bright NK cells represent about 30-40% of all intrahepatic NK cells, compared to about 10% in the peripheral blood. Liver NK cells reside mostly in the liver sinusoids [62]. Here, several liver cell types express a number of chemokines that have been suggested to retain NK cells in the tissue [76,77].

Recently, NK cell precursor stages were identified in human liver tissues. The early NK cell precursor stages found in liver tissue did not differ from the precursor stages found in peripheral blood, but later precursor stages differed from their peripheral blood counterparts in several characteristics [14]. But while it was shown that these precursors are rapidly replaced by peripheral blood NK cell precursors when a liver is transplanted into a new recipient, some NK cells of the organ donor were still found multiple months after the transplantation inside the liver tissue [63]. In murine studies, it has been shown that CD49a expressing NK cells residing in liver tissues exhibit memory-like characteristics and when transferred to naive mice can home to the site of antigen contact [78]. In humans, CD49a expressing cells have been described as a subset of liver resident NK cells [60]. A subset of human liver-resident $CD49a^+$ NK cells has been reported to express T-Bet, but not Eomes [60]. So far, no clear evidence of these cells exhibiting the same memory-like characteristics as their murine counterparts has been found.

Functionally, liver-resident NK cells seem to be close to peripheral blood NK cells, although some differences in both cytokine secretion and the type of secreted granzymes for cytotoxicity have been reported. But additional functions of liver-resident NK cells have been studied, such as killing liver stellate cells. Liver stellate cells can promote liver fibrosis in certain circumstances, thus their killing can protect from liver fibrosis [56,75,79]. On the other hand, overshooting killing of hepatocytes in viral infection settings can lead to liver damage and long-term liver disease [73,75].

1.1.4. Spleen residency

The spleen is a peripheral lymphoid organ located in the upper left abdomen. While it is a lymphatic organ, it has no central connection to the lymphatic system. Instead it has a high level of blood circulation and acts as a filter for many blood borne antigens, but also for microbes, antibody-antigen complexes and old erythrocytes. It consists of two main areas, the red and the white pulp. The red pulp acts mostly as a filter for old erythrocytes and thrombocytes, which are captured and removed from circulation. Around the smaller arteries inside the spleen are areas with a high number of lymphocytes, which form the islands of white pulp. A high number of T- and B-cells are located in the white pulp, but also other immune cells, including macrophages, immature dendritic cells and NK cells [57,80].

For a long time the spleen was considered an almost superfluous organ, as its removal during certain diseases or after traumatic injury did not seem to have a major effect on the human organism. But in recent years, closer examination of the spleen and studies on the effects of removal or loss of the spleen have revealed many important functions of this organ. Removal of the spleen is still one of the most efficient treatments for immune thrombocytopenia (ITP), as it is both of the main sites for anti-platelet antibody production and for filtering antibody-coated platelets from the circulation [81–83]. These platelets are digested by macrophages and presented to other cells of the immune system leading to the expansion of autoreactive B and T cells. Thus, when immunosuppressive therapies fail to suppress ITP, removal of the spleen is one of the alternative treatments. But the long-term effects of removing the spleen can be rather severe. A risk of overwhelming infections with encapsulated bacteria such as streptococcus pneumoniae has been well documented [81]. Due to the missing filtering of defective and old thrombocytes an increased risk of atherothrombotic events also stays with the patients, requiring lifelong thromboprophylaxis [81,83–85]. These findings have underlined the role of the spleen as part of the lymphatic system and have helped to understand some of spleen's functions in the human body.

Murine studies have shown that the spleen hosts a large number of NK cells, making the spleen a preferred organ to study murine NK cells [86]. But studies on the human spleen were especially difficult to perform, as taking sample from human spleens

carries a very high risk of rupturing the thin membrane around the organ, a complication requiring emergency surgery and associated with a high mortality rate. Thus, spleen samples are only available from patients undergoing organ removal for medical reasons (trauma or disease related) or from cadaveric donors. A recent study on spleen tissue samples obtained from organ donor material described a population of $CXCR6^+$ $CD69^+$ NK cell population found in the human spleen as well in bone marrow and lymph nodes, but not in peripheral blood [51]. These NK cells were described as lymphoid tissue NK cells (ltNK cells). While in the bone marrow, $CXCR6^+$ $CD69^+$ NK cells amounted to abound a third of all NK cells, in the spleen $CXCR6^+$ $CD69^+$ NK cells accounted for 45% of all splenic NK cells. While most these ltNK cells were $CD3^-$, $CD14^-$, $CD19^-$, $CD56^{bright}$ $CD16^-$ NK cells and expressed Eomes like peripheral blood NK cells, they differed from peripheral blood NK cells. They lacked the expression of the early differentiation marker CD127 but expressed NKG2A, which is usually downregulated during NK cells differentiation. At the same time most ltNK cells also lacked the expression of late differentiation markers. In regard to functional properties, stimulation with different cytokines shown that IFN-γ production by ltNK cells was comparable to peripheral blood CD56dim NK cells. In regard to cytotoxicty, they expressed similar levels of granyzyme B and perforin and similar expression of CD107a compared to peripheral blood CD56bright NK cells but less than peripheral blood CD56dim NK cells. Thus, while ltNK cells share some functional properties with both CD56bright and CD56dim peripheral blood NK cells, they also differ from both of these populations in some characteristics, suggesting that ltNK cells represent a separate population from peripheral blood NK cells. While these ltNK cells were defined by the expression of the tissue residency markers CD69 and CXCR6, which they share with tissue-resident NK cells from multiple other organs such as the liver, they lacked expression of CD49a and CD103, which have been used to describe tissue-resident NK cells in some publications [51,57].

1.1.5. Metabolism

In the human body the main molecule of energy currency is adenosine triphosphate (ATP). In can be generated from adenosine diphosphate (ADP) via a number of pathways and from a number of nutrients. The most prominent nutrients from which ATP can be generated are glucose, amino acids and fatty acids. With the exception of glycolysis, generating ATP from these nutrients also requires oxygen [87]. Studies on lymphocytes, which have been performed mostly on lymphocytes isolated from peripheral blood, have observed that some lymphocyte subsets seem to prefer fatty acids metabolization when resting, but activated lymphocytes rely mostly on metabolizing glucose, which has the advantage of being readily available in peripheral blood, as the blood glucose levels are usually tightly regulated and maintained at a high level [88–91]. Additionally, metabolizing glucose is usually faster than using amino acids of fatty acids to generate ATP [87]. But when studying tissue residency in immunity, it is important to consider the different conditions that lymphocytes might encounter in the respective tissues. While glucose levels in the blood are usually stable, the availability of glucose, and also other metabolites, can vary between blood tissues [92,93]. Additionally, the nutrient availability in tissues can differ from peripheral blood even more under certain pathologic conditions. Many tumor cells massively increase their glucose uptake, thus decreasing the availability of glucose in their environment [94–96]. Similarly, certain viruses e.g. hepatitis-C virus affect the uptake of glucose into infected cells, which can also affect the microenvironment around these cells [97,98]. Other factors, including fibrosis also affect nutrient availability in tissues by changing the replenishment of nutrients [99]. Thus, studying the metabolism of tissue-resident cells can not only further our understanding of the immune system, but also help develop new ways of managing many diseases.

In a resting state, lymphocytes metabolize glucose via glycolysis followed by oxidative phosphorylation in order to efficiently convert glucose to ATP, a process dependant on the presence of oxygen [100]. Hereby, one molecule of glucose is enough to generate 32 molecules of ATP. When activated, the metabolism of lymphocytes shifts towards the faster alternative, glycolysis without oxidative phosphorylation, even under the presence of sufficient oxygen („aerobic glycolysis”) [87]. During glycolysis, glucose is metabolized to pyruvate and then lactate, which in

turn is secreted from the cell. Glycolysis can provide many precursor molecules necessary for the generation of nucleotides, amino acids and lipids [100–102]. But only two molecules of ATP are produced from each molecule of glucose during glycolysis compared to the 32 molecules of ATP per molecule of glucose if pyruvate enters the citrate cycle for the process of oxidative phosphorylation after glycolysis instead of being secreted as lactate [87]. When using fatty acids for the production of ATP, the ATP yield per fatty acid is higher, but the process is dependant on oxygen and is generally slower [90,103].

As lymphocytes need to act quickly when activated and usually have a very high demand for energy when activated, lymphocytes need to either store high amounts of energy or they need a mechanism to replenish their energy stores quickly when the need arises. Using ATP as a currency for energy has the advantage that ATP can be directly used in many of the processes inside a cell, but due to the high number of cellular processes dependant on ATP and its instability in water, cellular ATP stores are usually exhausted quickly and regeneration of ATP from ADP is started when ATP is used [87]. But storing nutrients like glucose, amino acids and fatty acids inside the cell also has its disadvantages. Glucose is osmotically active, thus storing large amounts of glucose inside the cell would lead to an influx of water into the cell, which would be fatal for the cell. Storing glucose in bigger molecules like glycogen would partially circumvent this problem, but is more space consuming and is still osmotically active, although less so than glucose [103]. Storing energy as amino acids or fatty acids has other disadvantages, most notably the slower conversion into ATP, which would severely hamper the ability of lymphocytes to act quickly and defiantly when needed [89,90,104]. Thus, lymphocytes need to be able to replenish their nutrients from their surroundings when necessary in order to act quickly and efficiently but also as long as needed. This is usually done via a number of nutrient transporters on the cell surface, whose expression on the cell surface can be increased and decreased to dynamically adapt the nutrient uptake to the cells current demand [100,105].

The glucose transporter 1 (Glut1) is one of a family of 14 glucose membrane transporter proteins and is responsible for the transport of glucose into the cell [106,107]. It is expressed on most human cells, though the highest expression has

been found on erythrocytes, endothelial cells of the blood-brain barrier, in the kidney and on many malignant cells, including hepatocellular carcinoma. In T-effector cells, expression of Glut1 on the cell surface was shown to be a critical factor for rapid proliferation and cytokine production [106]. Recently, it was demonstrated that cytokine stimulation of murine and human NK cells upregulated cell surface expression of Glut1, as well as increasing the rate of glycolysis, oxidative phosphorylation and IFN-y production [105].

CD98 is a transmembrane protein that can form a neutral amino acid transport system when linked with other L-type amino acid transport proteins [108–110]. It is expressed on virtually all cell type apart from platelets. Resting lymphocytes express very low amounts of CD98, but CD98 has been shown to be highly expressed on activated and proliferating lymphocytes [111–113] as well as a large number of tumors [108,109]. Studies suggest that CD98 regulates amino acid transport activity, but the underlying mechanisms are still unknown.

Another critical metabolite for most living cells is iron. Under most conditions, circulating iron is bound to transferrin in humans [103,114]. Transferrin-bound iron is taken up into the cell via receptor-mediated endocytosis after binding to the cell surface transferrin receptor protein 1 (TfR1) or CD71 and is subsequently released from transferrin [114–116]. As important as cellular iron is for the human body, it is highly toxic in higher concentrations [114]. Thus a mechanism for regulating iron import into the cell is a valid strategy to prevent toxicity by high iron concentration but at the same time allow for sufficient iron to be available during times of higher demand. After uptake, cellular iron is used in a wide array of cellular functions including in DNA replication, protein function and mitochondrial respiration [114,117,118]. The role of CD71 in NK cell function has not been thoroughly examined until recently, where a study reported an upregulation of CD71 on cytokine stimulated NK cells in parallel with an upregulation of CD98 and Glut1 [105]. But while both CD71 and CD98 have been used as surrogate markers of lymphocyte activation and proliferation, what is known about their biological role so far suggests that their upregulation is necessary to provide the metabolites necessary for the cells increased demand during activation and proliferation [109,112,119,120].

1.1.6. Question and problems to be addressed

In recent years, the characteristics and functions of NK cells have been studied intensely and many additional roles of NK cells in different compartments of the body have been elucidated.

The aim of my work was to further our understanding of different NK cell populations residing in tissues and in peripheral blood.
We focussed on examining the proliferative potential, the expression of a number of transcription factors in NK cells and the expression of a number of nutrient transporters on tissue resident NK cells compared to other NK cell populations.

Thus, we isolated NK cells from blood, liver and spleen samples, subjected them to a number of different conditions and examined how the different NK cell populations from the different compartments reacted to those conditions.

1.2. Materials and Methods

For these studies, we collected tissue samples from a number of study cohorts. Liver tissue and matching blood samples were collected from patients either undergoing liver explantation (tissue samples of the explanted livers) or undergoing tumor resection (tissue samples from the resection border). Spleen tissue and matching blood samples were collected from patients undergoing splenectomy either due to proximity to a malignant tumor or due to immune thrombocytopenia (ITP). Peripheral blood samples from healthy donors were drawn from the HHCH donor cohort. After collection, samples were directly processed.

Peripheral blood was processed via density centrifugation using Ficoll/Percoll.

Liver tissue was cut into smaller pieces and subsequently further mechanically dissociated via a gentleMACS Octo Dissociator (Milteny). After this step, the liver tissue was then manually pushed through a series of filters (500µm/300µm/100µm/70µm/40µm, Greiner Bio-One GmbH). Afterward, the liver cells were either frozen for later use, set aside for experiments or further processed via Optiprep density gradient medium (Sigma-Aldrich).

Spleen tissue was directly manually pushed through a series of filters (500µm/300µm/100µm/70µm/40µm, Greiner Bio-One GmbH). After washing, erythrocytes were lysed using ACK lysis buffer (Biozym) and washed again.

After preliminary processing, samples were either frozen for later use or stained with antibodies following standard flow-cytometry staining protocols. Intracacellular flow cytometry stainings were performed using adapted staining protocols. These adapted protocols and the protocols for the experiments following sample collection/thawing of previously collected samples are detailed in the materials and methods sections of the publications appended in the sections 4, 5 and 6 of this document. The samples were then either measured directly or sorted via flow-cytometer for further experiments.

Flow cytometry was performed at the HPI flow cytometry core facility. Samples were acquired on a LSR Fortessa (BD Biosciences). Cell sorting was performed on a FAC-SAria Fusion (BD Biosciences). Results were analyzed using FlowJo software version 10.

1.3. Results

1.3.1. Hobit expression by a subset of human liver-resident CD56^{bright}CD16^{-} Natural Killer cells [27]

In this study we investigated the native expression of the transcription factor Hobit by human NK cells and its role in regulating tissue residency of NK cells from liver samples and matched blood samples taken during liver transplantation surgery.
We found Hobit to be expressed in almost all CD56^{dim}CD16^{+} NK cells regardless of their origin. But CD56^{bright}CD16^{-} NK cells isolated from human livers expressed significantly higher levels of Hobit than CD56^{bright}CD16^{-} NK cells isolated from peripheral blood and Hobit was expressed on a significantly higher percentage of CD56^{bright}CD16^{-} NK cells from the liver than on CD56^{bright}CD16^{-} NK cells in peripheral blood.

We also found CD56^{bright}CD16^{-} NK cells to be major subpopulation of NK cells in liver tissue compared to peripheral blood, where it is only a small subpopulation of all NK cells. These liver CD56^{bright}CD16^{-} NK cells had higher percentage of CD69^{+}, CXCR6^{+} and CD49^{+} NK cells than liver CD56^{dim}CD16^{+} NK cells. Additionally CD56^{bright}CD16^{-} Hobit^{+} NK cells expressed higher levels of CD69, CXCR6 and CD49a than CD56^{bright}CD16^{-}Hobit^{-} NK cells.

Additionally, we examined the expression of the transcription factors T-bet, Eomes and Blimp-1 on NK cells isolated from peripheral blood and from livers. Eomes and Blimp-1 expression was lower in liver NK cells compared to peripheral blood NK cells, while T-bet was expressed significantly higher on liver CD56^{bright}CD16^{-} NK cells compared other NK cell populations. When comparing Hobit^{+} and Hobit^{-} NK cells from the liver, we found CD56^{bright}CD16^{-}Hobit^{+} NK cells to contain a significantly higher percentage of Blimp-1^{+} and T-bet^{+} NK cells compared to CD56^{bright}CD16^{-}Hobit^{-} NK cells.

Taken together, we found NK cell populations from peripheral blood and livers to differ in their expression of a number of transcription factors and factors associated with tissue residency in murine studies

1.3.2. Proliferative capacity exhibited by human liver-resident CD49a^{+}CD25^{+} NK cells [63]

In this study, we examined the expression of activation and differentiation factors on NK cells isolated from human livers and matched peripheral blood samples.

We found intrahepatic NK cells to differ from peripheral blood NK cells in a wide array of surface markers. While some differences in the distribution of CD56^{bright}CD16^{-} and CD56^{dim}CD16^{+} were found, we found more pronounced differences when examining tissue residency markers. CD49a expression was almost exclusively found on intrahepatic NK cells. Intrahepatic NK cells were found to express CD34 more often and CD57 less often, as well as CXCR4, than PB NK- cells. These results suggest that intrahepatic NK cells tend to be less differentiated than their peripheral blood counterparts.

When further studying NK cells from livers, we found that CD56^{bright}CD16^{-} NK cells expressed CD49a to a significantly higher percentage compared to CD56^{dim}CD16^{+} NK cells. We found that CD49a^{+} NK cells contained significantly higher proportions of CD25^{+}, CD34^{+} and CXCR3^{+} cells, regardless of CD56 and CD16 expression.

The expression of CD34 on CD49a^{+} expressing NK cells suggests immaturity and a potential for self-renewal of liver resident NK cells. The expression of CD25 on liver resident NK cells suggests a potential for proliferation after stimulation with IL-2. We demonstrated that, in contrast to CD49^{+}CD25^{-} and CD49^{-} NK cells from liver tissue, CD49a^{+} CD25^{+} NK cells have the capability to proliferate in response to low-dose IL-2 stimulation, a cytokine that has been shown to be elevated in many inflammatory liver diseases.

In conclusion, we demonstrated that liver-resident NK cells contain subpopulations with different phenotypes and functional properties. In addition, they have a higher capability to proliferate in response to a proinflammatory stimulus compared to NK cells from peripheral blood.

1.3.3. Tissue-resident NK cells differ in their expression profile of the nutrient transporters Glut1, CD98 and CD71 [121]

In this study, we examined NK cells isolated from human spleens, human livers and peripheral blood samples matched to the respective tissue samples. The NK cells isolated from these compartments were characterized and classified according to their CD16, CD56 expression as well as the expression of the tissue residency marker CXCR6. These subpopulations were then examined for their expression of the nutrient transporters Glut1, CD98 and CD71 both directly after isolation as well as following cytokine stimulation in vitro.

In line with our previous studies and in line with the literature, we found $CD56^{bright}CD16^{-}$ NK cells to be enriched in both liver and spleen tissue compared to peripheral blood samples. $CD56^{bright}CD16^{-}$ NK cells in tissue exhibited a high frequency of CXCR6 expression compared to $CD56^{dim}CD16^{+}$ NK cells from tissue or NK cells from peripheral blood. When comparing tissue resident ($CXCR6^{+}$), tissue derived ($CXCR6^{-}$) and peripheral blood $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ NK cells in regard to their nutrient transporter expression, we noted a number of differences between the populations both before and after cytokine stimulation.

In general, $CD56^{dim}CD16^{+}$ NK cells had a higher expression of Glut1 at baseline, but after stimulation peripheral blood $CD56^{bright}CD16^{-}$ NK cells upregluated Glut1 significantly, while $CD56^{bright}CD16^{-}$ NK cells from tissue expressed significantly lower amounts of Glut1. On the other hand, CD98 expression after cytokine stimulation was the highest in $CD56^{bright}CD16^{-}$ NK cells from tissues, regardless of their CXCR6 expression. In regard to CD71 expression after stimulation, we also noted a difference between $CXCR6^{+}$ and $CXCR6^{-}$ NK cells from tissues with $CXCR6^{+}$ $CD56^{dim}CD16^{+}$ NK cells expressing significantly more CD71 than $CXCR6^{-}$ $CD56^{dim}CD16^{+}$ NK cells.

In conclusion, we have shown that tissue resident, tissue derived and peripheral blood NK cells and their two main subpopulations differ significantly in their expression of nutrient transporters, both at baseline and after cytokine stimulation.

1.4. Discussion

1.4.1. Transcription factors Hobit, Eomes and T-Bet their role in regulating tissue residency in NK cells

In this project, we were able to show that the expression of multiple transcription factors, which are critical factors in NK cell maturation and function, differs significantly between NK cells from peripheral blood and liver samples. We observed that Hobit and T-Bet are expressed more frequently in NK cells from the liver than from peripheral blood. The most striking difference was the higher expression of Hobit in $CD56^{bright}CD16^{-}$ NK cells from the liver compared to $CD56^{bright}CD16^{-}$ NK cells from peripheral blood. $CD56^{bright}CD16^{-}$ NK cells are a minor population in peripheral blood and only make up about 10% of all peripheral blood NK cells, but in certain tissues $CD56^{bright}CD16^{-}$ NK cells are highly enriched. On the other hand, Eomes and also Blimp-1 are expressed more frequently on peripheral blood cells compared to NK cells from liver samples. But while Blimp-1 expression was lower in liver NK cells compared to peripheral blood NK cells, when focusing on $Hobit^{+}$ NK cells, Blimp-1 expression was significantly higher than in $Hobit^{-}$ NK cells. It has been shown in murine studies that, while most peripheral blood and bone marrow NK cells express Eomes, an Eomes-independent NK cell lineage with a high expression of T-Bet can be found in certain tissues [19,20]. It has been shown in mice that Hobit and Blimp-1 regulate the expression of genes linked to tissue retention and egress.

Our results suggest that, similar to results from murine experiments, tissue residency of human NK is a process in part controlled by a complex program of expression of certain transcriptions factors and the lack of expression of other transcription factors. In conclusion, we showed that liver tissue harbors a population of $Hobit^{+}$ $CD56^{bright}CD16^{-}$ NK cells that differ from other, previously described liver-resident NK cells in multiple characteristics, including the expression of several key transcription factors.

Our experiments were only designed to characterize the distribution of several markers previously identified to be associated to tissue residency, both from mouse models and studies in different human tissues. Currently still unknown markers may additionally play a role in this process. A closer examination of a program that

defines tissue residency would have to integrate data on gene expressions, preferably sequentially in different NK-cell populations.

1.4.2. Tissue resident NK cells and their potential for proliferation

In another part of this project we assessed the proliferative capabilities of tissue resident NK cells. We were able to identify a subpopulation of tissue-resident $CD49^+CD25^+$ NK cells with the ability to proliferate in response to a low-dose IL-2 stimulus. IL-2 is a proinflammatory cytokine that has been found to be significantly increased in inflamed liver and is considered as a biomarker with a negative prognostic risk for patients with increased levels of IL-2 in liver and serum [24,32,122]. Expression of CD25 on NK cells has been demonstrated to be affected by the cytokines IL-12, 15 and 18, all of which can be produced by Kupffer cells, which reside inside of liver tissue, in response to liver injury [36,63,75]. This suggests the presence of a reservoir of NK cells residing inside liver tissue with the potential to proliferate when liver damage occurs, although this is only a small subpopulation of what is currently defined as tissue-resident NK cells [60,63,75]. As our samples were taken from livers that were either in end-stage liver disease or undergoing tumor resection surgery, the possibility of an enrichment of these $CD49^+CD25^+$ NK cells in the liver due to the disease situation has to be considered. Our tumor resection samples were taken from the limits of the resections with a safety distance to the tumor and no significant difference between tumor-resection livers and other liver samples could be observed. But as the tissue around tumors can affected by the tumor as well, we cannot exclude the possibility that this population of $CD49a^+CD25^+$ NK cells is locally expanded or has been recruited into the liver via a local increase of IL-2 secretion. Due to ethical concerns, no samples from healthy human livers could be procured, as such a healthy control group could not be established.

We were also able to identify a second population of NK cells with a possible potential to proliferate when subjected to the correct stimulus, a population of $CD49a^+$ $CD34^+$ $CD25^-$ NK cells. CD34 is expressed early during NK cell differentiation and suggests that at least some NK cell differentiation independent of IL2-CD25 stimulation can take place in the liver [123]. We were not able to differentiate whether these early $CD34^+$ NK cell precursors reside inside the liver since embryogenesis and generate their own lineage of tissue-resident NK cell

progenitors or if they are recruited to the liver via engagement of CD49a and then differentiate into more mature NK cells there. Studies have shown that certain NK cells can reside in the liver over several years [59,63], while other studies have shown that at least some liver-resident NK cells immigrate from the circulation [7,59,62], but this does not tell us if these cells immigrate into the liver and then reside there long-term or if these are two distinct NK cell populations has not been answered so far. Finding $CD49a^{+}CD34^{+}$ cells in peripheral blood would be one way to answer this question, but so far this effort was unsuccessful, either because these cells do not circulate freely or due to the scarcity of $CD49a^{+}$ NK cells in peripheral blood, the low percentage of $CD34^{+}$ NK cells in blood and tissue and limitations of the currently available techniques to identify extremely rare subpopulations of circulating cells. Studies in humanized mouse models or once new, suitable technologies are available might answer the question if a reservoir of self-replenishing tissue-resident NK cells exists or if tissue-resident NK cells are replenished only via NK cell progenitors immigrating into these tissues and then further differentiate there.

1.4.3. Tissue residency and metabolism

In the third part of this project, NK cells from liver and spleen samples were analyzed in parallel to NK cells from matched peripheral blood samples. We were able to show that NK cells from tissues differ from peripheral blood NK cells in regard to their expression of several nutrient transporters. Both $CXCR6^{+}$ and $CXCR6^{-}$ NK cells from tissue differed significantly from peripheral blood NK cells. This could be shown for both main NK cell subpopulations, for $CD56^{bright}CD16^{-}$ NK cells as well as for $CD56^{dim}CD16^{+}$ NK cells.

We found tissue resident NK cells to differ from peripheral blood NK cells in their expression of Glut1, a glucose transporter, CD98, an amino acid transporter and CD71, a receptor for transferrin both at baseline and after stimulation. Glut1 expression in tissue resident $CD56^{dim}CD16^{+}$ NK cells was lower at baseline compared to peripheral blood $CD56^{dim}CD16^{+}$ NK cells and tissue resident $CD56^{bright}CD16^{-}$ NK cells expressed significantly less Glut1 after cytokine stimulation compared to peripheral blood $CD56^{bright}CD16^{-}$ NK cells. On the other hand, we observed a robust upregulation of CD98 on all NK cells after cytokine stimulation,

with tissue resident NK cells expressing the highest amount of CD98 after stimulation.

Glucose is usually the preferred source of energy for most lymphocytes due to glucose being abundantly available in peripheral blood and due to a quick metabolization pathway until ATP is generated [89,100,124]. A shift away from glucose metabolism towards amino acids suggests that tissue-resident NK cells are adapted to their tissue environment and situations when glucose might not be available. Our observations were made in vitro, where glucose in our used media is readily available. This persisting shift away from glucose metabolization despite glucose being available suggests that tissue resident NK cells are pre-programmed to cover part of their metabolic demand via glucose independent pathways regardless of what is available at the time of need. This could mean that tissue resident NK cells are pre-equipped for situations when glucose might be scarce.
Alternatively, this could be an effect of our study cohort, as the tissue samples in this study were taken during tumor resections or from heavily damaged livers from liver transplant recipients, meaning that the tissue resident NK cells we studied might have first-hand experience with nutrient shortages and thus have been forced to shift their metabolism away from glucose metabolism.
In contrast, spleen samples were, for the most part, taken during resection of spleen-adjacent malignant tumors as a safety precaution against invasive growth and not macroscopic tissue reorganisation was visible in these samples. Thus, these spleens samples should not be affected by the donor's disease. But as a control, studies into this topic with more suitable organs, such as spleens removed after traumatic injury, might be necessary. On the other hand any examination of samples from healthy tissue poses risk to patients and might not be feasible for ethical reasons.

In our experiments cytokine stimulation not only led to upregulation of several common tissue-residency markers but also made it difficult to identify some subpopulations following stimulation. Thus, we were unable to determine whether $CXCR6^-$ NK cells from tissue that we observed behaving differently than $CXCR6^+$ NK cells, but also differently than peripheral blood NK cells in regard to changes of nutrient transporter expression (see Results 1.3.3.) were a separate population of tissue-resident NK cells that expressed different tissue residency markers or if they

were NK cells from peripheral blood that were passing through or transiently residing in tissue at the time the samples were taken. If they were peripheral blood NK cells only transiently in the tissue, the questions arise how long they were in the tissue, how quickly and most importantly via which mechanisms their metabolism was affected in so far that they retained these properties after isolation and in vitro cultures. Further studies on this topic might give critical insight into tissue-resident NK cells, peripheral blood NK cells and how tissues can affect NK cell function.

Again, the limitations of our study are clear. Taking tissue samples from individuals without a medical need and without benefit for the donor are practically impossible due to ethical concerns and thus limit further experiments with human samples. Sampling tissue for scientific research is thus limited to situations when the organ in question is being resected or operated on for health-related issues.

These questions can be addressed in the future in different experimental models, such as mice, humanized mice or organoids.

1.4.4. Conclusion

In this project, I demonstrated that tissue-resident NK cells differ from peripheral blood NK cells on multiple levels, including the expression of transcription factors, their function and in their expression of several important metabolic transporters on their cell surface. The breadth of differences suggests that tissue-resident NK cells either differentiate from precursor cells that at some point during embryogenesis of our lives migrated into tissues and are a completely separate population of NK cells, or that tissue-resident NK cells are derived from mature peripheral blood NK cells which undergo major reprogramming when they migrate into tissues. Our results, as well as earlier studies, have demonstrated that tissue-resident NK cells tend to have a less differentiated, less mature phenotype than peripheral blood NK cells [7,10,12], which to me is a strong argument to the former model with NK cells maturing and fully differentiating in the tissue instead of peripheral blood NK cells migrating into tissues and then transforming into tissue-resident NK cells. Further studies into this topic will hopefully be able to provide an answer to this, although at the current time this was not possible due to the complexity of the remaining questions and the many hurdles, which need to be overcome on the way, technically, biologically and ethically.

2. Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Blimp-1	B lymphocyte-induced maturation protein 1
CD	Cluster of differentiation
$CD56^{bright}CD16^{neg}$	CD56bright CD16 negative NK cells
$CD56^{dim}$ $CD16^{+}$	CD56dim CD16 positive NK cells
EBV	Epstein-Barr-Virus
Eomes	Eomesodermin
GLUT1	Glucose transporter 1
HCMV	Human cytomegalovirus
Hobit	Homologue of Blimp-1 in T cells
IFN	Interferon
IL	interleukine
ILC	Innate lymphoid cell
ITP	Immune thrombocytopenia (ITP),
KIR	Killer cell immunoglobulin-like receptors
ltNK cell	Lymphoid tissue Natural killer cell
MHC-1	Major histocompability complex-class I molecules
MCMV	Murine cytomegalovirus
NK cell	Natural killer cell
NKT	Natural killer T cells
PLZF	Promyelocytic leukaemia zinc finger protein
Rag1	Recombination activating gene 1
S1P	Spinhosine-1-phospate
S1P1	Sphingosine-1-phosphate receptor 1
SIV	Simian immunodeficiency virus
TF	Transcription factor
TfR1	Transferrin receptor protein 1 or CD71
TNF- α	Tumor-necrosis factor alpha

3. Literature

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4. Publication 1

Hobit expression by a subset of human liver-resident CD56^{bright} Natural Killer cells.

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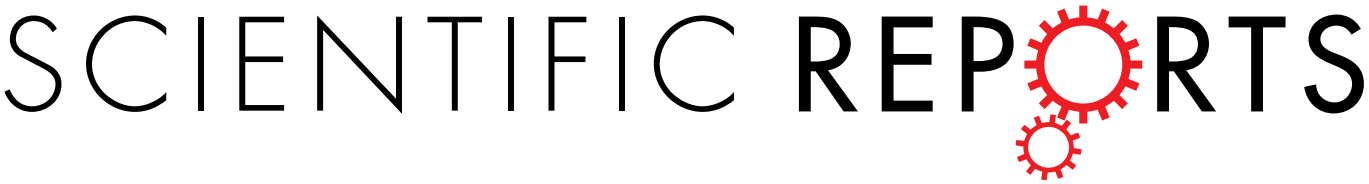

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Hobit expression by a subset of human liver-resident CD56^{bright} Natural Killer cells

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Sebastian Lunemann[1], Gloria Martrus[1], Hanna Goebels[1], Tobias Kautz[1], Annika Langeneckert[1], Wilhelm Salzberger[1], Martina Koch[2], Madeleine J. Bunders[1], Björn Nashan[2], Klaas P. J. M. van Gisbergen[3] & Marcus Altfeld[1]

Immune responses show a high degree of tissue specificity shaped by factors influencing tissue egress and retention of immune cells. The transcription factor Hobit was recently shown to regulate tissue-residency in mice. Whether Hobit acts in a similar capacity in humans remains unknown. Our aim was to assess the expression and contribution of Hobit to tissue-residency of Natural Killer (NK) cells in the human liver. The human liver was enriched for CD56^{bright} NK cells showing increased expression levels of the transcription factor Hobit. Hobit^{pos} CD56^{bright} NK cells in the liver exhibited high levels of CD49a, CXCR6 and CD69. Hobit^{pos} CD56^{bright} NK cells in the liver furthermore expressed a unique set of transcription factors with higher frequencies and levels of T-bet and Blimp-1 when compared to Hobit^{neg} CD56^{bright} NK cells. Taken together, we show that the transcription factor Hobit identifies a subset of NK cells in human livers that express a distinct set of adhesion molecules and chemokine receptors consistent with tissue residency. These data suggest that Hobit is involved in regulating tissue-residency of human intrahepatic CD56^{bright} NK cells in a subset of NK cells in inflamed livers.

The quality of immune responses is influenced by a plethora of factors. There is mounting evidence that tissues are shaping immune responses to serve their specific needs through interactions between immune and tissue cells. Tissue homing, retention and egress of immune cells are important in ensuring that the correct immune microenvironment for each tissue is established and maintained. The underlying mechanisms influencing tissue specificity and residency are slowly being unraveled and will improve our understanding of this essential part of immunology.

Natural Killer (NK) cells are part of the innate immune system and play a pivotal role in the early control of infections[1] and malignancies[2]. NK cells can be divided into two main subsets of CD56^{dim} and CD56^{bright} NK cells, based on their expression of CD56 and CD16[3]. In general, CD56^{bright} NK cells act by producing cytokines, while CD56^{dim} NK cells exert their effector functions through secretion of perforin and granzyme[2]. In the peripheral blood, CD56^{dim} NK cells make up roughly 90% of the NK cells pool, with CD56^{bright} NK cells contributing the remaining 10%. In contrast, CD56^{bright} NK cells represent the dominant population in lymphoid and non-lymphoid tissues[4], and are also found in increased frequencies in inflamed and cancer tissues[5]. Tissue-resident NK cells have now been identified in uterus, liver and lymphoid tissues[4,6], and appear to play important roles not only in the defense against foreign pathogens and cancers[4], but also in tissue remodeling and regeneration[4,7]. While in mice several markers have been identified to define tissue-resident NK cells and it was shown that these tissue-resident NK cells are not circulating through the periphery[8], the factors regulating tissue residency in humans are less well defined[9].

Recent studies in humans have shown that some of the markers used to identify tissue-resident NK cells in murine livers, namely CD49a[9] and CXCR6[10,11], are also expressed on human NK cells within the liver. Additionally, it was shown that CXCR6+ NK cells in human livers exhibited an Eomes^{hi} T-bet^{lo} phenotype[10,11], which is in contrast to the Eomes^{lo} T-bet^{int} phenotype initially described for CD49a+ NK cells in the liver[9]. This might suggest that several heterogeneous subsets of liver-resident NK cells exist in human livers. Further evidence

[1]Department of Virus Immunology, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany. [2]Department of Hepatobiliary Surgery and Transplantation, University Medical Center Hamburg, Hamburg, Germany. [3]Department of Experimental Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands. Sebastian Lunemann and Gloria Martrus contributed equally to this work. Correspondence and requests for materials should be addressed to M.A. (email: marcus.altfeld@hpi.uni-hamburg.de)

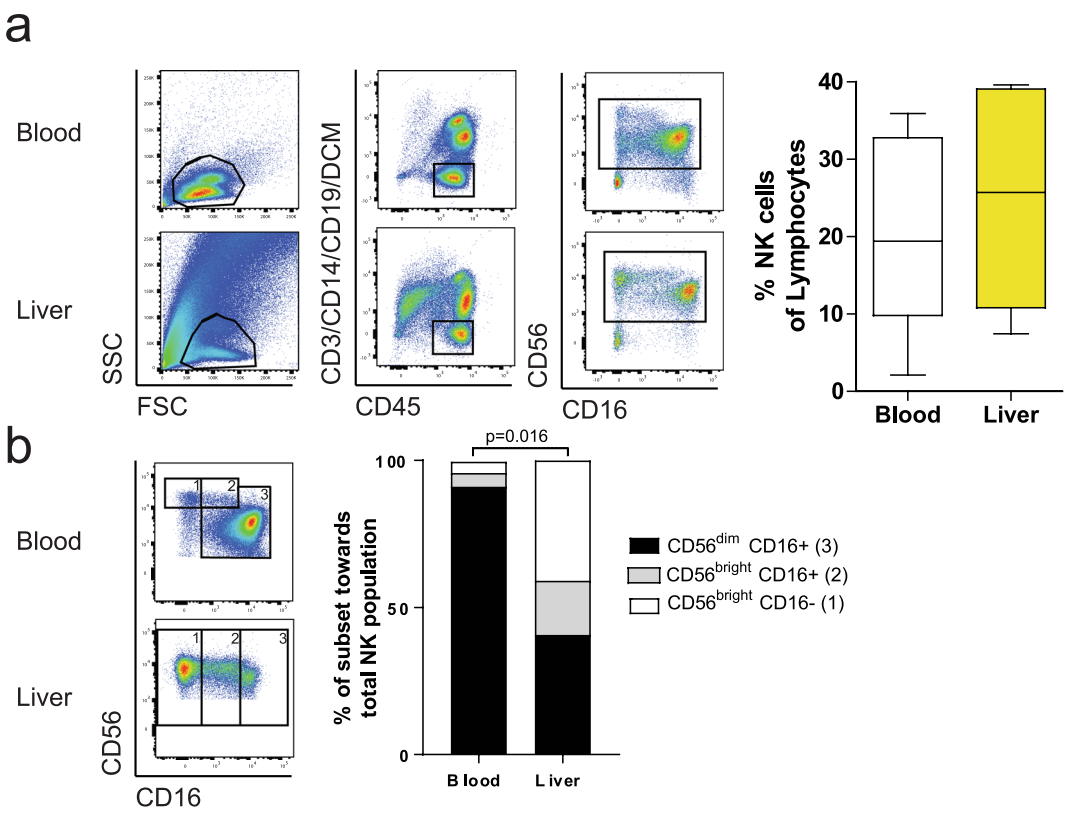

Figure 1. CD56^{bright} NK cells are enriched in human liver tissue. (**a**) Gating strategy (left panel) for identification of NK cells in peripheral blood (upper row) and liver tissue (lower row). Summarization of the data (right panel) comparing frequency of NK cells in blood and liver from matched patient samples (n = 7). Box and whiskers (Tukey) plots was used. (**b**) Identification of CD56^{dim} (3), CD56^{bright} CD16+ (2) and CD56^{bright} CD16- (1) NK cells (left panel) in peripheral blood (upper plot) and liver tissue (lower plot). Summarization of the data (right panel) comparing CD56^{dim},CD56^{bright} CD16+ and CD56^{bright} CD16- NK cells in the blood and liver of matched patient samples (n = 7). Bars show the median for all individuals, Wilcoxon matched pair sign rank test was used to determine statistical differences.

for this was provided by a recent study describing CD49e as a marker almost exclusively expressed on NK cells derived from the human blood, whereas more than 50% of NK cells in the liver lack expression of CD49e[12]. A subset of Eomes^{hi} NK cells in human livers was shown to persist for up to 13 years in a transplantation setting[13]. However, if and how the turn-over of these liver-resident NK cell subsets is shaped by different transcriptional programs remains unknown. In mice, it was recently described that the transcription factor Hobit (homolog of Blimp1 in T cells or ZNF683), a zinc finger protein, acts in concert with Blimp-1 to serve as a master regulator of tissue-residency for lymphocytes[14]. Hobit was initially found to regulate NKT cell effector differentiation[15] and subsequently also used to identify effector-type lymphocytes in humans[16]. Hobit acts together with Blimp-1 in regulating expression of genes involved in tissue retention and egress[14], thereby shaping the lymphocyte compartment of the tissue, and Hobit knockout mice exhibited less tissue-resident NK cells in their liver[14]. Whether Hobit is also playing a role in regulating tissue-residency of human NK cells remains unknown. Here we investigated Hobit expression by human NK cells and its role in regulating tissue-residency of intrahepatic NK cells.

Results

CD56^{bright} NK cells are enriched in human liver tissue. Matched liver and blood samples were obtained from individuals undergoing liver transplantation at the Department of Hepatobiliary and Transplant Surgery of the University Medical Center Hamburg. Basic clinical data of the study cohort is summarized in the supplement (Suppl. Table 1). All individuals had advanced liver disease requiring liver transplantation, including alcoholic liver disease, hepatitis C infection, hepatocellular carcinoma and cholangiocarcinoma. Flow cytometric analysis of the samples revealed that the overall frequency of bulk NK cells within the lymphocyte population was slightly higher in human liver samples (Fig. 1a) compared to matched peripheral blood. While this increase was not significant, the distribution of NK cell subsets was notably shifted between liver and blood samples (Fig. 1b), with a marked increase of both CD56^{bright} CD16- and CD56^{bright} CD16+ NK cells and a corresponding decrease of CD56^{dim} CD16+ NK cells (p = 0.016) in liver tissues.

Increased frequency and expression of Hobit on CD56^{bright} liver NK cells. Next, we investigated the expression of the transcription factor Hobit in peripheral blood and liver-derived NK cells. Hobit was expressed

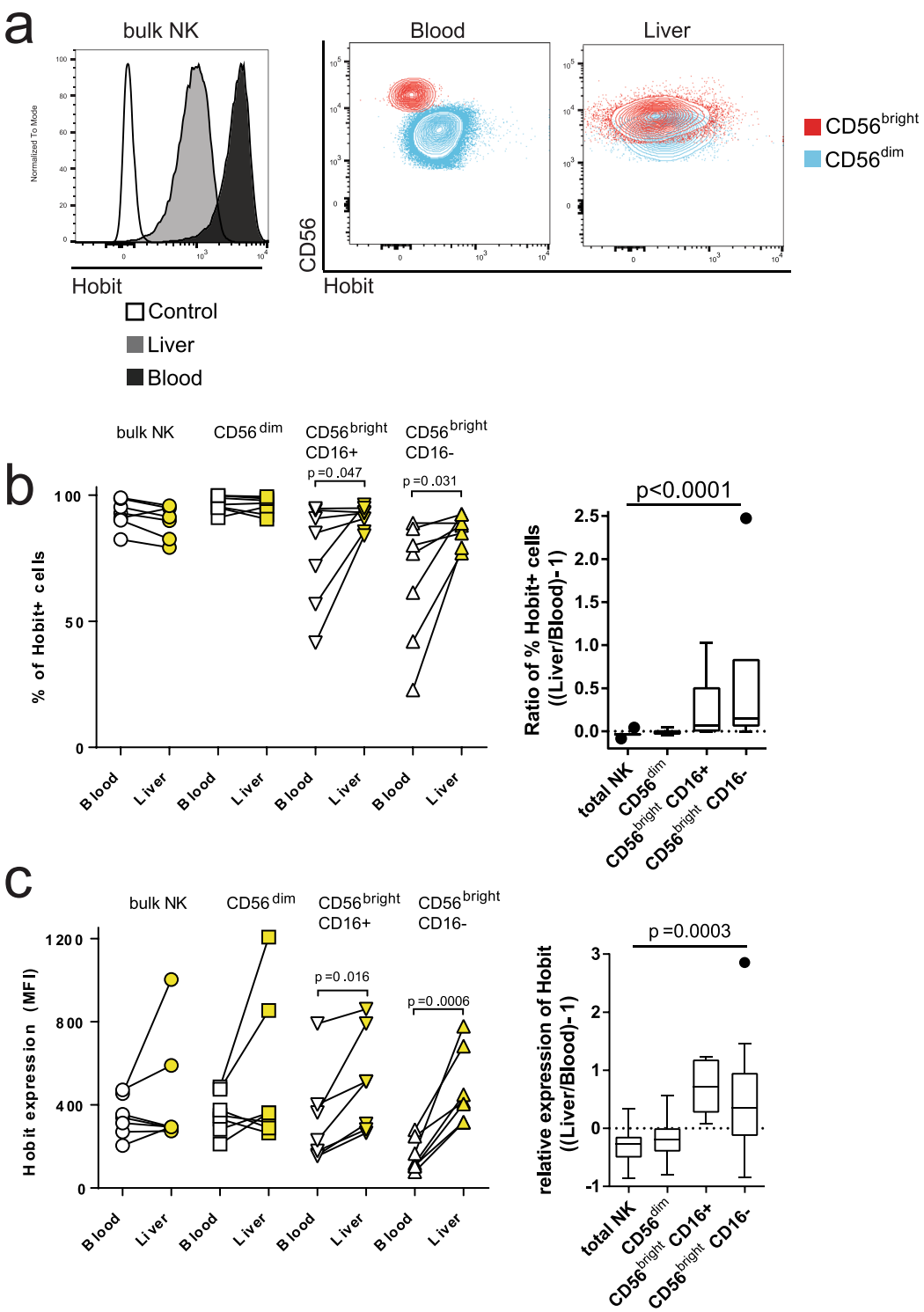

Figure 2. Increased frequencies and expression of Hobit on CD56^{bright} liver NK cells. (**a**) Histogram (left panel) showing Hobit expression on bulk NK cells in the blood (dark grey), the liver (light grey) and a FMO control (white). Contour plots showing the expression of Hobit on CD56^{dim} (blue) and CD56^{bright} (red) NK cells in the blood and liver. (**b**) Frequency (left panel) of Hobit^{pos} bulk, CD56^{dim},CD56^{bright} CD16+ and CD56^{bright} CD16- NK cells in the blood (open symbols) and liver (yellow symbols). Ratio of the frequency (right panel) of Hobit^{pos} cells in each subset between liver and peripheral blood. (**c**) Expression (left panel) of Hobit on bulk, CD56^{dim}, CD56^{bright} CD16+ and CD56^{bright} CD16- NK cells in the blood (open symbols) and liver (yellow symbols). Ratio of the expression (right panel) of Hobit in each subset between liver and peripheral blood samples. Individual data points are blotted in the graphs on the left side. Box and whiskers (Tukey) was used to depict the expression ratio between liver and blood. Wilcoxon matched pair sign rank test was used to determine statistical differences in the scatter plots and Friedman test was used for box plots.

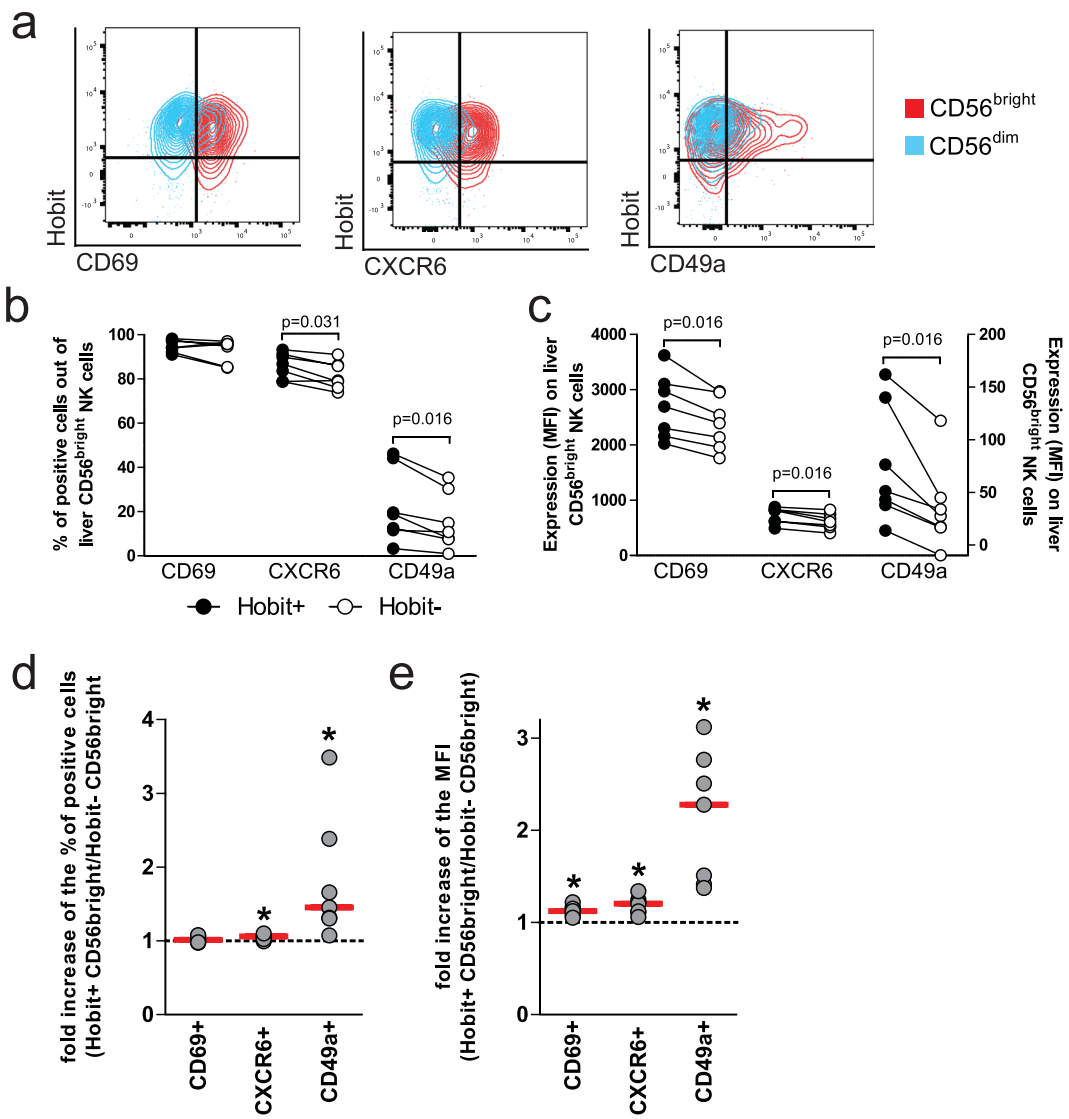

Figure 3. Hobit^{pos} CD56^{bright} liver NK cells express high levels of markers related to tissue residency. (**a**) Contour plots showing expression CD69 (left plot), CXCR6 (middle plot) and CD49a (right plot) compared to Hobit on CD56^{bright} (red) and CD56^{dim} (blue) NK cells in the liver. Frequency (**b**) and expression levels (**c**) of CD69, CXCR6 and CD49a comparing Hobit^{pos} (full symbols) to Hobit^{neg} (open symbols) CD56^{bright} NK cells from the liver. (**d**) Fold change (left panel) of CD69-, CXCR6- and CD49a-positive CD56^{bright} liver NK cells comparing Hobit^{pos} to Hobit^{neg} cells. (**e**) Fold increase of the MFI (right panel) of CD69, CXCR6 and CD49a on CD56^{bright} liver NK cells comparing Hobit^{pos} with Hobit^{neg} cells. Wilcoxon matched pair sign rank test was used to determine statistical differences in the scatter plots.

on bulk liver-derived NK cells, albeit to a lower level than in matched blood samples (Fig. 2a). While almost all bulk and CD56^{dim} NK cells, independent of the compartment they were derived from, expressed Hobit (Fig. 2b), CD56^{bright} CD16^{neg} liver NK cells contained a significantly higher frequency of Hobit^{pos} cells when compared to CD56^{bright} CD16^{neg} NK cells derived from the blood (Fig. 2b, $p = 0.031$). Furthermore, these CD56^{bright} NK cells in the liver also expressed significantly higher levels of Hobit compared to their blood counterparts (Fig. 2c, $p = 0.0006$). CD56^{bright} CD16^{pos} NK cells in the liver showed an intermediate phenotype, both for the %-Hobit+ (Fig. 2b) and expression levels of Hobit (Fig. 2c), when compared to CD56dim and CD56bright CD16- NK cells. Taken together, these data demonstrate that CD56^{bright} NK cells represent a large fraction of the NK cells in the liver, and that these CD56^{bright} NK cells contain a higher frequency of Hobit^{pos} cells expressing higher levels of Hobit compared to CD56^{bright} NK cells in the peripheral blood.

Hobit^{pos} CD56^{bright} liver NK cells express markers related to tissue residency. To determine whether Hobit expression was associated with the expression of other markers previously linked to tissue-residency of NK cells, we subsequently investigated the expression of the surface markers CD69, CXCR6, and CD49a on Hobit^{pos} NK cells[9–11]. We observed that CD69, CXCR6 and CD49a were all expressed on liver-derived NK cells, with higher levels on CD56^{bright} NK cells compared to CD56^{dim} NK cells (Fig. 3a).

Furthermore, liver Hobit^{pos} CD56^{bright} NK cells contained a significantly higher frequency of CXCR6+ ($p = 0.031$) and CD49a+ ($p = 0.016$) cells (Fig. 3b and d), and exhibited a higher expression of CD69, CXCR6 and CD49a ($p = 0.016$ for all comparisons) compared to Hobit^{neg} CD56^{bright} NK cells (Fig. 3c and e). Overall, Hobit^{pos} CD56^{bright} NK cells in the liver showed a significantly higher expression of adhesion molecules and chemokine receptors related to tissue-residency compared to Hobit^{neg} CD56^{bright} NK cells.

Altered expression of transcription factors in Hobit^{pos} CD56^{bright} liver NK cells. Following the observation that Hobit^{pos} NK cells in the liver expressed markers of tissue-residency, we next assessed whether other transcription factors were also differentially expressed on CD56^{bright} NK cells in human livers. We therefore analyzed the expression of Blimp-1, T-bet and Eomes comparing bulk, CD56^{dim} and CD56^{bright} NK cells (Fig. 4a) derived from either peripheral blood or matched liver tissue. Blimp-1 was decreased in liver-derived bulk and CD56^{dim} NK cells compared to peripheral blood (Fig. 4b, left panel, $p = 0.047$, respectively). While the expression of Blimp-1 showed a tendency to be lower in liver-derived CD56^{bright} NK cells compared to peripheral blood (Fig. 4b, left panel, $p = 0.3$), this decrease in Blimp-1 expression was less pronounced compared to the relative decrease for bulk and CD56^{dim} NK cells, as reflected by the ratios of Blimp-1 expression between liver- and blood-derived NK cells (Fig. 4b, right panel, $p = 0.031$). Eomes expression was lower on liver-derived bulk, CD56^{dim} and CD56^{bright} NK cells, albeit only reaching significance for the CD56^{dim} NK cells from the liver (Fig. 4c, $p = 0.016$). T-bet was expressed at similar levels among bulk and CD56^{dim} NK cells comparing to blood and liver NK cells, but was significantly increased in liver-derived CD56^{bright} NK cells (Fig. 4d, $p = 0.016$). We subsequently focused the analysis of the expression of these different transcription factors on the Hobit^{pos} CD56^{bright} NK cell population in the liver, and observed higher frequencies of Blimp-1^{pos} and T-bet^{pos} cells compared to to Hobit^{neg} CD56^{bright} NK cells (Fig. 4e, $p = 0.031$, respectively). Expression levels of Blimp-1 and T-bet followed a similar pattern (Fig. 4f, $p = 0.02$, respectively), while Eomes expression did not differ significantly, neither for frequency of positive cells ($p = 0.4$) nor expression levels ($p = 0.16$). Taken together, liver- and peripheral blood-derived NK cells exhibited distinct expression patterns of transcriptions factors, and this was most apparent for liver-derived Hobit^{pos} CD56^{bright} NK cells.

Discussion

Dissecting the factors that influence tissue-residency and shape the local immune microenvironment is crucial to improve our understanding of tissue-specific immunity. Several recent studies, mainly in mouse models, have demonstrated that liver-resident NK cells differ from NK cells circulating in the peripheral blood in expression of surface markers and transcription factors that regulate their trafficking behavior. In particular, the transcription factor Hobit has been shown to play a pivotal role in instructing tissue-residency of murine lymphocytes in conjunction with Blimp-1[14]. Hobit was furthermore shown to be expressed at high levels by peripheral blood NK cells[16,17] and iILC1s from human tonsils[18]. In this study we investigated Hobit expression by human NK cells in matched samples from peripheral blood and explant liver tissues. We demonstrate that CD56^{bright} NK cells in the liver expressed higher levels of Hobit compared to their blood counterparts, and that Hobit-expression was accompanied by an increased expression of tissue residency markers and changes in the expression of transcription factors.

In mice, the transcription factors Hobit and Blimp-1 acted synergistically to regulate the expression of a specific set of genes linked to tissue retention and egress[14] of lymphocytes, thereby shaping tissue-specific immunity. Hobit/Blimp-1 double-knockout mice showed a down regulation of *Klf2*, *S1pr1*, *Tcf7* and *CCR7*, all genes previously liked to tissue egress or the formation of circulating immunity[19–23]. While Hobit was shown to be expressed by human NK cells circulating in the peripheral blood, it was almost absent in NK cells derived from human tonsils[16], raising the question whether Hobit is also involved in regulating tissue-specific localization of human lymphocytes. A subsequent study showed that Hobit gene expression was high in iILC1 derived from human tonsils relative to other innate and adaptive lymphocyte subsets[18]. The differences observed in these two studies might be based on differences in the lymphocyte subsets studies, namely CD56+ NK cells[16] versus CD56+ NKp44 + CD103+ iILC1s[18]. In line with the study by Vieira Braga *et al.*[16], we observed Hobit expression by the majority of human NK cells circulating in the peripheral blood. Interestingly, Hobit was also expressed by NK cells derived from livers, in contrast to what had been described for NK cells derived from tonsils[16]. This demonstrates that expression of transcription factors by human NK cells differs between different tissues, as also suggested by recent studies of innate lymphoid cells in mice[24].

While we did not observe differences in the frequency of Hobit^{pos} bulk NK cells or Hobit-expression levels among bulk and CD56^{dim} NK cells derived from peripheral blood or liver, CD56^{bright} NK cells obtained from liver tissues exhibited a significantly increased frequency of Hobit^{pos} cells and Hobit-expression levels compared to CD56^{bright} NK cells in the peripheral blood. These data suggest that CD56^{bright} NK cells, that have been shown to be enriched in tissues, might be required to reduce Hobit-expression in order to egress tissues and enter the peripheral circulation. However, the detection of Hobit^{pos} NK cells in the peripheral blood in humans in our study and the previous study by Vieira-Braga *et al.*[16], while Hobit-expression is almost absent in circulating NK cells in mice, suggests clear differences in the function of Hobit between mice and humans. In humans, high Hobit-expression appears to be compatible with the circulation of CD56^{dim} NK cells in the peripheral blood, as also described for human CD45RA+ effector CD8 T cells, which express Hobit and circulate[16], while Hobit-expression was reduce in CD56^{bright} NK cells circulating in the peripheral blood. In line with these differences in Hobit-expression in circulating lymphocytes between mice and humans, a number of Hobit-regulated genes are differentially expressed between mice and humans, including TCF1[25], CD62L[26] and granzyme B[27]. This indicates that different pathways may be required in mice and humans to facilitate tissue residency, and emphasizes the need to better understand the factors regulating tissue-entry, retention and egress of lymphocytes in humans. In this context it is important to highlight that the tissues used in our study were obtained from patients suffering from end-stage liver diseases

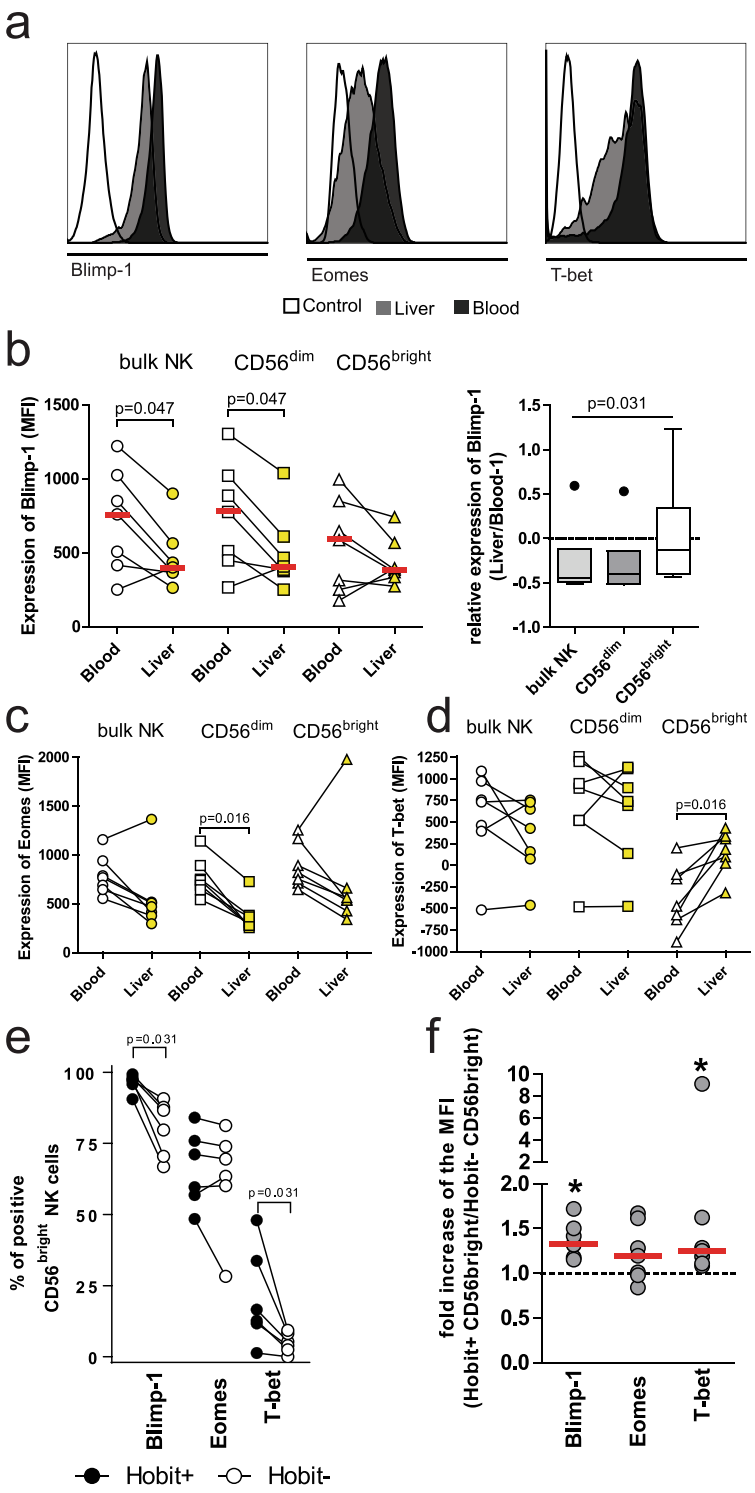

Figure 4. Altered expression of transcriptions factors in Hobit^{pos} CD56^{bright} liver NK cells. (**a**) Histogram showing expression of Blimp-1 (left panel), Eomes (middle panel) and T-bet (right panel) on bulk NK cells in the blood (dark grey) compared to the liver (light grey) and a FMO control (white). (**b**) Expression (left panel) of Blimp-1 on bulk, CD56^{dim} and CD56^{bright} NK cells in the peripheral blood (open symbols) and liver (yellow symbols). Ratio of the expression (right panel) of Blimp-1 in each subset between Liver and Blood. (**c**) Expression of Eomes on bulk, CD56^{dim} and CD56^{bright} NK cells in the blood (open symbols) and liver (yellow symbols). (**d**) Expression of T-bet on bulk, CD56^{dim} and CD56^{bright} NK cells in the blood (open symbols) and liver (yellow symbols). (**e**) Frequency of Blimp-1^{pos}, Eomes^{pos} and T-bet^{pos} cells among CD56^{bright} liver NK cells comparing Hobit^{pos} (black symbol) with Hobit^{neg} (open symbol). (**f**) Fold change of the expression (MFI) of Blimp-1, Eomes and T-bet on CD56^{bright} liver NK cells comparing Hobit^{pos} with Hobit^{neg} cells. Wilcoxon matched pair sign rank test was used to determine statistical differences in the scatter plots and Friedman test was used for box plots.

of different etiologies, and might therefore not reflect the situation in healthy tissue. Despite the variability in the underlying diseases, we observed consistent differences in Hobit-expression between CD56^{bright} NK cells in livers and the peripheral blood, suggesting that Hobit-expression was involved in the determination of liver-residency of this NK cell subset.

The expression of several surface molecules involved in cell adhesion or chemotaxis have been associated with liver-resident NK cells in humans[9–12,28]. Expression of CD49a, the alpha part of the $\alpha 1\beta 1$ integrin complex which facilitates binding to collagen and laminin, is a critical marker for liver-residency in mice[8], and was also shown to be almost exclusively expressed on human liver-derived NK cells and absent on peripheral blood NK cells[9]. CXCR6 has been described to be required to retain NK cells in the liver tissue[28], and to be expressed by a subset of T-bet^{lo} Eomes^{hi} CD56^{bright} NK cells residing in the liver[10,11]. Part of the Eomes^{hi} NK cells have been shown to persist up to 13 years in the liver in a transplantation setting[13] and can be replenished by the Eomes^{lo} NK cells from the periphery. Very recently, the lack of CD49e was revealed as another possible marker to discriminate between tissue-resident and circulating NK cells[12]. We observed that Hobit^{pos} CD56^{bright} NK cells in livers contained an increased frequency of CD49a^{pos} and CXCR6^{pos} cells and that Hobit^{pos} CD56^{bright} NK cells in livers expressed higher levels of these markers associated with liver-residency on their surface compared to Hobit^{neg} CD56^{bright} NK cells and CD56^{bright} NK cells in the periphery. In addition, Hobit^{pos} CD56^{bright} NK cells also expressed higher levels of CD69, which has been described to be a hallmark marker of human tissue-resident memory T cells[29] and NK cells[10]. CD49a expression showed the most pronounced difference between Hobit^{pos} and Hobit^{neg} CD56^{bright} NK cells in the liver, thereby being an important contributor to the tissue-resident phenotype. Differences in the expression of CD69 and CXCR6 were less dominant but still significant. These data suggest that Hobit^{pos} CD56^{bright} NK cells represent a tissue-resident population retained in livers by interactions with cell adhesion molecules and chemotaxis, in line with data obtained in Hobit/Blimp-1 knockout mice showing down-regulation of genes related to tissue egress[14].

Expression of the transcription factor T-bet together with Eomes has been described for human liver-resident CD56^{bright} NK cells in several recent studies[9–11,13]. In line with these studies, we observed that Hobit^{pos} CD56^{bright} NK cells in the liver expressed T-bet, which is, together with IL-15, involved in regulating the expression of Hobit[14]. Furthermore, while Eomes expression was lower among liver NK cells in general, Eomes expression was higher in Hobit^{pos} CD56^{bright} NK cells compared to Hobit^{neg} CD56^{bright} NK cells within the liver. In addition to expression levels also the frequency of Blimp-1^{pos} and T-bet^{pos} cells was increased among Hobit^{pos} CD56^{bright} NK cells. However, Hobit^{pos} NK cells in the liver did not include the T-bet^{lo} Eomes^{hi} subset of liver-resident NK cells recently described in two studies[10,11,13], indicating that Hobit-positivity might define an independent subset of tissue-resident NK cells. Which might be predisposed to be recruited towards inflamed livers, due to the high expression of CD49a. One of the hallmarks of liver inflammation is fibrosis that is accompanied by an increase of collagen production, which acts as a ligand for CD49a[30]. CD49a^{pos} NK cells might therefore have a specific function in the context of liver inflammation, and it has been suggested that NK cells can prevent fibrosis by killing activated stellate cells[31], one of the main drivers of liver fibrosis.

In conclusion, we show that the human liver harbors a subset of Hobit^{pos} CD56^{bright} NK cells expressing chemokine receptors and adhesion molecules associated with liver residency. Hobit^{pos} CD56^{bright} NK cells in livers expressed a distinct pattern of transcription factors differing from previously described intrahepatic NK cells. These data suggest that Hobit is involved in regulating tissue-residency of human intrahepatic CD56^{brigt} NK cells and that Hobit-expression can define a distinct subset of liver-resident NK cells in inflamed livers.

Methods

Study Design. The objective of this study was to investigate whether Hobit is expressed by human intrahepatic NK cells and whether it influences tissue-residency of NK cells. *Ex vivo* phenotyping using flow cytometry was performed on matched liver and blood samples from individuals who underwent liver transplantation. Randomization and blinding were not used in this study.

Patient Cohort. Individuals undergoing liver transplantation at the Department of Hepatobiliary and Transplant Surgery in the University Medical Center Hamburg were included in this study. Reasons for transplantation varied and included alcoholic liver diseases (n = 2), hepatitis C virus infection (n = 2), hepatocellular carcinoma (n = 2) and Cholangiocarcinoma (n = 1). Informed consent was obtained from all patients. This study has been approved by the ethics committee of the Ärtzekammer Hamburg. All experiments were performed in accordance with the relevant guidelines and regulations. Matched blood samples were obtained, either during surgery or directly before. Samples of the explanted livers were collected during surgery, immediately transferred to the Heinrich Pette Institute and processed. Individuals undergoing repeated liver transplantations or individuals in whom no blood samples were obtained were excluded from the analysis.

Sample processing. PBMCs were isolated from blood samples using density centrifugation with Ficoll/Percoll. Liver tissue samples were cut into small pieces using scalpels and subsequently mechanically dissociated using a gentleMACS Octo Dissociator (Miltenyi). No enzymes were used to assist with digestion of the tissue, due to their effects on the surface expression of NK cell markers. Liver tissue samples were subsequently serially filtered using decreasing filter sizes from 100 µm to 70 µm and 40 µm.

Flow Cytometry. The following antibodies were used from Biolegend: anti-Blimp-1-PE, anti-CD16-BV785 (3G8), anti-T-bet-BV711 (4B10), anti-CD69-BV510 (FN50), anti-IgM-BV421 (RMM-1), anti-CD14-AlexaFluor700 (M5E2), anti-CD19-AlexaFluor700 (HIB19), anti-CD3-AlexaFluor700 (UCHT1), Zombie NIR Fixable Viability Kit, Zombie Aqua Fixable Viability Kit, anti-CXCR6-PerCP-Cy5.5 (K041E5), anti-CD56-BV605 (HCD56), anti-CD14-BV510 (M5E2), anti-CD19-BV510 (HIB19), LiveDead-Aqua,

anti-CD45-APC-Cy7 (HI30), anti-CD3-BV510 (UCHT1). From BD Bioscience: anti-CD3-PE-CF594 (UCHT1), anti-CD45-BUV395 (HI30) and anti-CD56-BUV737 (NCAM16.2). Additional antibodies used were anti-CD49a-PE-Vio770 (Miltenyi), anti-Eomes-APC (R&D Systems) and anti-CD2-Qdot605 (S5.5, life technologies). The Hobit antibody was kindly provided by Klaas van Gisbergen[14].

Stainings of cells (10^6 PBMCs or 2×10^6 liver-derived lymphocytes) were performed as follows: after washing in PBS with 2% (v/v) FBS and 0.5 M EDTA, cells were stained with an antibody mastermix prepared in the same buffer for 30 min at 4 °C in the dark. After two washing steps, cells were incubated in freshly prepared Fix/Perm solution (eBioscience) for 30 min at 4 °C in the dark. Following one washing steps, with freshly prepared Permwash (eBioscience), cells we stained with the Hobit antibody (1:10 dilution) for 30 min at 4 °C in the dark. Cells were subsequently washed two times and then stained with anti-IgM-BV421 for 30 min at 4 °C in the dark. After washing ones with both Permwash and PBS containing 2% (v/v) FBS and 0.5 M EDTA, cells were fixed in 4% (w/v) PFA. All samples were acquired on a BD LSRFortessa (BD Bioscience). The data was analyzed using FlowJo v10 Software (Treestar).

Statistical Analysis. Statistical analyses were performed using Excel (Microsoft Corp.) and Prism 5 (GraphPad Software Inc.). Due to the small sample size, data were not assumed to be normally distributed and non-parametrical tests were performed. As matched peripheral blood and liver samples were obtained from the same individual, matched analyses were performed. Comparisons between two groups were performed using the Wilcoxon matched pair sign rank test to determine statistical differences. For comparisons between three or more groups, the Friedman test was used. If the p-value is not depicted as number it is abbreviated with *, where * represents p values below 0.05, ** below 0.01 and *** below 0.001.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Author Contributions

S.L., G.M., T.K., H.G., W.S., A.L. performed the experiments. S.L., G.M. and M.A. analyzed the data. S.L., G.M., M.K., M.B., B.N., K.G. and M.A. drafted the paper. All authors reviewed the manuscript. S.L. and G.M. contributed equally to this work.

Additional Information

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Suppl. Table 1. Summary of clinical data for all patients used in the study.

Number	7
Gender (f/m; %)	1/6 (86%)
Age (years)	55 (25-63)
Weight (kg)	82 (64-132,5)
Size (cm)	176 (154-182)
AST (U/L)	38 (23-110)
ALT (U/L)	31 (15-85)
CMV (p/n, % positive)	4/3 (57%)

All clinical data is shown as median with minimum to maximum.

5. Publication 2

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

Martrus G, PLoS One. 2017

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RESEARCH ARTICLE

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

Glòria Martrus[1☯], Tobias Kautz[1☯], Sebastian Lunemann[1], Laura Richert[1,2], Laura Glau[3], Wilhelm Salzberger[1], Hanna Goebels[1], Annika Langeneckert[1], Leonard Hess[1], Tobias Poch[4], Christoph Schramm[4], Karl J. Oldhafer[5], Martina Koch[6], Eva Tolosa[3], Björn Nashan[6], Marcus Altfeld[1]*

1 Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany, **2** Univ. Bordeaux, Inserm, Bordeaux Population Health Research Center, team SISTM, UMR1219 and Inria, Bordeaux, France, **3** Department of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, **4** First Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, **5** Department of General & Abdominal Surgery, Asklepios Hospital Barmbek, Semmelweis University of Medicine, Asklepios Campus, Hamburg, Germany, **6** Department of Hepatobiliary and Transplant Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

☯ These authors contributed equally to this work.
* marcus.altfeld@leibniz-hpi.de

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Abstract

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

Introduction

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

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

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

Materials and methods

1. Study design and study population

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

2. Cell preparation

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

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

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

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

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

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3. Antibody staining and flow cytometry

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

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

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

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

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

4. Cell sorting and proliferation assay

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

5. t-SNE analysis

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

6. Statistical methods

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

Results

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

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

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

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

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

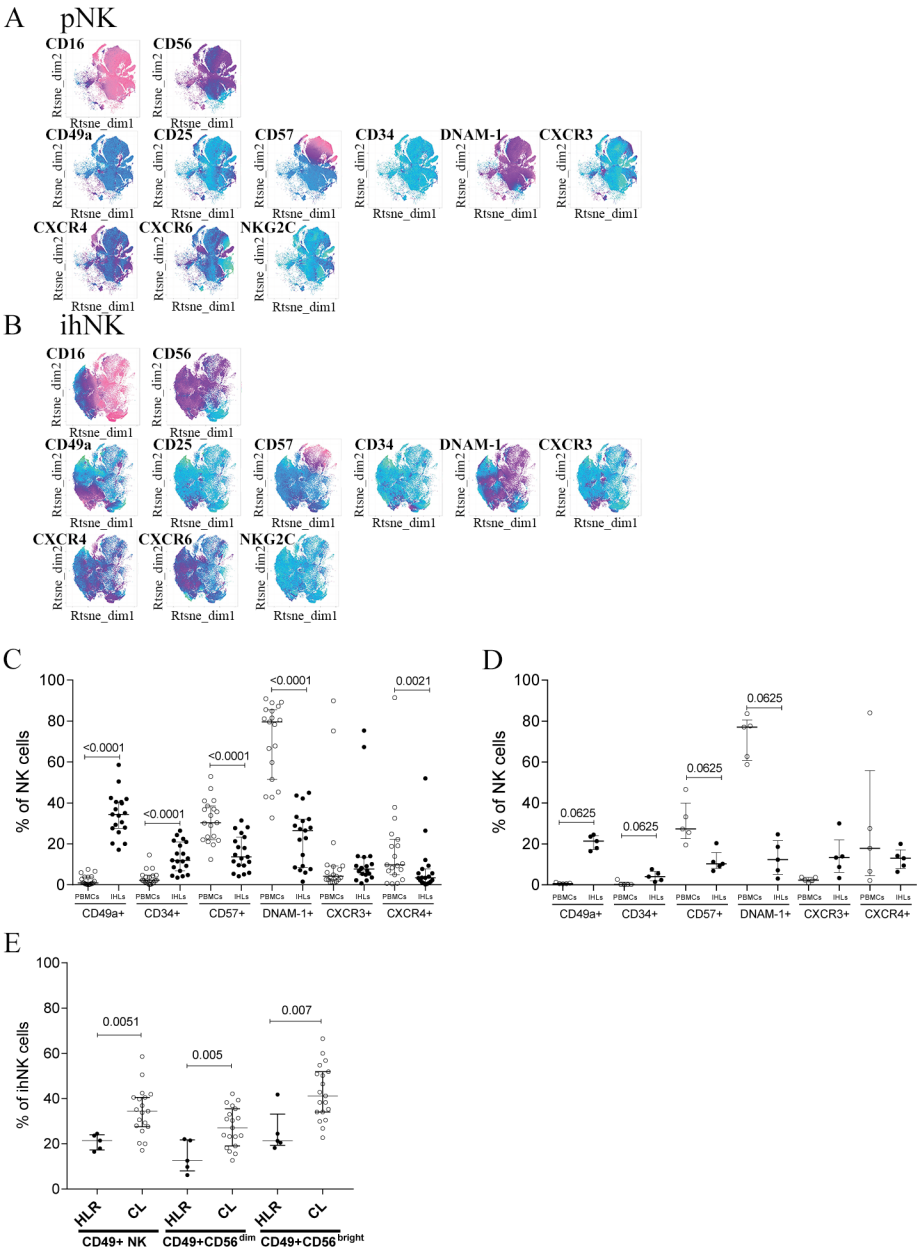

Fig 1. Immune phenotyping of combined peripheral and intrahepatic NK cells. Gated NK cells from 19 donors were concatenated and represented in t-SNE maps for the expression of chemokine receptors, activation and residency markers. **(A)** peripheral and **(B)** intrahepatic NK cells are shown. Color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

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

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

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

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

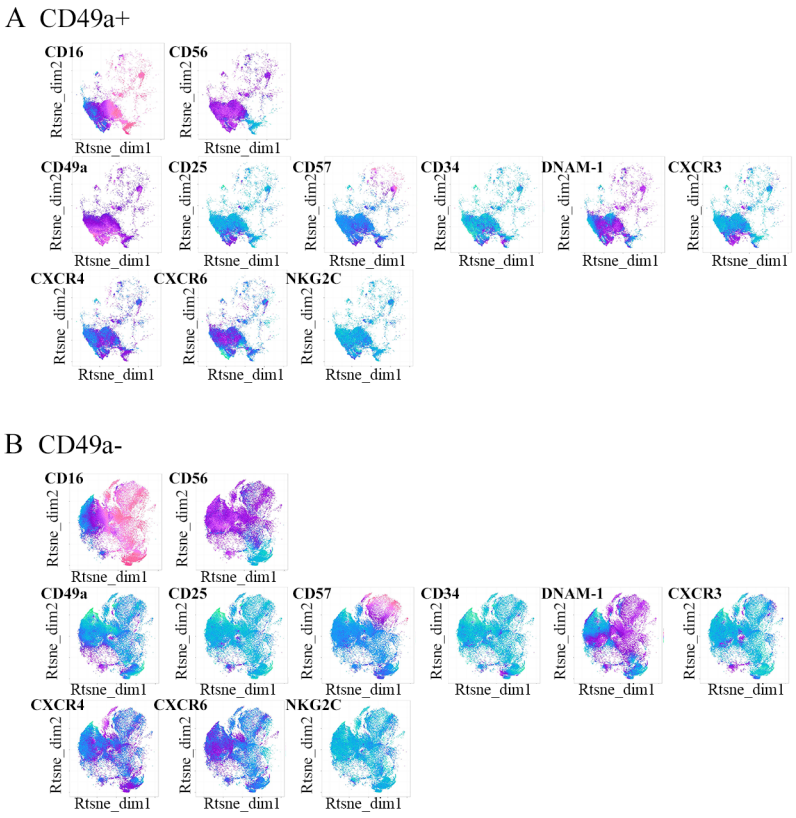

Fig 2. Unsupervised analysis of intrahepatic CD49a+ and CD49a- NK cells. (A) Gated CD49a+ and **(B)** CD49a- NK cells from 19 donors were concatenated and represented in t-SNE maps for the expression of chemokine receptors, activation and residency markers. Color coding indicates the expression intensity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

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

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

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

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

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

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

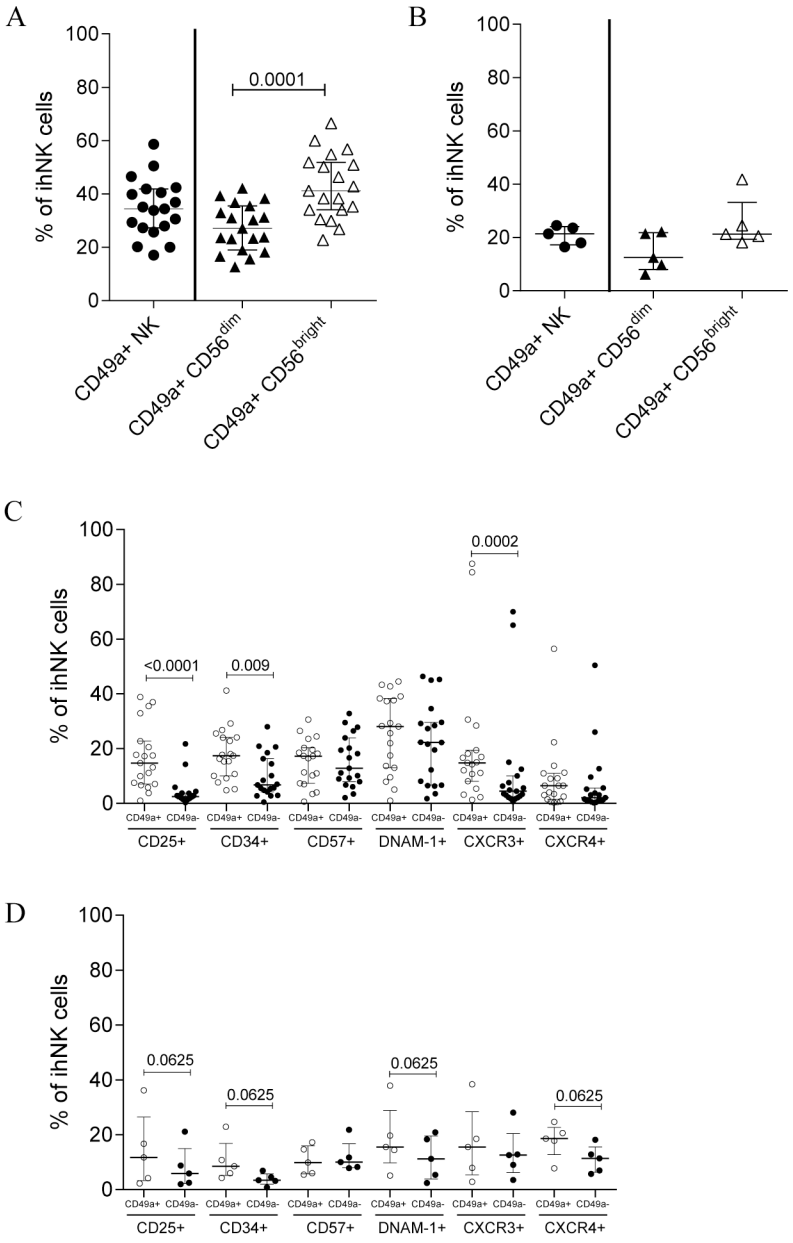

Fig 3. Immunophenotyping of intrahepatic CD49a+ and CD49a- NK cells. CD49a expression on bulk, $CD56^{dim}$ and $CD56^{bright}$ NK cells in the liver transplantation cohort **(A)** and in the tumor-free liver resection cohort **(B)**. Proportion of cells expressing specific markers in ihNK cells once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

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

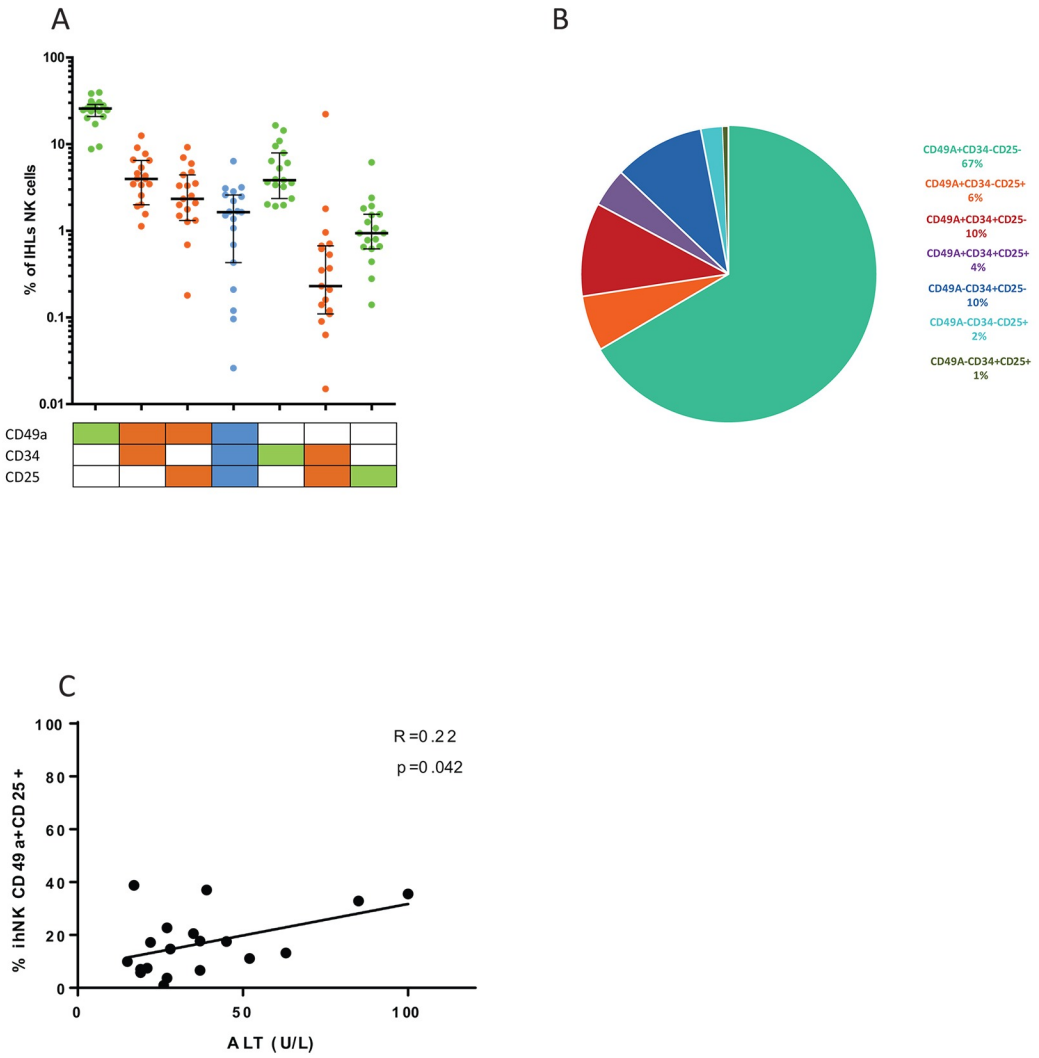

Fig 4. Relevance of CD49a+CD25+ ihNK cells. (A) Boolean gating of CD49a, CD25 and CD34 markers on ihNK cells. **(B)** Pie chart representing the frequency of each of the 7 possible combinations of the 3 markers, CD49a, CD25 and CD34. The median percentage of each population is represented. **(C)** Alanine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

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Discussion

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

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

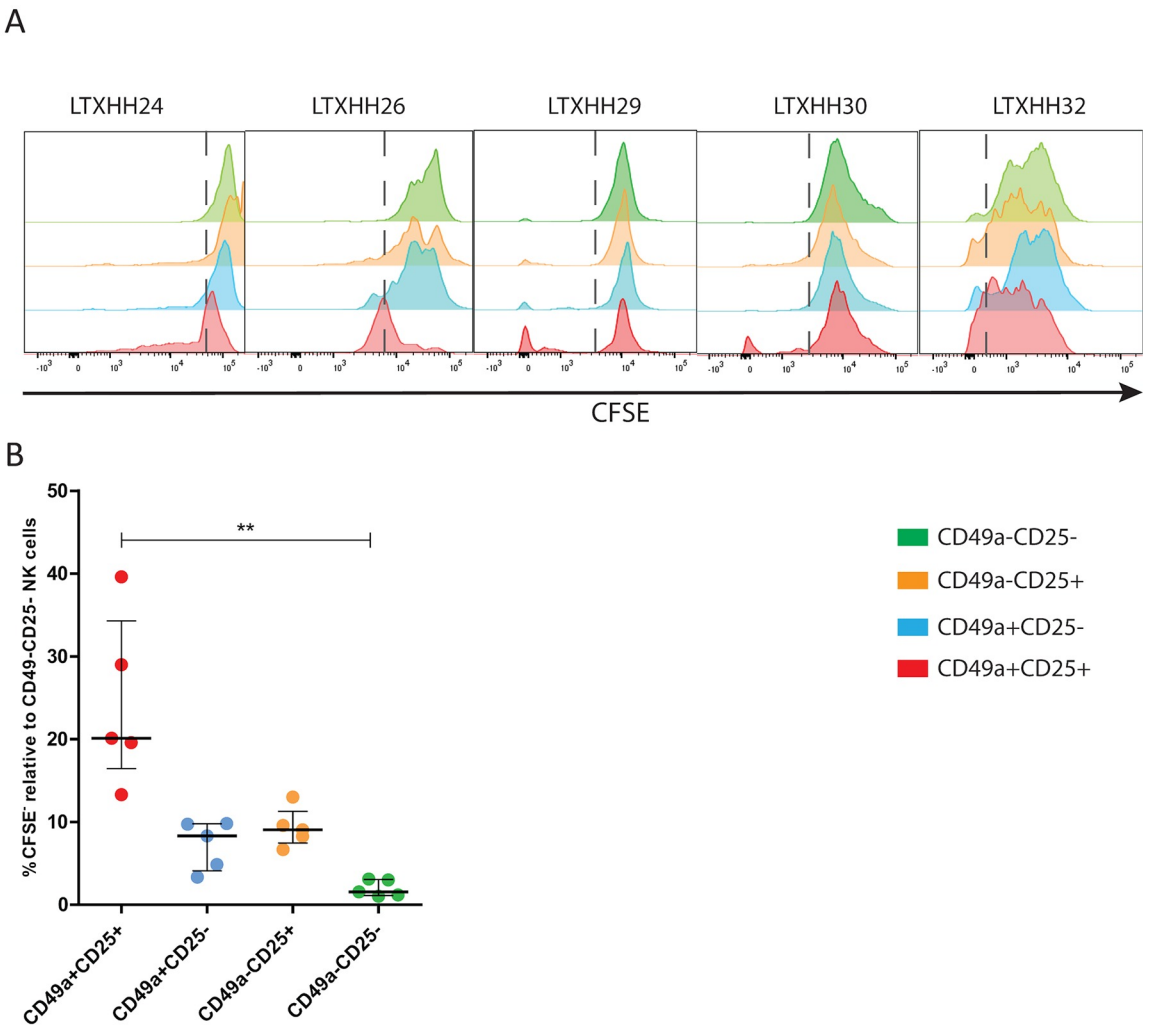

Fig 5. Functional assessment of liver-resident CD49a+ CD25 ihNK cells. (A) Representative histograms 7 days post-cell tracing with CFSE on 5 livers, LTXHH24, LTXHH26, LTXHH29, LTXHH30 and LTXHH32 (gated on live cells). Intrahepatic NK cells were sorted using a BD FACSARIA FUSION according to their expression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

https://doi.org/10.1371/journal.pone.0182532.g005

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

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

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

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

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

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

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

Supporting information

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

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

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

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

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Author Contributions

Conceptualization: Glòria Martrus, Tobias Kautz, Marcus Altfeld.

Data curation: Glòria Martrus, Tobias Kautz, Sebastian Lunemann, Wilhelm Salzberger, Hanna Goebels, Annika Langeneckert, Leonard Hess, Tobias Poch.

Formal analysis: Glòria Martrus, Tobias Kautz, Laura Richert, Laura Glau, Eva Tolosa, Marcus Altfeld.

Funding acquisition: Christoph Schramm.

Investigation: Glòria Martrus.

Methodology: Glòria Martrus, Sebastian Lunemann.

Resources: Karl J. Oldhafer, Martina Koch, Björn Nashan.

Software: Laura Richert, Laura Glau, Eva Tolosa.

Supervision: Marcus Altfeld.

Validation: Glòria Martrus.

Writing – original draft: Glòria Martrus, Christoph Schramm, Karl J. Oldhafer, Martina Koch, Björn Nashan, Marcus Altfeld.

Writing – review & editing: Glòria Martrus, Tobias Kautz, Sebastian Lunemann, Laura Richert, Laura Glau, Wilhelm Salzberger, Hanna Goebels, Annika Langeneckert, Leonard Hess, Tobias Poch, Christoph Schramm, Karl J. Oldhafer, Martina Koch, Eva Tolosa, Björn Nashan, Marcus Altfeld.

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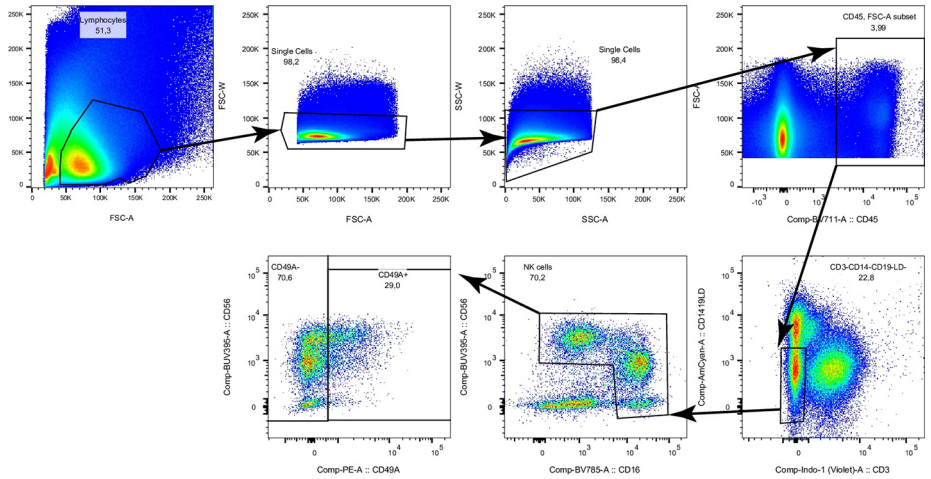
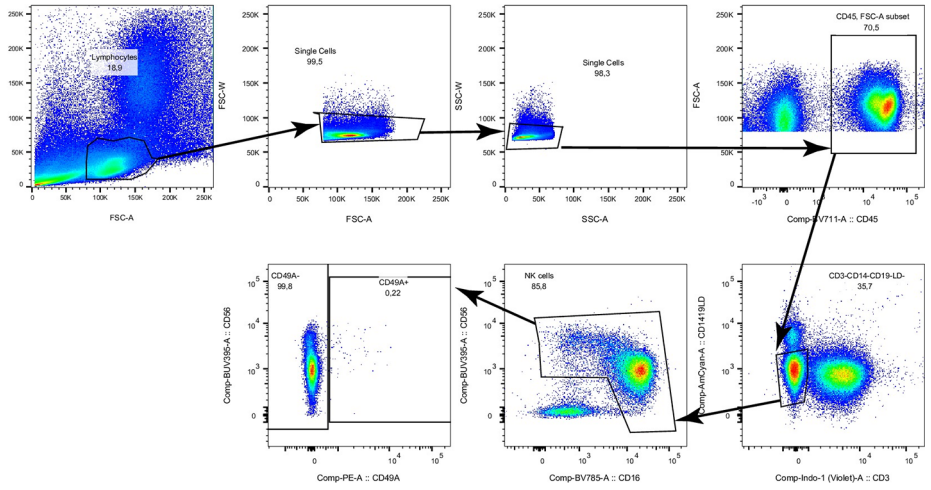
S1 Fig

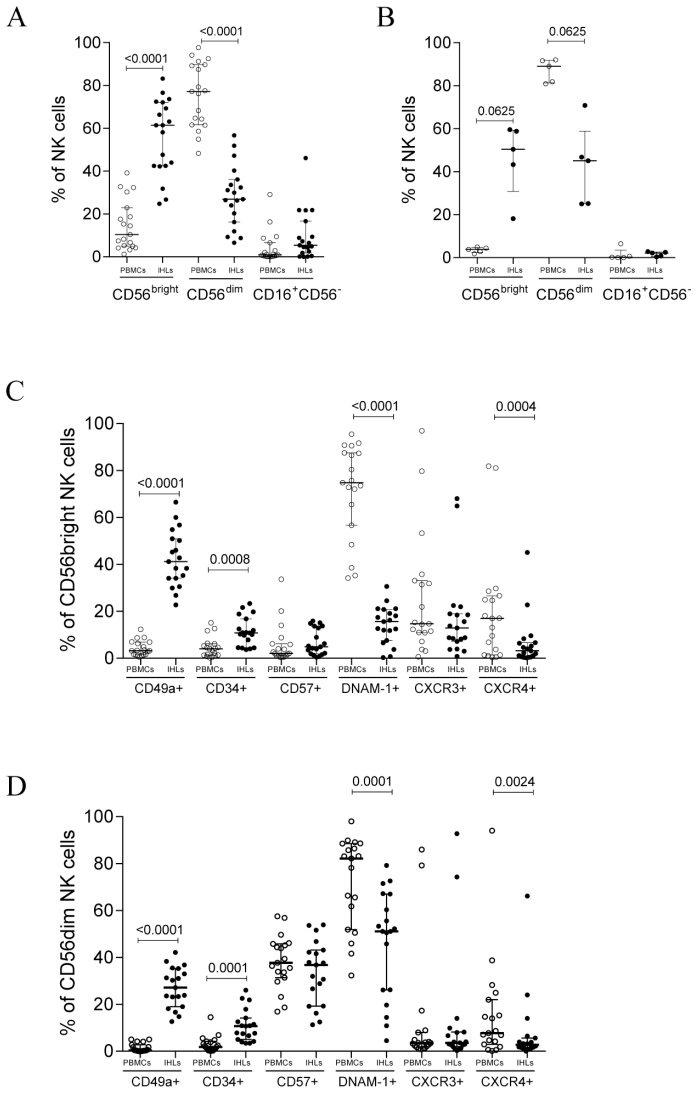
A.


Lymphocytes
51,3
FSC-A
FSC-W
Single Cells
98,2
FSC-A
SSC-W
Single Cells
98,4
SSC-A
FSC-A
CD45, FSC-A subset
3,99
Comp-BV711-A :: CD45
CD49A-
70,6
CD49A+
29,0
Comp-PE-A :: CD49A
Comp-BUV395-A :: CD56
NK cells
70,2
Comp-BV785-A :: CD16
Comp-BUV395-A :: CD56
CD3-CD14-CD19-LD-
22,8
Comp-Indo-1 (Violet)-A :: CD3
Comp-AmCyan-A :: CD14/19LD

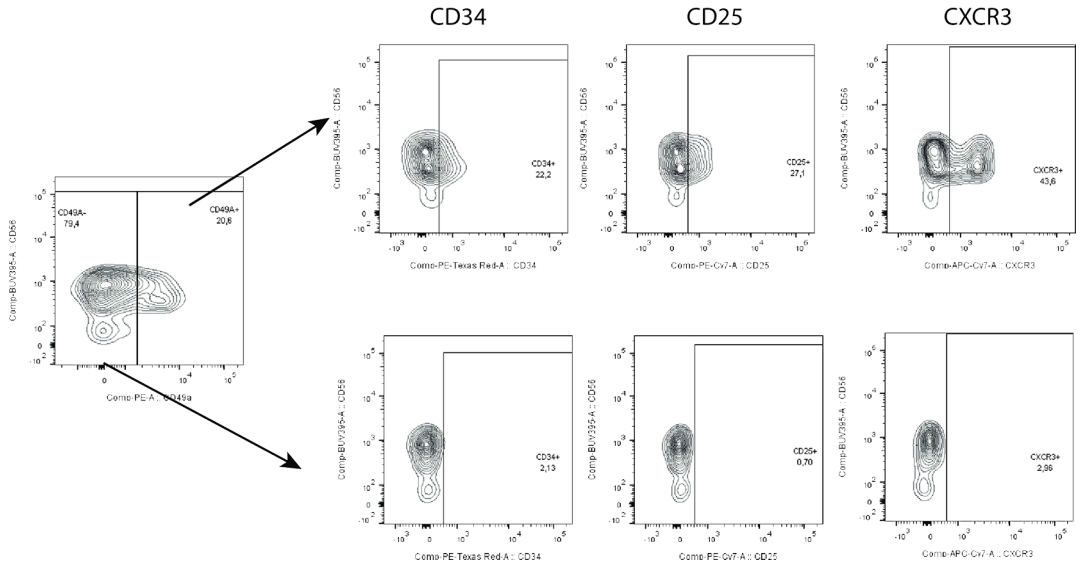
B.


Lymphocytes
18,9
FSC-A
FSC-W
Single Cells
99,5
FSC-A
SSC-W
Single Cells
98,3
SSC-A
FSC-A
CD45, FSC-A subset
70,5
Comp-BV711-A :: CD45
CD49A-
99,8
CD49A+
0,22
Comp-PE-A :: CD49A
Comp-BUV395-A :: CD56
NK cells
85,8
Comp-BV785-A :: CD16
Comp-BUV395-A :: CD56
CD3-CD14-CD19-LD-
35,7
Comp-Indo-1 (Violet)-A :: CD3
Comp-AmCyan-A :: CD14/19LD

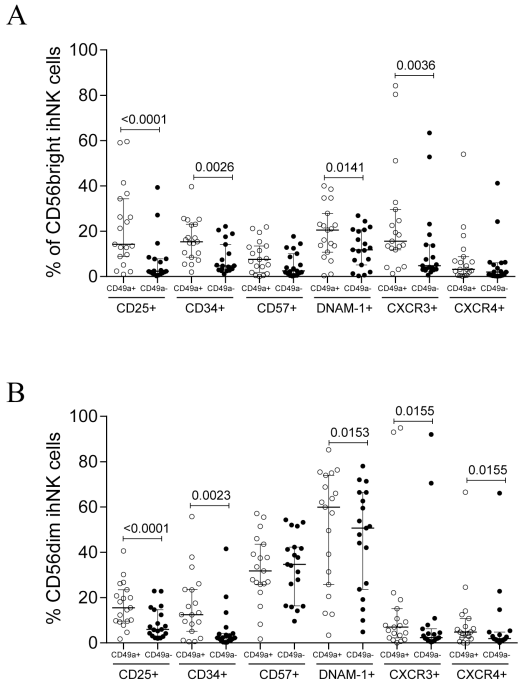
S2 Fig


A
B
C
D
% of NK cells
% of CD56bright NK cells
% of CD56dim NK cells
PBMCs
IHLs
CD56bright
CD56dim
CD16+CD56-
CD49a+
CD34+
CD57+
DNAM-1+
CXCR3+
CXCR4+
<0.0001
0.0625
0.0008
0.0001
0.0004
0.0024

S3 Fig


CD34
CD25
CXCR3
CD49A-
79,4
CD49A+
20,8
CD34+
22,2
CD25+
27,1
CXCR3+
43,6
CD34+
2,13
CD25+
0,70
CXCR3+
2,96

S4 Fig


A
% of CD56bright ihNK cells
100
80
60
40
20
0
<0.0001
0.0026
0.0141
0.0036
CD49a+ CD49a-
CD25+
CD34+
CD57+
DNAM-1+
CXCR3+
CXCR4+
B
% CD56dim ihNK cells
100
80
60
40
20
0
<0.0001
0.0023
0.0153
0.0155
0.0155
CD49a+ CD49a-
CD25+
CD34+
CD57+
DNAM-1+
CXCR3+
CXCR4+

6. Publication 3

Tissue-resident NK cells differ in their expression profile of the nutrient transporters Glut1, CD98 and CD71.

Salzberger W, PLoS One. 2018

PLOS ONE

RESEARCH ARTICLE

Tissue-resident NK cells differ in their expression profile of the nutrient transporters Glut1, CD98 and CD71

Wilhelm Salzberger[1], Gloria Martrus[1], Kai Bachmann[2], Hanna Goebels[1], Leonard Heß[1], Martina Koch[3], Annika Langeneckert[1], Sebastian Lunemann[1], Karl J. Oldhafer[4], Caroline Pfeifer[1], Tobias Poch[5], Laura Richert[6], Christoph Schramm[5,7], Ramez Wahib[2], Madeleine J. Bunders[1], Marcus Altfeld[1]*

1 Department of Viral Immunology, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany, **2** Department of General, Visceral and Thoracic Surgery, University Medical Center Hamburg, Hamburg, Germany, **3** Department of Hepatobiliary and Transplant Surgery, University Medical Center Hamburg, Hamburg, Germany, **4** Department of General & Abdominal Surgery, Asklepios Hospital Barmbek, Semmelweis University of Medicine, Hamburg, Germany, **5** First Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, **6** INSERM U1219, INRIA SISTM, Bordeaux University, Bordeaux, France, **7** Martin Zeitz Centre for Rare Diseases, University Medical Center Hamburg, Hamburg, Germany

* marcus.altfeld@leibniz-hpi.de

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Abstract

Metabolism is a critical basis for immune cell functionality. It was recently shown that NK cell subsets from peripheral blood modulate their expression of nutrient receptors following cytokine stimulation, demonstrating that NK cells can adjust to changes in metabolic requirements. As nutrient availability in blood and tissues can significantly differ, we examined NK cells isolated from paired blood-liver and blood-spleen samples and compared expression of the nutrient transporters Glut1, CD98 and CD71. $CD56^{bright}$ tissue-resident ($CXCR6^{+}$) NK cells derived from livers and spleens expressed lower levels of Glut1 but higher levels of the amino acid transporter CD98 following stimulation than $CD56^{bright}$ NK cells from peripheral blood. In line with that, $CD56^{dim}$ NK cells, which constitute the main NK cell population in the peripheral blood, expressed higher levels of Glut1 and lower levels of CD98 and CD71 compared to liver $CD56^{bright}$ NK cells. Our results show that NK cells from peripheral blood differ from liver- and spleen-resident NK cells in the expression profile of nutrient transporters, consistent with a cell-adaptation to the different nutritional environment in these compartments.

Introduction

Natural Killer (NK) cells were first described in 1975 as immune cells able to spontaneously kill tumor cells [1]. Since this first description, it has been shown that NK cells have a key role in several immune-regulation mechanisms, including the ability to recruit other immune cells via cytokine secretion and to kill virus-infected and malignantly transformed cells [2,3]. NK

scope of the originally provided informed consent. Written requests may be sent to vorstandsreferat@leibniz-hpi.de.

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cells furthermore play an important role in reproduction and autoimmunity [4,5]. Classically, NK cells are defined by a lack of CD3, CD14 and CD19 expression combined with expression of CD56 and/or CD16. NK cells are further divided into two main populations, $CD56^{bright}$ $CD16^{neg}$ NK cells and $CD56^{dim}$ $CD16^{+}$ NK cells [6]. In peripheral blood, $CD56^{bright}$ NK cells constitute 10–20% of the NK cell population, and have been suggested to be the precursors of $CD56^{dim}$ NK cells [6]. Upon stimulation, $CD56^{bright}$ NK cells produce high amounts of cytokines and chemokines, which recruit other immune cells and modulate their function [5,7]. In contrast, $CD56^{dim}$ NK cells represent the major NK cell population in peripheral blood, constituting 80–90% of circulating NK cells. $CD56^{dim}$ NK cells are capable of killing target cells via the secretion of perforin and granzymes, and can also produce cytokines such as IFN-γ upon activation, although to a lesser extent than $CD56^{bright}$ NK cells [4,5]. A vast majority of studies on human NK cells have been performed using NK cells derived from peripheral blood. Although convenient to gather functional and phenotypical data, this approach has some limitations, as the major part of the NK cell compartment in the human body resides in tissues [7,8]. A specific population of interest are tissue-resident NK cells, which can reside in tissue for years and execute local tasks. Studies of tissue-resident NK cells from human livers [7–10], lungs [10,11], lymph nodes [12,13], uterus [14], kidneys [15] and spleens [13,16] have elucidated how tissue-resident NK cells are distinct from NK cells circulating in the peripheral blood, showing crucial differences in phenotypical and functional characteristics.

Tissue-resident NK cells are usually defined by the expression of tissue-residency markers, including CD49a, CD69 or CXCR6 [9,17]. Tissue NK cells are therefore divided into two populations, those expressing tissue-residency markers (tissue-resident or TR NK cells) and those NK cells derived from tissues lacking expression of tissue-residency markers (tissue-derived or TD NK cells). Parabiosis studies in mice suggest that the lack of tissue-residency molecules allows NK cells to egress from tissues and re-enter the circulation [18]. Nonetheless, as the microenvironment differs between tissue and blood, these tissue-derived NK cells may acquire some characteristics of tissue-resident NK cells and differ from NK cells observed in peripheral blood, representing a population of transiently tissue-resident NK cells [19]. Tissue-resident NK cells, depending on their specific tissue location, face different nutritional conditions and metabolic requirements than NK cells in peripheral blood. NK cells in peripheral blood usually rely on an abundance of glucose and oxygen, as blood glucose and oxygen levels are tightly regulated in healthy individuals. In contrast, glucose and oxygen levels in tissues need to be constantly replenished via the bloodstream, a process that can be affected by blood circulation, inflammation or tumor infiltration, resulting in local differences of nutrient levels [20,21]. Many tumor cells possess the ability to significantly increase their glucose uptake, thus decreasing glucose availability for immune cells in their surroundings as a mechanism of immune evasion [22]. Similarly, certain viruses such as hepatitis B and C virus can modulate the uptake of glucose into infected cells, which also affects the nutritional microenvironment [23,24]. It still remains unknown how tissue-resident NK cells adjust to these changes in available nutrients to fulfill their functional requirements.

In a resting state, lymphocytes rely on an oxygen-dependent process in the mitochondria, oxidative phosphorylation (OxPhos), in order to efficiently convert one molecule of glucose into 30 molecules of adenosine triphosphate (ATP) [25–28]. When activated, the metabolism of lymphocytes shifts towards glycolysis, even in the presence of oxygen, a process called aerobic glycolysis. During glycolysis, glucose is metabolized to pyruvate and then lactate, which is finally secreted from the cell. Apart from generating ATP, aerobic glycolysis provides precursor molecules essential for the generation of nucleotides, amino acids and lipids [29–31]. However, aerobic glycolysis is less efficient in providing cellular energy, as it only generates two molecules of ATP from each molecule of glucose compared to the 30 molecules of ATP

produced per molecule of glucose during OXPHOS [28,29,31]. Inhibition of glycolysis in murine NK cells was shown to prevent proliferation, production of cytotoxic proteins and affected NK cell adhesion [32]. This suggests that tissue-resident NK cells might face the challenge of having high energy requirements during pathological stages while at the same time the availability of glucose can be limited, which might severely inhibit the NK cells functionality.

To measure the metabolic status of lymphocytes, several markers are of interest. Firstly, the glucose uptake from the surrounding microenvironment is controlled in lymphocytes by the glucose transporter 1 (Glut1). Expression of Glut1 was shown to be a critical factor for rapid proliferation and cytokine production in T effector cells [33,34]. A recent study demonstrated that cytokine stimulation of murine splenic and human peripheral blood NK cells upregulated the cell surface expression of Glut1 and increased the rate of glycolysis, oxidative phosphorylation and IFN-γ production [26,35]. However, as most effector functions of NK cells take place in tissues, studies of tissue-derived NK cells are required to understand metabolic pathways impacting NK cell function in tissues. Next to glucose, amino acids can be used for energy generation. Furthermore amino acids are critical building blocks for protein syntheses. CD98 is a transmembrane protein forming a neutral amino acid transport channel when linked with other L-type amino acid transport proteins. Its expression is detected on all cells except from platelets and is upregulated on activated and proliferating lymphocytes [36–39], however the distribution on tissue derived-NK cells is not known. In addition to nutrients for energy production, other nutrients, such as iron, are critical for proliferation and function of lymphocytes. For example intracellular iron is important both in DNA replication but also the expression of costimulatory molecules on the lymphocyte cell surface impacting activation of lymphocytes [40–42]. Due to its toxicity at higher concentrations, cellular iron content is tightly regulated [40]. Cell surface transferrin receptor protein 1 (TfR1) or CD71 is required for uptake of diferric transferrin via receptor-mediated endocytosis [40,41]. CD71 expression can be further used as a marker for activation or proliferation of T cells [41,43] and was recently reported to be upregulated on cytokine-stimulated NK cells derived from peripheral blood in parallel with an upregulation of CD98 and Glut1 [35]. In this study, we examine whether NK cells from spleen and liver tissues differ from peripheral blood NK cells in their expression profile of the nutrient transporters Glut1, CD98 and CD71 in steady state, and in their ability to upregulate these nutrient transporters in response to cytokine stimulation.

Methods

Patients and ethics

Matched liver and paired blood samples (n = 12) were obtained from individuals undergoing liver transplantation in the Department of Hepatobiliary and Transplant surgery at the University Medical Center Hamburg-Eppendorf or individuals undergoing liver resection in the process of abdominal tumor excision surgery at the Asklepios Hospital, Barmbek (Table 1). Matched spleen and blood samples (n = 11) were obtained from individuals undergoing abdominal excision surgery at the Department of General, Visceral and Thoracic surgery at the University Medical Center Hamburg-Eppendorf (Table 2). All samples used in this study were obtained from patients undergoing surgery for medical reasons. All study participants provided informed written consent according to the guidelines by the Institutional Review Board of the medical faculty at the University of Hamburg. This study received ethics approval under the ethic proposals PV3548 (spleen samples) and PV4898 (liver samples) by the Ärztekammer Hamburg.

Table 1. Relevant clinical data of the liver cohort.

Internal Code	Sample Type	Gender	Age	Diagnosis
L1	Liver+Blood	M	73	Malignant neoplasia of the liver after rectal carcinoma
L2	Liver+Blood	M	63	Hepatocellular carcinoma
L3	Liver+Blood	M	64	Retransplant after autoimmune hepatitis / primary sclerosing cholangitis
L4	Liver+Blood	M	57	Hepatocellular carcinoma (state after hepatitis E infection)
L5	Liver+Blood	M	59	Hepatocellular carcinoma
L6	Liver+Blood	M	64	Hepatocellular carcinoma
L7	Liver+Blood	M	43	Retransplant after hydropic decompensation of transplant after Morbus Wilson
L8	Liver+Blood	M	54	Decompensated livercirrhosis after hepatitis B and C infection
L9	Liver+Blood	M	58	Hepatocellular carcinoma after hepatitis C infection
L10	Liver+Blood	F	46	Primary biliary cholangitis
L11	Liver+Blood	F	51	Polycystic liver disease
L12	Liver+Blood	F	33	Drug induced acute liver failure
Median (IQR)			57 (47–63)	

https://doi.org/10.1371/journal.pone.0201170.t001

Isolation of mononuclear cells from blood and tissue

Peripheral blood mononuclear cells (PBMCs) were isolated from full blood via density centrifugation using Ficoll/Percoll. After counting, cells were frozen in 90%FBS/10% DMSO and stored in liquid nitrogen for later use. Mononuclear cells from spleen tissue (SMCs) were isolated by cutting the fresh tissue into pieces of approximately 0.5x0.5x0.5cm. Spleen tissue pieces were then manually pushed through a series of filters (5003bcm/300μm/100μm/70μm/40μm, Greiner Bio-One GmbH) with a plunger while irrigating the samples with Hanks solution when needed. After washing, the cells underwent erythrocyte lysis using ACK lysis buffer (Biozym) for 3 minutes and were subsequently washed twice with Hanks solution. Cells were then counted with a TC20 automated cell counter (Biorad) and their viability was assessed with trypan blue staining before cryopreservation for later use. Mononuclear cells from liver tissue (LMCs) were isolated by manually cutting tissues into pieces of approximately 0.5x0.5x0.5cm. Liver tissue pieces were then mechanically dissociated with a gentleMACS Octo Dissociator (Milteny). After the mechanical dissociation, cells were manually pushed through a series of filters (500μm/300μm/100μm/70μm/40μm). After washing, LMCs were isolated via a series of density centrifugation steps with Optiprep density gradient medium (Sigma-Aldrich), counted and frozen for later use.

Table 2. Relevant clinical data of the spleen cohort.

Internal Code	Sample Type	Gender	Age	Diagnosis
S1	Spleen+Blood	M	52	Gastrointestinal stromal tumor
S2	Spleen+Blood	F	68	Intraductal papillary mucinous neoplasia of the pancreas
S3	Spleen+Blood	M	79	Non-Hodgkin B-Cell Lymphoma
S4	Spleen+Blood	M	80	Diffuse gastric cancer
S5	Spleen+Blood	F	74	Pancreatic adenocarcinoma
S6	Spleen+Blood	F	75	Gastroesophageal junctional adenocarcinoma
S7	Spleen+Blood	M	51	Gastrointestinal stromal tumor
S8	Spleen+Blood	F	31	Chronic immune thrombocytopenic purpura
S9	Spleen+Blood	F	35	Steroid-refractory immune thrombocytopenic purpura
S10	Spleen+Blood	F	73	Immune thrombocytopenic purpura with splenomegaly with Non-Hodgkin B Lymphoma
S11	Spleen+Blood	F	44	Dilated lymphatic vessels
Median (IQR)			68 (44–75)	

https://doi.org/10.1371/journal.pone.0201170.t002

Culturing and stimulation

All cells were cultured in RMPI 1640 (Life Technologies GmbH), supplemented with 10% heat inactivated FBS Superior (Biochrom AG). Cells were stimulated with 5ng/mL of recombinant human IL-12 and 2.5ng/mL of recombinant IL-15 (both from Pepro Tech EC GmbH).

Flow stainings

Surface antibody stainings were performed in PBS supplemented with 2% heat inactivated FBS for 20 minutes at room temperature. Glut1.- receptor dinding protein (RBD)-eGFP labeling was done in RPMI1640 + 10%FBS + 5% NaN_3 +0.5mM EDTA for 30 minutes at 37˚C. The eBioscience Foxp3/Transcription Factor Staining Buffer Set (product protocol B) was used for intracellular staining. The monoclonal mouse anti-human antibodies anti-CD16-BUV395 (clone 3G8, BD Horizon™), anti-CD56-BUV737 (clone NCAM16.2, BD Horizon™), anti-CD71-BV711 (clone M-A712, BD Horizon™), anti-CD57-BV605 (clone NK-1, BD Horizon™), anti-CD186 (CXCR6)-PE/Cy7 (clone K041E5, Biolegend), anti-CD127-PE-CF594 (clone HIL-7R-M21, BD Horizon™), anti-CD45-BV785 (clone HI30, Biolegend), anti-CD3-AlexaFluor700 (clone UCHT1, Biolegend), anti-CD14-BV510 (clone M5E2, Biolegend), anti-CD19-BV510 (clone HIB19, Biolegend), as well as recombinant human anti-CD98-APC-Vio770 (clone REA387, Milteny Biotec GmbH), Zombie Aqua™(Biolegend) and anti-human GLUT1 (SLC2A1)-eGFP RBD were used for staining. NK cells were defined as CD3 ^{neg}, CD14 ^{neg}, CD19 ^{neg}, CD45+, and were divided into $CD56^{bright}$ CD16 ^{neg} NK cells (referred to as $CD56^{bright}$ NK cells) and $CD56^{dim}$ CD16+ NK cells (referred to as $CD56^{dim}$ NK cells).

Analysis

Samples were acquired on a LSR Fortessa (BD Biosciences) and results were analyzed using Flowjo software version 10. Percentages of subpopulations and median fluorescence intensity (MdFI) values of subpopulations from paired samples were compared using Wilcoxon matched-pairs signed rank tests using Graphpad Prism version 6. Test multiplicity was controlled for by a false discovery rate (FDR) method accounting for dependency among statistical tests, using SAS software, version 9.3 (SAS Institute, Cary, North Caroline, USA)[44]. A p-values reported in this manuscript are FDR-adjusted and considered statistically significant if $p < 0.05$. Cytobank services were used for viSNE analysis.

Results

Phenotypic characteristics of NK cells from liver tissue, spleen tissue and peripheral blood

NK cells in peripheral blood are predominantly $CD56^{dim}CD16^{+}$ NK cells and only a very low percentage of NK cells express tissue-residency markers such as CD49a, CD69 or CXCR6. NK cells from liver and spleen tissues on the other hand have been reported to contain a sizable population of $CD56^{bright}CD16^{neg}$ NK cells as well as NK cells expressing tissue residency markers [7,8,13]. To identify tissue-resident and circulating NK cells, we analyzed the distribution of $CD56^{dim}$ and $CD56^{bright}$ NK cells as well as the expression of CXCR6, a marker for tissue residency [45], in peripheral blood, liver tissue and spleen tissue samples. We separated NK cells into two subsets, $CD56^{bright}$ and $CD56^{dim}$ NK cells. $CXCR6^{+}$ NK cells from spleen and liver tissues were classified as tissue-resident (TR) NK cells. $CXCR6^{neg}$ NK cells derived from spleen and liver tissues were classified as tissue-derived (TD) NK cells, $CXCR6^{neg}$ NK cells from the blood were classified as peripheral blood (PB) NK cells. An enrichment of the proportion of $CD56^{bright}$ NK cells in tissue samples compared to peripheral blood samples was

Table 3. Expression of CXCR6 in NK cells in tissues.

		%CD56^{bright} NK cells	%CXCR6^{+} among CD56^{bright} NK cells	%CXCR6^{+} among CD56^{dim} NK cells
Liver (n = 12)	**Tissue**	37.3% (30.0–45.2%)	76.8% (52.0–86.5%)	22.8% (4.8–36.3%)
	Blood	4.9% (3.4–6.8%)	8.2% (2.4–24.0%)	1.6% (0.9–2.8%)
Spleen (n = 11)	**Tissue**	29.1% (18.2–42.7%)	67.3 (35.8–74.3%)	8.8% (5.0–10.2%)
	Blood	3.7% (1.4–5.2%)	6.6% (1.6–17.5%)	0.6% (0.4–1.1%)

Median and interquartile range (IQR) of %CD56^{bright} NK cells, %CXCR6^{+} among CD56^{bright} NK cells and %CXCR6^{+} among CD56^{dim} NK cells in tissue and blood of liver and spleen tissue donors

https://doi.org/10.1371/journal.pone.0201170.t003

observed (PB vs. TR liver: p = 0.02; PB vs. TR spleen: p = 0.02, Table 3). CD56^{bright} NK cells from liver and spleen tissues were predominantly CXCR6^{+} tissue-resident NK cells compared to peripheral blood (blood vs. liver: p = 0.02; blood vs. spleen: p = 0.03; Table 3), while CD56^{dim} NK cells from liver and spleen tissues were predominantly CXCR6^{neg} tissue-derived NK cells, although there were significantly more CD56^{dim} CD16^{+} CXCR6^{+} NK cells present in both liver and spleen tissues compared to peripheral blood (PB vs. TR liver: p = 0.03; PB vs. TR spleen: p = 0.03; Table 3). In peripheral blood, only a small fraction of NK cells expressed CXCR6 (Table 3). No significant differences in the expression of the differentiation markers CD57 and CD127 between CD56^{bright} CD16^{neg} from liver, spleen and peripheral blood or CD56^{dim} CD16^{+} NK cells from these compartments were observed (Fig 1A and data not shown). Taken together, these results confirmed previous studies [5,8,46] showing that peripheral blood contains mostly CXCR6^{neg} CD56^{dim} NK cells, while both spleen and liver tissues contain CD56^{bright} and CD56^{dim} NK cells, including a significant number of NK cells expressing the tissue-residency marker CXCR6.

Expression of nutrient transporters on NK cells from liver, spleen and peripheral blood

The expression of transporters for nutrients can vary between different NK cell subsets [35]. In peripheral blood, nutrients are usually readily available, while nutrient levels in tissues depend on replenishment via the blood and *in situ* neogenesis, and can vary depending on blood flow and local metabolic conditions [20]. We examined the expression of Glut1, an important transporter for glucose into cells, CD98, a transporter for amino acids, and CD71, a transporter for transferrin, on NK cells derived from peripheral blood and patient-matched liver and spleen tissues.

In general, a lower expression of Glut1 on CXCR6^{+} compared to CXCR6^{neg} CD56^{dim} NK cells isolated from liver and spleen tissues was observed (liver: p = 0.02; spleen: p = 0.05; Fig 1B). Glut1 was also expressed at lower levels on tissue-resident CXCR6^{+} CD56^{dim} NK cells from tissues compared to peripheral blood CD56^{dim} NK cells, although these differences did not reach statistical significance (liver: p = 0.1; spleen: p = 0.2). In contrast, no differences in Glut1 expression were observed between CD56^{bright} NK cells derived from blood, liver and spleen, neither in the CXCR6^{+} nor CXCR6^{neg} CD56^{bright} NK cell populations. However, when assessing CD56^{bright} and CD56^{dim} NK cells from the same tissue compartments, a significantly lower expression of Glut1 on CD56^{bright} NK cells compared to CD56^{dim} NK cells was observed for all NK cell subsets in livers (CXCR6^{+}: p = 0.02; CXCR6^{neg}: p = 0.02; PB: p = 0.01; Fig 1B) and in spleens for CXCR6^{neg} CD56^{bright} NK cells (p = 0.03; Fig 1B). While CD98 expression differed slightly between different NK cell subsets, these differences represented only minor shifts in the MdFI of CD98 (Fig 1C). Furthermore, no significant differences in the expression of CD71 were observed between CXCR6^{+} or CXCR6^{neg} NK cells derived from tissues and

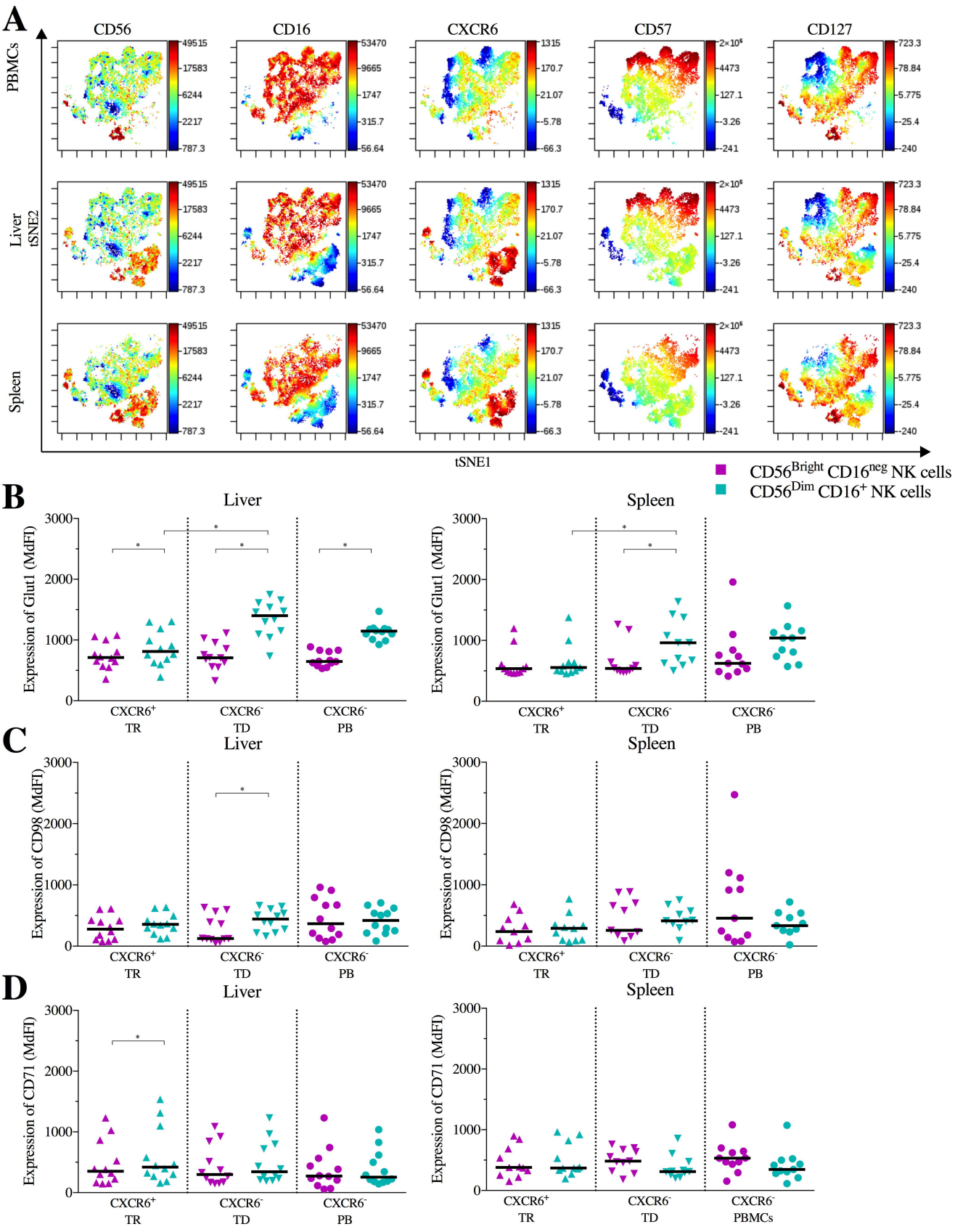
A
CD56
CD16
CXCR6
CD57
CD127
PBMCs
Liver
Spleen
tSNE2
tSNE1
CD56Bright CD16neg NK cells
CD56Dim CD16+ NK cells
B
Liver
Spleen
Expression of Glut1 (MdFI)
C
Expression of CD98 (MdFI)
D
Expression of CD71 (MdFI)
CXCR6+ TR
CXCR6- TD
CXCR6- PB
CXCR6- PBMCs

Fig 1. NK cell phenotype and baseline nutrient receptor expression: Samples were compared using Wilcoxon matched-pairs signed rank tests and multiplicity was controlled for by FDR testing. Bars indicate the median, significance was defined as $p \leq 0.05$ (*). A. viSNE representation of peripheral blood- (PBMC, top row), liver- (middle row) and spleen (bottom row) derived NK cells and their expression of CD56, CD16, CXCR6, CD57 and CD127. B. Expression (Median fluorescence intensity, MdFI) of Glut1 on $CD56^{bright}CD16^{-}$ (purple) and $CD56^{dim}CD16^{+}$ (teal) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples. C. Expression (MdFI) of CD98 on $CD56^{bright}CD16^{-}$ (purple) and $CD56^{dim}CD16^{+}$ (teal) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples. D. Expression (MdFI) of CD71 on $CD56^{bright}CD16^{-}$ (purple) and $CD56^{dim}CD16^{+}$ (teal) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.

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peripheral blood NK cells at baseline, apart from a slightly higher expression of CD71 on $CXCR6^{+}$ $CD56^{dim}$ compared to $CXCR6^{+}$ $CD56^{bright}$ NK cells derived from livers (p = 0.02; Fig 1D). In conclusion, and in contrast to prior reports [35], we consistently observed a higher expression of Glut1 at baseline, without any stimulation, on $CD56^{dim}$ compared to $CD56^{bright}$ NK cells in most investigated tissue compartments. Furthermore, tissue-resident $CXCR6^{+}$ $CD56^{dim}$ NK cells expressed significantly lower levels of Glut1 compared to tissue-derived $CXCR6^{neg}$ $CD56^{dim}$ NK cells.

Effect of cytokine stimulation on NK cell phenotype and Glut1 expression

We next determined the ability of peripheral blood and tissue NK cell subsets to regulate the expression of nutrient transporters, as it was previously reported that stimulation of NK cells induces shifts in their expression profiles [35]. We first studied the effect of cell culture and cytokine stimulation on NK cell subset distributions and phenotypes. Overnight incubation, including stimulation with low amounts of cytokines (5ng/mL IL-12 and 2.5ng/mL IL-15), induced no significant changes in the proportion of $CD56^{bright}$ and $CD56^{dim}$ NK cells derived from all tissue compartments (Fig 2B and S2 Table). In the overnight cell cultures lacking cytokines, a significant decrease of $CXCR6^{+}$ $CD56^{bright}$ NK cells was observed (liver: p = 0.01; spleen: p = 0.04; S2 Table), while the percentage of $CXCR6^{+}$ $CD56^{bright}$ and $CD56^{dim}$ NK cells derived from peripheral blood increased (p = 0.01 for both subsets; S2 Table). Incubation in cytokine-free medium did not induce any significant changes in Glut1-expression on any NK cell population (Part A in S1 Fig). Taken together, these data (Fig 1B and Part A in S2 Fig) show that, in the absence of any stimulation, tissue-resident $CD56^{dim}$ NK cells expressed lower levels of Glut1 than peripheral blood and tissue-derived $CD56^{dim}$ NK cells, while $CD56^{bright}$ NK cells did not differ in their expression of Glut1 irrespective of tissue-residency.

To determine the impact of cytokine stimulation on nutrient transporter expression profiles of NK cells, we next investigated the expression of Glut1 after overnight stimulation with IL-12 and IL-15 (Fig 2A). Cytokine stimulation with 5ng/mL IL-12 and 2.5ng/mL IL-15 over 18 hours led to a significant upregulation of Glut1-expression on all NK cell subsets compared to their unstimulated counterparts (Fig 2B and Part A in S2 Fig). Glut1 expression upon stimulation was higher on $CD56^{dim}$ NK cells compared to $CD56^{bright}$ NK cells in tissues, for example liver-resident $CD56^{dim}$ NK cells compared to liver-resident $CD56^{bright}$ NK cells (p = 0.02; Fig 2B). However, when assessing the fold change as a measure of capacity to upregulate Glut1 after cytokine stimulation, in particular peripheral blood-derived $CD56^{bright}$ NK cells were more effective in upregulating Glut1 after cytokine stimulation compared to $CXCR6^{+}$ or $CXCR6^{neg}$ $CD56^{bright}$ NK cells from liver ($p < 0.05$ for all comparisons, Fig 2B). Peripheral blood $CD56^{bright}$ NK cells also expressed significantly higher Glut1 levels after cytokine stimulation compared to tissue-resident $CXCR6^{+}$ or tissue-derived $CXCR6^{neg}$ $CD56^{bright}$ NK cells from liver ($p < 0.05$ for all comparisons, Fig 2B). Liver-resident $CXCR6^{+}$ $CD56^{bright}$ NK cells showed a significantly higher upregulation of Glut1 when compared to liver-derived $CXCR6^{neg}$ $CD56^{bright}$ NK cells (p = 0.04), but overall a significantly lower upregulation of

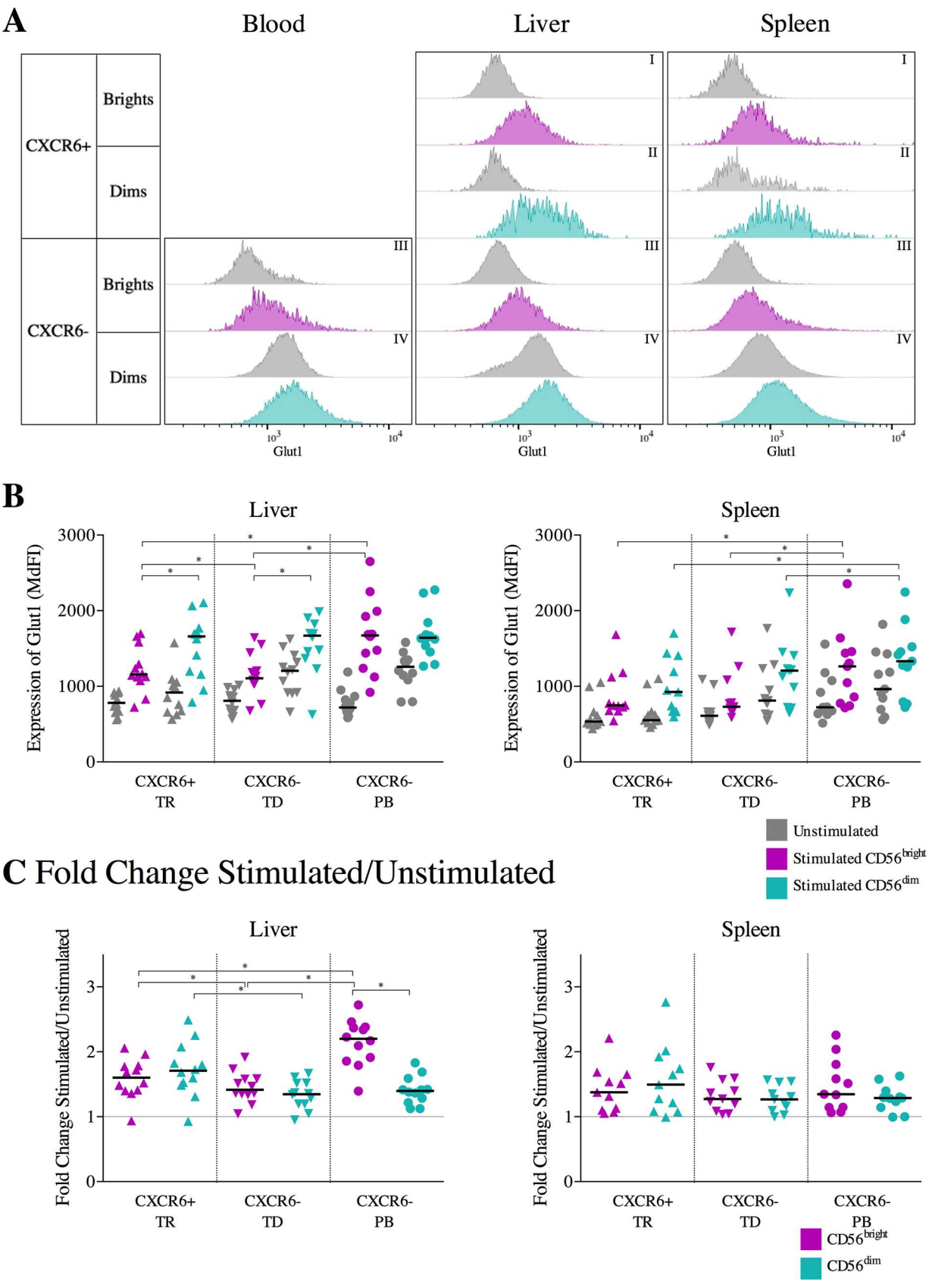
A
Blood
Liver
Spleen
CXCR6+
Brights
Dims
CXCR6-
Brights
Dims
I
II
III
IV
Glut1
B
Liver
Spleen
Expression of Glut1 (MdFI)
CXCR6+ TR
CXCR6- TD
CXCR6- PB
Unstimulated
Stimulated CD56bright
Stimulated CD56dim
C Fold Change Stimulated/Unstimulated
Fold Change Stimulated/Unstimulated
CD56bright
CD56dim

Fig 2. Effects of cytokine stimulation on Glut1 expression: Samples were compared using Wilcoxon matched-pairs signed rank tests and multiplicity was controlled for by FDR testing. Bars indicate the median, significance was defined as $p \leq 0.05$ (*). A. Representative histograms of Glut1 expression on unstimulated and stimulated $CD56^{bright}$ $CXCR6^{+}$ (I, grey: unstimulated; purple: stimulated), $CD56^{dim}$ $CXCR6^{+}$ (II, grey: unstimulated; teal: stimulated), $CD56^{bright}$ $CXCR6^{-}$ (III, grey: unstimulated; purple: stimulated) and $CD56^{dim}$ $CXCR6^{-}$ (IV, grey: unstimulated; teal: stimulated) NK cells from blood (left), liver (middle) and spleen (right) samples. B. Expression (MdFI) of Glut1 on unstimulated (grey) and $CD56^{bright}CD16^{-}$ (purple) and $CD56^{dim}CD16^{+}$ (teal) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples. C. Differences (fold change) in Glut1 expression (MdFI) between stimulated and unstimulated $CD56^{bright}CD16^{-}$ (purple) and $CD56^{dim}CD16^{+}$ (teal) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.

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Glut1 than peripheral blood $CD56^{bright}$ NK cells (p = 0.03, Fig 2C). Furthermore, even after cytokine stimulation, within the liver, $CD56^{bright}$ NK cells still expressed significantly less Glut1 than $CD56^{dim}$ NK cells (p = 0.02 for both TR and TD; Fig 2B). A similar tendency was observed for NK cells derived from spleens, with $CD56^{dim}$ NK cells expressing higher levels of Glut1 than $CD56^{bright}$ NK cells, irrespectively of the tissue compartment (TR: p = 0.5; TD: p = 0.07; Fig 2B). In addition, $CXCR6^{+}$ and $CXCR6^{neg}$ $CD56^{dim}$ NK cells derived from spleens expressed significantly less Glut1 after cytokine stimulation compared to peripheral blood NK cells (p = 0.02 for both comparisons; Fig 2B). Overall, $CD56^{dim}$ NK cells had a higher expression of Glut1 than $CD56^{bright}$ NK cells, however after stimulation, peripheral blood $CD56^{bright}$ NK cells were most effective in upregulating Glut1 while the large population of $CD56^{bright}$ NK cells from tissues expressed significantly lower amounts of Glut1.

Effect of cytokine stimulation on expression of CD98 on NK cells from liver, blood and spleen

We next examined changes in the expression of CD98 on NK cells derived from livers, spleens and peripheral blood stimulated with IL-12 and IL-15 (Fig 3A). Incubation in cytokine-free medium induced a significant but small increase of CD98 expression in the majority of NK cell subsets (Part B in S1 Fig). In contrast, overnight cytokine stimulation led to a significant upregulation of CD98 (Fig 3B and Part B in S2 Fig) expression in all NK cell subsets. Tissue-resident, tissue-derived and peripheral blood $CD56^{bright}$ NK cells had a higher expression of CD98 than tissue-resident, tissue-derived and peripheral-blood $CD56^{dim}$ NK cells from liver (TR: p = 0.04; TD: p = 0.02; PB = 0.02; Fig 3B) and spleen donors (TD: p = 0.02; PB: p = 0.02; Fig 3B), although this did not reach significance for spleen resident NK cells. Furthermore, tissue-resident $CXCR6^{+}$ $CD56^{dim}$ NK cells from liver and spleen had a higher expression of CD98 compared to tissue-derived $CXCR6^{neg}$ (liver: p = 0.02; spleen: p = 0.02; Fig 3B). Taken together, cytokine stimulation led to a robust upregulation of CD98 on NK cells, with highest levels in tissue-resident and tissue-derived $CD56^{bright}$ NK cells in the liver.

Effect of cytokine stimulation on expression of CD71 on NK cells from liver, blood and spleen

Lastly, we assessed the expression of the transferrin transporter CD71 on NK cells from blood, liver and spleen tissue after overnight incubation with and without cytokines (Fig 4A). Incubation in cytokine-free medium induced no significant changes in CD71 expression (Part C in S1 Fig). However, stimulation with cytokines also led to a significant upregulation of CD71 in all NK cell subsets (Fig 4B and Part C in S2 Fig). Overall, $CD56^{bright}$ NK cells had the highest CD71 expression compared to $CD56^{dim}$ NK cell populations, and this was most pronounced amongst spleen-derived $CXCR6^{neg}$ cells (p = 0.02, Fig 4B). A similar trend was observed in liver-derived $CXCR6^{neg}$ NK cell population (p = 0.02, Fig 4B). Thus CD71 expression was

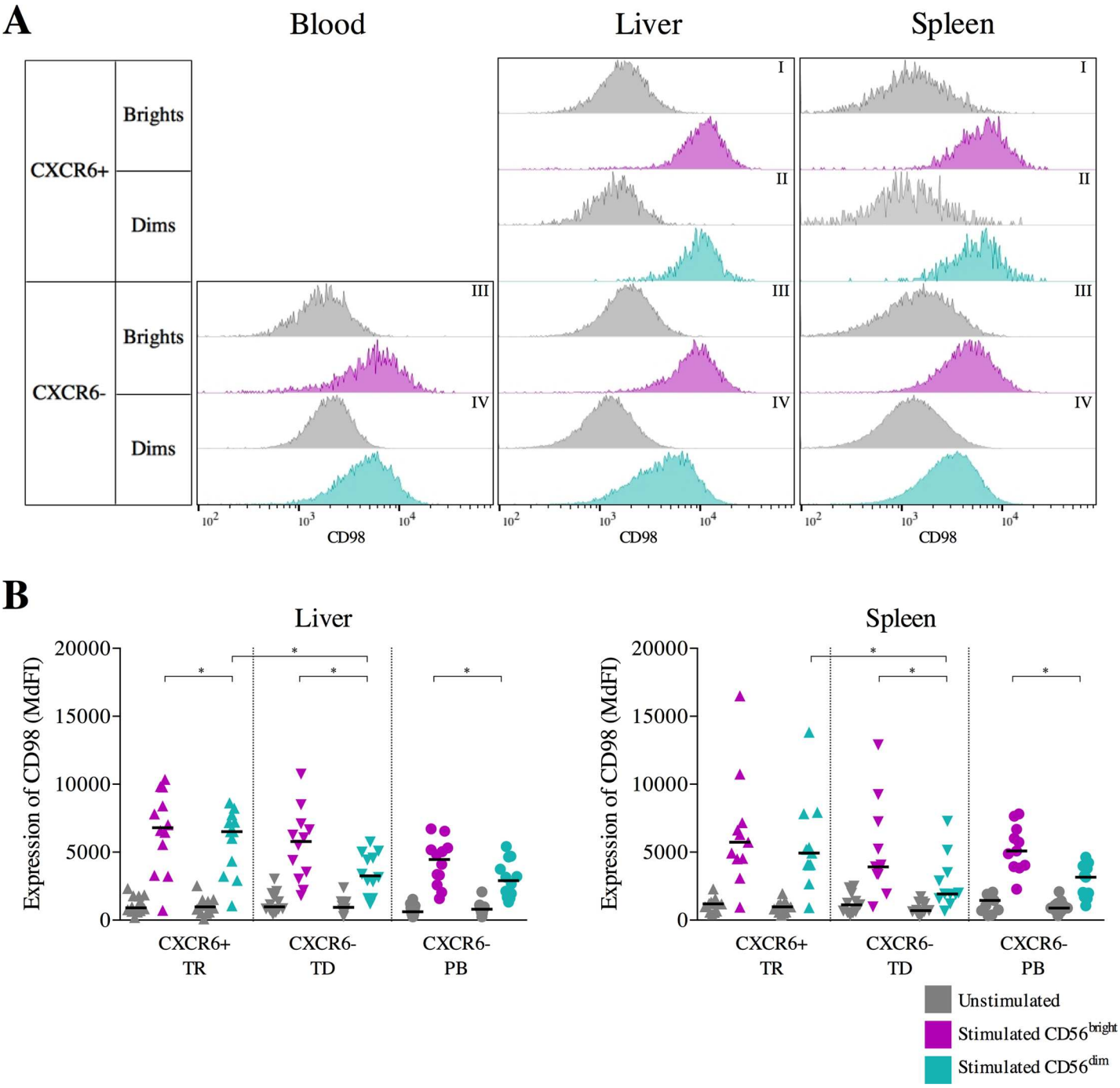

Fig 3. Effects of cytokine stimulation on CD98 expression: Samples were compared using Wilcoxon matched-pairs signed rank tests and multiplicity was controlled for by FDR testing. Bars indicate the median, significance was defined as $p \leq 0.05$ (*). A. Representative histograms of CD98 expression on unstimulated and stimulated $CD56^{bright}$ $CXCR6^{+}$ (I, grey: unstimulated; purple: stimulated), $CD56^{dim}$ $CXCR6^{+}$ (II, grey: unstimulated; teal: stimulated), $CD56^{bright}$ $CXCR6^{-}$ (III, grey: unstimulated; purple: stimulated) and $CD56^{dim}$ $CXCR6^{-}$ (IV, grey: unstimulated; teal: stimulated) NK cells from blood (left), liver (middle) and spleen (right) samples. B. Expression (MdFI) of CD98 on unstimulated (grey) and $CD56^{bright}CD16^{-}$ (purple) and $CD56^{dim}CD16^{+}$ (teal) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.

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higher on tissue-resident $CXCR6^{+}$ $CD56^{dim}$ NK cells compared to tissue-derived $CXCR6^{neg}$ $CD56^{dim}$ NK cells, indicating a difference between tissue-resident and tissue-derived $CD56^{dim}$ NK cells in their capacity for nutrient uptake.

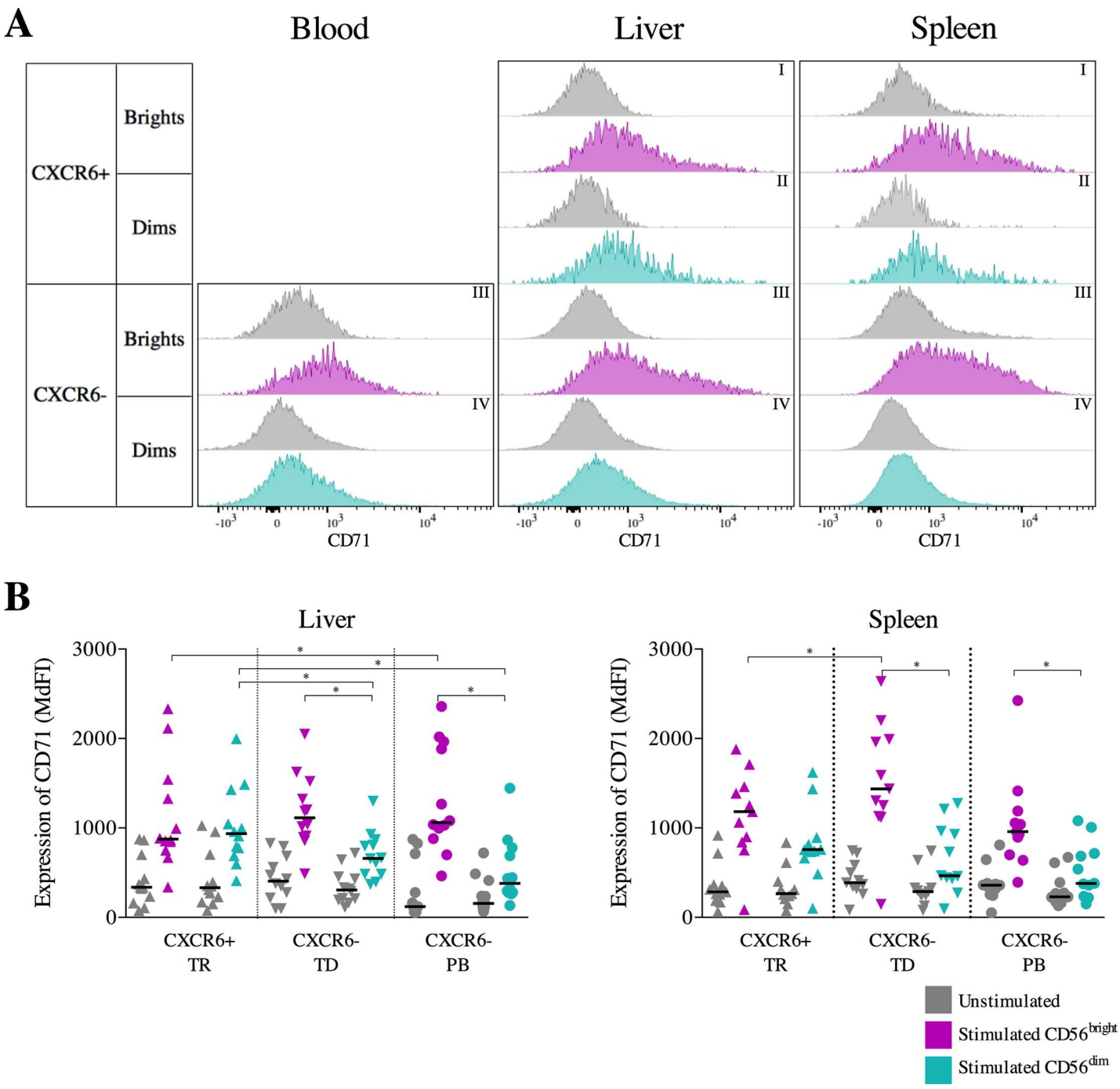

Fig 4. Effects of cytokine stimulation on CD71 expression: Samples were compared using Wilcoxon matched-pairs signed rank tests and multiplicity was controlled for by FDR testing. Bars indicate the median, significance was defined as $p \leq 0.05$ (*). A. Representative histograms of CD71 expression on unstimulated and stimulated $CD56^{bright}$ $CXCR6^{+}$ (I, grey: unstimulated; purple: stimulated), $CD56^{dim}$ $CXCR6^{+}$ (II, grey: unstimulated; teal: stimulated), $CD56^{bright}$ $CXCR6^{-}$ (III, grey: unstimulated; purple: stimulated) and $CD56^{dim}$ $CXCR6^{-}$ (IV, grey: unstimulated; teal: stimulated) NK cells from blood (left), liver (middle) and spleen (right) samples. B. Expression (MdFI) of CD71 on unstimulated (grey) and $CD56^{bright}CD16^{-}$ (purple) and $CD56^{dim}CD16^{+}$ (teal) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.

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Discussion

Immune cell metabolism is a critical determinant of the function of tissue-resident immune cells, as nutrient availability in tissues can largely vary depending on the specific tissue characteristics and other factors influencing local nutrient availability, such as viral infections [23,24,47] and malignancies [22,48,49]. Infections and malignancies affect the nutritional microenvironment by changing nutrient supply to tissues, resulting from fibrosis or modulated vascularization [50,51], or by consuming nutrients themselves [48]. When activated, human immune cells, including NK cells, are able to radically increase their nutrient uptake in order to act efficiently and quickly. On the other hand, it was demonstrated that inhibition of glycolysis can decrease NK cell functionality against viral targets [32]. In the last years, important differences in phenotypes and functions of NK cell subsets from different tissue compartments have been elucidated [8,9,12,14,17]. However, no study on differences in the expression of nutrient transporters on NK cells derived from different tissues has been performed to date. In this study, we characterized the expression of the metabolic transporters Glut1, CD98 and CD71 on unstimulated and stimulated NK cell subsets derived from peripheral blood, liver and spleen obtained from individuals undergoing liver transplantation, liver resection in the process of abdominal tumor excision surgery, or spleen resection for medical reasons. We observed significant differences between tissue-resident, tissue-derived and peripheral $CD56^{dim}$ and $CD56^{bright}$ NK cells in regard to their expression of nutrient transporters, both before and after stimulation with cytokines.

To characterize the expression of nutrient transporters on tissue-resident and tissue-derived NK cells, we initially studied NK cells from liver and spleen tissues and compared these samples to paired blood samples without any prior incubation or stimulation. Glut1 is one of the transporters for glucose into cells and expressed on virtually all human cells. Its expression level on T cells has been linked to their functional capacity [52] and Glut1 expression has been demonstrated to differ between NK cell subsets in peripheral blood [35]. When comparing tissue and peripheral blood NK cells without any prior incubation or stimulation, we observed a higher expression of Glut1 on $CD56^{dim}$ NK cells compared to $CD56^{bright}$ NK cells in peripheral blood and the $CXCR6^{neg}$ population of NK cells derived from tissues. Tissue-resident $CXCR6^{+}$ NK cells on the other hand had a low expression of Glut1, irrespective of their expression level of CD56. We next examined the effect of overnight incubation with IL-12 and IL-15 on the expression of Glut1 on NK cells from livers, blood and spleens. Previous studies had shown that peripheral blood NK cells can upregulate Glut1 expression in response to a combination of these cytokines, which went in hand with functional changes, such as IFNγ production [26,35]. While the differences in Glut1 expression between NK cell subsets were modest, our data show that NK cells from peripheral blood either expressed high levels of Glut1 already in the resting state ($CD56^{dim}$ NK cells) or upregulated Glut1 expression to a high degree when stimulated ($CD56^{bright}$ NK cells). On the other hand, NK cells derived from tissues, especially $CD56^{bright}$ NK cells, did not express similar levels of Glut1 after stimulation, suggesting that these tissue-resident NK cells might be less dependent on the ability to upregulate their glucose uptake upon activation and instead use other metabolites. In peripheral blood, glucose levels are usually maintained at a high level [53], enabling peripheral blood NK cells to rely in their metabolism on glucose when needed. NK cells from tissues on the other hand are more likely to encounter situations in which glucose might not be readily available [53,54], resulting in metabolic challenges that could impair NK cell functions. Tissue-resident NK cells therefore might be already adapted to conditions in which glucose is rare by using other nutrients than glucose in order to maintain their anti-viral and anti-tumor activity.

One important alternative source of energy apart from glucose are amino acids. CD98 is a molecule composing a transporter system for amino acids into the cell [55]. Recently, it was reported that CD98 is upregulated along with Glut1 and CD71 on cytokine-stimulated peripheral blood NK cells [35]. Additionally, it was demonstrated that murine splenic NK cells possess the ability to switch from glycolysis towards non-glucose based oxidative phosphorylation when glycolysis was inhibited *in vitro* [36,56]. While no differences in expression levels of CD98 were observed between $CD56^{dim}$ and $CD56^{bright}$ NK cells stained without any prior incubation or stimulation, peripheral blood $CD56^{bright}$ NK cells expressed significantly higher CD98 levels than peripheral blood $CD56^{dim}$ NK cells upon cytokine stimulation, in line with previously published results [35]. We furthermore observed a higher expression of CD98 on stimulated liver- and spleen-resident $CXCR6^{+}$ $CD56^{dim}$ NK cells compared to tissue-derived $CXCR6^{neg}$ $CD56^{dim}$ NK cells. Our results showed that $CD56^{bright}$ NK cells derived from livers and spleens tissues preferentially expressed CD98 post-cytokine stimulation compared $CD56^{dim}$ NK cells. Moreover, we observed that tissue-resident $CD56^{dim}$ NK cells from the liver expressed significantly higher CD98 levels than tissue-derived and peripheral blood $CD56^{dim}$ NK cells, although they expressed less CD98 than $CD56^{bright}$ NK cells from the respective compartments. Taken together, $CD56^{bright}$ tissue-resident NK cells, which expressed lower amounts of Glut1 expressed high amounts of CD98 after stimulation. In contrast, NK cell subsets expressing high Glut1 levels at baseline or after cytokine stimulation did not significantly upregulate CD98 expression. These findings support a model in which peripheral blood NK cells mainly cover their energy demands through readily available glucose, while $CD56^{bright}$ NK cells, and in particular that large population of tissue-located $CD56^{bright}$ NK cells, obtain part of their energy demands via amino acids instead of glucose when activated.

Lastly, we examined the expression of CD71, a transporter for transferrin. CD71 has been described as a marker for T cell activation or proliferation [40]. Additionally, loss of *TFRC*, the gene encoding for CD71, has been demonstrated to cause a combined immunodeficiency in two families [41]. Prior to any stimulation, NK cells from tissues and peripheral blood had very similar expression levels of CD71, but post-cytokine stimulation, we observed a significantly higher expression of CD71 on peripheral blood and tissue-derived $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells from the same compartments. In liver samples and their paired blood samples, we observed that $CXCR6^{+}$ $CD56^{dim}$ NK cells in tissues had a higher expression of CD71 after stimulation compared to $CXCR6^{neg}$ NK cells in tissues or $CD56^{dim}$ NK cells from the peripheral blood. While the exact role of CD71 and iron in NK cell function remains unknown, parallels to T cell function suggest that iron might be required for expansion of NK cells, but also for their activation. We observed a higher expression of CD71 on $CD56^{bright}$ NK cells than $CD56^{dim}$ cells irrespective of the tissue compartment. As iron has been shown to be a critical cofactor in DNA synthesis [42] and as peripheral blood $CD56^{bright}$ NK cells have been suggested to be less differentiated precursors of peripheral blood $CD56^{dim}$ NK cells [5], the increased expression of CD71 on $CD56^{bright}$ NK cells after stimulation might be a reflection of the proliferative capacity of these cells and the proliferative effect of IL-12 and IL-15 stimulation. Additionally, liver-resident NK cells might have to compete with hepatocytes, one of the main storage sites for iron in the human body apart from hemoglobin, and thus express higher levels of CD71. Taken together, our findings suggest that CD71 and iron metabolism might have an important role in NK cell function and tissue residency, which will merit further research in the future.

In conclusion, our data show that tissue-resident, tissue-derived and peripheral blood NK cells differ in their baseline expression of nutrient transporters and their ability to upregulate these transporters in response to low-dose cytokine stimulation. Limitations of this study are that samples were obtained from individuals that underwent liver transplantation, liver resection or spleen resection for medical indications, and it remains unclear how these findings

translate into findings in healthy individuals. Furthermore, only changes in three nutrient transporters were assessed, and immune cells can express a large number of different nutrient receptors, including receptors involved in the transport of other nutrients such as lipids. The observed differences between peripheral blood NK cells and tissue-derived CXCR6^{neg} NK cells furthermore suggest that either a population of tissue-resident NK cells within this tissue-resident population is skewing our results or that tissues can affect cells passing through them in a more profound way than was previously anticipated. These data highlight the importance of studying immune cells in different tissue compartments, as the different nutritional conditions in these compartments might have critical effects on the immune cells that can be found in these tissues. Future studies should include more comprehensive assessments of the large number of nutrient transporters to obtain a more complete picture of the metabolic adaptations of immune cells in different compartments, both in health and disease.

Supporting information

S1 Fig. Expression of Glut1, CD98 and CD71 at baseline and after incubation without cytokines: Samples were compared using Wilcoxon matched-pairs signed rank tests and multiplicity was controlled for by FDR testing. Bars indicate the median, significance was defined as $p \leq 0.05$ (*).
A. Expression (Median fluorescence intensity, MdFI) of Glut1 on unincubated ("Fresh") and incubated but unstimulated ("Rested") CD56^{bright}CD16^{-} (left) and CD56^{dim}CD16^{+} (right) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.
B. Expression (Median fluorescence intensity, MdFI) of CD98 on unincubated ("Fresh") and incubated but unstimulated ("Rested") CD56^{bright}CD16^{-} (left) and CD56^{dim}CD16^{+} (right) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.
C. Expression (Median fluorescence intensity, MdFI) of CD71 on unincubated ("Fresh") and incubated but unstimulated ("Rested") CD56^{bright}CD16^{-} (left) and CD56^{dim}CD16^{+} (right) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.
(TIFF)

S2 Fig. Expression of Glut1, CD98 and CD71 at after incubation without cytokines and with cytokines: Samples were compared using Wilcoxon matched-pairs signed rank tests and multiplicity was controlled for by FDR testing. Bars indicate the median, significance was defined as $p \leq 0.05$ (*).
A. Expression (Median fluorescence intensity, MdFI) of Glut1 on unstimulated ("Rested") and stimulated CD56^{bright}CD16^{-} (left) and CD56^{dim}CD16^{+} (right) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.
B. Expression (Median fluorescence intensity, MdFI) of CD98 on unstimulated ("Rested") and stimulated CD56^{bright}CD16^{-} (left) and CD56^{dim}CD16^{+} (right) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.
C. Expression (Median fluorescence intensity, MdFI) of CD71 on unstimulated ("Rested") and stimulated CD56^{bright}CD16^{-} (left) and CD56^{dim}CD16^{+} (right) tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from paired liver-blood (left diagram, n = 12) and spleen-blood (right diagram, n = 11) samples.
(TIFF)

S1 Table. Median and interquartile range (IQR) of the median fluorescence intensity (MdFI) of Glut1, CD98 and CD71 expression on tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells from liver and spleen donors.
(XLSX)

S2 Table. Median and interquartile range (IQR) of %CD56bright NK cells, %CXCR6 + among CD56bright NK cells and %CXCR6+ among CD56dim NK cells in tissue and blood of liver and spleen tissue donors after overnight incubation without ("Rested") or with 5ng/mL of IL-12 and 2.5ng IL-15/ml.
(XLSX)

S3 Table. Median and interquartile range (IQR) of the median fluorescence intensity (MdFI) and fold difference of Glut1 expression on tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells incubated without ("rested") or with ("stimulated") cytokines from liver and spleen donors.
(XLSX)

S4 Table. Median and interquartile range (IQR) of the median fluorescence intensity (MdFI) and fold difference of CD98 expression on tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells incubated without ("rested") or with ("stimulated") cytokines from liver and spleen donors.
(XLSX)

S5 Table. Median and interquartile range (IQR) of the median fluorescence intensity (MdFI) and fold difference of CD71 expression on tissue-resident (TR), tissue-derived (TD) and peripheral blood (PB) NK cells incubated without ("rested") or with ("stimulated") cytokines from liver and spleen donors.
(XLSX)

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Author Contributions

Conceptualization: Wilhelm Salzberger, Caroline Pfeifer, Madeleine J. Bunders, Marcus Altfeld.

Data curation: Wilhelm Salzberger.

Formal analysis: Wilhelm Salzberger, Laura Richert.

Funding acquisition: Wilhelm Salzberger, Sebastian Lunemann, Christoph Schramm, Marcus Altfeld.

Investigation: Wilhelm Salzberger, Gloria Martrus, Kai Bachmann, Hanna Goebels, Leonard Heß, Martina Koch, Annika Langeneckert, Sebastian Lunemann, Karl J. Oldhafer, Tobias Poch.

Methodology: Wilhelm Salzberger, Gloria Martrus, Hanna Goebels, Leonard Heß, Martina Koch, Annika Langeneckert, Sebastian Lunemann, Caroline Pfeifer, Tobias Poch, Laura Richert, Christoph Schramm, Ramez Wahib, Madeleine J. Bunders, Marcus Altfeld.

Project administration: Wilhelm Salzberger, Marcus Altfeld.

Resources: Gloria Martrus, Tobias Poch, Christoph Schramm, Ramez Wahib, Marcus Altfeld.

Software: Wilhelm Salzberger, Laura Richert.

Supervision: Gloria Martrus, Madeleine J. Bunders, Marcus Altfeld.

Validation: Laura Richert.

Visualization: Wilhelm Salzberger.

Writing – original draft: Wilhelm Salzberger.

Writing – review & editing: Gloria Martrus, Madeleine J. Bunders, Marcus Altfeld.

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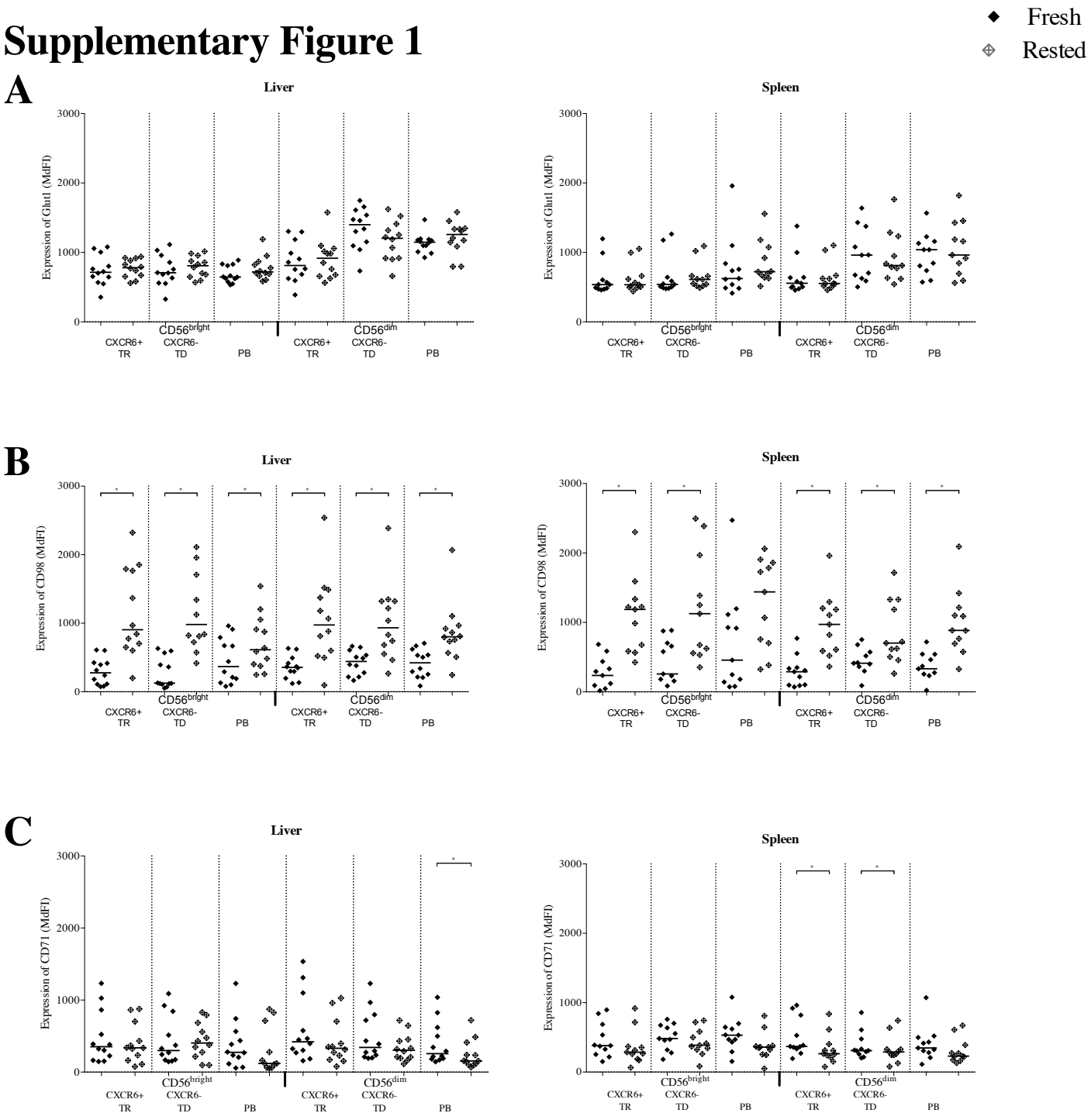
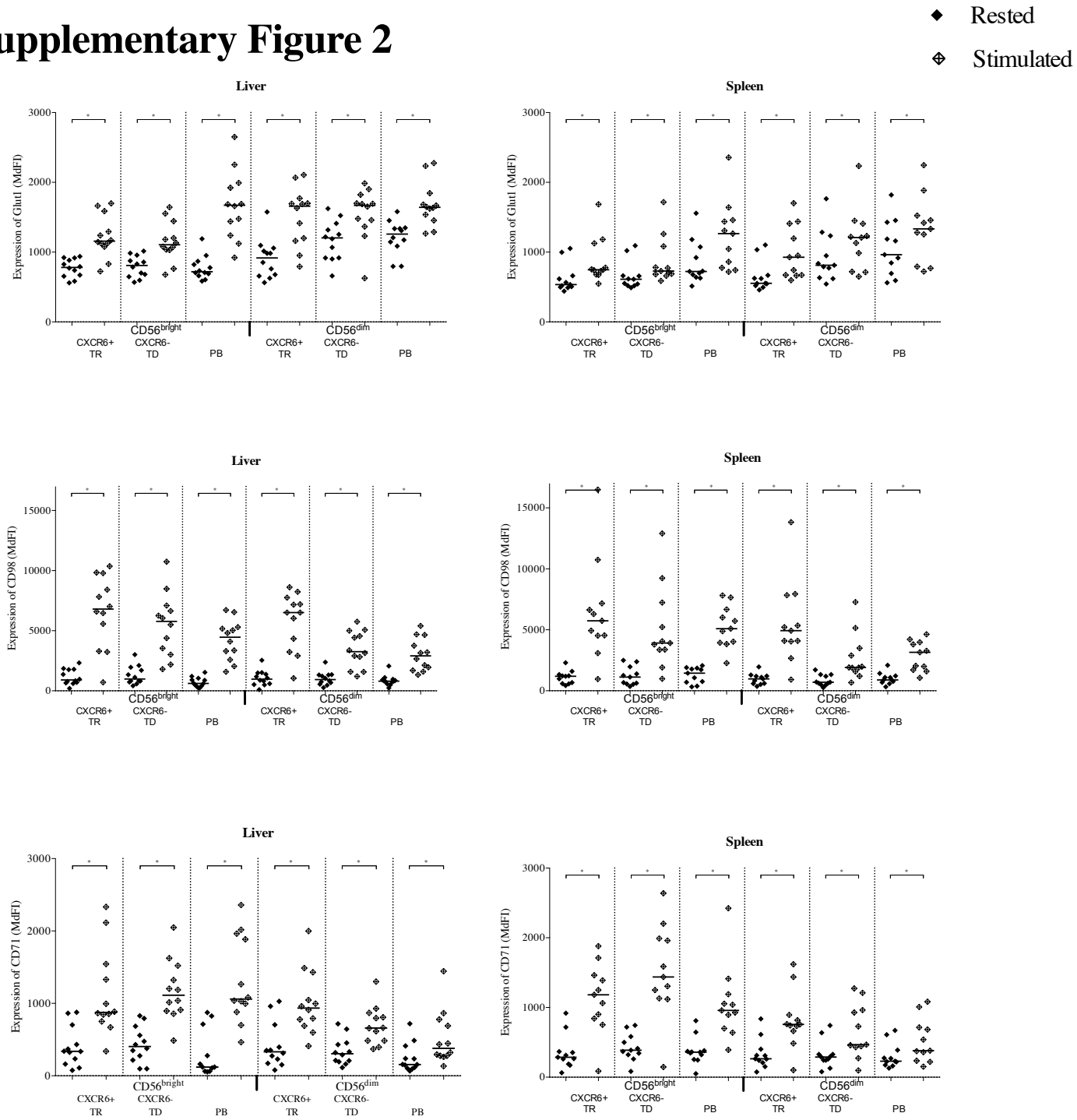
Supplementary Figure 1



Supplementary Figure 2



Supplementary table 1

		Glut1		CD98		CD71	
		Liver	Spleen	Liver	Spleen	Liver	Spleen
TR	$CD56^{bright}$	713 (587-953)	537 (474-604)	277 (110-422)	237 (46-437)	353 (180-779)	380 (254-687)
	$CD56^{dim}$	812 (640-1139)	556(497-641)	356 (221-474)	290 (98-350)	421 (277-969)	370 (335-821)
TD	$CD56^{bright}$	707 (578-932)	540 (490-643)	125.5 (110.5-524)	258 (157-703)	300 (169-761)	483 (320-674)
	$CD56^{dim}$	1400 (1110-1593)	962 (627-1377)	443 (232-590)	412 (299-573)	343 (218-779)	310 (258-477)
PB	$CD56^{bright}$	647 (589-826)	624 (489-841)	367 (145-763)	457 (142-1114)	274 (138-533)	531 (433-647)
	$CD56^{dim}$	1147 (1032-1186)	1040 (739-1159)	421 (224.8-600)	333 (233-541)	256 (184-589)	348 (281-499)

Supplementary table 2

		Rested			Stimulated		
		$\%CD56^{bright}$ NK cells	$\%CXCR6^{+}$ among $CD56^{bright}$ NK cells	$\%CXCR6^{+}$ among $CD56^{dim}$ NK cells	$\%CD56^{bright}$ NK cells	$\%CXCR6^{+}$ among $CD56^{bright}$ NK cells	$\%CXCR6^{+}$ among $CD56^{dim}$ NK cells
Liver	Tissue	37.3% (30.0-45.3%)	37.8% (14.4-44.8%)	14.3% (2.7-27.2%)	25.6% (19.8-35.0%)	40.7% (19.7-60.2%)	21.8% (4.8-34.2%)
	Blood	7.3% (4.0-11.2%)	1.4% (0.6-3.4%)	1.0% (0.7-1.2%)	8.5% (3.1-15.2%)	9.6% (7.8-15.9%)	10.0% (7.5-13.4%)
Spleen	Tissue	22.6% (16.3-39.0%)	20.7% (11.8-42.2%)	5.4% (2.6-9.9%)	27.1% (16.9-36.2%)	24.8% (13.4-32.2%)	5.9% (3.2-10.7%)
	Blood	3.6% (1.3-6.1%)	3.9% (0.7-8.7%)	0.8% (0.3-1.5%)	4.9% (1.7-8.7%)	8.7% (5.4-14.4%)	4.2% (1.9-10.9%)

Supplementary Table 3

		Liver			Spleen		
		MdFI rested	MdFI stimulated	Fold Change	MdFI rested	MdFI stimulated	Fold Change
TR	CD56bright	782 (659-908)	1158 (1089-1514)	1.6 (1.4-1.8)	535 (498-661)	747 (679-1125)	1.4 (1.1-1.7)
	CD56dim	918 (663-1047)	1659 (1170-1753)	1.7 (1.5-2.0)	553 (520-668)	926 (671-1409)	1.6 (1.1-1.9)
TD	CD56bright	809 (656-936)	1106 (1031-1384)	1.4 (1.3-1.6)	611 (521-661)	730 (682-1083)	1.3 (1.1-1.6)
	CD56dim	1205 (918-1389)	1670 (1390-1791)	1.3 (1.2-1.5)	812 (633-1234)	1209 (720-1407)	1.3 (1.1-1.5)
PB	CD56bright	720 (669-857)	1673 (1289-1975)	2.2 (1.9-2.4)	723 (639-1075)	1265 (775-1460)	1.3 (1.1-1.8)
	CD56dim	1258 (1101-1346)	1642 (1476-1804)	1.4 (1.2-1.5)	963 (694-1428)	1333 (793-1524)	1.3 (1.1-1.4)

Supplementary table 4

		Liver			Spleen		
		MdFI rested	MdFI stimulated	Fold Change	MdFI rested	MdFI stimulated	Fold Change
TR	CD56bright	905 (662-1785)	6803 (3866-9449)	5.6 (4.6-7.2)	1188 (585-1335)	5737 (4518-7173)	5.4 (4.8-6.8)
	CD56dim	975 (542-1460)	6516 (3511-7612)	5.8 (5.1-7.5)	972 (590-1204)	4934 (4049-7834)	5.1 (4.1-6.7)
TD	CD56bright	982 (746.3-1895)	5782 (3147-6983)	4.3 (3.6-6.1)	1125 (558-1968)	3918 (3366-7237)	3.8 (3.2-5.4)
	CD56dim	934 (585-1322)	6516(3511-7612)	3.8 (2.8-4.6)	702 (504-1330)	1917 (1576-3490)	2.7 (2.6-3.9)
PB	CD56bright	613 (383-1017)	4460 (2760-5269)	6.7 (3.3-8.7)	1440 (703-1860)	5093 (3934-6674)	5.3 (3.2-6.7)
	CD56dim	803 (609-955)	2906 (1759-4428)	3.9 (2.4-4.6)	886 (694-1213)	3151 (1705-3983)	2.8 (2.0-3.8)

Supplementary table 5

		Liver			Spleen		
		MdFI rested	MdFI stimulated	Fold Change	MdFI rested	MdFI stimulated	Fold Change
TR	CD56bright	338 (180-635)	876 (774-1491)	2.7 (2.3-8.0)	287 (188-367)	1183 (842-1462)	4.0 (1.9-5.3)
	CD56dim	331 (186-628)	935 (711.5-1333)	2.3 (2.1-6.1)	265 (209-402)	758 (663-894)	2.5 (1.8-3.4)
TD	CD56bright	404 (231-654)	1113 (898.8-1471)	2.6 (2.4-7.0)	388 (322-583)	1437 (1130-1991)	3.6 (2.2-5.1)
	CD56dim	305 (198-448)	660 (483.3-853.5)	2.1 (1.8-3.2)	289 (244-328)	467 (430-964)	1.8 (1.6-2.4)
PB	CD56bright	404 (231-654)	1060 (909-1944)	6.7 (2.8-8.6)	359 (255-381)	958 (700-1190)	3.1 (2.1-3.8)
	CD56dim	156 (106-369)	378.5 (277-756.8)	2.0 (1.8-2.5)	229 (175-389)	380 (236-715)	1.5 (1.3-1.8)

7. Zusammenfassung/Summary

7.1. Zusammenfassung

Das Ziel dieser Arbeit war es, unser Verständnis über die Unterschiede zwischen NK Zellen aus Leber- und Milz-Gewebe und NK Zellen aus dem peripheren Blut zu erweitern. Studien in den letzten Jahren haben unterschiedliche Rollen von NK Zellen aus verschiedenen Geweben aufgezeigt und wie diese NK Zellen mit anderen Zellen interagieren können. Aber ein kritischer Faktor für die korrekte Funktion von NK Zellen in Geweben wurde bisher ignoriert, ihr Metabolismus. Neue Studien haben mehrere Transporter für Metabolite und deren Rolle in NK Zell Funktionalität Untersucht, insbesonders Glut1, CD98 und CD71.

In diesem Projekt haben wir eine Reihe von Faktoren untersucht, die sich zwischen von NK Zellen in Gewebe und NK Zellen im peripheren Blut Unterscheiden. Wir demonstrierten dies für proliferativen Fähigkeiten von $CD49a^+$ $CD25^+$ NK Zellen aus Lebern. Zudem zeigten wir Unterschiede in der Expression einer Reihe von Transkriptionsfaktoren zwischen NK Zellen aus Gewebe und peripherem Blut auf. Diese Faktoren sind in der Reifung und in der Regulation von Geweberesidenz von NK Zellen involviert. Im finalen Teil des Projekts konnte ich aufzeigen, dass NK Zellen aus Gewebe und Blut signifikante Unterschiede in der Expression von Glut1, CD98 und CD71 aufzeigen, sowohl in Ruhe als auch nach Stimulation.

Ich interpretiere dies als einen Hinweis auf zwei mögliche Szenarien. Entweder sind NK Zellen im Gewebe darauf vor-programmiert, um im Krankheitsfall und einer daraus erfolgenden Mangel-Situation in ihren jeweiligen Geweben gerüstet zu sein, um dennoch ihre Funktion ausüben zu können. Alternativ sind die von uns gemessenen Veränderungen eine Folge von Veränderungen in den entnommenen Geweben, die aus Patienten mit Schäden in oder um die betroffenen Organe entnommen wurden. Im Falle der Milz Proben sollte dieser Effekt allerdings nur begrenzt zum Tragen kommen, da diese während Tumor-Resektionen in angrenzenden Geweben entnommen wurden.

Zusammengefasst konnten wir zeigen, dass es signifikante Unterschiede zwischen NK Zellen aus Geweben und peripherem Blut gibt, sowohl auf transkribtioneller, funktioneller und metabolischer Ebene. Dies zeigt, dass Geweberesidenz von NK Zellen auf vielen Ebenen reguliert ist und wiederum auf vielen Ebenen die Funktion der Zellen beeinflusst

7.2. Summary

In this project, my aim was to expand our knowledge on how tissue resident NK cells from human livers and spleens differ from their peripheral blood counterparts. Studies in recent years have elucidated many roles of tissue-resident NK cells and their interactions with other cells in the human bodies. But so far, one critical factor for the correct function of tissue-resident NK cells has been ignored, their metabolism. Recent studies have demonstrated several metabolic transporters critically involved in NK cell function, most notably Glut1, CD98 and CD71.

In this project, we studied several factors differentiating certain populations of tissue-resident NK cells from peripheral blood NK cells, including the proliferative capacities of $CD49a^+$ $CD25^+$ NK cells from livers. We also showed how tissue-resident NK cells differ in the expression of multiple transcription factors, which are known regulators in NK cell development or are involved in the regulation of tissue residency.

Lastly, I was able to shown that tissue-resident NK cells differ significantly from peripheral blood in their expression of Glut1, CD98 and CD71, both at baseline and after cytokine stimulation.

I believe this is either a sign of an imprinted program of tissue-resident NK cells, preparing these cells for pathogenic situations inside their respective tissue when nutrients might be rare or that this is a reaction to the pathogenic situation inside the tissue at the time of sample extraction. Although for spleen samples, this effect is likely only to be a minor factor as spleen samples were taken during tumor resections in adjacent organs and no macroscopic spleen remodeling was observed.

In conclusion, we showed significant differences between tissue-resident and peripheral blood NK cells, both in regard to their transcriptional program, their function and their metabolism, demonstrating that tissue-residency in NK cells is regulated on multiple levels and affects NK cell function on multiple levels.

8. Declaration of my own contributions to the publications

8.1. Hobit expression by a subset of human liver-resident CD56^{bright} Natural Killer cells

In this study, I participated in collecting and processing of tissue and blood samples. Furthermore, I participated in preliminary measurements and preliminary analysis. I provided feedback in the study design. I also provided feedback on multiple steps of the writing process of the manuscript.

I was one of the authors for the ethic proposal for collecting peripheral blood and liver tissue and I participated in writing an addendum for the ethic proposal.

8.2. Proliferative capacity exhibited by human liver-resident CD49a^{+}CD25^{+} NK cells

In this study, I was part of the team collecting and processing the samples after collection. I performed a number of the preliminary measurements. I participated in sorting and subsequently stimulating NK cells from peripheral blood and liver tissue. I provided feedback in the study design. I also provided feedback on multiple steps of the writing process of the manuscript.

I was one of the authors for the ethic proposal for collecting peripheral blood and liver tissue and I participated in writing an addendum for the ethic proposal.

8.3. Tissue-resident NK cells differ in their expression profile of the nutrient transporters Glut1, CD98 and CD71

This study and the experiments performed in this study were designed by me with the assistance of my supervisor. I was one of the authors for the ethic proposal for collecting peripheral blood and liver tissue and I participated in writing an addendum for the ethic proposal allowing. I was the author of the addendum for an ethic proposal allowing us to study NK cells from spleen tissue and matched peripheral blood collected from a cohort of the department of general surgery of the University Hospital Eppendorf.

I was part of the team collecting and processing matched liver and blood samples. I collected and processed the matched spleen and blood samples. I performed experiments and measurements. Data analysis and statistical analysis were

performed by me with the assistance of our statistician. The manuscript for this publication was written by me with the feedback of my co-authors.

9. Acknowledgments

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10. Curriculum vitae

Lebenslauf entfällt aus datenschutzrechtlichen Gründen

11. Eidesstattliche Erklärung

I hereby formally declare that I wrote this dissertation independently. No other sources or materials outside of those declared were used. I clearly marked any citations used in this study, both verbatim and paraphrased. All citations are clearly labeled and the original source is given.

Furthermore, I confirm that I did not submit this thesis in whole or in part to any other university or institute for review or grading. I used none of the data I collected during this project to apply for a doctorate at any other university.
I declare that this thesis is an original report of my research. Collaborative contributions have been indicated clearly and acknowledged.
I agree to this dissertation being checked for plagiarism by the deanery of the medical faculty.

04.05.2021

Wilhelm Friedrich Salzberger