Hepatic NK cells in liver transplantation

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Hepatic NK cells in liver transplantation

Lever NK cellen in levertransplantatie

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To Periklis,
Who had patience to wait
Who gave me strength to continue.

Now I won't go away without you.

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Chapter 1

General introduction and outline of the thesis

1. General introduction

The liver is the second largest organ in the human body (the first being the skin) and is responsible for numerous key biological functions among which metabolism of nutrients, detoxification of toxic substances, blood filtration, synthesis of blood-clotting components and bile production. Anatomically the liver is divided into lobes, each containing thousands of lobules, and is served by a system of two distinct blood supplies. The hepatic artery conveys oxygenated blood to the liver and constitutes around 25% of the total blood supply; the remaining 75% derives from the portal vein, which conveys nutrient-rich blood from the stomach and the intestines. The blood conveying from the bowel via the portal vein is rich in antigens such as toxic dietary compounds, food antigens and bacterial components from the gut. This implies that the liver is constantly exposed to a large antigenic load and needed to evolve a mechanism to avoid unnecessary responses to harmless antigens. To this aim, the liver has developed a complex system to tightly control the local immune response thus creating a particular tolerogenic environment [1-5]. While protecting the liver from a state of constant inflammation, hepatic tolerogenicity exposes the organ to a higher risk of developing tumor metastases and of being the target of chronic viral and parasitic infections.

The key players in this finely tuned balance between tolerance and immune reactivity in the liver are the local components of the immune system. It is known that the liver has a peculiar composition of resident leukocytes, which differs from peripheral blood (Figure 1). Specifically, the liver contains selectively enriched populations of CD8+ T cells, Natural Killer (NK) cells and $CD56^+$ T cells, together with populations of liver-resident macrophages, called Kupffer cells [2, 6-9]. In addition, besides the classical hematopoietic antigen presenting cells (APC), the liver contains multiple populations of non-hematopoietic cells, including sinusoidal endothelial cells, stellate cells and liver parenchymal cells that can function as antigen presenting cells.

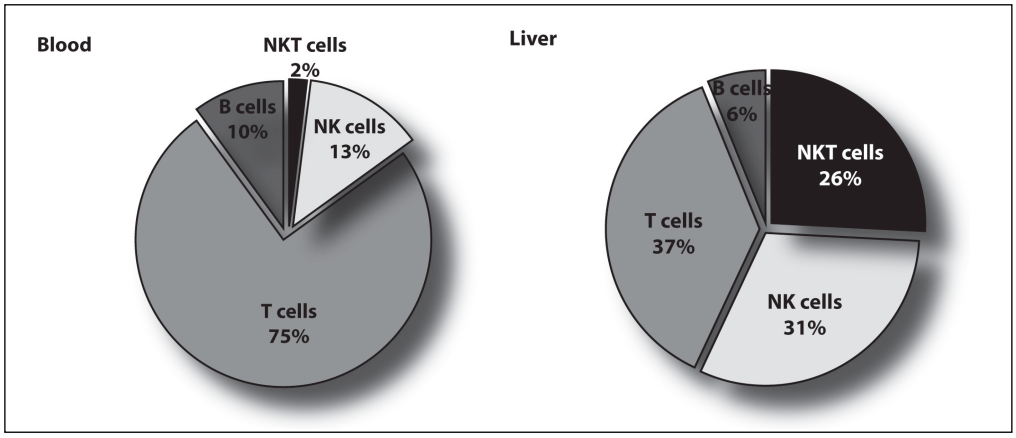

Figure 1. Distribution of human lymphocytes in blood and liver.
Elaborated from the original figure of Doherty and O'Farrelly, Immunological Reviews, 2000

This elaborated immunological milieu in the liver represents a sophisticated system for restricting the local immune reactivity. At the same time, this system reflects the complexity of any attempt aimed at distinguishing the roles of each cell type in maintaining the balance between tolerance and immune regulation.

2. Liver transplantation and tolerance

Having such a large number of pivotal functions, the liver is an essential organ in the human body and advanced stages of liver dysfunction can be life threatening. Liver transplantation (LTX) is the only life-saving procedure for several end-stage liver diseases, among which viral hepatitis, hepatocellular carcinoma and liver cirrhosis due to alcohol abuse or autoimmune diseases. The most common LTX procedure is the replacement of the diseased organ with a healthy liver from a deceased organ donor. Although liver transplantation is a procedure that has been performed for several decades a main challenge still remains: allograft rejection. From an immunological point of view, allograft rejection is caused by the alloreaction of recipient T cells towards the allogeneic graft. To prevent organ rejection, liver-transplanted patients require life-long immunosuppression. However, long-term use of immunosuppressive drugs leads to nephrotoxicity, metabolic disorders and manifestations of aspecific immunological suppression, such as opportunistic

infections and cancers [10, 11]. In terms of frequency of organ rejection, however, liver transplantation constitutes an exception: compared to other solid organ allografts, liver transplants are characterized by a low incidence of rejection and a high percentage of patients that can be weaned off immunosuppression. Specifically, it is estimated that about 20% of LTX-recipients can be withdrawn from immunosuppressive therapy without incurring in graft rejection [12]. This phenomenon is called *operational tolerance* and is presumably related to the unique hepatic microenvironment, promoting tolerance rather than immunity. In some animal models, such as mice and rats, liver grafts are spontaneously accepted across totally major histocompatibility complex (MHC)-incompatible barriers [13, 14]. Additionally, liver transplants in these animals induce donor-specific tolerance in immune-competent recipients that otherwise reject other organ grafts from the same donor (skin, heart, kidney) [14, 15]. The mechanisms responsible for this relatively tolerogenicity of the liver have only been partially understood and many hypotheses still need to be tested. In general terms, it has been established that spontaneous liver tolerance is not achieved by immunological ignorance but rather by a number of active immunological processes inducing donor-specific tolerance [2, 3, 16]. Several mechanisms, such as T-cell stimulation by tolerogenic liver APCs, the persistence of passenger donor leukocytes, the secretion of donor-derived soluble MHC molecules and the presence of regulatory T cells may act in concert to induce and maintain allograft tolerance. Particularly, experiments in tolerant rat strains have suggested an important role for donor passenger leukocytes in liver allograft tolerance. In these animals, irradiation of the donor liver, leading to depletion of most donor leukocytes, converted spontaneous acceptance into rejection [13, 15]. Additionally, post-transplant administration of donor leukocytes (T cells, B cells, myeloid cells and NK cells) into the recipient reconstituted long-term survival after donor irradiation [17, 18]. Among these passenger leukocytes, a specific type of lymphocytes has been considered in this study: the NK cells.

3. Natural Killer cells

NK cells are large granular lymphocytes that can kill malignant cells or virus-infected cells without the need of prior sensitization [19, 20]. Immunophenotypically, human NK cells are defined as $CD3^{-}CD56^{+}$ cells. NK cells constitute around 5-15% of peripheral blood mononuclear cells (PBMC) and are subdivided in two subsets on the basis of their CD56 and CD16 expression: $CD56^{bright}CD16^{-/low}$ (hereon called $CD56^{bright}$) and $CD56^{dim}CD16^{+}$ (hereon called $CD56^{dim}$). In peripheral blood, the $CD56^{dim}$ NK cells are the most abundant subset (90% of NK cells) and are characterized by high cytotoxicity and poor cytokine production. The $CD56^{bright}$ NK cells constitute the remaining 10% of peripheral blood NK cells and are the cytokine-producing subset with poor cytotoxic capacity. Most of our current knowledge about NK cells derives from studies performed on circulating cells from peripheral blood, however recent evidence has shown that organ-specific NK cells can be found in several peripheral tissues such as lymph nodes, tonsils, placenta, liver and spleen [19-21].

In the adult human liver, NK cells account for about 30-40% of hepatic mononuclear cells (MNC). Hepatic NK cells were first described by Wisse et al. in 1976 and while originally given the name of "Pit cells" later on they were functionally defined as liver-associated NK cells [22, 23]. It is not known whether these cells derive form circulating NK cells that migrate into the liver or originate from local precursors, as already shown for secondary lymphoid tissues and intestine [24, 25]. Hepatic NK cells reside in the lumen of hepatic sinusoids and adhere to endothelial cells [22, 23] (Figure 2). The typical fenestrations of the sinusoidal endothelium allow hepatic NK cells to extend pseudopodia, enter the space of Disse and make contact with the microvilli of hepatocytes [22, 23]. In earlier years the technical difficulties of obtaining NK cells from human liver tissue has limited the number of studies describing their characteristics. Therefore, few groups until now have described the functional properties of human hepatic NK cells [7, 9, 22, 26]. More recently a new source of human hepatic mononuclear cells has been used: the liver graft perfusate [27]. A liver graft perfusate is the liquid used to flush out the liver graft during clinical transplantation, just before the donor organ is connected to the recipient circulation. Liver perfusates constitute a precious and easily-accessible material for the study of liver-resident cells [27-29]. A relevant part of the present study has been performed on NK cells obtained from liver perfusates.

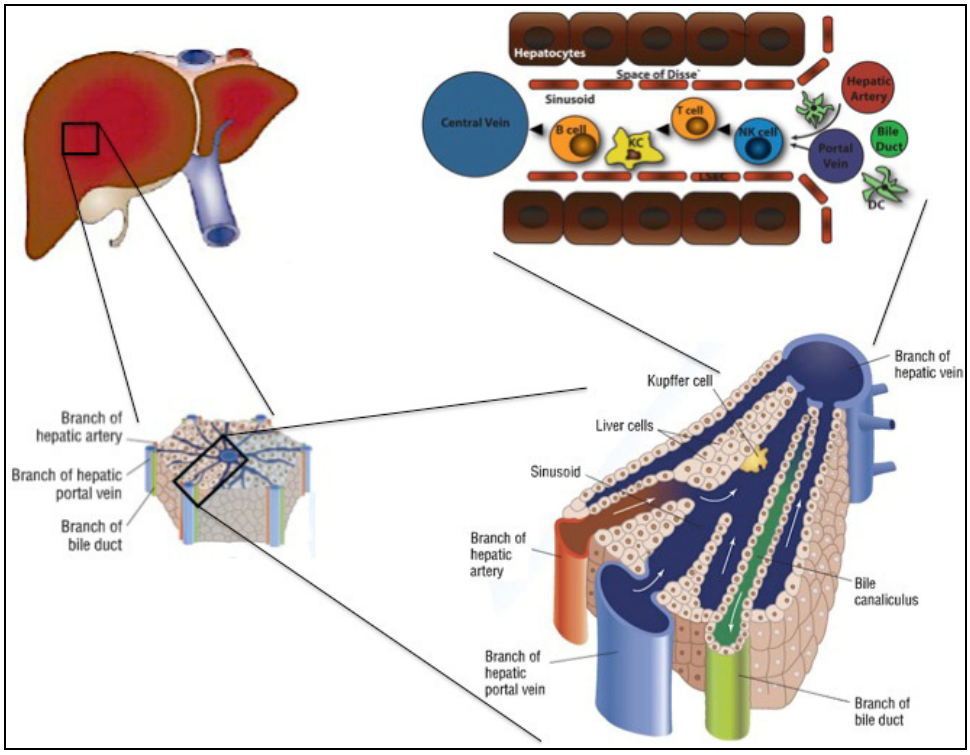

Figure 2. Schematic representation of the intrahepatic cell populations. Within each hepatic lobe, the liver contains thousands of lobules with a similar structure, which constitute the functional units of the liver. The blood enters the lobules through branches of the portal vein (conveying nutrient-rich blood) and of the hepatic artery (conveying oxygenated blood), then flows through the sinusoids to reach a branch of the hepatic vein, located in the center of the lobule. The healthy liver comprises: hepatocytes (about 60-80% of all cells), biliary cells, liver sinusoidal endothelial cells (LSECs) which are lining the liver sinusoids and hepatic stellate cells (HSC) involved in liver fibrosis and localized in the space of Disse between hepatocytes and LSECs. A large number of immune cells are also found in liver sinusoids, such as Kupffer cells (KC), dendritic cells (DCs), T cells, NK cells and B cells (here depicted). *Elaborated from the original figures of Crispe, Nature Reviews Immunology, 2003 and Racanelli and Rehermann, Hepatology, 2006.*

4. NK cell allorecognition

NK-cell effector responses include both cytokine release (e.g. IFN-γ) and direct cytotoxicity mediated through perforin, granzymes, FasL and TRAIL. NK cell activation can be triggered by cytokines (including IFNs, IL-2, IL-18, IL-12, and IL-15) or by the imbalanced expression of stimulatory and/or inhibitory ligands on the surface of target cells. With regard to this latter mechanism, a complex balance of signals from inhibitory and activating receptors on the surface of NK cells regulates their reactivity [30, 31]. When these receptors engage with specific ligands on target cells, they will transduce intracellular signals that will either trigger or limit the activation of NK cells, depending on the inhibitory or activating nature of the receptor engaged. The net result derived from the combination of these intracellular signals will determine the final activation state of NK cells.

In humans, NK cells express two main classes of inhibitory receptors: the lectin-like receptors, such as the heterodimer CD94:NKG2A, and the Immunoglobulin-like receptors, which are distinguished in two families, the Leukocyte Ig-like receptors (LILRs or ILT) and the killer Ig-like receptors (KIRs) [31]. The lectin-like receptor CD94:NKG2A recognizes the non-classical HLA-E class I molecule loaded with peptides cleaved from most of the classical HLA-A, -B and -C allotypes. Thus lectin-like receptors are used as sensors for the overall HLA class I expression level of a cell. With a similar function, the LILRs are broadly reactive toward HLA-A, -B and -C allotypes and recognize the non-classical HLA-G molecule. By contrast, KIRs represent a family of receptors with finer specificity for classical MHC class I molecules. Each KIR can recognize one specific group of HLA-A, -B or -C allotypes [32-34]. As KIR receptors constitute a significant part of the focus of this thesis we provide a more detailed description of these receptors in the next section.

Activating receptors in humans include some members of the CD94/NKG2 family and some of the KIR family. The heterodimeric CD94/NKG2C receptor constitutes the activating correspondent of CD94/NKG2A; these two receptors compete for the recognition of HLA-E. The activating components of the family of KIR receptors have similar specificities as the inhibiting ones; however their affinity for HLA ligands is believed to be lower than their inhibitory counterparts [35-37]. In addition to these two classes, NK cells express activating receptors such as the natural cytotoxicity receptors (NCR: NKp30, NKp44 and NKp46) and the homodimeric form of NKG2D. While the biological activity of NCRs is not completely unraveled (mainly due to the

lack of known ligands for these receptors), NKG2D has specificity for two types of ligands (MIC A/B and ULBPs) that are induced on cells by stress, viral infection or malignant transformation [20, 38, 39].

Table 1. HLA class I specificities of the main KIRs expressed by human NK cells.

KIR	HLA-class I specificity
KIR2DL1	Group 2 HLA-C alleles (-Cw2, -Cw4, -Cw5, -Cw6) [Asn77, Lys80]*
KIR2DL2/3	Group 1 HLA-C alleles (-Cw1, -Cw3, -Cw7, -Cw6) [Ser77, Asn80]*
KIR3DL1	HLA-Bw4 alleles (e.g. HLA-B27)
KIR3DL2	HLA-A3, -A11

KIR= Killer Immunoglobulin-like Receptor

* The two groups of HLA-C alleles can be distinguished on the basis of alternative amino acid sequence motif at position 77 and 80 of the α1 helix. Site-directed mutagenesis unequivocally demonstrated that these residues are crucial for KIR-mediated recognition.

Adapted from Velardi et al., Trends in immunology, 2002

4.1. The KIR/HLA system

The human KIR gene cluster is located within the leukocyte receptor complex on chromosome 19 and segregates independently from the HLA locus, which is located on chromosome 6. Only the KIR genotype, but not the HLA genotype, determines the repertoire of KIR genes expressed by NK cells. KIRs are stochastically expressed on NK cells implicating that a large number of possible combinations can form, in each individual, a repertoire of NK cells with different specificities for HLA class I molecules [33]. Until recently, it was believed that all NK cells expressed at least one inhibitory receptor for self-MHC class I molecules [40, 41]. Conversely, recent evidence has shown that in both humans and mice a substantial number of circulating NK cells lack specific inhibitory receptors for self-MHC class I molecules [42, 43]. These NK cells maintain self-tolerance by exhibiting a state of hyporesponsiveness determined during their "education". The process of "NK cell education" is required for the development of fully functional NK cells and is determined by the strength of

interactions between inhibitory receptors and self-MHC class I molecules [42-46]. Based on this principle, four main models have been proposed to explain "NK cell education": the arming (or licensing) model, the disarming model, the *cis*-interaction model and the rheostat model (all reviewed by Höglund and Brodin in [47]). Each of these models explains how, during "education", NK-cell responsiveness is modulated by self-HLA/KIR interactions. To appreciate the mechanisms of NK cell education it is necessary to understand the main signaling pathways triggered upon engagement of an inhibitory or activating receptor. Inhibitory receptors are characterized by immunoreceptor tyrosine-based inhibitory motifs (ITIM) that recruit intracellular tyrosine phosphatases (SHP-1, SHP-2 and SHIP) mediating the inhibition of cytotoxicity and cytokine production [48-51]. Conversely, activating receptors act by associating intracellularly with the DAP-12 molecule, which contains an immunoreceptor tyrosine-based activating motif (ITAM) [52]. It is believed that the integration of these positive and negative signals determines the final responsiveness of NK cells (Figure 3).

As already mentioned, by means of HLA/KIR interactions an NK cell can distinguish between self and non-self targets (Figure 3). According to the original "missing self" hypothesis [53, 54], in the presence of the specific MHC class I molecule that binds to an inhibitory receptor on the NK cell, the target cell will be sensed as self and NK cell cytotoxicity will be inhibited [30, 55, 56]. Alternatively, NK cells can become activated in the absence of an inhibitory signal due to low or no expression of the specific MHC class I molecule (as is the case for non-self cells or for some transformed or virus-infected cells that down-regulate their MHC class I expression). Similarly, overexpression of activating ligands synergize with the absence of inhibitory signals to induce NK cell activation [30, 49]. Overall, KIR recognition is one of the main tools detecting not only transformed and virus-infected cells, but also recognizing missing-self in allogeneic transplantation settings.

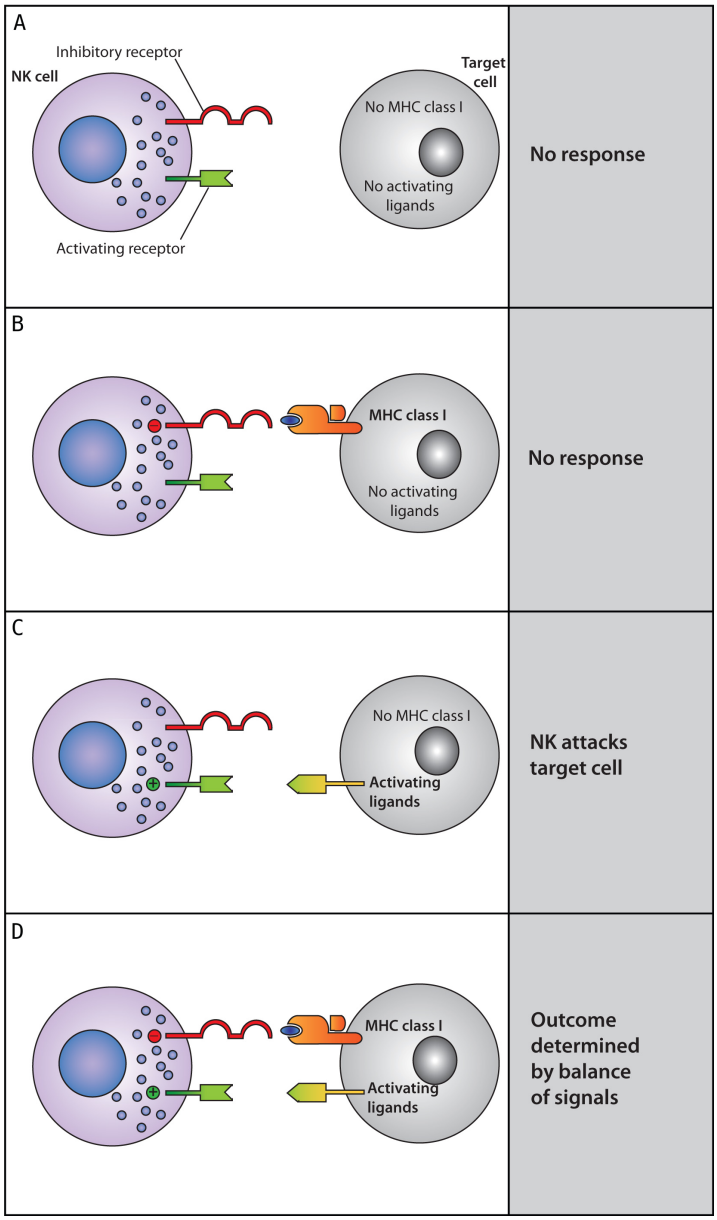

Figure 3. Schematic representation of the most common model of NK-cell alloreactivity against a target cell, according to the revisited "missing-self" model. In A, NK cells do not react to the target cells since no receptor is engaged. In B, NK-cell alloreactivity is kept on hold by the interaction of their inhibitory receptor with the MHC class I molecule on target cells. In C, the absence of MHC class I molecules on target cells determines a lack of inhibition of NK cells; in addition, the presence of activating ligands on the targets triggers NK-cell activation, which will stimulate NK cells to attack their targets. In D, both activating and inhibitory ligands are present on target cells and are recognized by receptors on NK cells; the outcome will be determined by the nature and the number of the ligand/receptor interactions. *Elaborated from the original figure of L.L.Lanier, Annu. Rev. Immuol., 2005.*

5. The changing role of NK cells in transplantation

For many years the field of transplant immunology has focused on cells of the adaptive immune system. Until the "rediscovery" of the innate immunity and its critical role in shaping the adaptive response, in fact, the largest part of research was focused on targeting the T cell-mediated mechanisms of allograft rejection [57, 58]. Only recently the discussion concerning allograft rejection and tolerance induction has been broadened to include the cellular components of the innate immune system, among which the NK cells. NK cells possess two important characteristics which, in combination, make them uniquely relevant to transplantation: they are able to distinguish allogeneic cells from self and they have potent cytolytic effector mechanisms that can be exerted without prior activation [59].

5.1. The dichotomy of NK cells in organ transplantation

Until now, the role of NK cells in solid organ transplantation has been considered only in the recipient-versus-donor direction [57-59]. Overall, research in this field has led to the conclusion that NK cells, like DCs, may have dual roles in transplantation [57, 60-62]. On the one hand, results emerged from a number of studies have implied that NK cells may not be sufficient to reject a solid organ allograft directly but may promote acute allograft rejection by facilitating the activity of alloreactive T cells [59, 63, 64]. The mechanisms at the basis of this induction of rejection are poorly understood but may refer to the capacity of NK cells to kill immature DCs (while sparing mature DCs) and to release pro-inflammatory cytokines, including IFNγ and TNFα. These cytokines are known to induce MHC class II expression and antigen presentation by APCs that, in turn, promote T-cell alloreactivity [61, 65]. On the other hand, NK cells have been implicated in tolerance induction. Evidence of their role in allograft tolerance has emerged from studies in islet [66] and skin transplantation [67]. The mechanisms that have been enumerated to explain the role of NK cells in allograft tolerance refer to the modulation of the host immune response [66] and to the control of T-cell activation via reduction of the donor APC activity [67, 68]. Secretion of the immunoregulatory cytokine IL-10 has also been proposed as a possible mechanism of tolerance induction [69]. Taken together, these studies indicate that the challenge of reconciling the inflammatory role of NK cells with their regulatory function in the context of solid organ transplantation is still open. It is

believed that the tolerant or non-tolerant effect of NK cells may largely depend upon the type of NK cell subset activated, the type of inhibitory/activating NK cell receptors engaged, the nature of the transplant and the immunosuppressive regimen [61, 65].

5.2. NK cells in liver transplantation

A discussion about the role of NK cells in LTX requires a separate section, given the unique characteristics that distinguish this organ from other transplanted organs. As mentioned earlier, liver allografts are not only more easily accepted than other solid organ grafts but are also unique in terms of number and functional features of leukocytes that are transferred by LTX. Interestingly, classical HLA-matching, indicating T-cell alloresponse, does not predict the risk of rejection after LTX [70]. Until now, the few studies published on the role of recipient NK cells in LTX have led to contradictory results. The first study suggesting that NK cells favor acute rejection episodes in LTX reported a significant correlation between acute rejection episodes and donor-recipient KIR receptors mismatches [71]. However, others have found no correlation between predicted NK-cell alloreactivity and acute liver allograft rejection, despite the fact that recipients with predicted NK-cell alloreactivity did have an expanded population of circulating NK cells one month post-LTX compared to recipients without predicted NK-cell alloreactivity [72]. In 2008, a transcriptional profiling study of peripheral blood lymphocytes from LTX recipients revealed that patients tolerant to their liver grafts up-regulated the expression of a number of genes specific to NK cells and $\gamma\delta$ T cells [73]. In the same year, a new study showed that the presence of the HLA-C group 2 allele in the donor was associated with improved long-term graft and patient survival after LTX [74]. Tran et al. rapidly contradicted these data by demonstrating that, in a larger cohort, donor HLA-C2 genotype had no impact on 10-year graft or patient survival [75].

Until now the results presented here referred to the recipient-versus-donor component of a donor/recipient immunological interaction. However, as the basis of transplant immunology consists of the so-called "two-way paradigm" in which two types of interactions (recipient-versus-donor and donor-versus-recipient) coexist [76], it is worth considering also the other component. Given the large number of donor leukocytes transferred by LTX into the recipient and given the existence, although very rare, of cases of graft-versus-host disease after LTX [77, 78], the liver represents a

system in which donor-versus-recipient immunological interactions may have a relevant role. Until now, however, studies considering the donor-versus-recipient component have been performed only in the field of allogeneic hematopoietic stem cell transplantation (HSCT) but not in LTX. In HSCT, a number of studies have shown how donor-versus-recipient NK-cell alloreactivity could prevent leukemia relapse and graft rejection and protect patients against graft-versus-host disease [34, 79-81]. In the present study we will consider, as a novel approach, the effect of donor NK-cell alloreactivity on LTX outcome.

6. HLA-G in liver transplantation

HLA-G expression is one of the factors that have been recently included among the possible mechanisms contributing to liver allograft tolerance [70]. HLA-G is a non-classical MHC class I molecule with unique immunoregulatory functions and restricted tissue distribution. In healthy individuals, HLA-G expression is restricted to extravillous trophoblasts of the fetal placenta, which invade the decidualized maternal uterus [82]. Additionally, ectopic expression has been suggested in diseased tissues and organ allografts [83-85]. Seven different isoforms characterize HLA-G expression: four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7). Among these, the full-length membrane-bound HLA-G1 and soluble HLA-G5 isoforms are known to be expressed, stable and functional [86-88]. The structure of these two isoforms is similar to that of classical HLA class I molecules, with three globular domains non-covalently bound to β2-microglobulin and a nonapeptide. By contrast, all the other isoforms lack one or more globular domains and should not present peptides. In addition to this peculiar pattern of isoforms derived from alternative splicing, shed HLA-G molecules can be generated by proteolysis of the HLA-G membrane-bound forms. Three main receptors have been found to bind HLA-G (ILT2, ILT4 and KIR2DL4) and to be expressed on a wide range of immune cells. While ILT2 and ILT4 can bind ligands other than HLA-G and are inhibitory receptors [89, 90], KIR2DL4 selectively interacts with HLA-G [91] and can have both inhibiting and activating effects [92, 93]. KIR2DL4 is expressed almost exclusively by NK cells [92, 94] and, importantly, is present in all KIR haplotypes [95].

In general terms, HLA-G is known for its immunoregulatory effect on a wide range of immune cells: engagement of ILT2 and ILT4 inhibitory receptors on NK cells, T cells

and APCs reduce the proliferation and cytotoxic activity of NK and T cells [88, 96] while limiting maturation and antigen presentation of APCs [97-99]. However, in the context of human pregnancy, it has been shown that the soluble form of HLA-G interacts with KIR2DL4 and selectively stimulates uterine and peripheral NK-cell proliferation and IFN-γ production, while leaving unaffected their cytotoxic capacity [91, 100]. Similarly, membrane-bound HLA-G stimulated proliferation and cytokine production by uterine NK cells [101]. These peculiar effects may constitute the main functions of HLA-G during pregnancy: to modulate the production of cytokines and angiogenic factors by uterine NK cells as to alter trophoblast invasion and differentiation or tissue remodeling [102]. The initial role attributed to HLA-G during pregnancy, however, was that of protecting the fetal trophoblasts from maternal NK cell-mediated lysis [103]. However, this hypothesis is not supported by more recent studies [104-106].

While the role of HLA-G has been mostly studied in the context of maternal-fetal tolerance, more controversial is the role of HLA-G in solid organ transplantation. Despite some evidence that HLA-G can be expressed by allografts and can correlate with better graft acceptance, the observations are still too scattered and the conclusions of different studies are too fragmentary to allow the extrapolation of a general mechanism of action for HLA-G in transplantation. In the context of heart transplantation and combined liver-kidney transplantation, few studies have found that HLA-G was expressed *in situ* by cells that are the primary targets of the immune system during rejection [84, 85, 107, 108]. Higher HLA-G expression in those patients correlated with better graft acceptance. In the same types of transplantation, plasmatic levels of the soluble form of HLA-G were also found to correlate with graft acceptance. These observations led to the attractive hypothesis that monitoring HLA-G serum levels could constitute a successful screening method, discriminating patients expected to better accept their allografts [85, 107, 109, 110]. However, there is considerable controversy on these data since many anti-HLA-G antibodies have now been shown to cross-react with classical MHC class I molecules, which share 80% amino acid sequence identity in their extracellular domain with HLA-G [82].

In the context of LTX only few papers reported the expression of membrane-bound or soluble HLA-G after LTX in adults [111-113]. These few findings leave large uncertainties in the field of HLA-G in solid organ transplantation and especially in LTX, leaving much space for further research. In addition to the uncertain possibility of

exploiting the function of HLA-G in patients, our understanding of the biology of HLA-G is still relatively limited. Despite the increasing number of publications, a shared consensus is still lacking on many aspects of HLA-G: its precise tissue distribution, its binding receptors, the possible differential effects of the membrane-bound forms and the soluble forms, etc. In this thesis we will provide some insights on the relevance of HLA-G in clinical LTX.

AIMS AND OUTLINE OF THE THESIS

The liver is an organ that has driven large attention for its tolerogenic properties. Being at the interface between the external environment (stomach, intestine) and the internal environment, the liver has to control unwanted immune-responses towards harmless antigens such as food products or components of the gut bacteria. An additional example of hepatic immune tolerance is the relatively weak immunogenicity of liver transplants. This has been shown both in animal models and in clinical settings. However, the mechanisms of this relative tolerogenicity have been only partially explored. Considerable attention has been paid to the possible pro-tolerogenic properties of hepatic APCs such as Kupffer cells, sinusoidal endothelial cells and hepatic dendritic cells [28, 114-116]. However, little is known about the functions of the most abundant hepatic immune cell type, i.e. NK-cells [117, 118].
In this thesis we studied liver-resident NK cells and assessed their role in human liver transplantation. The main focus of this thesis is to establish whether donor NK cells play any role after LTX and if their activity can be predicted by KIR/HLA mismatches. The possible association of NK-cell alloreactivity with LTX outcome may represent a potent tool for the identification of donor/recipient pairs with favorable transplantation outcome.

First, we investigated the immunophenotypical and functional properties of liver-resident NK cells and compared them with blood-derived ones. We also established whether hepatic NK cells are transferred into the recipient upon LTX and we compared these results to kidney-transplanted patients (**chapter 2**). We then considered the role of donor NK cells in LTX by two independent approaches. We first analyzed the effect of NK-cell alloreactivity in terms of HLA/KIR mismatches between donor and recipient on LTX outcome (**chapter 3**). Secondly we analyzed the effect of donor NK cell depletion on transplant tolerance in a rat model of LTX (**chapter 4**). We then tested whether hepatic NK cells are a population of cells derived from peripheral blood or if they originate from a local precursor. In addition, we established if $CD34^+$ hepatic precursors could generate functional NK cells *in vitro* (**chapter 5**). Finally we considered HLA-G, a non-classical MHC class I molecule that has been shown to induce tolerance during pregnancy, and the effects of its expression on tolerance induction after LTX (**chapter 6**). The final chapter (**chapter 7**) discusses and summarizes the content of this thesis.

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Chapter 2

Liver grafts contain a unique subset of NK cells that are transferred into the recipient after liver transplantation

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ABSTRACT

In contrast to other solid organ transplantations, liver grafts have tolerogenic properties. Animal models indicate that donor leukocytes transferred into the recipient after liver transplantation (LTX) play a relevant role in this tolerogenic phenomenon. However, the specific donor cell types involved in modulation of the recipient alloresponse are not yet defined. We hypothesized that this unique property of liver grafts may be related to their high content of organ-specific NK and $CD56^+$ T cells. Here we show that a high proportion of hepatic NK cells that detach from human liver grafts during pre-transplant perfusion belong to the $CD56^{bright}$ subset, and are in an activated state ($CD69^+$). Liver NK cells contained perforin and granzymes, exerted stronger cytotoxicity against K562 target cells when compared with blood NK cells, and secreted interferon-γ, but no IL-10 or T-helper 2 cytokines, upon stimulation with monokines. Interestingly, while the $CD56^{bright}$ subset is classically considered as non-cytolytic, liver $CD56^{bright}$ NK cells showed a high content of cytolytic molecules and degranulated in response to K562 cells. After LTX, but not after renal transplantation, significant numbers of donor $CD56^{dim}$ NK and $CD56^+$ T cells were detected in the recipient circulation for approximately two weeks. In conclusion, after clinical LTX activated and highly cytotoxic NK cells of donor-origin are transferred into the recipient, and a subset of them mixes with the recirculating recipient NK cell pool. The unique properties of the transferred hepatic NK cells may enable them to play a role in regulating the immunological response of the recipient against the graft and therefore contribute to liver tolerogenicity.

INTRODUCTION

It is generally recognized that after clinical liver transplantation (LTX) the incidence of chronic rejection is lower than after transplantation of other organ grafts. Furthermore, in about 20% of LTX-recipients immunosuppressive therapy can be withdrawn without occurrence of graft rejection [1]. Various animal models are spontaneously tolerant to LTX, while rejecting other organs [2, 3]. Furthermore co-transplantation of a liver allograft can prevent rejection of other organ grafts from the same donor [4, 5].

The mechanisms responsible for this relative tolerogenicity of the liver have only been partially elucidated. A number of observations in animal models indicate that the immune cells present in the liver graft may play a relevant role in the induction of tolerance. With LTX, so-called passenger leukocytes from the donor, are transferred into the recipient and can establish a condition of chimerism of variable proportions and duration [6, 7]. Independent studies from different groups have shown that in rat models acceptance of liver grafts is strongly associated with the presence and the abundance of these passenger leukocytes [2, 3, 8-13]. Depletion of passenger leukocytes from liver grafts abrogates tolerance, whereas their reconstitution restores the organ's tolerogenic potential [2, 12]. One of the possible mechanisms by which migrating donor leukocytes promote acceptance of liver grafts is by inducing apoptosis of alloreactive T cells in recipient lymphoid tissues [2, 14]. Also in humans, a few studies have shown that after LTX a variable number of donor lymphocytes are transferred into the recipient and can be detected, *in situ* or in the recipient circulation, for variable time periods [6, 7, 15, 16]. An early study by Rao et al. [17] showing how cell migration and chimerism were observed more dramatically after LTX compared to other organ transplantations, suggested that this phenomenon may explain why the liver is more tolerogenic than the kidney. This observation concurred with the initial suggestion from Starzl et al. that already in 1993 proposed that donor hepatic leukocytes were responsible for the enhanced tolerogenicity of liver grafts [18]. In brief, although there are multiple observations indicating that the transfer of donor leukocytes may positively influence liver graft survival, the specific donor cell types involved in the induction of tolerance are not yet defined.

We observed that donor-derived myeloid dendritic cells migrate from liver grafts into LTX recipients. However, these liver-derived dendritic cells appeared to be stimulators

of allogeneic T cell proliferation and pro-inflammatory cytokine production, thus suggesting that they represent a major player in the induction of acute rejection, instead of acting as promoters of tolerance [15, 19].

A cell type that is abundantly present within the hepatic lymphocyte pool is the natural killer (NK) cell. While NK cells in peripheral blood account for 10-15% of lymphocytes, hepatic NK cells comprise 30-40% of all lymphocytes present in a normal adult liver. These cells, originally called *pit cells*, were first described by Wisse et al. in 1976 [20] and later functionally defined as liver-associated NK cells [21]. Doherthy et al. have shown that this population, resident in the liver sinusoids, is highly cytotoxic [22]. Until now most of the work that has been performed on alloreactive NK cells in transplantation was dedicated to the study of recipient NK cells rather than the donor derived populations. Although NK cells are classically regarded as effector cells contributing to rejection of allogeneic grafts, recent studies have shown their involvement in graft acceptance [23-25]. NK cells seem to be involved in tolerance induction by either targeting donor-derived antigen presenting cells or recipient alloreactive T cells [25]. Both mechanisms are actually not mutually exclusive but rather complementary and may be dependent on the model analyzed and the local environment in which NK cells are activated [25]. After allogeneic bone marrow transplantation between selected donor-recipient pairs donor-derived NK cells have been shown to reduce Graft-versus-Host Disease by killing of recipient antigen presenting cells, to promote graft acceptance by killing recipient alloreactive T cells and to prolong leukemia-free survival of patients by eliminating residual leukemic cells [26, 27].

In addition to their well-known innate functions, there is emerging evidence of the extensive cross-talk between NK cells and the adaptive arms of the immune system [25, 28]. Although part of the innate immune system, NK cells are now becoming known also for their capacity to modulate adaptive immune responses. Therefore, if in the heterologous settings of transplantation donor NK cells – which are able to distinguish allogeneic cells from self – migrate into the recipient, they may be involved in modulating the host immune response against the graft.

In this study we determined the extent and duration of donor-derived NK cell migration into recipients after clinical LTX, and compared this to the NK cell chimerism after renal transplantation (RTX). We characterized donor NK cells that detach from human liver grafts in terms of both immunophenotypical and functional properties and compared them with blood NK cells. Finally we determined the

expression of a number of key integrins and chemokine receptors indicating their migratory potential in liver graft recipients.

MATERIALS AND METHODS

Peripheral blood and liver graft perfusate collection

To determine the numbers of donor-derived NK cells and $CD56^+$ T cells in the recipient circulation, we selected 7 $HLA\text{-}A2^-$ LTX-recipients, and 7 $HLA\text{-}A2^-$ renal transplant (RTX) recipients that had been transplanted with a graft from an $HLA\text{-}A2^+$ donor. HLA-A2 was used as a marker to distinguish between donor and recipient cells. All LTX-procedures considered in this study were performed from a post-mortem donor. Peripheral blood samples were collected from LTX and RTX-recipients immediately before and one day after transplantation. To evaluate for how long donor lymphocytes were detectable in the host circulation, we selected five additional LTX recipients that satisfied the same conditions described above and peripheral blood samples were collected before transplantation and during each one of the following time intervals after LTX: 1 to 5 days, 6 to 10 days, 11 to 15 days, 16 to 20 days, 21 to 30 days, 1 to 6 months and 6 months to 1 year. Peripheral blood mononuclear cells (PMBC) were prepared by Ficoll density gradient centrifugation. For this first part of the study isolated PBMC were stored in liquid nitrogen and thawed right before usage. Perfusates were collected from human liver grafts during the back table procedure. Upon arrival at the hospital, grafts were perfused through the portal vein with 1 to 2 liters of University of Wisconsin (UW)-solution to remove residual blood from the vasculature. Immediately before transplantation, the donor livers were perfused once more with 200-500 ml of human albumin solution. These latter perfusates were collected from the vena cava, and used to study hepatic NK cells. Mononuclear cells (MNC) from fresh liver graft perfusates were isolated by density gradient centrifugation. For immunophenotypical and functional analysis of blood NK cells, PBMC were isolated from healthy individuals. The Ethical Committee of the Erasmus MC approved the study protocol, and written informed consent was obtained from each patient.

Detection of donor-derived NK cells and $CD56^+$ T cells

Donor-derived NK cells and $CD56^+$ T cells were detected in the recipient circulation using a FITC-conjugated anti-HLA-A2 mAb (BD Biosciences, San Jose, CA, USA), in combination with CD3-PE (BioLegend, San Diego, CA, USA) and CD56-APC

(Beckman Coulter Immunotech, Marseille, France) mAb. Cryopreserved samples of recipient PBMC were used for this first part of the study. Dead cells were excluded from analysis by using 7-AAD (BD Biosciences Pharmingen, San Diego, USA). Binding of an appropriate isotype-matched FITC-conjugated control mAb was subtracted from binding of the anti-HLA-A2 mAb. Analysis was performed using FACS Canto II flowcytometer (BD Biosciences, San Jose, CA, USA) equipped with BD FACSDiva™ flow cytometry software 6.1.1 (BD Biosciences, San Jose, CA). At least $1x10^6$ events were acquired from each sample.

Phenotypic analysis of NK cells

MNC from liver graft perfusates, from blood of healthy individuals, LTX-donors or LTX-recipients were thawed in FCS and stained with mAb to assess differences in the immunophenotypic properties of the $CD3^-CD56^+$ NK cells. The following mAbs were used: CD107a-PE, CD16-Pacific Blue, CD56-PE, CD62L-APC, CD69-APC, anti-Granzyme A-PE, anti-Perforin-PE, CD14-PE, anti-α4-PCy5, anti-β7-PE, anti-CXCR4-PE (BD Biosciences, San Jose, CA, USA); CD158a,h-APC, CD158b1/b2,j-APC, CD94-PE, anti-NKG2A-PE, anti-NKG2D-PE, anti-NKp30-PE, anti-NKp44-PE, CD11a-FITC, CD19-PE (Beckman Coulter Immunotech, Marseille, France); anti-BDCA1-PE (Miltenyi Biotec, Bergisch Gladbach, Germany); CCR7-PE (R&D systems, Abingdon, United Kingdom); CD16-FITC, CD3-PerCP-Cy5.5 (ExBio, Praha, Czech Republic); anti-Granzyme B-PE (Sanquin, Amsterdam, The Netherlands); anti-CCR9-PE, anti-CXCR3-APC, anti-CCR5-PE, anti-CCR1-PE (R&D Systems, Minneapolis, USA). The following mAbs were used as isotype matched controls: mouse IgG1-PE, IgG1-APC, IgG2a-FITC, IgG2b-FITC, IgG2b,k-PE, IgG1k-PCy5, IgG2a-PE, and rat IgG2a-PE. Optimal dilutions of all mAb used were established in preliminary experiments. Flow cytometric analyses were performed using FACS Calibur or FACS Canto II (both from BD Biosciences,San Jose, CA, USA).

Isolation of NK cells from peripheral blood and from perfusates

$CD3^-CD56^+$ NK cells were isolated from fresh liver perfusate MNC and from fresh PBMC by negative selection using the NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Briefly, non-NK cells were firstly labeled with a cocktail of biotin-conjugated antibodies against lineage-specific markers, and subsequently with Anti-Biotin MicroBeads. In addition to this,

during NK cell isolation from perfusate MNC, CD15 MicroBeads (Miltenyi Biotec) were added for complete granulocyte depletion. The entire cell suspension was then loaded onto a MACS LD column that was placed in a magnetic field and retained only the antibody-conjugated cells. All the reagents used for the isolation were diluted in calculated proportions of MACS buffer (PBS from BioWhittaker, Lonza, Belgium, supplemented with and 2mM EDTA and 2.5% BSA both from Sigma, St Louis, MO, USA). Purity of isolated NK cells and proportion of the two subtypes were evaluated by flow cytometry using CD3-PE, CD56-APC and CD16-FITC mAb. Viability was determined by flow cytometry using 7-AAD.
The average purity of the enriched NK cell population was 99.6±0.5, while the average viability was 97±1%.

Cytotoxicity and degranulation assays with K562 cell line

Flow cytometry was used for a combined assessment of degranulation and cytotoxicity. The first was quantified by measuring the surface expression of CD107a, a molecule detectable on cells after degranulation, the latter was assessed by CFSE-labeling of the targets and quantification of cell death. Freshly purified blood or perfusate NK cells were cultured overnight at 37°C in RPMI medium supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml; both from Gibco BRL Life Technologies, Breda, The Netherlands). K562 target cells lacking MHC class I expression were labeled with CFSE (10nM, Molecular Probes, OR, USA) and cultured overnight at the same conditions. The following day K562 cells were co-incubated with NK cells in an effector:target ratio of 1 to 1. CD107a-PE mAb, or alternatively IgG1-PE mAb, was added at the beginning of the culture to the wells to determine the proportion of NK degranulation. After 4 hours of incubation at 37°C, the cells were washed twice with PBS and then stained with CD56 and CD16 mAb. The proportion of viable K562 was determined by using 7AAD; to quantify their absolute number a fixed amount of beads (CaliBRITE unlabeled beads, BD Biosciences, San Jose, CA, USA) was added to each sample and measured by flow cytometry. For each well the absolute number of living K562 was calculated as a proportion to the number of beads; the percentage of specific cytotoxicity was then estimated as follows:

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

Four replicas of each sample for each condition were measured for every assay.

NK cell stimulation for cytokine production

Cryopreserved purified NK cells (5x10 ^{4}cells/well) were stimulated with combinations of PMA (250ng/ml) and Ionomycin (500ng/ml), or IL-12 (10ng/ml) and IL-15 (100ng/ml), or IL-12 (10ng/ml) and IL-18 (100ng/ml) or IL-2 (100ng/ml) and IL-21 (50ng/ml). Supernatants were collected after 48 hours of culture and assayed in duplicate using enzyme-linked immunosorbent assay (ELISA) for production of IFN-γ (Invitrogen BioSource, Nivelles, Belgium), IL-10 and IL-13 (eBioscience, San Diego, USA).

Statistical analysis

All data are presented as means ± SEM, unless differently specified. The Mann-Whitney U test was used to analyze whether differences between unrelated groups were significant. For paired comparisons the Wilcoxon signed rank test was used, alternatively, when appropriate, the paired T test was applied. A two-sided p-value <0.05 was considered as significant.

RESULTS

Donor NK and CD56$^+$ T cells migrate into the recipient after LTX, but not after renal transplantation

PBMC from HLA-A2$^-$ patients that received a liver or renal transplantation from an HLA-A2$^+$ donor were used to analyze migration of donor-derived CD56$^+$CD3$^-$ NK and CD3$^+$CD56$^+$ T cells into recipients. Flow cytometric analysis of PBMC obtained before TX confirmed the absence of HLA-A2$^+$ cells in the recipient, while donor's splenocytes showed complete HLA-A2 positivity. One day after LTX donor-derived mononuclear cells in the recipient circulation could be identified as a separate cloud of cells expressing HLA-A2. Within this population, NK cells were defined as CD3$^-$CD56$^+$HLA-A2$^+$ and CD56$^+$ T cells as CD3$^+$CD56$^+$HLA-A2$^+$. At day 1 after LTX an average of 7.0% (range 1.4-17.3%) of the circulating NK cell population was of donor origin (1A). Donor-derived NK cells were characterized by a high proportion of the CD56^{dim} CD16$^+$ subtype (Figure 1A and 1B) and a low activation profile (the early activation marker CD69 was expressed by 23.1±5.8% of CD56^{dim} cells and by 22.0±6.5% of CD56^{bright} cells). Donor CD56$^+$ T cells were also observed in the circulation (average 12.6%; range 6.1-31.3% of all circulating CD56$^+$ T cells). Notably, donor-derived NK cells and CD56$^+$ T cells were only detected in LTX recipients, while in RTX recipients lymphocyte chimerism was below the detection level of flow cytometry (each lymphocyte subset being <0.1%) (Figure 1C).

The average time-span in which these donor-derived cells were circulating in the recipient was determined in five LTX patients. Relevant clinical data of these patients are shown in Table 1. Although the proportions of donor NK cells varied among different LTX patients, in all cases these cells were detectable in the circulation for an average period of 15 days (Figure 2A). In the case of donor CD56$^+$ T cells a few differences were observed. Firstly, the cells were detectable in the recipient in a wider range of proportions varying between 1.1 and 13.2% of the total CD56$^+$ T cell population (Figure 2B). Secondly, in two out of five patients, circulating donor CD56$^+$ T cells were detectable in high numbers up to 16-20 days after LTX.

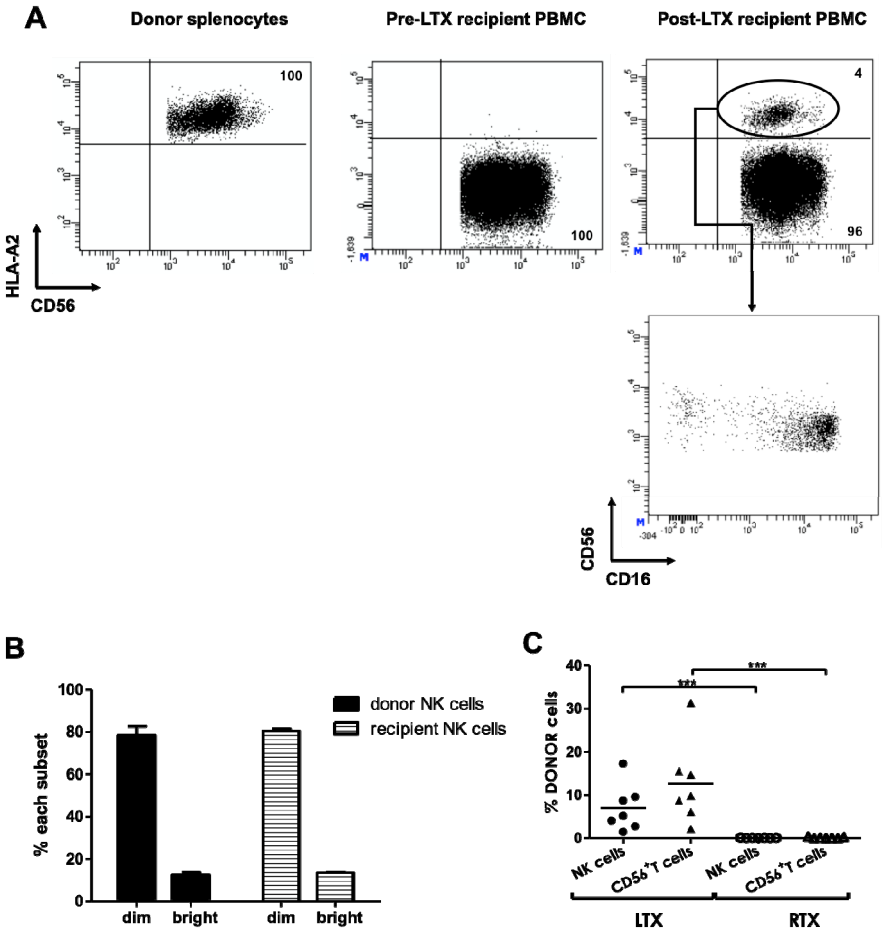

Figure 1. Detection of donor NK and CD56⁺ T cells in the circulation of recipients one day after liver (LTX) or renal transplantation (RTX). Combinations of HLA-A2⁻ LTX-recipients or RTX-recipients that had been transplanted with a graft from an HLA-A2⁺ donor were selected. **(A)** All dot plots here represented are showing NK cells gated as $CD3^{-}CD56^{+}$. Before transplantation complete HLA-A2 positivity was confirmed for donors (left panel) and HLA-A2 negativity was verified for all recipients (central panel). At day 1 after transplantation part of the NK cells detected in the recipient's circulation were of donor origin, as demonstrated by their HLA-A2 positivity (circled in the right panel). Gates were set by use of an appropriate Isotype control mAb matched with the HLA-A2 mAb and percentages of cells in each quadrant are indicated in the plots. The lower panel shows donor NK cells plotted according to their CD16/CD56 expression and indicate a tipical distribution of the $CD56^{dim}$ and $CD56^{bright}$ subsets (lower panel). **(B)** Quantification of proportions of $CD56^{dim}$ and $CD56^{bright}$ NK cells within the donor derived NK cells or within recipient NK cells detectable in the recipient's circulation early after LTX. Data are shown as means ± SEM of 4 independent experiments. **c.** Donor derived NK and CD56⁺ T cells were quantified for seven LTX recipients and seven RTX recipients. Percentages indicate the proportion of donor cells within the cell type specified. Horizontal lines on the graph represent the calculated average for each cell type.

Table 1. Relevant clinical data of the LTX-patients for whom NK and CD56⁺ T cell chimerism was measured at sequential time-points.

Patient	Diagnosis	Induction therapy (anti-IL-2R mAb)	Maintenance immune suppression	Acute Rejection	Ischemia times (min)	
					cold	warm
1	HBV	Yes	STE/TAC	No	270	36
2	HBV	Yes	STE/TAC	No	435	42
3	PBC	Yes	STE/CsA	No	793	34
4	Post-alcoholic liver cirrhosis	No	STE/AZA /CsA/END	No	839	96
5	Insulinoma with liver metastasis	No	STE/TAC	No	390	72

STE=Steroids; TAC=Tacrolimus; CsA=CyclosporinA; AZA=Azathioprine; END=Endoxan

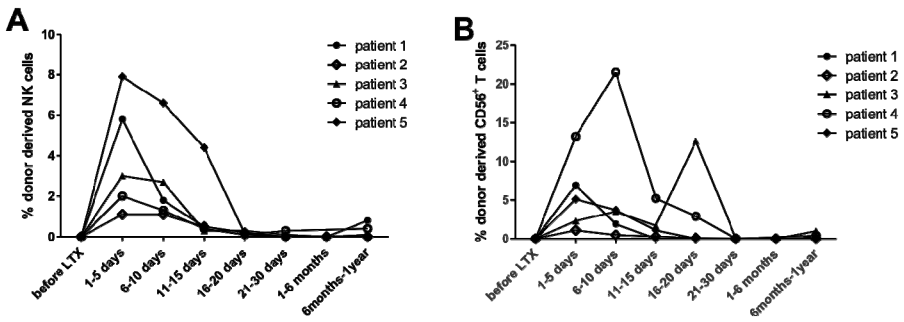

Figure 2. Longitudinal course of donor-derived NK and CD56⁺ T cells in the recipient circulation after LTX. Five HLA-A2⁻ patients that received a liver transplantation from an HLA-A2⁺ donor were selected and peripheral blood samples were collected before transplantation and during each one of the following time intervals after LTX: 1 to 5 days, 6 to 10 days, 11 to 15 days, 16 to 20 days, 21 to 30 days, 1 to 6 months and 6 months to 1 year. Donor derived NK cells **(A)** and CD56⁺ T cells **(B)** were quantified for each time-point. Percentages on the *y* axis indicate the proportion of donor cells within the cell type specified.

Liver NK cells show differences in their subtype composition and their phenotypical features compared with blood NK cells

To characterize the donor NK cells that detach from liver grafts we collected perfusates during regular vascular reperfusion of grafts with human albumin solution before transplantation. Perfusate MNC contained on average 44±12% NK cells. Classical NK cell subsets were gated on the basis of the surface expression of CD56 and CD16 (Figure 3) according to the work of Cooper and colleagues [29]. Among the NK cells that detached from liver grafts, the $CD56^{bright}$ subset was enriched as compared to its counterpart in peripheral blood: while in blood only 9.9±3.2% of NK

cells (n=8) belonged to the CD56^{bright} population, this subset accounted for 45.8±6.5% of the NK cells in perfusate (n=17). Both in perfusate and blood the majority of CD56^{bright} cells were CD16^{-}, while all CD56^{dim} NK cells expressed CD16.

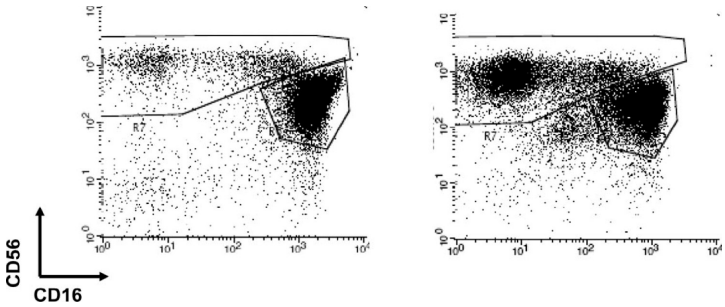

Figure 3. CD56/CD16 phenotype of NK cells from blood and from liver perfusate. Representative dot plot of freshly isolated liver and blood NK cells showing the gates applied to distinguish the CD56^{dim} and CD56high subsets. Upon labeling with CD56 and CD16 mAb, the CD56^{bright} and CD56^{dim} subsets were gated as shown in the dot plots. Blood NK cells are shown in the left panel while perfusate NK cells are represented in the right panel.

Immunophenotypical analysis of other surface markers was then performed in a comparative analysis between blood and liver NK cells (Table 2). The activation marker CD69 was expressed on the majority (93.8±2.6%) of CD56^{bright} liver NK cells while the same subset in blood showed a significantly lower expression (23.0±3.6), indicating a physiological activation status of NK cells detaching from the liver. To test whether this activated profile was caused by the exposure of hepatic cells to University of Wisconsin (UW) preservation solution during graft storage, we incubated PBMC or total blood from healthy individuals in UW preservation solution for 24 hours at 4°C, mimicking the storage conditions of liver grafts before utilization. In none of the two cases NK cell activation could be observed. In contrast, CD69 expression on NK cells was slightly decreased after preservation in UW solution (data not shown). To exclude that the activation of NK cells is an artifact related to the pre-morbid state of the donor, we tested the expression of CD69 on paired samples of fresh perfusate and donor blood collected at the time of LTX. The results confirmed a physiological activation of the CD56^{bright} subtype of hepatic NK cells and a low expression of CD69 on samples of donor blood (for liver NK cells: 96.4±1.3% of CD56^{bright} cells CD69^{+}, 7.6±0.9% of CD56^{dim} cells expressing CD69; for blood NK cells: 3.9±0.5% of CD56^{bright} cells CD69^{+}, 5.5±3.2% of CD56^{dim} cells expressing CD69).

The major inhibitory receptors on NK cells are the heterodimeric receptor NKG2A/CD94, binding HLA-E loaded with leader peptides from MHC class I molecules, and part of the Killer Ig-like Receptors (KIRs) recognizing different MHC class I alleles [30]. As in blood, $CD56^{bright}$ NK cells from liver are characterized by a higher expression of NKG2A/CD94, and by a lower expression of KIRs compared with $CD56^{dim}$ NK cells (Table 2). KIR analysis was performed using two mAbs commercially available, CD158a and CD158b. These mAbs are able to distinguish sets of KIRs that are specific for a certain HLA-C group; however they do not allow discrimination between activating and inhibiting receptors. CD158a mAb recognizes KIR2DL1 (inhibitory) and KIR2DS1 (activating), which are both receptors specific for HLA-C group 2 alleles. CD158b mAb detects KIR2DL2, KIR2DL3 (both inhibitory) and KIR2DS2 (activating), which are all receptors that can bind ligands of the HLA-C group 1. Data reported in Table 2 on KIR expression are not meant to compare blood and perfusate samples, since differences in KIR expression largely reflect the genetic variability among individuals. Therefore we compared the presence of these receptors on paired samples of blood and perfusate material collected from the same donor (data not shown). The results indicated that there is no significant difference between NK cells derived from the two compartments.

Concerning the activating receptors, on average 60% of blood NK cells and 72% of liver NK cells expressed the C-type lectin receptor NKG2D (Table 2). Likewise, hepatic and blood NK cells expressed comparable amounts of NKp30, belonging to the NK-specific family of Natural Cytotoxicity Receptors (NCR). Interestingly, while part of the $CD56^{bright}$ NK cells in perfusates expressed the activation-induced NCR NKp44, the same receptor was not present on $CD56^{bright}$ NK cells in blood. This result confirms that the hepatic $CD56^{bright}$ NK cells are activated.

Expression of CD11a, the a-chain of the integrin LFA-1, was higher on liver $CD56^{bright}$ NK cells compared with the same subset in blood. This adhesion molecule is highly expressed on all hepatic lymphocytes, mediating their adhesion to ICAM-1 on Kupffer cells and liver sinusoidal endothelial cells [31, 32], further confirming the hepatic origin of perfusate NK cells.

Intracellular staining was used to detect the content of perforin and granzymes A and B as an indication of the potential cytotoxic capacity of NK cells. Most $CD56^{dim}$ NK cells in liver and blood expressed these cytolytic molecules, although the proportion of hepatic $CD56^{dim}$ cells expressing perforin was lower compared to its

counterpart in blood (Figure 4). However, in contrast to CD56^{bright} NK cells in blood, the majority of hepatic CD56^{bright} cells expressed granzymes A and B, and about 50% of them ^{expressed} perforin. These results indicate that liver CD56^{bright} NK cells have all prerequisites to exert cytotoxicity, while in blood and lymphoid tissues this subset has consistently been described as poorly cytotoxic [30, 33].

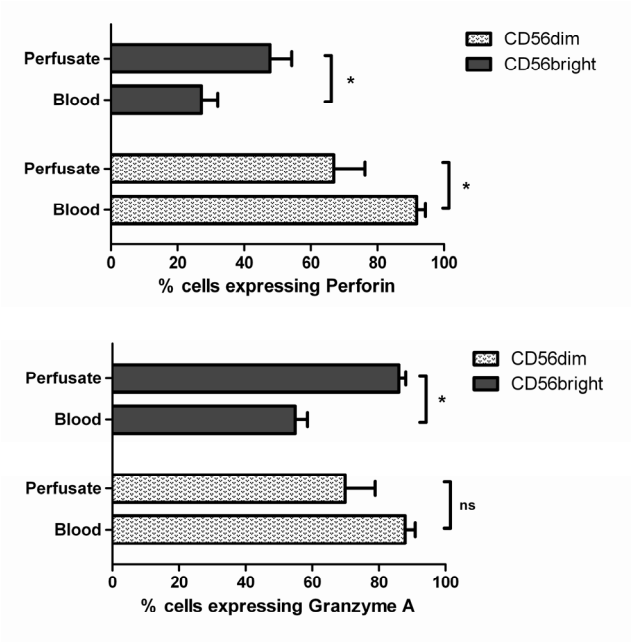

Figure 4. Comparison of intracellular expression of perforin and granzymes in blood and perfusate NK cells. Intracellular staining was performed on MNC derived from liver perfusates or peripheral blood from healthy controls. The results indicate differences in the content of perforin and granzyme A, while analysis of granzyme B did not show any significant difference. Data refer to 7AAD$^-$CD3$^-$CD56$^+$ cells gated on the CD56^{dim} and CD56^{bright} subsets and are shown as the mean percentage ± SEM of six different perfusates and six blood samples.

Table 2. Immunophenotypical comparison of NK cells in blood and liver perfusate.

	Total NK cells or subset	Blood NK cells	Perfusate NK cells	P value
CD69	total	26.6±4.8	68.5±4.3	* 0.0007
	$CD56^{dim}$	27.4±4.8	34.0±4.8	ns
	$CD56^{bright}$	23.0±3.6	93.8±2.6	* 0.0007
NKp44	total	0.1±0.0	2.2±0.8	* 0.0006
	$CD56^{dim}$	0	0	ns
	$CD56^{bright}$	4.3±1.1	9.2±3.5	ns
NKG2D	total	60.3±5.2	72.5±5.5	ns
	$CD56^{dim}$	62.1±5.9	65.4±8.1	ns
	$CD56^{bright}$	90.6±3.7	96.4±1.7	ns
CD94	total	28.2±7.3	49.2±7.2	ns
	$CD56^{dim}$	25.4±7.5	32.9±5.9	ns
	$CD56^{bright}$	90.3±2.0	76.9±5.6	* 0.0207
NKG2A	total	49.7±7.5	52.6±5.1	ns
	$CD56^{dim}$	49.3±7.4	41.0±5.5	ns
	$CD56^{bright}$	90.9±2.4	72.3±6.1	* 0.0148
NKp30	total	45.3±6.4	34.3±7.3	ns
	$CD56^{dim}$	50.4±6.9	45.9±8.0	ns
	$CD56^{bright}$	67.0±3.3	50.9±6.2	*0.0379
CD158a (KIR2DL1 KIR2DS1)	total	20.3±3.6	12.7±2.7	$
	$CD56^{dim}$	22.4±3.6	24.4±2.7	$
	$CD56^{bright}$	3.0±0.5	4.7±1.9	$
CD158b (KIR2DL2 KIR2DL3 KIR2DS2)	total	30.9±4.3	17.1±3.8	$
	$CD56^{dim}$	33.8±4.2	28.9±4.3	$
	$CD56^{bright}$	4.4±1.0	9.0±4.1	$
CD11a (Mean Fluorescence Intensity)	total	68.6±5.6	111.4±6.9	*0.0003
	$CD56^{dim}$	73.4±7.0	92.7±4.7	ns
	$CD56^{bright}$	40.3±3.1	138.9±6.3	*0.0003

Data refer to gated $7AAD^{-}CD3^{-}CD56^{+}$ cells and are shown as the mean percentage ± SEM of eight different perfusates and eight blood samples. $= P value is not reported because differences in KIR expression are largely genetically determined and not comparable between non-paired samples.

Cytotoxicity of liver NK cells is enhanced while IFN-γ production is comparable to blood NK cells

To compare the cytotoxic capacity of liver and blood NK cells, freshly purified NK cells either from liver perfusates or blood were co-incubated for 4 hours with CFSE-labeled MHC class I-devoid K562 cells in an effector:target ratio of 1 to 1. No activating cytokines were added in the culture system in order to obtain observations not biased by any *in vitro* activation. When surface expression of CD107a was quantified as a direct measurement of degranulation activity, hepatic NK cells showed a similar response compared with blood NK cells (Figure 5A). Notably, when the two classical NK cell subtypes were gated, the $CD56^{bright}$ liver NK cells showed the highest degranulation activity (data not shown). To asses the cytotoxic capacity of NK cells, numbers of CFSE-labeled K562 where quantified by flow cytometry after incubation in the presence or absence of the effector NK cells (Figure 5B). The average killing capacity of hepatic NK cells was two-fold higher than their blood counterparts (Figure 5C) indicating that perfusate NK cells are highly cytotoxic.

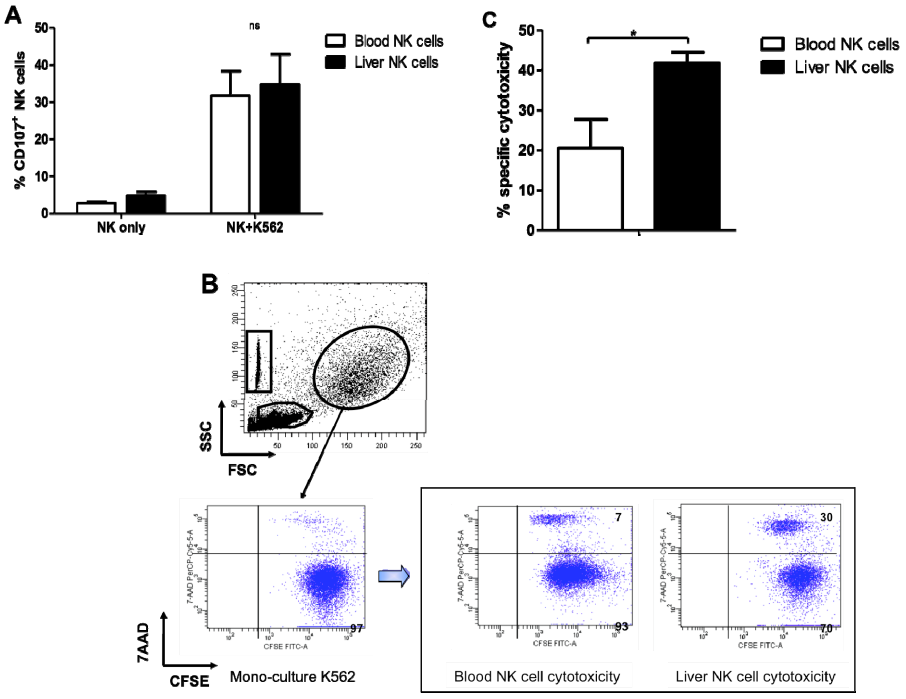

Fig 5. Comparison of degranulation capacity and cytotoxicity between liver and blood NK cells. Purified NK cells from blood or from perfusate were co-incubated with K562 cells in an E:T ratio of 1 to 1. **(A)** After 4 hours degranulation of the effector NK cells was quantified by detection of CD107a. Data are shown as means ± SEM of 4 independent experiments. **(B)** Example of a typical FSC and SSC plot obtained at the end of the 4-hour co-incubation of purified NK cells and K562 target cells Beads used for normalization are gated in the squared gate, purified NK cells are gated in the irregular-shaped gate, while the circle gate represents K562 cells. K562 target cells, labeled with CFSE, are then plotted against 7AAD to discriminate living and dead cells. Representative examples of dot plots used for this quantification are here reported: K562 in single culture (left panel), K562 co-cultures with blood or liver NK cells (right panels, in the box). Numbers in the plots indicate percentages of cells in the quadrants **(C)** By normalization with beads the absolute numbers of living K562 after incubation were obtained and specific cytotoxicity was calculated. Here shown are average ± SEM of percentages of K562 cells killed by blood or perfusate NK cells in 4 independent experiments (*: p=0.026).

As NK cell stimulation by K562 cells does not induce production of cytokines, purified NK cells were cultured with different combinations of stimuli and supernatants were assayed for the presence of cytokines. IFN-γ was induced in some of the conditions tested: the highest amount of IFN-γ was produced in response to a combination of IL-12 and IL-18, and no significant difference was found between liver and blood NK cells (Figure 6). Similarly the combination of IL-12 and IL-15 induced significant amounts of IFN-γ and, as for the previous condition, large variability among samples was observed. By contrast both the stimulation by PMA and ionomycin and by IL-2 and IL-21 did not induce any IFN-γ production. Furthermore both liver and blood NK

cells were unable to produce IL-10 and IL-13 in all the conditions here tested (data not shown).

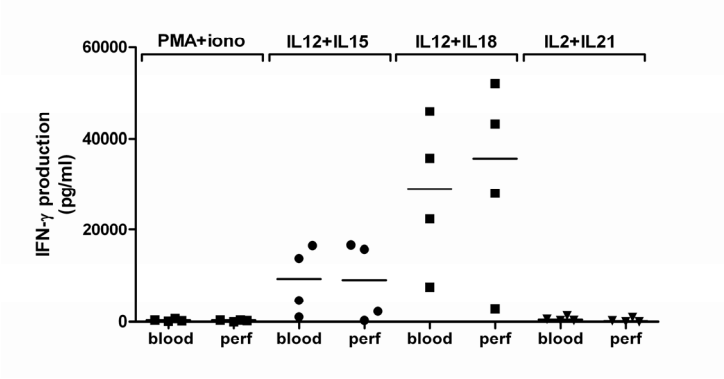

Figure 6. Quantification of IFN-γ production by purified liver or blood NK cells upon monokine stimulation. Purified NK cells were stimulated for 48h in four different conditions with the following combination of stimuli: PMA and ionomycin, IL-12 and IL-15, IL-12 and IL-18, IL-2 and IL-21. Supernatants collected from four samples of blood NK cells and four of liver NK cells were tested for IFN-γ production. The results here reported are expressed in pg/ml and horizontal bars indicate median values.

Donor NK cells in the recipient express L-selectin and the integrin α4/β7

To estimate the homing potential of donor NK cells that detach from liver grafts, we measured the expression levels of a number of relevant chemokine receptors, selectin and integrins both on NK cells that detach from the grafts during reperfusion and on donor-derived NK cells found in the circulation of the recipient.

Firstly perfusate NK cells were analyzed (Figure 7A). The chemokine receptors CCR1, CCR5 and CXCR3 involved in homing into inflamed tissues showed no detectable expression on liver NK cells (N=4), suggesting that they have no capacity to migrating into inflamed tissues. Expression of the chemokine receptor CXCR4, selected to identify potential homing to bone marrow, was generally low. Around 15% of hepatic NK cells expressed the gut-homing receptor CCR9, and 38% expressed the two chains of the integrin α4/β7, a ligand for Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1), which is expressed on the high-endothelial venules of Peyer's patches, and sinus-lining cells of the splenic marginal zone. For both markers the expression was higher on the $CD56^{bright}$ subset than on the $CD56^{dim}$. L-selectin (CD62L) and the chemokine receptor CCR7, both involved in selective migration into secondary lymphoid organs, were detected on small proportions of both $CD56^{dim}$ and $CD56^{bright}$ liver NK cells. In contrast, in blood 37.6±5.2% (n=7) of NK cells are

CD62L-positive. Moreover, although CCR7 is not expressed on peripheral $CD56^{dim}$ cells, 66.9±4.0% of $CD56^{bright}$ cells in blood are CCR7-positive (n=7).

Expression of homing receptors on migrating HLA-$A2^+$ donor NK cells in peripheral blood obtained from patients shortly after LTX was comparable to that of circulating HLA-$A2^-$ recipient NK cells (Figure 7B). High expression of α4/β7 characterized both donor (47.2±9.4%) and recipient NK cells (35.8±10.8%), while CCR9 was detectable but on a lower proportion of circulating NK cells. CD62L-positivity was observed on significantly higher percentages of circulating donor than perfusate NK cells (10.1±3.8% on perfusate NK cells; 32.4±8.3% on donor NK cells, p=0.031; 36.5±6.0% on recipient NK cells, p=0.016). In summary, these data indicate that a large proportion of donor-derived NK cells that migrate in the circulation of LTX- recipients express the α4/β7 integrin, allowing re-allocation into the recipient spleen, and a minority co-express CCR9, allowing homing to gut-associated lymphoid tissues.

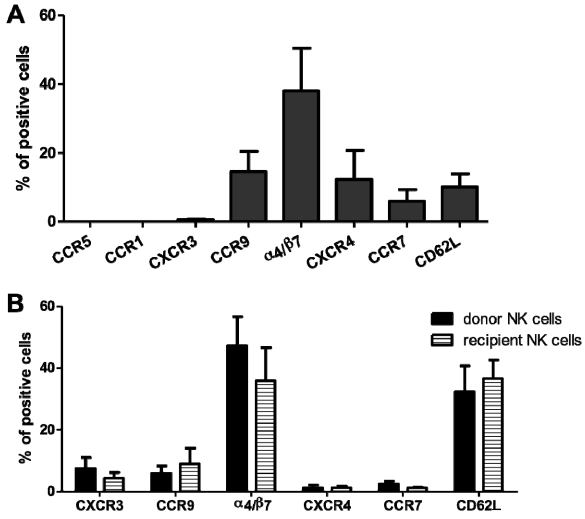

Figure 7. Expression of relevant integrins, selectins and chemokine receptors on NK cells from liver perfusate and donor NK cells circulating in the recipient peripheral blood after LTX. (A) NK cells in liver perfusates were tested for expression of CCR5, CCR1, CXCR3, CCR9, α4/β7, CXCR4 (n=4) CCR7 and CD62L (n=14). Mean percentages and SEM are reported. **(B)** PBMC from LTX patients were tested for the expression of homing receptors on both donor-derived NK cells (black bars) and recipient NK cells (dashed bars). For all samples mean percentages of NK cells positive for the indicated marker and SEM values are shown (n=7).

DISCUSSION

This study demonstrates that a large proportion of hepatic NK cells that are transferred with a human liver graft belong to the $CD56^{bright}$ subset, and exhibit a physiologically activated profile. Our *in vitro* functional assays indicate that they can kill their targets more efficiently than their counterpart in blood and secrete IFN-γ, but not Th2-cytokines or IL-10, upon stimulation with monokines. During LTX these hepatic NK cells are transferred into the recipient and their unique properties may enable them to play a role in regulating the immunological response of the recipient against the graft by exerting alloreactivity against recipient immune cells entering the liver. Moreover, we show that after LTX, but not after RTX, significant numbers of donor NK cells (mainly belonging to the $CD56^{dim}$ subset) and $CD56^{+}$ T cells mix with the recirculating pool of recipient NK cells for approximately two weeks. The donor NK cells in the recipient bloodstream express chemokine receptors and integrins that indicate that a minority has the potential to be relocated into spleen or gut-associated lymphoid tissues of the recipient.

It is not known what determines the release of donor lymphocytes from the liver graft during perfusion, but one major trigger has been suggested being the cold preservation of the liver before the graft is transplanted. The preservation conditions can determine an injury to the adhesion molecules of sinusoidal-lining cells thus favoring the release of viable cells into the sinusoidal lumen [34, 35]. This hypothesis is supported by the demonstration of the large number of leukocytes –ranging between 10^6 and 10^8– in the liquid collected after reperfusion of the liver at the end of the cold storage period (our data and [15, 16, 36]. Although this might represent the main mechanism triggering the release of hepatic leukocytes, no clear correlations were found between the grade of chimerism and the duration of cold or warm ischemia times in the cases considered in this study (data not shown).

Our data confirmed that liver NK cells are phenotypically and functionally different from the correspondent cell type in blood. Firstly hepatic NK cells are enriched for $CD56^{bright}$ cells, which represent about 50% of the total liver NK cell population. By contrast, in blood the $CD56^{bright}$ cells account for about 10% of the total population. Secondly, while it was already known that intrahepatic lymphocytes are

characterized by a high activation state [15, 36-39], we here have shown that it is specifically the hepatic CD56^{bright} subset that expresses the early activation marker CD69 and the activation-induced NCR NKp44. We excluded that this liver-specific activation could represent an artifact due to the pre-morbid state of the donor or due to the exposure to low temperatures (4°C) or UW preservation solution. Similarly, other authors have already shown that the isolation procedure of perfusate leukocytes, including prolonged exposure to cold UW solution, did not promote activation of mononuclear cells nor did it enhance their stimulatory capacity in MLRs [36, 38].
Thirdly CD11a expression was elevated on the CD56^{bright} subset. CD11a is an adhesion molecule highly expressed on all hepatic lymphocytes mediating their adhesion to ICAM-1 on Kupffer cells and liver sinusoidal endothelial cells.

Functionally, freshly isolated liver NK cells killed K562 target cells more efficiently than blood NK cells. This effect could be observed without any stimulation by external cytokines and at a very low E:T ratio. While several studies in rodents have already demonstrated that hepatic NK cells are more cytotoxic than the same cell type in blood or spleen [40-42], few studies describe the cytotoxic properties of human liver NK cells [22, 43]. The group of O'Farrelly reported that NK cells isolated from biopsy specimens from normal human liver tissue were able to kill K562 target cells but that high E:T ratios were necessary – clear cytotoxicity was distinguishable from a 50:1 E:T ratio – [22]. This difference from our results may be attributed to different methods used for quantification of cytotoxicity, i.e. the conventional Chromium-release assay, which is known to be less sensitive compared to the flow cytometric technique used in the present study. Similarly the group of Asahara showed that IL-2 activated human liver NK cells were more cytotoxic against an hepatocellular carcinoma cell line compared with blood NK cells; however purified and unstimulated liver NK cells were not tested in these experiments [43]. Our results demonstrate that freshly isolated NK cells from human liver kill target cells more efficiently compared to their counterparts in blood, without the need of prior activation *in vitro*. While in blood the CD56^{bright} subset of NK cells is mainly considered as cytokine producing [29, 30, 44-46], we here demonstrated that in liver this same subtype shows clear cytotoxic degranulation, as measured by detection of CD107a on their surface, in response to target cells. Additionally, intracellular staining of the three most common cytolytic enzymes – perforin, granzymes – showed a higher proportion of hepatic CD56^{bright}

cells containing these molecules compared to the same subtype in blood. In addition, liver NK cells secreted IFN-γ but not IL-10 nor IL-13 upon stimulation with monokines.
Hepatic NK cells differ also from NK cells resident in lymph nodes [33]. Although an enrichment of the $CD56^{bright}$ subset has been described also for lymph nodes, these cells show a resting immunophenotype (CD69negative), do not express the NCRs NKp30 and NKp44, are perforin negative and are unable to kill target cells; furthermore IL-2 stimulation is required for induction of perforin expression and cytolytic activity [33].

By LTX liver-resident leukocytes are intrinsically transferred from the donor into the recipient, leading to the establishment of a variable grade of chimerism that may positively affect the induction of graft tolerance [6, 7, 34, 47, 48]. In this study we show that, in addition to T cells, B cells and MDC [7, 16, 19, 34], donor-derived NK and $CD56^+$ T cells migrate into the recipient circulation. Although previous studies have already shown that $CD56^+$ cells [7] and $CD16^+$ cells [34] are transferred into recipients after LTX, the authors did not discriminate between NK and $CD56^+$ T cells. Our data show that all LTX recipients are chimeric in NK and $CD56^+$ T cell lineages for about two weeks after transplantation. In contrast to NK cells detaching from liver grafts during pre-transplantation perfusion, the majority of donor-derived NK cells that mix with the recirculating pool of recipient NK cells belonged to the $CD56^{dim}$ subtype. This discrepancy can be explained by two distinct hypotheses: first, the $CD56^{bright}$ subset may be preferentially retained within the liver sinusoids by adhesion molecules, such as CD11a, which is highly expressed on this hepatic subset (Table 2). Alternatively hepatic $CD56^{bright}$ NK cells, which are highly activated and have a higher expression of the α4/β7 integrin, rapidly relocate from the recipient circulation to other compartments, such as gut-associated lymphoid tissue, thus determining their absence from the bloodstream as early as few hours after LTX [49].

The fact that donor lymphocyte chimerism can be more or less prominent among different patients is influenced by a number of factors [34]. The total number of cells released from the liver, the rate of clearance of the allogeneic cells and their migration to other tissues are all aspects that can play a significant role in this context. A previous study excluded that allogeneic lymphocytes migrating in recipient circulation after LTX are derived from blood transfusions administered during

the transplantation procedure, thus confirming that leukocyte chimerism after LTX is due to the intrinsic transfer of such cells by liver transplantation procedure [7].

Analysis of selectin, integrin and chemokine receptor expression on perfusate and migrating donor NK cells gave few indications of where these cells may be homing after detaching from the donor liver. No detectable expression was observed for three chemokine receptors, CCR1, CCR5 and CXCR3, involved in lymphocyte homing into inflamed sites [50-52]. While CXCR4, the receptor for homing to bone marrow [53, 54], was detectable at low levels on perfusate NK cells, donor-derived NK cells in the recipient circulation were CXCR4negative, suggesting that these cells are not homing to bone marrow. CCR7 and L-selectin (CD62L) were used to test the potential migration properties to lymph nodes. While CCR7 is a receptor responding to CCL19 (MIP-3-beta) and CCL21, CD62L can bind to addressins on high endothelial venules to facilitate extravasation of lymphocytes from the circulation. Notably, while expression of both molecules was low on perfusate NK cells, we detected a significantly higher expression of CD62L on donor NK cells circulating in the recipient, but CCR7 remained low, suggesting that migrating donor NK cells have limited potential to enter recipient lymph nodes.

Under normal conditions expression of the ligands for CCR9 (CCL25) and for α4/β7 (MAdCAM-1) is restricted to the gut [55, 56]. Therefore, the minority of migrating donor NK cells that co-express CCR9 together with the integrin α4/β7 may selectively home into gut-associated lymphoid tissue [55, 56]. Interestingly, homing of donor lymphocytes to gut-associated lymphoid tissue after LTX was recently observed in an animal model [49].

Finally, while in animal models donor NK cells have been found to migrate into the host spleen after LTX [57], in humans the mechanism of leukocyte homing to spleen is not yet completely unraveled. While localization of lymphocytes in the red pulp of the spleen has been described as a passive process [58], migration into the white pulp is independent of interactions with high endothelial venules but rather involves the expression of MAdCAM-1 by the sinus-lining cells [59]. Therefore, the 40-50% of migrating donor NK cells that expressed the α4/β7 integrin but did not co-express the gut-homing receptor CCR9, may be able to enter the white pulp of the recipient spleen.

Although the functional role of donor lymphocytes transferred into the recipient is yet not clear, most of the studies indicate that they have a protective role against rejection of the transplanted liver. This hypothesis is clearly supported by numerous studies in animal models in which spontaneous tolerance to liver grafts, which is observed in a number of fully allogeneic rat strains combinations, can be abrogated by reduction of passenger leukocytes [2, 12, 13]. Mechanisms proposed to explain the immunoprotective role of liver grafts include the induction of apoptosis of recipient T cells within the graft [60, 61] or in recipient lymphoid organs [2, 14]. We propose that among transferred donor-derived leukocytes, hepatic NK cells in particular may be instrumental in inducing apoptosis of alloreactive T cells of the recipient. Indeed, NK cells have been shown to kill allogeneic MDC *in vivo* in experimental transplantation models [23, 24, 62]. Upon experimental allogeneic bone marrow transplantation donor-derived NK cells protect against graft rejection by killing recipient T cells and antigen presenting cells [26]. We propose that a similar mechanism may be effective after LTX. Unfortunately, with current techniques it is not possible to detect killing of allogeneic leukocytes by freshly isolated NK cells *in vitro* [26]. To study NK alloreactivity *ex vivo*, prolonged activation in the presence of appropriately mismatched allogeneic feeder cells is needed [63-66], or use of NK-cell clones [26, 67-69]. Similarly, only *in vitro* activated, but not resting, NK cells are able to kill monocyte-derived immature DC *in vitro* [70]. Demonstrating cytotoxicity of *ex vivo* cultured or cloned liver NK cells against allogeneic leukocyte targets does not answer the question whether donor derived liver NK cells can kill recipient leukocytes *in vivo*. This question can therefore only be answered in an appropriate experimental animal liver transplantation model, which is beyond the scope of the present study.

Taken together, our data show that LTX in humans results in transfer of potentially cytotoxic, mainly $CD56^{bright}$, NK cells of donor origin into the recipient. In addition $CD56^{dim}$ NK cells from the donor mix with the recirculating pool of recipient NK cells for approximately two weeks after LTX. Upon encounter with the recipient immune system, either within the graft or in recipient tissues, these NK cells may inhibit the recipient's response to the liver graft by killing recipient MDC or T cells. By analyzing associations between rejection-free liver graft survival and KIR – KIR ligand mismatching in donor to recipient direction, the influence of donor NK cells on the recipient's alloresponse in human LTX may be elucidated.

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Chapter 3

Donor and recipient HLA/KIR genotypes do not predict liver transplantation outcome

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ABSTRACT

Whether NK cells affect the immune response to solid organ allografts is still controversial. Main determinants of NK-cell activation are specific HLA/KIR interactions that, in transplantation, may induce NK-cell alloreactivity. So far, in liver transplantation (LTX) donor-versus-recipient alloreactivity has not been investigated; additionally, studies of predicted recipient-versus-donor NK-cell alloreactivity have led to contradicting results. We typed a cohort of LTX donors and recipients for HLA-C/Bw4 and KIRs. We estimated the effect of NK-cell alloreactivity, as predicted by classically used models, in the donor-versus-recipient direction. The results indicate that HLA/KIR mismatches in the donor-versus-recipient direction do not predict graft rejection nor graft or patient survival, suggesting that donor-derived NK cells do not play a major role in LTX outcome. Additionally, when considering predicted NK-cell alloreactivity in the reverse direction (recipient-versus-donor), we first confirmed that donor HLA-C genotype was not associated with acute rejection, graft or patient survival, and secondly we found that none of the models describing NK-cell alloreactivity could predict LTX outcome. Overall our observations suggest that, in contrast to what shown in hematopoietic stem cell transplantation, donor-derived NK cells may not contribute in preventing liver graft rejection, and that recipient-versus-donor NK-cell alloreactivity does not predict LTX outcome.

INTRODUCTION

The role of the adaptive immune response in organ transplantation has been clearly established [1, 2], however the effects of the innate response are far less clear. Natural killer (NK) cells are part of the innate immunity and can distinguish between self and non-self tissues by use of a variety of receptors that recognize specific alleles of MHC class I molecules expressed on cell surfaces. The main receptors involved in self-recognition are the killer-cell immunoglobulin-like receptors (KIRs). By binding specific MHC class I alleles on target cells (Table 1 in reference [3]), KIRs constitute an effective tool detecting both transformed and virally-infected cells as well as recognizing "missing self" in transplantation settings. The most relevant MHC class I alleles linked to NK-cell activity are HLA-A3 and -A11, HLA-Bw4, and HLA-C [3]. HLA-C has recently raised interest as a possible factor involved in prediction of transplantation outcome. Importantly, all of the >250 alleles of HLA-C are recognized by KIRs and can be divided into one of two groups (HLA-C1 and HLA-C2) with regard to their ability to bind KIRs [3].

Results from animal models did not indicate a role for NK cells in solid organ transplantation [4-6]. Nevertheless, recent studies introduced the novel concept that recipient NK cells participate in both acute and chronic rejection after solid organ transplantation by modulating the host immune response rather than directly affecting the transplanted organ [6-11]. Importantly, a new perspective in the analysis of the role of NK cells in transplantation has been introduced by studies in hematopoietic stem cell transplantation (HSCT) [3, 12-14]. In this context, donor-versus-recipient NK-cell alloreactivity prevents graft rejection besides inducing additional beneficial effects for the recipient [12]. Similarly to what observed in HSCT we hypothesized that donor NK cells may have a protective role in liver transplantation (LTX) on the basis of two main observations. First, donor-versus-recipient alloreactivity has been occasionally observed in the context of LTX, since cases of graft-versus-host disease have been recorded [15, 16]. Secondly, a previous publication from our group [17] has shown that highly cytotoxic donor NK cells derived from the graft are consistently transferred into the recipient upon LTX.

With regard to the effect of recipient-versus-donor NK-cell alloreactivity only few studies have addressed this issue in solid organ transplantation in humans [18, 19]. In kidney transplantation missing HLA-C or HLA-Bw4 KIR-ligands in the recipient-versus-donor direction has no impact on either acute rejection [20, 21] or graft survival [22]. Importantly, contradictory results have been published so far for LTX. In one study, HLA-C disparity between recipient and donor was found to be correlated with a higher risk of acute rejection of the liver graft [23], but this was not confirmed by other groups [24-26]. Moreover, while Hanvesakul et al. [24] provided evidence that the presence of HLA-C2 in the donor was associated with improved long-term graft and patient survival after LTX, Tran et al. [27] found no impact of donor HLA-C2 genotype on 10-years graft or patient survival. Therefore, the effect of HLA/KIR genotypes and recipient NK cells on liver graft rejection and survival is still an unresolved issue.

In the present study we explored, for the first time, the effect of donor-versus-recipient NK-cell alloreactivity on LTX outcome. For this purpose we used common models describing NK-cell alloreactivity to estimate the effects of donor NK cells on acute rejection, graft and patient survival. In addition, by analyzing our study cohort in terms of recipient-versus-donor alloreactivity, we aimed at adding new evidence to the existing data on the effects of HLA/KIR matching on LTX outcome.

PATIENTS AND METHODS

Patients

This study includes 348 liver transplantations performed at the Erasmus Medical Center in Rotterdam (the Netherlands) between 1987 and 2008. All LTX were first grafts, while re-transplantations or multi-organ transplantations were excluded. Only patients with graft survival of >7 days were included. From 322 paired donors and recipients HLA-Bw4 typing was available. From 260 pairs DNA of both donor and recipient was archived, and used for typing of HLA-C. A smaller cohort of 153 donor/recipient pairs was typed for the KIR genes (Figure 1). To exclude that partial typing of the cohort had determined a selective exclusion of donors or recipients we compared the baseline characteristics of the groups with complete or non-complete typing and verified that they did not differ in terms of age and gender. The Ethical Committee of the Erasmus MC approved the study.

Clinical data

All clinical data considered as endpoints (e.g. graft failure, acute/chronic rejection, biliary complications) were re-evaluated for each case by an experienced transplant hepatologist (HJM). Acute rejection was defined as an episode with increased liver enzymes together with histopathological evidence (Rejection Activity Index-score ≥5) combined with a biochemical response to the treatment by high dose corticosteroids or another change in immunosuppressive treatment. Graft loss was defined as graft-related patient death or graft failure requiring re-transplantation. For the analysis of patient survival, patient loss was defined as patient death for any cause. Ischemic bile duct damage was defined as the development of diffuse intra- and/or extra-hepatic biliary strictures and dilatations in the presence of normal hepatic arterial circulation and in the absence of other diagnosis such as recurrence of primary sclerosing cholangitis. Chronic rejection was defined as deteriorating liver graft functions accompanied by loss of small bile ducts in 50% or more of the portal tracts in sequential needle biopsy specimens. Standard immunosuppression included a combination of calcineurin inhibitors (either cyclosporine or tacrolimus) and steroids, supplemented with either azathioprine or induction therapy with an anti-IL-2 receptor blocking antibody.

HLA and KIR genotyping

The presence of HLA-Bw4 was deducted from the serological typing routinely performed before LTX. For HLA-C and KIR genotyping, DNA was extracted by the classical salting out method. Molecular typing for HLA-C was performed by polymerase chain reaction with sequence-specific oligonucleotide probes using LABType® SSO C Locus kit (One-Lambda, Canoga Park, USA) according to ASHI standards. Differentiation into HLA-C1/C2 was made on the basis of Lysine or Asparagine at position 80 of HLA-C. Inhibitory and activating KIRs (14 in total; Figure 1) were typed in donor and recipient samples using sequence specific primers according to the protocol previously described [28].

Models predicting NK-cell alloreactivity

Predictions of NK-cell alloreactivity were initially performed on basis of two main models: "missing self" and "missing ligand" model. The definition of the "missing self" model was based on two recent studies [22, 29]. Briefly, for prediction of NK-cell alloreactivity in donor-versus-recipient direction, donor/recipient combinations were divided into 3 groups: (i) "C1/2–Bw4 matched" group, if donor and recipient shared the same HLA-C1, C2 and Bw4 epitopes; (ii) "C1/2–Bw4 mismatched compatible" group, if donor and recipient were mismatched for C1, C2 or Bw4 epitopes, but the donor's KIR ligands were not missing in the recipient's HLA genotype; (iii) "C1/2–Bw4 mismatched incompatible" group, if the recipient's HLA genotype did not include a C1, C2 or Bw4 epitope that was present in the donor.

The "missing ligand" model in donor-versus-recipient direction states that for each inhibitory KIR expressed in the donor, its ligand needs to be present in the recipient so as to avoid NK-cell alloreactivity. Missing ligand combinations were defined positive when the recipient was missing at least one of the KIR ligands for which the donor had a KIR [13, 14]. Following previous observations [30-32], "unlicensed NK cells", meaning donor NK cells that possess a certain KIR for which the corresponding ligand is not present in the donor genotype, were considered as potentially able to be activated by the absence of their ligand in the recipient.

In addition to the previous analysis, we considered two other models, both named "strength of inhibition": the first including only inhibitory KIRs and the second including both inhibitory and activating KIRs. As the activation of NK cells is known to be the net result of signals from activating and inhibitory KIRs we included the donor KIR-

gene repertoire as to predict NK-cell alloreactivity. As for the inhibitory KIRs, there is a hierarchy in the strength of inhibition that defines how the combination of KIR2DL1 with HLA-C2 leads to the strongest NK-cell inhibition, whereas KIR2DL2 with HLA-C1 gives an intermediate inhibition and KIR2DL3 with HLA-C1 confers the least inhibition [20], [33]. To perform this analysis (strength of NK inhibition, predicted by donor inhibitory KIRs and recipient HLA) we classified donor/recipient pairs according to the three abovementioned categories of strength of inhibition and we determined their association with graft survival, patient survival and acute rejection. Next, in the final model we aimed at testing the combined effect of inhibitory and activating donor KIRs, and recipient HLA genotype, on LTX outcome. With regard to the activating receptors, two groups of KIR-haplotypes can be distinguished: while most stimulatory KIR genes are present in the haplotype B, the haplotype A contains only one stimulatory receptor (KIR2DS4) [34]. Accordingly, individuals carrying the AA KIR genotype will exhibit a lower NK-cell activation originating from activating KIRs compared to individuals with the AB or BB KIR genotypes [18, 35]. To perform the analysis of the combined effect of inhibitory and activating KIRs we categorized the donor/recipient pairs on basis of the combination of their strength of inhibition (determined by inhibitory KIRs) and the KIR haplotype (determined by activating KIRs). Donor/recipient pairs were therefore divided in six possible combinations, correspondent to different degrees of inhibition, e.g. in case of donor-versus-recipient alloreactivity, the combination with the strongest inhibition was given by an homozygous HLA-C2/C2 recipient, with a donor possessing KIR2DL1 and AA haplotype.

Statistical analysis

Survival data were analyzed using the Kaplan-Meier method and the log-rank test. Multivariate Cox regression was used to verify that single factors were independently associated with graft or patient survival. Crosstabs were used for all correlations with acute rejection and were tested by use of the Pearson Chi-Square test. Probability (p) values of less than 0.05 were considered significant. All statistical analyses were performed using SPSS.

RESULTS

Donor-versus-recipient NK-cell alloreactivity, as predicted by the missing self model, does not affect LTX outcome

Patients' demographics and their associations with graft survival, patient survival and acute rejection are reported in Table 1A, while causes of graft and patient loss are listed in Table 1B and 1C.

We here explored the effect of hepatic donor NK-cell alloreactivity on three LTX outcomes: acute rejection, graft failure and graft survival. For this purpose we estimated the level of donor NK-cell activation as predicted by a number of classically used models. We first analyzed our study cohort by using the "missing self" model, for both HLA-C and -Bw4, to predict donor-versus-recipient NK-cell alloreactivity. The analysis was performed by stratifying donor/recipient pairs into three groups (see Patients and Methods). No differences in terms of graft survival (p=0.13), patient survival (p=0.13) or incidence of acute rejection (p=0.71) were found among the three groups (Table 2A). The two groups "C1/2-Bw4 matched" and "C1/2-Bw4 mismatched compatible" represent all cases of KIR-ligand compatible transplants, while the "C1/2–Bw4 mismatched incompatible" group identifies the KIR-ligand incompatible transplants. This additional classification showed that "missing self" in donor to recipient direction had no effect on LTX outcome compared to compatible donor/recipient pairs (Table 2A).

Likewise, analysis of the "missing self" model by considering donor-versus-recipient disparities only in the HLA-C epitope or the HLA-Bw4 molecule did not show any effect on LTX outcome (Table 2A).

Table 1. Demographics and relevant genetic characteristics of the study population (A); causes of graft (B) and patient (C) loss.

A

	n=348	**Graft survival[a]**	**Patient survival[b]**	**Acute rejection**
Number of events		*graft loss=41/348*	*patient loss=71/348*	*acute rejection=81/348*
Characteristics	*Number (range or percentage)*	*p-Value[c]*	*p-Value[c]*	*p-Value[c]*
Recipient age	47.1 (16-69)	**<0.001**	**<0.001**	
Recipient gender M:F	203:145	0.53	0.16	**0.02**
Donor age	41.7 (11-72)	**0.009**	0.83	
Donor gender M:F	170:178	0.26	0.46	0.69
Ethnicity *Caucasian* *Black* *Asian*	 300 (86.2%) 29 (8.3%) 19 (5.5%)	0.11	0.12	0.08
Gender mismatch *no mismatch:mismatch*	191:157	**0.04**	0.21	0.16
Diagnosis *Viral hepatitis* *Auto-immune etiology[d]* *Alcohol abuse* *Acute fulminant hepatitis* *All other causes*	 86 (24.7%) 100 (28.7%) 39 (11.2%) 27 (7.8%) 96 (27.6%)	0.16	0.45	**<0.001**

[a] Median graft survival time was of 7.4±5.2 years (range 0–22.1)

[b] Median patient survival time was of 7.8±5.0 years (range 0–22.1)

[c] P-values indicate a univariate association of the specific factor with graft survival, patient survival or acute rejection

[d] Auto-immune etiology includes: primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and auto-immune hepatitis (AIH)

B

Causes of graft loss (graft-related death or re-LTX) (n=41)	n (%)
Ischemic type biliary lesions	20 (48.8%)
Chronic rejection	8 (19.5%)
Recurrence of original disease (HCV, PSC, AIH)	8 (19.5%)
Vascular complications	4 (9.8%)
Recurrence of HCC	1 (2.4%)

C

Causes of patient loss (n=71)	n (%)
Not graft related	40 (56.4%)
Ischemic type biliary lesions	2 (2.8%)
Chronic rejection	4 (5.6%)
Recurrence of disease (HCV, PSC, AIH)	5 (7.0%)
Vascular complications	1 (1.4%)
Recurrence of HCC	9 (12.7%)
De novo tumor	10 (14.1%)

re-LTX = re-liver transplantation; HCV = hepatitis C virus; PSC = primary sclerosing cholangitis; AIH = auto-immune hepatitis; HCC = hepatocellular carcinoma

LTX outcome is not influenced by KIR-ligand mismatching or donor KIR gene repertoire

For this study we genotyped 153 donor/recipient pairs for 14 different KIR genes. The KIR gene distribution in both donors and recipients is depicted in Figure 1 and did not differ from the frequencies observed in healthy populations [21, 36]. Analysis of our cohort by use of the "missing ligand" model (see Patients and Methods) revealed that missing a KIR-ligand in the donor-versus-recipient direction had no effect on LTX outcome (Table 2A). In addition, analysis of potential donor-versus-recipient NK-cell alloreactivity in terms of hierarchy in strength of inhibition (see Patients and Methods) showed no significant association either with graft or patient survival or with acute rejection (Table 2A). Inclusion of the donors' KIR haplotype (accounting for the number of activating KIRs in the donor genotype) in this model did not result in any significant association with LTX outcome (Table 2A). Altogether our results indicate that none of the current models describing NK-cell alloreactivity in the donor-versus-recipient direction could predict LTX outcome.

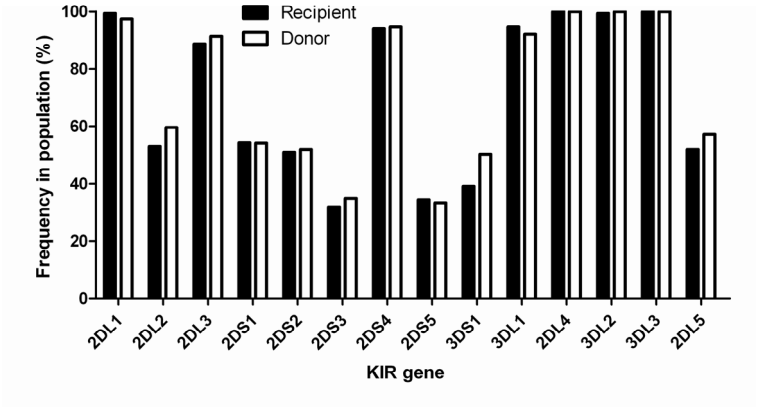

Figure 1. KIR gene distribution in our cohort of liver transplant patients. KIR genotyping was performed on 153 recipients (black bars) and their paired donors (white bars). The KIR genes are indicated on the *x*-axis while the *y*-axis reports the frequencies for each donor and recipient KIR.

Recipient-versus-donor NK-cell alloreactivity

We here considered the most classical perspective for predicting LTX outcome: NK-cell alloreactivity in the recipient-versus-donor direction. For this purpose we applied the same set of models outlined above to explore potential NK-cell alloreactivity in the recipient-versus-donor direction. The majority of the models did not show an association with acute rejection, graft or patient survival (Table 2A), suggesting the absence of a major role of recipient-versus-donor NK-cell alloreactivity in LTX outcome. However, analysis of the "missing self" model by considering the single HLA-Bw4 molecule was significantly associated with better graft survival in univariate analysis (p=0.01; Table 2A). Nevertheless, the effect was completely abrogated in multivariate analysis (p=0.69; Table 2B) when considering all factors univariately associated with graft survival (see next paragraph).

Table 2. p-Values of the univariate analyses performed considering the most relevant models predicting NK-cell alloreactivity (A) and of Cox regression multivariate analysis of all factors univariately associated with liver transplantation outcomes (B).

A

	DONOR versus RECIPIENT reactivity			REC versus DONOR reactivity		
	Graft survival	**Patient survival**	**Acute rejection**	**Graft survival**	**Patient survival**	**Acute rejection**
Model	*p-Value*	*p-Value*	*p-Value*	*p-Value*	*p-Value*	*p-Value*
MISSING SELF HLA-C and -Bw4 (3 categories)	0.13	0.13	0.71	0.69	0.86	0.49
MISSING SELF – compatible LTX vs incompatible LTX[a]	0.21	0.13	0.61	0.42	0.58	0.28
MISSING SELF – only HLA-C	0.65	0.54	0.87	0.97	0.64	0.97
MISSING SELF – only HLA-Bw4	0.85	0.46	0.85	**0.01**	0.69	0.51
MISSING LIGAND	0.48	0.12	0.79	0.11	0.76	0.75
STRENGTH of NK INHIBITION (inhibitory KIRs)	0.47	0.97	0.51	0.75	0.49	0.61
STRENGTH of NK INHIBITION (inhibitory and activating KIRs)	0.57	0.49	0.58	0.49	0.21	0.79
Presence of DONOR HLA-C2	0.48	0.64	0.61	0.06	0.18	0.66
DONOR HLA-C genotype	0.63	0.88	0.26	**0.05**	**0.01**	0.79

Statistically significant associations are indicated in bold. Associations with graft or patient survival were analyzed by using the Log-rank test. Associations with acute rejection were performed using the Pearson Chi-Square test.

[a] Compatible LTX vs incompatible LTX= "C1/2-Bw4 matched" and "C1/2-Bw4 mismatched compatible" versus "C1/2-Bw4 mismatched incompatible".

B

	Multivariate analysis for all factors univariately associated with graft survival		
	Significance	**Hazard ratio**	**95% CI**
Recipient age (for a 10-year increase in age)	**0.01**	0.97	0.94–0.99
Donor age (for a 10-year increase in age)	**0.02**	1.03	1.00–1.06
Gender mismatch	**0.05**	0.45	0.21–0.99
Missing HLA-Bw4 (REC vs DON)[a]	0.96	0.00	0.00–8.8E+254
Donor HLA-C genotype	0.75	1.08	0.67–1.73

	Multivariate analysis for all factors univariately associated with patient survival		
	Significance	**Hazard ratio**	**95% CI**
Recipient age (for a 10-year increase in age)	**0.03**	1.03	1.00–1.06
Donor HLA-C genotype	0.07	1.48	0.96–2.26

Statistically significant associations are indicated in bold.
[a] Compared with "non-missing HLA-Bw4" donor/recipient pairs

Effect of donor HLA-C genotype on graft and patient survival

We then completed our study by analyzing the possible association between LTX outcome and donor HLA-C genotype (homozygous C1C1: 102 (39.2%); heterozygous C1C2: 124 (47.7%); homozygous C2C2: 34 (13.1%)). In our cohort, the donor HLA-C genotype (p=0.05) or the presence of HLA-C2 in the donor (p=0.06) were not associated with improved graft survival (Figure 2A, Table 2A). More specifically, the 10-year graft survival was 79.4±3.9% in the presence and 90.7±3.0% in the absence of the donor HLA-C2 allele (p=0.06). Comparing graft survival rates between the three possible donor HLA-C genotypes revealed a trend towards a gene dose effect (overall log rank p-value=0.05). HLA-C2 homozygous donors led to the lowest graft survival rate (HLA-C2 versus HLA-C1 homozygous donors, p=0.02), whereas transplants from HLA-C1C2 heterozygous donors showed an intermediate rate of graft loss. However, in multivariate analysis this effect was abrogated and only recipient age, donor age and gender mismatch were associated with graft failure (Table 2B).

In univariate analysis, donor HLA-C genotype was significantly associated with patient survival (p=0.01) (Table 2A); conversely, the presence of HLA-C2 in the donor did not affect patient survival (p=0.18) (Figure 1B, Table 2A). Homozygous HLA-C2 donors showed the lowest patient survival rate (HLA-C2 versus HLA-C1 homozygous donors, p=0.007; homozygous HLA-C2 versus HLA-C1C2 donors, p=0.01). However, in multivariate Cox regression analysis recipient age abrogated the effect of donor HLA-C genotype on patient survival (Table 2B). To eliminate a possible bias related to the inclusion of multiple ethnicities we repeated the analysis limiting our study group to Caucasian patients typed for HLA-C (n=224). The results indicated a trend similar to the one described for the total cohort (Figure 2C, D).

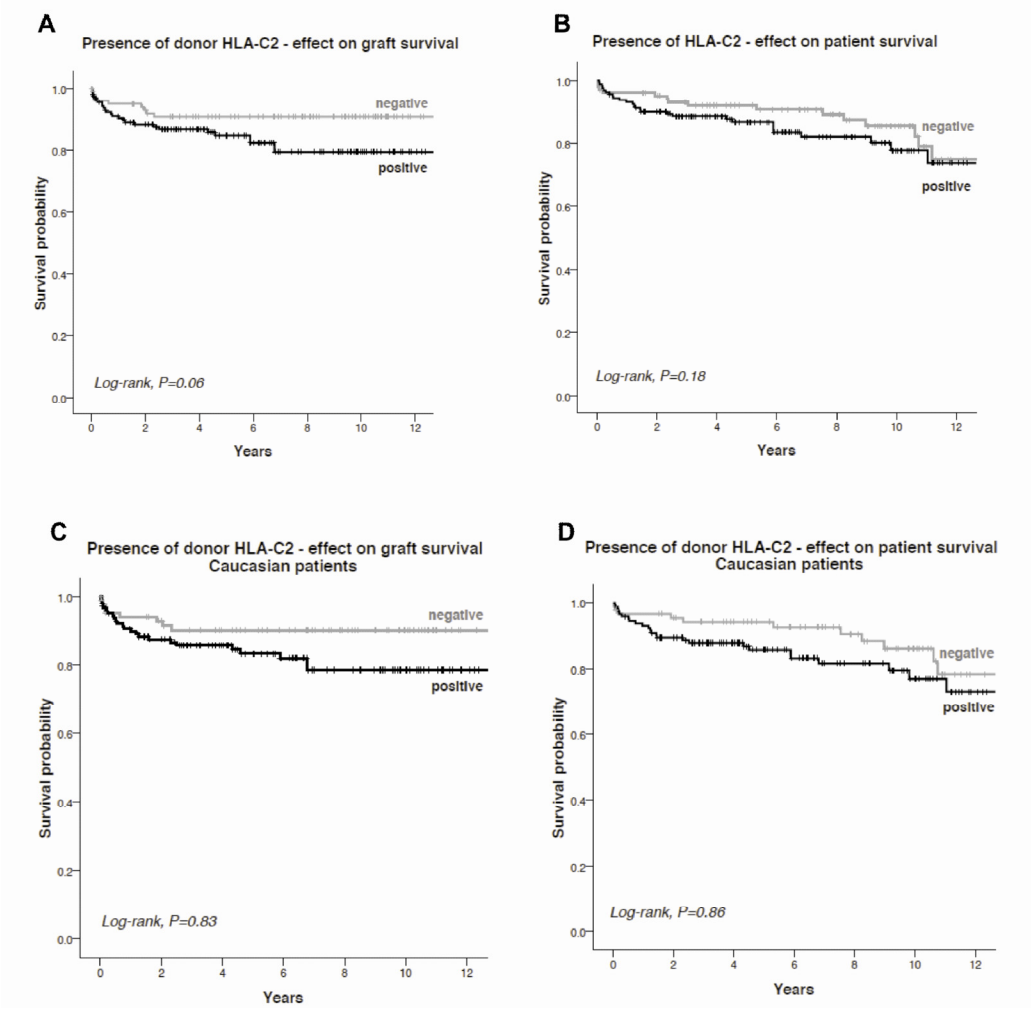

Figure 2. Effect of donor HLA-C2 on graft and patient survival after liver transplantation. Kaplan-Meier survival curves of graft **(A, C)** and patient **(B, D)** survival after liver transplantation on basis of the presence (positive) or absence (negative) of HLA-C2 in the donor genotype. **A, B**: complete cohort. **C, D**: Caucasian patients only.

Donor HLA-C genotype does not correlate with the type of graft injury nor with acute graft rejection

Here we analyzed the association between the presence of donor HLA-C2 and graft injury. Specifically, we tested the effect of the donor genotype on chronic rejection and biliary complications. Our results suggest that donor HLA-C2 may correlate (although not reaching statistical significance) with an increased incidence of chronic rejection (Figure 3A). No clear effect of donor HLA-C2 was observed on

biliary complications (Figure 3B). Finally, the presence of HLA-C2 in the donor did not influence the incidence of acute rejection (Table 2A).

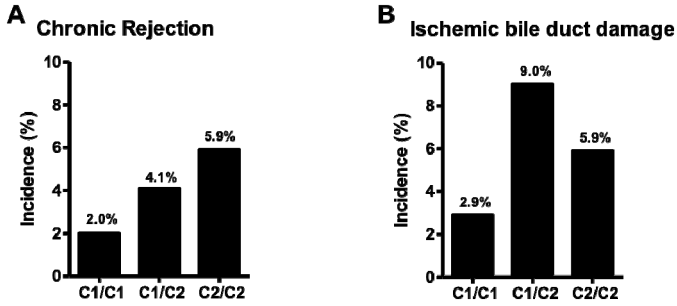

Figure 3. Impact of donor HLA-C genotype on causes of histological features of chronic injury. All cases of chronic rejection **(A)** and ischemic bile duct damage **(B)** (not only associated with graft loss) are grouped on basis of the donor genotype to evaluate a possible effect on hepatic injury. Donor HLA-C genotype is not significantly associated with incidence of chronic rejection (p=0.49) or ischemic bile duct damage (p=0.17). However the trend suggests a negative effect of donor HLA-C2 on chronic rejection (C1/C1, n=2/102; C1/C2, n=5/122; C2/C2, n=2/34), whereas no effect could be evidenced on ischemic bile duct damage (C1/C1, n=3/102; C1/C2, n=11/122; C2/C2, n=2/34; for 4 patients, diagnosed with ischemic bile duct damage, donor HLA-C typing was not available). Statistical analysis was performed using Pearson Chi-Square test.

DISCUSSION

Our results indicate that HLA/KIR mismatches between donor and recipient do not predict LTX outcome. None of the models here used to predict NK-cell alloreactivity has indicated a prominent role of donor or recipient NK cells in graft rejection, graft or patient survival. In addition, our results show that the possession of HLA-C2 by the donor does not influence graft survival, patient survival or acute rejection, confirming what reported by Tran and colleagues [27].

Donor-versus-recipient NK-cell alloreactivity has been demonstrated for the first time in HSCT [3, 12-14]. In this context the authors have shown that donor NK-cell alloreactivity could prevent leukemia relapse and graft rejection and protect patients against graft-versus-host disease [12]. The existence of a donor-versus-recipient alloreactivity was also occasionally observed in the context of LTX, since cases of graft-versus-host disease have been recorded [15, 16]. However the specific cell types contributing to the onset of this alloreaction have not been clarified. A previous publication from our group has introduced the hypothesis that, similarly to HSCT, a tolerogenic effect due to donor NK-cell alloreactivity may also apply to LTX [17]. This concept was supported by the observation that highly cytotoxic graft-derived NK cells are transferred into the recipient upon LTX [17]. Seen the universally recognized tolerogenic effects of liver grafts we hypothesized that, similarly to HSCT, the large number of donor NK cells transferred into the recipient may be an important factor contributing to graft acceptance by specifically targeting the recipient antigen presenting cells and alloreactive T cells. Here we report that the classical models used to predict NK-cell alloreactivity did not indicate a prominent role for donor NK cells in the outcome of LTX.

The role of donor and recipient HLA/KIR interactions in recipient-versus-donor alloreactivity is poorly defined in solid organ transplantation due to conflicting data from clinical and *in vitro* studies as well as animal models [18, 37-40]. In the context of LTX, conflicting data have been reported. Two recent publications [24, 27] reported largely contradicting results on the effects of HLA-C on LTX outcome: while Hanvesakul and colleagues [24] showed improved graft and patient survival when the donor possessed at least one HLA-C2 allele, Tran and colleagues [27] found that

the same donor allele had no impact on either graft or patient survival. Additionally, contradicting results were published with regard to the effect of NK-cell alloreactivity on liver graft rejection [23-26]. Here we substantially add to this discussion by showing that, in our cohort, the possession of HLA-C2 by the donor does not independently affect graft survival, patient survival or acute rejection, confirming what reported by Tran and colleagues.

A few differences between the three studies can be enumerated for a better comparison of the outcomes. As the group of Hanvesakul [24], we also considered patients from one single center, while the cohort analyzed by Tran [27] was multicenter. An additional difference refers to patients' ethnicity: the cohort analyzed by Tran included only Caucasians patients, whereas the original study from Hanvesakul was unclear on this aspect, but most probably analyzed a multiethnic cohort. Our cohort was composed of multiple ethnicities (Table 1A) and restriction of our study group to Caucasian patients did not change the results as concerning the effect of donor HLA-C2 on LTX outcome (Figure 2). Finally, while the study from Hanvesakul excluded patients with graft survival of <30 days, Tran included all patients available, both primary LTX and re-LTX procedures (which represented 6.4% of the whole cohort). For our study we included only patients with primary LTX and graft survival of >7 days, since graft loss in the first week is, in general, mostly due to surgical complications and less related to immunological factors. The decision of excluding re-LTX cases from our cohort was based on the evidence that re-transplanted patients, having a significantly worse survival than primary transplants [41, 42], may add a confounding effect to the outcome of the analysis.

Further analyses were performed to estimate the association of NK-cell alloreactivity, as predicted by accepted models such as "missing self" or "missing ligand", with LTX outcome. While "missing self" considering both HLA-C and -Bw4 (or each single epitope considered alone) did not significantly associate with LTX outcome, we initially observed a univariate association with better graft survival when the donor missed the HLA-Bw4 allele present in the recipient's genotype. This trend, however, was completely abrogated in multivariate analysis. Our observation that missing-self considering HLA-C alone is not associated with acute rejection after LTX confirms similar results from three other groups [24-26].

Several levels of complication are intrinsically characterizing NK-cell biology and are still not completely elucidated: the licensing process of NK-cell education [43-45]; the dynamics determining that a certain KIR present at the DNA level is effectively expressed and functional at the cell surface [46-48]; the kinetics determining the stronger/weaker affinity of each inhibitory KIR compared to its activating counterpart [49]; the hierarchy determining the strength of inhibition of different HLA alleles [33, 50-52]. Additionally, the attempt to investigate the effects of NK-cell alloreactivity on LTX outcome is further complicated by the variation of a number of clinical parameters that differ among patients, such as the underlying disease and the immunosuppressive treatment. Different underlying diseases may affect *a priori* the functionality of recipient NK cells and influence the basal functional competence of NK cells [53-55]. On the other hand, post-LTX immunosuppressive treatment may have an effect on NK cell function. In this sense, although immunosuppressive drugs appear to have a modest effect on NK cells [6, 19], few studies have reported an altered function of NK cells as a consequence of various immunosuppressants [56]. Lastly, interpreting correlations between HLA genotype and transplantation outcome is complicated by the existence of additional factors, such as viral infections, that *in vivo* can mask the direct effects of the sole genotype. Increasing evidence is indeed showing how these factors can either generate alloreactivity even when not predicted by HLA disparities [57, 58], or can promote immune regulation even in an unfavourable HLA mismatched environment [59, 60]. However, since current experimental techniques do not allow direct quantification of alloreactivity of bulk NK cells [12, 13], models that predict NK-cell alloreactivity on basis of KIR and HLA genotypes are presently the best approach available.

Overall, based on the current models of NK-cell alloreactivity, we could not find an association between predicted NK-cell alloreactivity and LTX outcome in both donor-versus-recipient and recipient-versus-donor direction. We support the concept that our current understanding of the biological mechanisms underlying NK-cell alloreactivity is still not complete and does not allow for the definite identification of their role, if any, in solid organ transplantation and LTX in particular. Ultimately, as already proposed by Tran and the editorial accompanying their article [61], this field remains quite "unresolved" and only further and more clinical/biological-integrated research will possibly lead to more clear indications.

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Chapter 4

No evidence for involvement of donor NK cells in liver transplant tolerance

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To the Editor:

We would like to report some new insights into the role of donor leukocytes in liver transplantation (LTX). Clinical LTX is known to be unique in the context of solid organ transplantation in that the incidence of chronic rejection is much lower compared to other organ grafts [1]. Approximately 20% of stable recipients can be weaned from immunosuppressive medication without developing graft rejection [2]. Rat models have shown that liver allografts are accepted without immunosuppression in many fully major histocompatibility complex (MHC)-mismatched strain combinations [3]. Furthermore, co-transplantation of a liver allograft can prevent rejection of other organ grafts from the same donor strain [3]. LTX from the fully MHC-mismatched PVG donor to DA recipient does not lead to rejection, whereas other organ allografts (skin, heart and kidney) in the same strain combination induce acute graft rejection [3]. Importantly, passenger leukocytes were found to be involved in this phenomenon of spontaneous liver allograft acceptance [4, 5], since irradiation of the donor liver, which resulted in depletion of most donor leukocytes, converted spontaneous acceptance to rejection [5]. Conversely, post transplant administration of donor leukocytes (T cells, B cells, myeloid cells and NK cells) into the recipient reconstituted long-term survival after donor irradiation [6, 7].

Liver-resident Natural Killer (NK) cells are among the pool of donor leukocytes that are transferred into the recipient by LTX. We observed that in clinical LTX large numbers of donor NK cells with high cytotoxic potential detach from the liver grafts and migrate into the recipient [8]. It is plausible that these donor NK cells may prevent liver graft rejection by killing recipient antigen presenting cells, thereby limiting the indirect pathway of alloantigen presentation. They could also inhibit rejection by killing recipient activated T cells, as is observed in allogeneic bone marrow transplantation [9].

We analyzed the role of donor NK cells in the tolerant PVG to DA rat LTX model by depleting NK cells in the donor prior to LTX. PVG ($RT1^c$) NK cells efficiently lyse DA ($RT1^a$) target cells [10], most likely because they express an inhibitory Ly-49 receptor unable to recognize $RT1^a$ [11], and may therefore kill recipient antigen presenting cells and/or T-cells upon transfer into DA rats. We hypothesized that, if NK cells were the main donor leukocyte subset responsible for liver allograft acceptance, depletion of these cells would lead to rejection. Intraperitoneal injection of 100µg of the anti-NKRP1 (CD161, clone 3.2.3, Thermo Fisher Scientific, Scoresby, Australia) monoclonal antibody effectively depleted $NKRP1^+TCR^-$ and $NKRP1^+TCR^+$ cells from

liver and spleen of PVG rats, as was previously reported [12, 13]. Twenty-four hours after injecting the anti-NKRP1 Ab, flow cytometry analysis revealed a decrease of 99% in NK cells in the liver (from 27.0 ± 6.0% of liver leukocytes in normal rats, n = 6, to 0.4 ± 0.1% in treated rats, n = 3) and 88% in NKT cells (from 4.1 ± 1.9% in normal rats, n = 6, to 0.5 ± 0.3% in treated rats, n = 3). Similarly, in the spleen the percentage of NK cells was reduced by 97% (from 8.7 ± 1.2% of splenocytes in normal rats, n = 6, to 0.3 ± 0.3% in treated rats, n = 5) and the percentage of NKT cells by 99% (from 2.7 ± 0.7 in normal rats, n = 6, to 0.03 ± 0.01 in treated rats, n = 5). Importantly, there was no depletion of other leukocyte subsets as T cells, B cells and myeloid cells were unaffected by this treatment. Proper controls with secondary antibody were performed to exclude the possibility that the apparent NK-cell depletion was due to epitope competition of the antibodies used for depletion and detection.

Twenty-four hours after i.p. injection of 100 µg of anti-NKRP1 Ab into PVG rats, their livers were transplanted into DA recipients (n=4). The recipient rats did not show any signs of distress during the follow-up period and survived for more than 100 days with functioning liver grafts, similarly to rats transplanted with non-NK cell depleted liver grafts [5]. These results suggest that donor liver NK cells are not vitally important for induction of liver transplant tolerance in the PVG to DA rat strain combination. These observations are in line with a recent study from our group [14] in which we found no evidence for a role of donor NK cells in the outcome of clinical LTX.

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Chapter 5

NK cells can generate from precursors in the adult human liver

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ABSTRACT

Hepatic natural killer (NK) cells constitute ~40% of hepatic lymphocytes and are phenotypically and functionally distinct from blood NK cells. Whether hepatic NK cells derive from precursors in the bone marrow or develop locally from hepatic progenitors is still unknown. Here we identify all five known sequential stages of NK-cell development in the adult human liver and demonstrate that $CD34^+$ hepatic progenitors can generate functional NK cells. While early NK-cell precursors (NKPs) were similar in liver and blood, hepatic stage 3 NKPs displayed immunophenotypical differences, suggesting the onset of a liver-specific NK-cell development. Hepatic stage 3 NKPs were $RORC^{neg}$ and did not produce IL-17 or IL-22, excluding them from the lymphoid tissue-inducer (LTi) subset. *In vitro* culture of hepatic NKPs gave rise to functional NK cells exhibiting strong cytotoxicity against K562 targets. To determine if hepatic NKPs are stably residing in the liver, we analyzed donor and recipient-derived cells in transplanted livers. Shortly after liver transplantation all donor NKPs in liver grafts were replaced by recipient-derived ones, indicating that hepatic NKPs are recruited from the bloodstream. Together, our results show that NKPs are continuously recruited from peripheral blood into the liver and can potentially differentiate into liver-specific NK cells.

INTRODUCTION

Natural killer (NK) cells are an important component of the innate immune system. Without prior activation, NK cells can kill infected or malignant cells and produce cytokines that contribute to shaping both innate and adaptive immune responses. Recent studies have defined 5 main stages of human NK-cell development [1, 2]. NK cells start from stage 1 (also named pro-NK: $CD34^+CD117^-CD94^-$), and proceed through stage 2 (or pre-NK: $CD34^+CD117^+CD94^-$) and stage 3 (or iNK: $CD34^-CD117^+CD94^-$), into stage 4 corresponding to the $CD56^{bright}$ NK cells ($CD3^-CD56^{bright}CD16^{-/dim}$), and stage 5 corresponding to the $CD56^{dim}$ NK cells ($CD3^-CD56^{dim}CD16^+$) NK cells [1-3]. These last 2 stages, which constitute the mature NK-cell subsets, are both fully differentiated but their plasticity enables $CD56^{bright}$ to convert into $CD56^{dim}$ cells [4-6].

Originally, NK cells were thought to develop from $CD34^+$ hematopoietic stem cells (HSCs) in bone marrow. However, recent data suggest that very early phases of development, such as the generation of stage 1 NKPs from HSCs, occurs in the bone marrow [1] whereas later phases may occur in peripheral organs, as shown for secondary lymphoid tissues [7]. Accordingly, a complete pathway of NK-cell differentiation comprising all 5 stages of NK-cell development has been found in bone marrow, peripheral blood, lymph nodes and tonsils [1, 2, 7], while the three final stages of NK-cell development were detected in human uterus [8]. In addition, *in vitro* studies have demonstrated that functional NK cells can be generated by stimulation of $CD34^+$ cells isolated from human cord blood, bone marrow, fetal liver, thymocytes [1, 9-13], secondary lymphoid tissues [1, 2, 7], intestine [14, 15] and uterus [8, 16].

The liver contains large numbers of leukocytes, with a peculiar lymphocyte distribution characterized by the enrichment of $CD8^+$T cells, NKT cells and NK cells when compared with that of peripheral blood [17, 18]. Hepatic NK cells, additionally, are phenotypically and functionally distinct from blood NK cells [17, 19] and have important functions in defense against viral infections [20, 21], tumor metastases [22], hepatocellular carcinoma [23] and prevention of liver fibrosis [24, 25]. It is not known

whether hepatic NK cells develop from local precursors or are of systemic origin. Similarly to secondary lymphoid tissues, hepatic NK cells are enriched for the $CD56^{bright}$ subset [17, 19], suggesting that liver NK cells may also develop locally. During fetal life the liver is the main site of hematopoiesis [26, 27]. Shortly after birth, however, the liver drastically reduces its hematopoietic activity and the bone marrow becomes the main site of hematopoiesis throughout normal adult life [26, 27]. Nevertheless, the adult liver maintains the potential to reconstitute hematopoiesis, as observed in pathological conditions resulting in bone marrow dysfunction in which the liver rehabilitates its hematopoietic activity [27, 28]. Additional evidence of this potential hematopoietic role of the adult liver derives from animal studies, in which isolated hepatic HSCs have been shown to successfully reconstitute hematopoiesis in lethally irradiated animals [26, 29].

Previous research has shown the presence of $CD34^+$ lymphoid progenitors in the adult human liver, nevertheless none of these studies has indicated whether these progenitors are stably resident in the liver or has investigated the local presence of intermediate NK-cell developmental stages or the potential to derive functional NK cells from hepatic $CD34^+$ progenitors [26-28]. In this study we investigated whether the liver is a possible site of NK-cell development by identifying hepatic NKPs, characterizing their origin and assessing their capacity to mature into functional NK cells *in vitro*.

MATERIALS AND METHODS

Isolation of mononuclear cells from liver graft perfusate, blood and liver biopsy

Perfusates were collected from human liver grafts during the back table procedure and MNCs were isolated as previously described [19]. MNCs from peripheral blood samples of either healthy controls or LTX patients were isolated by Ficoll density gradient centrifugation under standard conditions.

Liver graft biopsies were obtained from healthy grafts just before transplantation or from explanted liver grafts in patients who underwent re-LTX and were collected in University of Wisconsin (UW) preservation solution. Fresh biopsies were cut into small pieces and incubated with digestion medium: 1640 RPMI (Cambrex Bio Science, Verviers, Belgium) with collagenase type IV (0.5mg/mL; Gibco, Breda, The Netherlands) and DNase type I (0.02mg/mL; Roche Diagnostics, Manheim, Germany) for 40 minutes at 37°C. Subsequently the tissue pieces were mashed over a nylon mesh filter (200µm pore diameter) to obtain a single cell suspension. The suspension was centrifuged at 360 rpm for 2 minutes to allow sedimentation of hepatocytes. After hepatocytes removal, cells were washed and prepared for standard Ficoll gradient centrifugation to obtain MNCs.

Flow cytometry, cell sorting and qPCR

The following monoclonal Abs were used in this study: CD3-AmCyan, CD14-FITC, CD15-FITC, CD16-PacificBlue, CD34-APC, CD45-AmCyan, CD107a-PE, anti-HLA-A2-FITC, IgG1-PE-Cy7, anti-RORC-PE, IgG1-PE (BD Biosciences, San Jose, CA); CD3-FITC, CD56-APC, CD94-PE, CD117-PE-Cy7, CD158a-APC, CD158b-APC (Beckman Coulter Immunotech, Marseille, France); CD25-PacificBlue, CD19-FITC (BioLegend, San Diego, CA); CD127-APC-Cy7 and IgG1-APC (eBioscence, San Diego, CA); anti-HLA-Bw4-FITC (from One Lambda, Canoga Park, USA). NKPs were gated from viable cells by exclusion of $CD3^+$, $CD14^+$ and $CD19^+$ cells and according to the following combinations: stage 1: $CD34^+CD117^-CD94^-$; stage 2: $CD34^+CD117^+CD94^-$; stage 3: $CD34^-CD117^+CD94^-$. Mature NK cells were gated as $CD56^{bright}$ cells: $CD3^-CD56^{bright}CD16^{-/dim}$; $CD56^{dim}$ cells: $CD3^-CD56^{low}CD16^+$. All analyses were performed using FACS Canto II flow cytometer (BD Biosciences) equipped with BD FACS–Diva software, version 6.1.1. Non-viable cells were excluded using 7-AAD (BD Biosciences).

Flow cytometric cell sorting was used to freshly isolate NK-cell developmental stages for either qPCR analysis or *in vitro* cell culture. For gene-expression analysis, stage 1, 2 and 3 cells were sorted from 11 different fresh liver perfusates as described above. Mature NK cells were isolated from fresh perfusates by negative selection, using the NK-Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer instructions. Cell sorting was preceded by enrichment of NKPs from perfusate-derived MNCs by magnetic column depletion (Miltenyi Biotec, Bergisch Gladbach, Germany) of lin^+ cells (CD3, CD14, CD15 and CD56 cells). Upon labeling with CD34, CD117 and CD94 mAb, NKPs were sorted with BD FACSAria II Cell Sorter (BD Biosciences) following the scheme reported above. During FACS sorting, additional labeling with mAbs against lineage markers (CD3, CD56, CD14, CD15 and CD19) allowed maximal exclusion of committed $lineage^+$ cells and contaminating NK cells. Tonsils were collected during routine tonsillectomies contingent on informed consent. Tissue was cut into small pieces and cell suspensions were prepared by disrupting the tissue with a GentleMacs (Miltenyi Biotech) in the presence of 0.5mg/ml collagenase type IV (Sigma, St. Louis, MO). MNCs were isolated from Ficoll gradients. Prior to sorting, tonsil MNCs were enriched for LTi by labeling with anti-CD117 biotin (eBioscience) and positive selection of the $CD117^+$ cells using streptavidin microbeads and VarioMacs (Miltenyi Biotech). LTi cells were sorted as defined by expression of the following markers: $CD45^+CD3^-CD14^-CD19^-CD34^-CD117^+CD127^+NKp44^+$.

Total mRNA was extracted using the NucleoSpin RNA XS kit for small amounts of cells ($\leq 5x10^5$ cells) or the NucleoSpin RNA II kit (both from Macherey-Nagel, Düren, Germany) for larger amounts of cells (up to $5x10^6$ cells), according to the manufacturer instructions. Isolated mRNA was treated with rDNase to remove any contaminating genomic DNA. Quantification and purity of extracted mRNA was measured by Nanodrop (Thermo, Wilmington, USA). Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis kit (Bio-rad) according to the manufacturer instructions. QPCR was performed in duplicates using SYBRGreen (Quantace). The primer's sequences used in this study are here reported. Id2 forw, 5'-CTCGCATCC CACTATTGT-3'; Id2 rev 5'-TGCTTTGCTGTCATTTGA-3'; IL-17 forw 5'-GAAGGCAGGAATCACAATC-3'; IL-17 rev 5'-GCCTCCCAGATCACAGA-3'; IL-22 forw 5'-CCCATCAGCTCCCACTGC-3'; IL-22 rev 5'-GGCACCACCTCCTGCATATA-3'; RORC forw 5'-CCCGTCAGCAGAACTG-3'; RORC rev 5'-AGCCCCAAGGTGTAGG-3'; IFN-γ forw 5'-CCAGGACCCATATGTAAAAG-3'; IFN-γ rev 5'-TGGCTCTGCATTATTTTTC-3';

GAPDH forw 5'-TGCACCACCAACTGCTTAGC-3'; GAPDH rev 5'-GGCATGGACTGTGGTCATGAG-3'. The thermocycling conditions used are here reported: 10 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 10 sec at 58°C and 15 sec at 72°C. Data were analyzed with the Bio-rad IQ-5 to establish the Ct value for each sample. Only Ct values ≤35 were considered for the analysis and relative gene expression was calculated by normalization with the housekeeping gene GAPDH.

In vitro differentiation of NK cells

After sorting, cultures were initiated with 1×10^4 cells seeded in 96-well round-bottomed plates with 200µl of medium consisting of 1640 RPMI, 10% heat-inactivated human AB serum, 10% FBS (Hyclone, Logan, UT), penicillin (100U/mL) and streptomycin (100U/mL; both from Gibco, Breda, The Netherlands). The following cytokines were added to the culture medium: stem cell factor (SCF) (20ng/ml), FMS-like tyrosine kinase (FLT3-L) (20ng/ml), interleukin-7 (IL-7) (20ng/ml), IL-15 (20ng/ml) and IL-21 (20ng/ml) (all from PeproTech, NJ, USA). Half of the culture medium was replaced every 3–4 days**.** After 4 weeks of culture, cells were harvested and used for phenotypical or functional analysis as previously described by our group [19].

Identification of donor-derived NK cells and their precursors after LTX

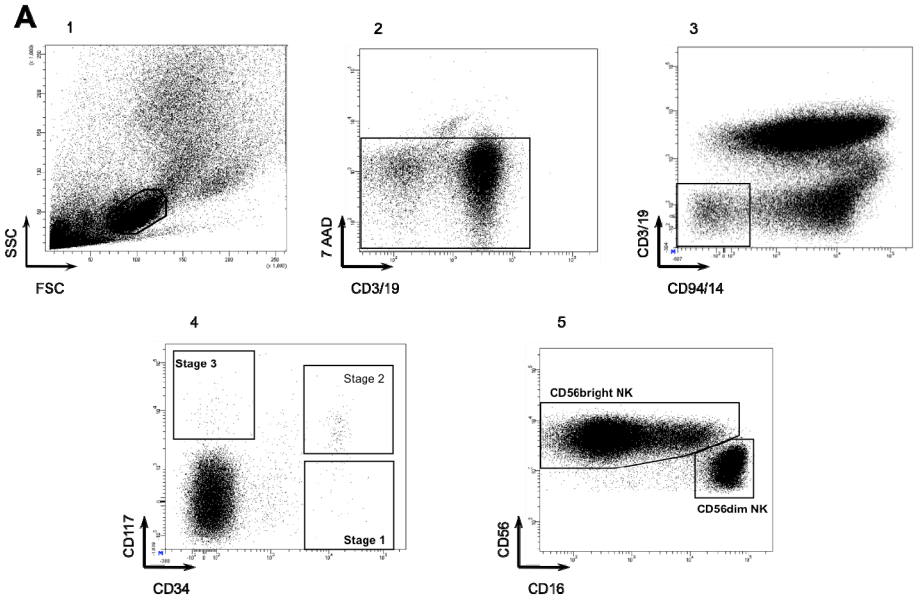
We selected donor/recipient pairs in which only one of the two parties had an HLA-A2 or HLA-Bw4 epitope. FACS analysis with a monoclonal Ab recognizing either HLA-A2 or HLA-Bw4 was used to distinguish between cells of donor and recipient origin in peripheral blood of LTX recipients or in biopsies from explanted first liver grafts in patients who underwent re-LTX. Multiple biopsies were obtained from different sites of the explanted graft. Biopsies were pooled together and processed as described above to isolate MNCs for FACS analysis.

The Ethical Committee of the Erasmus MC approved the study and all volunteers gave written informed consent. This project was funded by the Erasmus MC (Rotterdam, the Netherlands). The authors declare no conflict of interest.

RESULTS

Identification of NK-cell precursors within the adult human liver

To identify hepatic NKPs by flow cytometry we isolated mononuclear cells (MNCs) from fresh liver perfusates (n=15) and fresh liver biopsies (n=4) collected during liver transplantation (LTX) procedures. The first three developmental stages were initially gated from viable cells, negative for the lineage markers CD3, CD14, CD19 and for CD94 (Figure 1A, panels 1-3), and were then identified according to their expression of CD34 and CD117 (Figure 1A, panel 4). $CD56^{bright}$ and $CD56^{dim}$ NK cells were identified on basis of CD56 and CD16 expression within viable $CD3^{-}CD56^{+}$ NK cells (Figure 1A, panel 5).



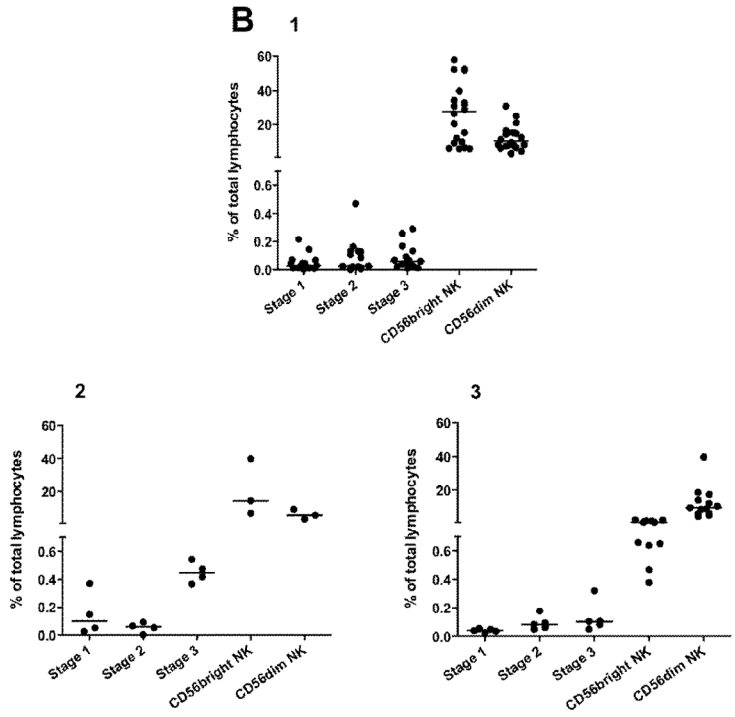


Figure 1. NKPs in liver graft perfusates, liver graft biopsies and peripheral blood. (A) Gating strategy for the identification of NKPs and NK cells. Stage 1-3 NKPs were identified among $CD45^+$ viable ($7AAD^-$) cells within the lymphocyte gate. $Lineage^+$ (CD3, CD14, CD19) cells and $CD94^+$ cells were excluded (1-3). Gating of NKPs (4) was based on isotype controls (for CD94, CD34 and CD117) and was obtained by recording at least $2x10^6$ events. $CD56^{bright}$ and $CD56^{dim}$ NK cells were gated within $CD3^-CD56^+$ cells on the basis of their CD56/CD16 expression (5). Dot plots represent liver graft perfusates, but similar profiles were observed for liver biopsies and peripheral blood. **(B)** Numbers of NKPs and NK cells in (1) liver perfusates (n=15), (2) liver biopsies (n=4) and (3) peripheral blood of healthy volunteers (n=5). NK cells were enumerated in additional samples of liver perfusates (total n=21) and blood from healthy volunteers (total n=13).

We identified all these sequential developmental stages in liver perfusate and liver biopsies (Figure 1B, panels1-2) and we confirmed their presence in peripheral blood (Figure 1B, panel 3). As expected, the number of cells in the first three stages of development (expressed as percentage of viable lymphocytes) was uniformly low, (Figure 1B). Additional control experiments (n=6) proved that stage 1-3 NKPs were $CD45^+$, thus excluding contamination by endothelial cells (data not shown). Expression of CD45RA, characterizing a subset of $CD34^+$ cells in bone marrow, blood and especially lymph nodes that can develop into $CD56^{bright}$ NK cells [7], was observed on 57±10% of stage 1 cells, 41±10% of stage 2 and 55±7% of stage 3 cells in liver perfusates (n=6). In liver biopsies we detected similar numbers of stage 1 and 2

NKPs compared to liver perfusates and higher proportions of stage 3 cells (0.45% of total hepatic lymphocytes, range 0.37-0.54, compared to 0.06%, range 0.01-0.29, in perfusate) (Figure 1B, panel 2). As expected, the frequency of hepatic NK cells was high and reflected the known 3:1 ratio between the $CD56^{bright}$ and $CD56^{dim}$ subsets [19] both in liver perfusates and biopsies (Figure 1A and 1B). The number of NKPs in peripheral blood was similar to what we found in liver and the proportion of $CD56^{bright}$ and $CD56^{dim}$ reflected the known 9:1 ratio (Figure 1B, panel 3).
We then analyzed CD94 expression on hepatic NK cells by gating on the following sequential subsets: $CD56^{bright}CD94^{high}$, $CD56^{dim}CD94^{high}$ and $CD56^{dim}CD94^{low}$ cells (Figure 2A) [6]. Our results indicated that all three subsets are present in both liver and peripheral blood, but that their distribution is different in the two compartments, possibly contributing to the hypothesis of a local hepatic differentiation into liver-specific NK cells.

Since NKPs are present in peripheral blood [1], we next aimed at detecting possible phenotypical differences between liver and blood-derived NKPs. To exclude that detected phenotypical differences between the tissues analyzed (liver from deceased organ donors and peripheral blood from healthy individuals) were related to the pre-morbid state of the organ donor, we hereon included also peripheral blood samples from organ donors obtained at the time of perfusate collection (n=4; Figure 1 Supporting Information). We compared the expression of CD127, CD25 and CD117 on liver and blood-derived NKPs (Figure 2B). Given the low frequency of NKPs, we only considered as reliable those experiments in which we measured at least 50 events for each developmental stage. Stage 1 and 2 cells showed similar immunophenotypes in the two tissues. However, a high percentage of liver-derived stage 3 cells were positive for CD127 (73.2±5.1%), while only 31.4±7.7% blood-derived stage 3 cells expressed CD127 ($p=0.001$). Moreover, CD25 expression was substantially higher on blood-derived stage 3 cells compared to their hepatic counterparts (61.0±7.6% of $CD25^{+}$ stage 3 cells in blood and 34.2±7.0% in liver, $p=0.02$). With regard to NK cells, only 5.0±2.5% of hepatic $CD56^{bright}$ NK cells was $CD127^{+}$, while 18.8±5.4% of blood-derived $CD56^{bright}$ NK cells expressed the same marker ($p=0.007$). Similarly, CD117 was expressed only by 0.8±0.2% of hepatic $CD56^{bright}$ NK cells while was present on 29.1±4.7% of $CD56^{bright}$ cells derived from peripheral blood ($p=0.0001$). Conversely, CD117 was completely absent from $CD56^{dim}$ NK cells of both tissues. Overall these immunophenotypical differences

indicate that hepatic NKPs differ from their blood counterparts from stage 3 onwards. Altogether, our results show that all NK-cell developmental stages, from multipotent CD34+ hematopoietic progenitor cells to mature NK cells, are present in the adult human liver.

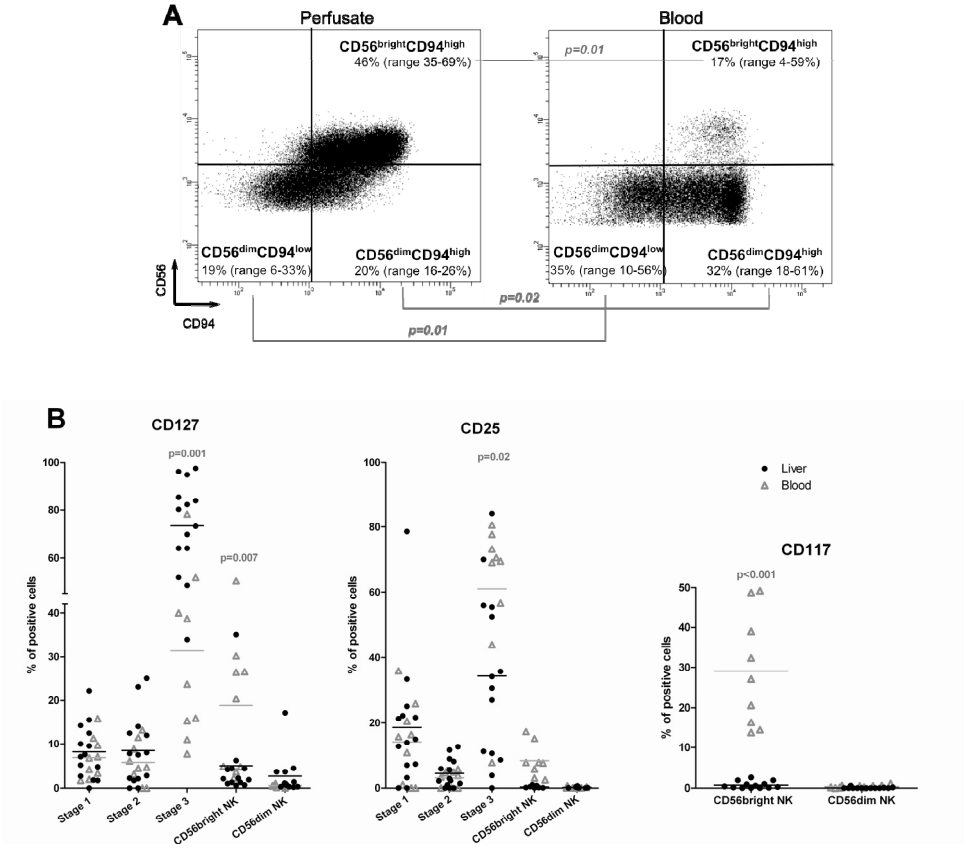


Figure 2. Expression of CD94, CD127, CD25 and CD117 by NKPs and NK cells from liver or peripheral blood. (A) Representative dot plot of the three subsets identified on the basis of CD56, CD16 and CD94 expression. Percentages indicate medians with ranges from 7 independent experiments for perfusates and 5 independent experiments for peripheral blood. p-values of differences in subset frequencies in the two tissues were calculated with the Mann–Whitney test. **(B)** Expression of CD127, CD25 and CD117 on NKPs and NK cells from liver (both perfusates and biopsies, closed circles) and peripheral blood (both healthy individuals and organ donors, open triangles). p-values of differences in the expression of these markers between liver and blood-derived cells were calculated with the Mann–Whitney test.

Hepatic stage 3 cells express CD127 but are not Lymphoid Tissue Inducer cells

To assess if NKPs in liver perfusates have characteristics of LTi cells (CD127+RORC+, IL-22 and IL-17 production [30-32]) we FACS sorted stage 1, 2 and 3 NKPs from fresh perfusates and measured by qPCR the mRNA levels of the transcription factors RORC

and ID2, as well as the cytokines IL-22, IL-17 and IFN-γ. RORC mRNA was not detected in NKPs nor NK cells from perfusates (Figure 3). Intracellular staining confirmed the absence of RORC in stage 1 and 2 cells, while only 6±3% of stage 3 cells were RORC^{pos} (n=3; data not shown). Adding to this, IL-17 (data not shown) and IL-22 mRNA were undetectable in NKPs and NK cells, while ID2 mRNA, essential for NK-cell development beyond stage 3 NKPs [33], was detected in all hepatic NKPs (Figure 3). These data indicate that hepatic NKPs, including stage 3 cells, are real NK-cell precursors and are not committed to the LTi lineage.

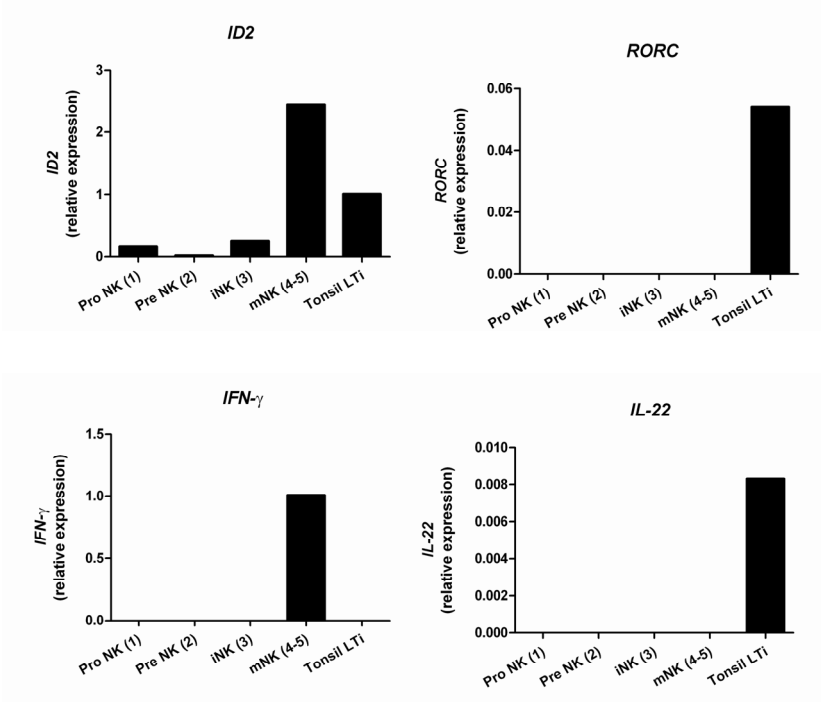


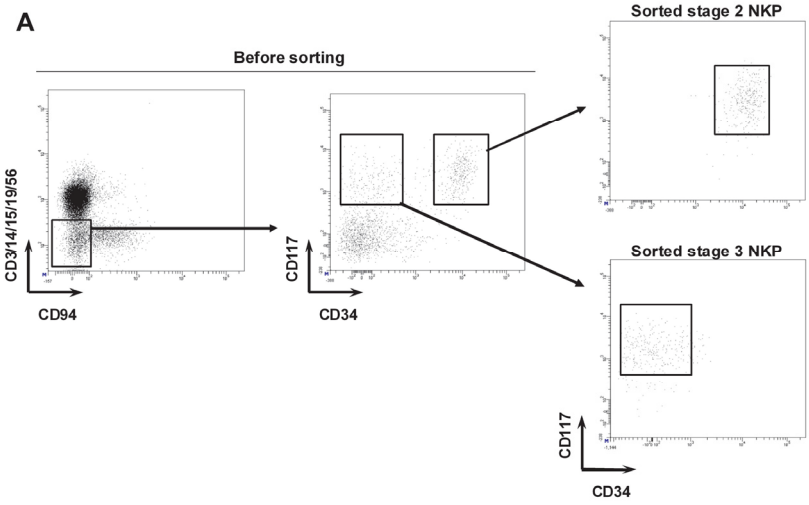
Figure 3. Expression of transcription factors and cytokines in hepatic NKPs and NK cells. qPCR analysis of ID2, RORC, IFN-γ and IL-22 in NKPs from fresh liver perfusates. mRNA of NK cells isolated from liver perfusate and LTi cells from tonsils were used as controls. Relative gene expression levels were normalized to GAPDH. Data shown are from one experiment representative of 2 independent qPCR analyses performed on mRNA extracted from: pools of NKPs isolated from 11 perfusates, NK cells from 3 perfusates and tonsil LTi cells from 3 tonsils.

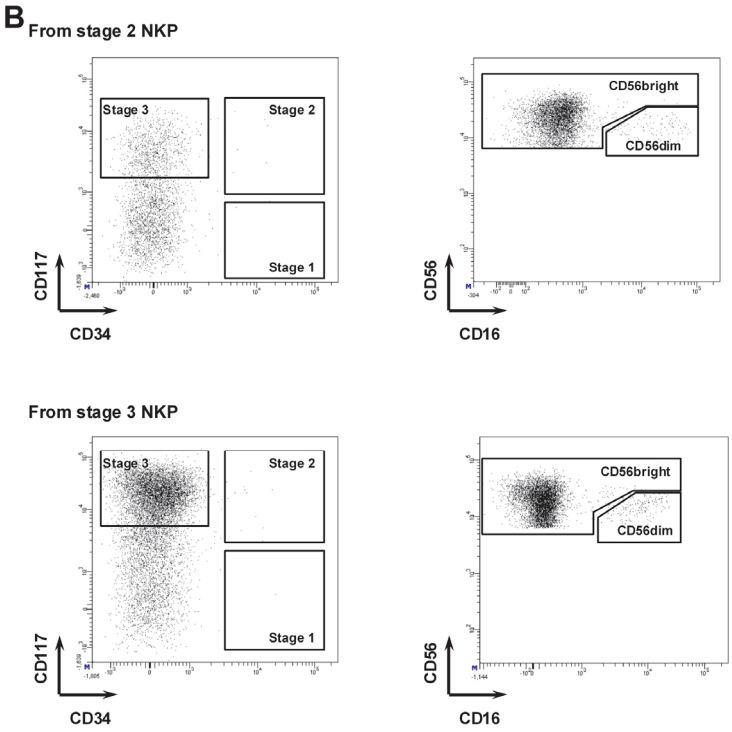
Hepatic NK-cell precursors develop in vitro into functional NK cells

Sorted stage 1, 2 and 3 from fresh liver perfusates were cultured, in the presence of cytokines that support NK cell differentiation *in vitro* [1, 11-13, 34], to assess their capacity to differentiate into NK cells. After cell sorting, stage 1 cells reached only 50-70% of purity and the large majority died during the first weeks of culture, most

probably indicating the need of a layer of feeder cells for appropriate expansion [2, 7, 31]. Conversely, cell sorting of stage 2 and 3 cells yielded highly pure populations (average purity of >97%; Figure 4A). After the first 2 or 3 weeks, the rate of expansion of stage 2 and 3 cells required redistribution of the cells into additional wells. After 4 weeks of culture, all stage 2 cells had developed into stage 3 NKPs (median of 21%; range 8-58%) or NK cells (75%; range 47-99%; n=8; Figure 4B). Of these latter NK cells, 76±9% belonged to the $CD56^{bright}$ subset and 21±8% to the $CD56^{dim}$ subset (Figure 4B). Importantly, in these cultures we did not detect stage 1 or stage 2 cells.
After 4 weeks of culture of stage 3 cells, none of the cells converted to stage 1 or 2, a variable proportion remained in stage 3 (24%; range 0.6-47%) and the majority had developed into mature NK cells (72%; range 51-90%, n=6, Figure 4B). Most of the stage 3-derived NK cells belonged to the $CD56^{bright}$ subset (81.1±7.6%), while 12.8±9.6% was $CD56^{dim}$. NK cells that developed *in vitro* from stage 2 and 3 lacked Killer Ig-like Receptors (KIRs) (data not shown). In addition, a large part of the *in vitro*-derived $CD56^{dim}$ NK cells expressed CD94 (93.9±2.2%), while a smaller fraction of the $CD56^{bright}$ NK cells was $CD94^{+}$ (59.5±9.9%). The expression of CD94 was also analyzed during the first weeks of culture and these data indicated that CD94 was sequentially upregulated during development (n=3; Figure 4C). Altogether these data confirm that freshly isolated stage 2 and 3 NKPs from adult human liver are able to give rise to $CD56^{bright}$ and $CD56^{dim}$ mature NK cells.

To assess if the NK cells we obtained *in vitro* from stage 2 and 3 NKPs are functionally competent we performed cytotoxicity assays with the classical K562 target cell line and measured CD107a expression on NK cells to assess their degranulation capacity. Stage 2-derived NK cells exhibited potent cytotoxicity (84±6% of K562 targets were killed in a 2:1 E:T ratio and 87±6% in a 5:1 ratio; Figure 4D and 4E) and an average degranulation of 43±4% of the cells (background of spontaneous degranulation was 13±2%; $p<0.001$; n=5; Figure 4E). Similarly, stage 3-derived NK cells killed most of the K562 targets (63±12% in 2:1 and 86±5% in 5:1 E:T ratios; Figure 4D) and exhibited an average degranulation of 52±10% (background of spontaneous degranulation was 18±3%; $p=0.009$; n=5).


A
Before sorting
CD3/14/15/19/56
CD94
CD117
CD34
Sorted stage 2 NKP
Sorted stage 3 NKP
CD117
CD34


B
From stage 2 NKP
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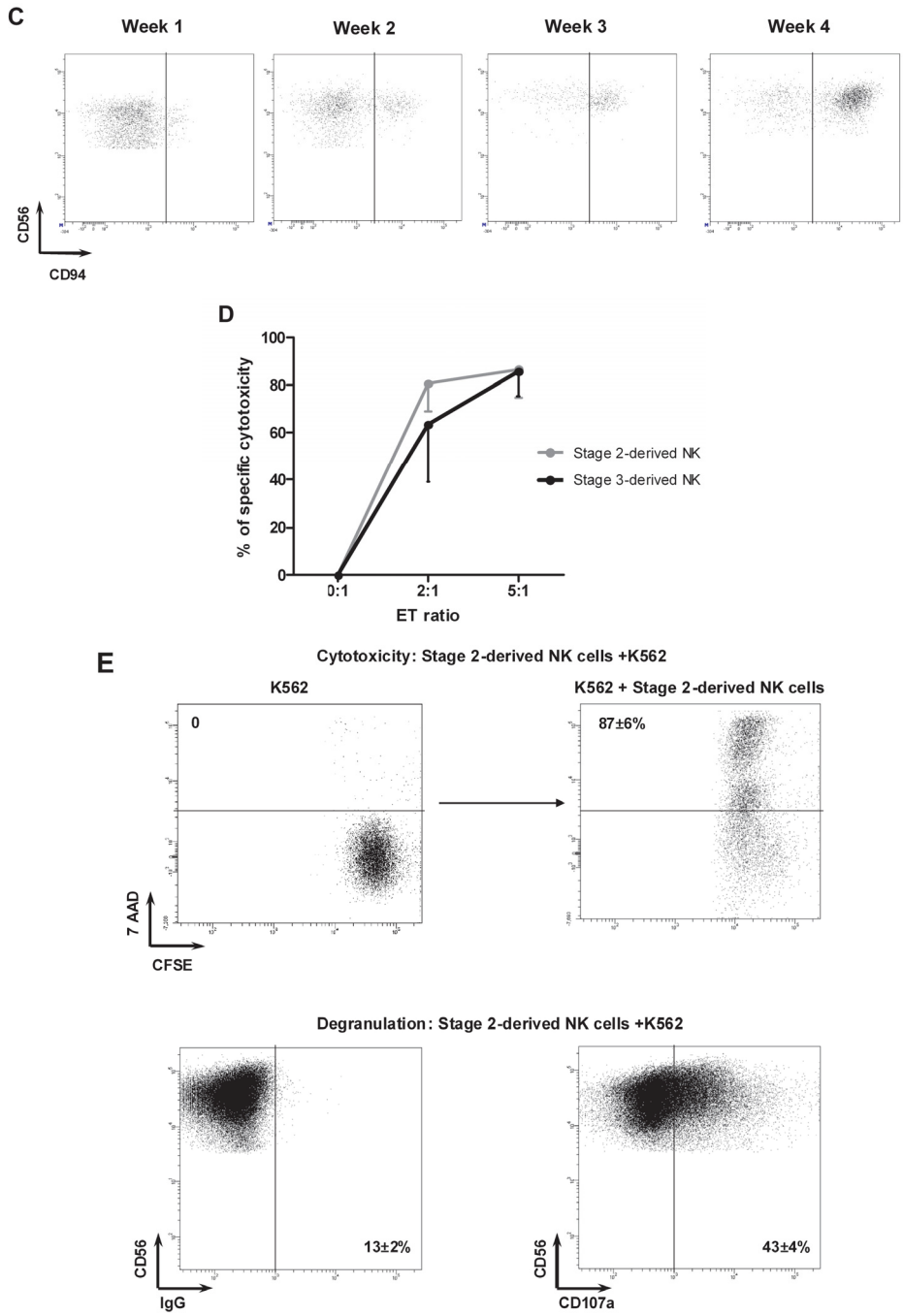

Figure 4. Differentiation of sorted hepatic NKPs into cytotoxic NK cells. (A) Representative example of FACS sorting of perfusate MNCs into stage 2 and 3 NKPs (purity >97%). Stage 2 and 3 NKPs were initially gated from $CD45^+$ viable ($7AAD^-$) cells within the lymphocyte gate. $Lineage^+$ (CD3, CD14, CD15, CD19, CD56) cells and $CD94^+$ cells were excluded and stage 2 and 3 NKPs were then sorted on the basis of their CD34 and CD117 expression. **(B)** Sorted stage 2 and 3 NKPs were cultured for 4 weeks, then harvested and phenotypically analyzed by flow cytometry. Dot plots are representative of 8 independent experiments for stage 2

cells and 6 independent experiments for stage 3 cells. **(C)** Progressive induction of CD94 expression during differentiation of stage 2 NKPs. Dot plots are representative of 3 independent experiments. **(D)** Cytotoxic capacity of *in vitro*-derived NK cells. After 4 weeks of culture, the NK cells obtained from hepatic stage 2 or 3 NKPs were co-incubated for 4h with K562 cells at 2 different E:T ratios (2:1 and 5:1). The percentage of K562 cells killed by NK cells was determined as previously described [19]. Results show means - SD of 5 independent experiments. **(E)** Cytotoxicity and degranulation of *in vitro*-derived NK cells. Representative dot plots showing the percentage of K562 targets killed upon co-incubation with NK cells (upper panel) and the amount of NK cells degranulating in the presence of K562 targets (lower panel). The plots shown depict stage 2-derived NK cells and are representative of 5 independent experiments; percentages of K562 killing and degranulation are expressed as means±SD.

Hepatic NKPs are rapidly replaced by precursors recruited from the circulation

To investigate whether the NKPs we detected in human liver are stably resident in the organ or are recruited from the circulation we collected biopsies from 5 liver grafts explanted during a re-transplantation (re-LTX) procedure 1 week to 2 years after the first transplantation. In these samples we determined the presence of NKPs and NK cells from the donor. In all 5 explanted liver grafts we detected only recipient-derived but not donor-derived NKPs (Table 1A), indicating that hepatic NKPs are replaced by blood-derived NKPs within 1 week after LTX. On the contrary, in all the biopsies donor-derived NK cells co-existed with recipient NK cells. To investigate whether donor-derived NK cells were also detectable in the recipient circulation late after LTX, we collected peripheral blood samples from 5 LTX patients between 10 and 17 months post-LTX. Donor-derived NK cells and their precursors were not found in any of these post-LTX blood samples where, instead, all detected NK cells and NKPs were derived from the recipient (Table 1B).

Table 1. Proportions of donor-derived NKPs and NK cells in transplanted livers and recipient peripheral blood after liver transplantation.

A

NKPs and NK cells as % of lymphocytes in liver grafts after LTX (% of donor-derived within each subset)					
Time post- LTX	Patient-1 7days	Patient-2 16days	Patient-3 1year	Patient-4 2years	Patient-5 2years
Stage 1	0.4 (0)	0.2 (0)	0.1 (0)	0.2 (0)	0.1 (0)
Stage 2	0.07 (0)	0.01 (0)	0.04 (0)	0.05 (0)	0.01 (0)
Stage 3	0.3 (0)	0.06 (0)	0.1 (0)	0.1 (0)	0.1 (0)
NK	10.1 (6.8)	11.6 (1.5)	42.8 (1.7)	7.9 (1.7)	32.2 (4.8)

B

NKPs and NK cells as % of total lymphocytes in peripheral blood after LTX (% of donor-derived within each subset)					
Time post-LTX	Patient-1 10months	Patient-2 14months	Patient-3 15months	Patient-4 15months	Patient-5 17months
Stage 1	0.3 (0)	0.2 (0)	0.2 (0)	0.2 (0)	0.5 (0)
Stage 2	0.05 (0)	0.03 (0)	0.09 (0)	0.02 (0)	0.07 (0)
Stage 3	0.01 (0)	0.01 (0)	0.05 (0)	0.03 (0)	0.06 (0)
NK	7.8 (0)	10.5 (0)	14.1 (0)	6.6 (0)	11.3 (0)

Proportions of NKPs and NK cells in **(A)** first liver grafts or **(B)** peripheral blood of recipients, at different time-points after LTX. Numbers of cells in each stage of NK-cell development are expressed as percentage of total viable lymphocytes. Numbers in parenthesis indicate the percentage of donor-derived cells within each stage.

DISCUSSION

In this study we demonstrate for the first time that the adult human liver contains precursors of NK cells that can develop into functional $CD56^{bright}$ and $CD56^{dim}$ mature NK cells. We have shown that all 5 stages of NK-cell development are detectable in the adult liver (both in biopsies and perfusates), that stage 2 and 3 NKPs can generate cytotoxic NK cells *in vitro* and that early NKPs are recruited from peripheral blood. As proposed by Freud and Caligiuri [1], the selective enrichment of $CD56^{bright}$ NK cells in a specific organ (as was shown for the human liver in our previous study [19]), together with the presence of the full range of developmental intermediates, here shown as spanning the continuum of differentiation from $CD34^{+}$ HSCs to $CD3^{-}$ $CD56^{+}$ NK cells, suggest that the specific organ may be a site of NK-cell development *in vivo*. Altogether our results support a model in which hepatic NKPs originate from circulating precursors (most probably derived from the bone marrow), relocate into the liver and hence can differentiate into a population of NK cells with liver-specific characteristics. Tissue-specific NK-cell development is possible since, in contrast to T-cell development (which requires a stage of selection within the thymus for the removal of auto-reactive T cells) NK-cell education ensuring self-tolerance can potentially take place in any tissue, as long as the local microenvironment is supportive [1]. Importantly IL-15, a key cytokine for NK-cell development, is present in the liver [26, 27, 35] (produced by Kupffer cells [36]) and IL-7, known to increase NK-cell proliferation, is produced by hepatocytes [27].

Our immunophenotypical definition of NKPs is derived from the model proposed by Caligiuri et al. which is based on the lack of lineage markers and CD94 and on the combined expression of CD34 and CD117 [2]. While stage 1 and 2 cells are still multipotent, full commitment to the NK cell lineage occurs at stage 3 of development [1, 2, 37, 38]. Therefore, the actual NKPs may be only a subset of stage 1 and 2 cells [2], as already suggested by studies in human lymph nodes which identified specific precursors of $CD56^{bright}$ NK cells within stages 1 and 2 as expressing CD45RA [7]. We found that around 60% of hepatic stage 1 cells and 40% of stage 2 cells expressed CD45RA, suggesting that these cell populations contain specific precursors of $CD56^{bright}$ NK cells.

The immunophenotypical definition of stage 3 cells can include also LTi cells [31]. LTi cells, required for the formation of lymph nodes during embryogenesis, exhibit the same phenotype as stage 3 cells but, in addition, express CD127 and RORC, and produce IL-22 and IL-17 [30-32]. Although a large proportion of hepatic stage 3 cells expressed CD127 we here have shown that they are real NKPs, since these cells had low or absent RORC expression, at both protein and mRNA level, and did not produce IL-22 or IL-17. The potential of hepatic NKPs to develop into NK cells was further supported by their expression of ID2. Altogether, our phenotypical analysis and qPCR data have shown that the liver contains specific stage 1, 2 and 3 NKPs.

When hepatic NKPs and NK cells from organ donors were compared with their counterparts in peripheral blood from healthy individuals, a number of differences emerged in terms of expression of CD25, CD127 and CD117. Confirmation that these differences were related to the tissue of origin rather than the pre-morbid state of the organ donor was obtained by comparing paired blood and liver samples from the same organ donors (Figure 1 Supporting Information). Interestingly, differences in expression of the abovementioned molecules were detected only from stage 3 of differentiation, suggesting that stage 3 cells are influenced by the hepatic environment and may constitute the first step towards the differentiation of liver-specific NK cells. Furthermore, hepatic $CD56^{bright}$ NK cells differed from their blood counterparts in that they exhibited lower expression of CD127 and complete absence of CD117. Additional differences between blood and liver NK cells were detected in terms of CD94 expression. The level of CD94 expression has been described to identify a functional intermediary between the classical $CD56^{bright}$ and $CD56^{dim}$ NK cells [6] that defines three sequential subsets: $CD56^{bright}CD94^{high}$, $CD56^{dim}CD94^{high}$ and $CD56^{dim}CD94^{low}$ cells (Figure 2A). Comparison of these three subsets in liver and blood shows a clear difference in their proportions, suggesting here again, as already postulated for decidua [39] and secondary lymphoid tissue [2], that specific types of NK cells may develop locally in different tissues.

We here show that stage 2 and 3 cells isolated from liver develop into functional NK cells *in vitro*. Our experiments showed that stage 2 cells progressed into stage 3 and, similarly to stage 3 cultures, ultimately differentiated into NK cells, largely belonging to the $CD56^{bright}$ subset. *In vitro*-derived NK cells progressively upregulated CD94

during the culture (Figure 4C). CD94 expression is known to be highly correlated with NKG2A levels [40], and the expression of the inhibitory dimeric receptor CD94/NKG2A, which occurs prior to KIRs expression, is generally linked to the acquisition of cytotoxic properties [41, 42]. In our experiments none of the *in vitro*-derived NK cells expressed KIRs after 4 weeks of culture, confirming previous *in vitro* observations [11, 12, 43, 44]. Despite the lack of KIRs, our *in vitro*-derived NK cells were highly functional: the cells degranulated in response to K562 targets, exhibiting a 1.5 to 2-fold higher cytotoxicity than our previously analyzed hepatic NK cells [19]. Besides demonstrating the cytotoxic potential of our *in vitro*-derived NK cells, these observations confirm that the lack of engagement of inhibitory KIRs is not the only mechanism triggering NK cell responses against MHC class $I^{negative}$ targets [45, 46].

Our observation that hepatic, donor NKPs are rapidly replaced after LTX by recipient-derived NKPs that infiltrate the graft, suggests that stage 1 and 2 NKPs from the bone marrow migrate into (or traffic through) the liver. This concept is supported by experiments in adult mice indicating that part of the HSCs from bone marrow enter the bloodstream and traffic to multiple peripheral organs (including the liver), where they reside shortly before entering draining lymphatics to return to the blood and, eventually, the bone marrow [47]. Importantly, a study by Massberg et al. [47] describes the liver as one of the peripheral organs in which tissue-resident HSCs are constantly replenished by blood-derived HSCs and are turned over within a matter of days. Our results indicating the presence of donor-derived NK cells in the grafted organ long time after LTX suggest at least two possible interpretations. First, hepatic NKPs transferred by LTX may displace to other sites, such as the bone marrow, and hence constitute a long-term source of donor cells, which may relocate again into the liver. This hypothesis is supported by data showing the regular release of HSCs from bone marrow to blood in humans [48]. Alternatively, the liver may contain a population of long-lived NK cells as demonstrated by recent experimental animal data showing that long-lived NK cells reside not only in lymphoid tissues but also in non-lymphoid tissues and particularly in the liver [49, 50]. Observations in mice indicate that NK cells, activated specifically by a chemical hapten [51] or a virus [49, 52] or non-specifically by a state of lymphopenia [53] or inflammatory cytokines [54], acquire classical characteristics of immune memory that include an extended lifespan, the ability to self-renew, and the capacity to mount robust and protective recall responses [52].

In light of the evidence we here report, we propose the following model for the NK-cell development in the adult liver: early NKPs (stage 1 and 2) are continuously recruited from peripheral blood into the liver, where they differentiate, under the influence of the local microenvironment, into the so called "liver-specific" NK cells. This hepatic population, enriched in $CD56^{bright}$ cells and highly cytotoxic [19], has important local functions such as the defense against viral infections and malignant cells and the protection from liver fibrosis. Besides contributing to our understanding of NK-cell biology, our results shed more light on the mechanisms determining prolonged intragraft NK-cell chimerism after LTX, which are possibly involved in induction of immunological tolerance to liver grafts [26, 55].

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Chapter 6

HLA-G in liver transplantation

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ABSTRACT

HLA-G is a non-classical HLA class I molecule with unique immunoregulatory properties and tissue distribution. While in the context of human pregnancy HLA-G has been shown to play a role in promoting maternal immunological tolerance towards the semi-allogeneic fetus, its role in solid organ transplantation is still debated. The aim of this study was to determine whether longitudinal changes in serum HLA-G levels are associated with graft acceptance after liver transplantation (LTX). Serum HLA-G levels were quantified in a cohort of 30 LTX patients before transplantation and at several time points during the first year after transplantation. Before transplantation, HLA-G serum levels in LTX patients were higher than in healthy controls and decreased after LTX. Serum HLA-G levels were positively correlated with serum transaminases and bilirubin. Patients with an acute rejection episode displayed elevated HLA-G compared to non-rejectors, during the time interval in which most acute rejections occurred. Interestingly, liver graft perfusion fluid contained high concentrations of HLA-G, indicating the release of HLA-G from deceased donor livers after cold storage. Overall our data do not support the hypothesis of a tolerogenic role for HLA-G in LTX and may rather suggest that HLA-G expression may associate with states of liver inflammation.

INTRODUCTION

HLA-G is a non-classical MHC class I molecule with unique immunoregulatory functions and low polymorphism [1]. HLA-G is expressed in seven different isoforms (HLA-G1 to -G7) that have a restricted tissue distribution. In physiological conditions only trophoblasts, thymus, cornea, pancreas and erythroid and endothelial precursors express HLA-G [1]. The mechanism by which HLA-G can inhibit the immune response is not completely understood. HLA-G has been shown to inhibit the proliferation and cytolytic function of NK cells [2-6], the cytotoxicity of CD8+ T cells [7-9], the alloproliferative response of CD4+ T cells [10, 11] and the maturation and function of dendritic cells [12, 13].

The role of HLA-G has been widely studied in the context of pregnancy, where it has been implicated in maternal-fetal tolerance [3]. Expression of HLA-G by fetal trophoblasts, which invade the maternal decidua during placentation, has been linked to maternal immunological tolerance for the semi-allogeneic fetus. Still controversial is the role of HLA-G in the context of solid organ transplantation. Despite the evidence that HLA-G can be expressed by allografts and may correlate with better graft acceptance, the role of HLA-G in solid organ transplantation has not been fully established. In heart and combined liver/kidney transplantation, HLA-G has been detected in both graft biopsies and serum [1, 14-16] and its detection correlated with better graft acceptance. In combined liver/kidney transplantations HLA-G expression was observed in 35% of the liver grafts, and expression on biliary epithelial cells correlated with absence of acute rejection [17, 18]. However, in patients with single liver transplants, the correlation between HLA-G expression on biliary epithelial cells and the incidence of acute rejection could not be demonstrated. Combined liver/kidney transplant recipients have higher soluble HLA-G levels in serum compared to single kidney or single liver transplant (LTX) recipients. Since combined liver/kidney transplant recipients experienced less acute rejection episodes, it was concluded that allograft acceptance is associated with HLA-G expression [19]. In kidney transplant recipients the absence of soluble HLA-G in serum was found to be associated with chronic rejection [20]. In pediatric LTX higher serum HLA-G levels were observed in recipients with stable graft function compared to recipients who experienced acute rejection or healthy individuals [21]. However,

none of these research groups investigated longitudinal changes in HLA-G serum levels.

The purpose of this study was to determine whether: (I) longitudinal changes in serum HLA-G levels are associated with rejection or operational tolerance after LTX; (II) expression of HLA-G in circulating leukocytes is associated with the outcome after LTX; (III) HLA-G is released from the donor liver after cold ischemic storage.

PATIENTS AND METHODS

Patients and sample collection

Our study cohort is composed of 30 patients who underwent LTX at the Erasmus MC in Rotterdam between 1998 and 2002, and from whom serum samples were available. We also included four patients who are considered to be "operationally tolerant" [22]. In these four patients immunosuppressive therapy was withdrawn (6 to 11 years after LTX) for medical causes (chronic norovirus infection, post-transplant lymphoproliferative disorder, Kaposi sarcoma and persistent Epstein-Barr viremia). For this study, peripheral blood samples were collected from these patients between 12 and 20 months after discontinuation of immunosuppressive drugs. The 24 healthy controls (12 men and 12 non-pregnant women) included in this study were selected among volunteers with an age between 25 and 60 years and were clinically healthy at the time of blood or serum collection. Blood samples were collected from LTX patients and healthy controls in clotting tubes and centrifuged for 5 min at 2000 rpm. The serum was collected and frozen at -80°C for ELISA analysis.

Samples of perfusates were obtained from 10 donor livers. Two types of liver graft perfusates (perfusate 1 and 2) were collected at the time of LTX as previously described [23]. To obtain cell-free liquid, perfusates were centrifuged at 1500 rpm for 10min at 4°C; supernatants were then collected and centrifuged again at 4000 rpm for 15min at 4°C. The supernatants obtained after this step were stored at -20°C for ELISA analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of LTX patients and healthy controls by Ficoll density gradient centrifugation [23]. All PBMC samples were frozen in 10% DMSO-containing medium and kept at -135°C before analysis.

Quantification of HLA-G in serum by ELISA

The amounts of soluble HLA-G1 and -G5 isoforms were quantified by ELISA using the capturing mAb Mem-G/9 and the detecting mAb 56B-biotin (both from ExBio, Praha, Czech Republic) [24]. Briefly, 96-well plates with flat-bottom (Nunc MaxiSorp, Langenselbold, Germany) were coated with capturing antibody for 1 hour at 37°C. Prior to the next incubation and in between each following incubation (except the last) a 5-time washing step was performed using a solution of PBS and 0.05% Tween-

20. The plates were then blocked using 5% skimmed milk or 2% BSA for 2 hours at room temperature. Then samples and recombinant soluble HLA-G standard [24] were applied and incubated for 90 min at 37°C. The biotinylated detection mAb was incubated for 1 hour at 37°C and the signal intensity was increased by using AMDEX Strep-HRP detection enhancer (Dako, Copenhagen, Denmark) for 1 hour at 37°C. As a substrate we used TMB Peroxidase Substrate and 0.02% H_2O_2 in Citric Acid buffer, freshly made prior to use, incubated for 10 min at room temperature. The enzymatic reaction was stopped by adding 2M H_2SO_4 to the wells without washing and optical density was measured at 450nm. The final concentrations were calculated by 4P (4 parameter algorithm with a weighed function ($1/y^2$)) curve fitting of the standard curve.

FACS analysis and mAb used for flow cytometry

After thawing in FBS (Hyclone, Logan, UT), PBMCs were washed with PBS and stained extracellularly with mAbs for 15 min in the dark at 4°C. Where indicated, additional steps were performed to carry out the intracellular staining using Fix&Perm solutions (An Der Grub Bio Research, Vienna, Austria) according to the manufacturer instructions. The following mAb were used in this study for flow cytometry: CD3-AmCyan, CD16-PacBlue and IgG1-PE (BD Biosciences, San Jose, CA); CD56-APC (Beckman Coulter Immunotech, Marseille, France); anti-HLA-G MEM-G/9 (ExBio, Prague, Czech Republic); CD20-PacBlue (eBioscience, San Diego, CA); CD14-PECy7 (BioLegend, San Diego, CA). All analysis were performed using FACS Canto II flow cytometer equipped with BD FACS-Diva software, version 6.1.1 (BD Biosciences, San Jose, CA). The gating strategy for the detection of HLA-G on lymphocytes was based on exclusion of dead cells by 7-AAD (BD Biosciences) and on consecutive gating as to define the specific cell type analyzed. For quantification of HLA-G+ cells the correspondent isotype control IgG1-PE was used to exclude unspecific binding.

Statistics

Statistical analysis was performed with GraphPad Prism (Version 5.01) and the type of statistical test used for each analysis is indicated in the Figure legends.

RESULTS

Increased serum HLA-G levels in patients with end-stage liver diseases

The amounts of the shed HLA-G1 and the soluble HLA-G5 isoforms (from here on HLA-G) were determined in serum before LTX and at several time points during the first year after LTX in a cohort of 30 patients. The cohort included 13 patients without graft rejection, 10 patients with an episode of acute rejection, 2 with chronic rejection within the first year after LTX and 5 patients who underwent a second LTX (re-LTX). Relevant clinical parameters of all the patients included in this study are listed in Table 1. Recipient age and gender were not significantly different among patients with different LTX outcomes (patients with chronic rejection were excluded from this analysis due to their low number), with the only exception that patients with a re-LTX included a significantly higher number of females than patients with acute rejection ($p=0.02$).

Pre-LTX HLA-G serum levels were significantly higher compared with those of a group of healthy individuals (n=24), whose age and gender did not differ significantly from our study cohort (Figure 1A). While 24 out of 28 LTX patients were HLA-G^+, 22 out of 24 of the healthy individuals were HLA-G^-. Additional pre-LTX serum samples, collected 1-10 months before LTX, were available from 12 patients. These latter samples showed that pre-LTX serum HLA-G levels in patients were stable in the time frame considered ($p=0.57$, calculated with Wilcoxon signed rank test). To assess if the variations in HLA-G expression correlated with the type of liver disease, we divided the patients into five groups according to their underlying disease: (I) cholestatic disease (PSC or PBC), (II) auto-immune hepatitis, (III) HBV or HCV-related cirrhosis, (IV) acute hepatitis or (IV) other causes of hepatic failure (Figure 1A). No difference was found in terms of serum HLA-G levels among patients with different underlying liver diseases. In addition, when we separated our cohort into patients with an acute or with a chronic type of liver disease requiring LTX (Table 1, "Indication"), no difference was found in their HLA-G levels ($p=0.1$).

In addition, we tested the correlation between serum HLA-G levels and liver function tests: AST and ALT as markers of liver inflammation, total bilirubin as a marker of liver function and γ-GT and alkaline phosphatase as markers of biliary damage. Our results showed the presence of a monotonic but relatively weak relationship (indicated by a statistically significant Spearman correlation, but with a low ρ coefficient) between

pre-LTX HLA-G levels and total bilirubin (Figure 1B). With regard to the post-LTX period, HLA-G serum levels correlated with ALT, AST and total bilirubin (Figure 1B). Altogether these results indicate that patients with end-stage liver disease have increased levels of HLA-G compared to healthy controls, but do not reflect differences in the type of liver disease. Additionally our results indicate that pre and post-LTX levels are correlated to the extent of liver function and dysfunction in bilirubin excretion.

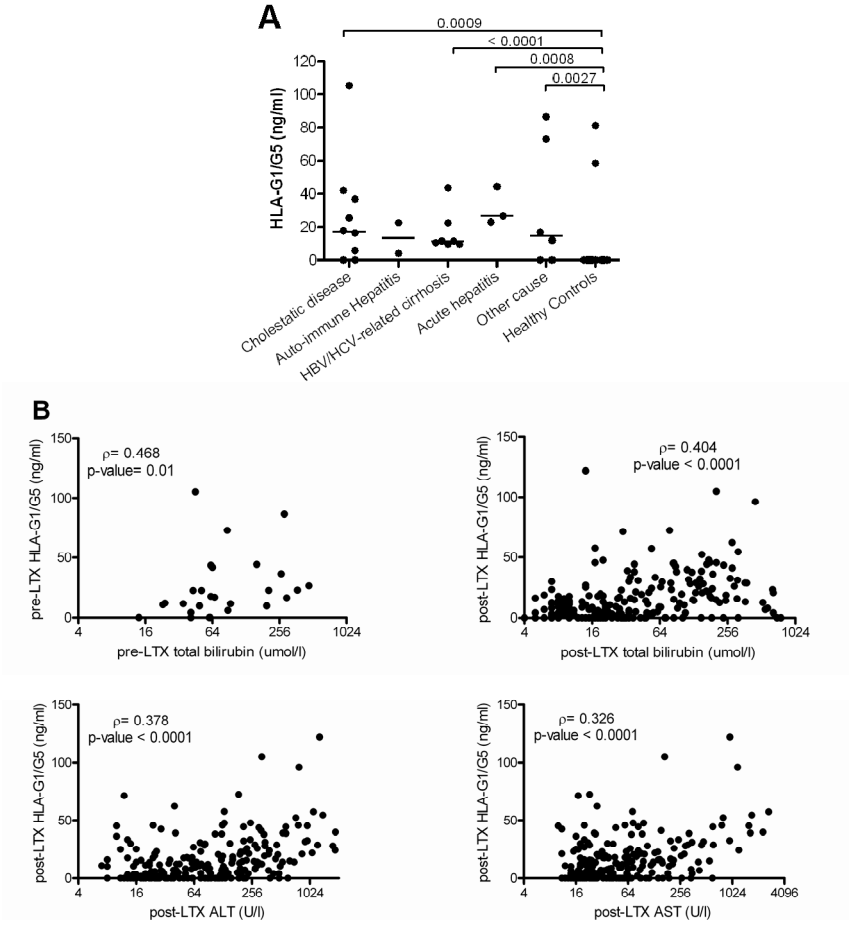

Figure 1. HLA-G serum levels in patients with different underlying diseases their correlation with liver function tests. (A) All patients in our cohort are here grouped on the basis of their underlying disease. Their HLA-G levels are compared both among different groups and with the HLA-G levels in healthy controls. Statistical significance was calculated by the Mann-Whitney test. Patients with auto-immune hepatitis were excluded from this analysis due to their low number. **(B)** Significant correlations between pre or post-LTX HLA-G serum levels and liver function tests. On the X-axis, values are reported in $\log_2$ scale. Correlations are estimated by Spearman rank correlation (ρ) and p-values indicate statistical significance.

Table I. Relevant clinical parameters of all patients included in the study.

Patient study nr	LTX date	LTX outcome*	Gender recipient	Gender donor	Underlying disease	Indication §	Cause of re-LTX	Pre-LTX ALT	Pre-LTX AST	IS therapy				Pre-LTX serum present	Post-LTX PBMC samples
										IL-2 blocker induction	High-dose steroids induction	Maintenance: Tac/CyA	Combined with: Aza/Ster/MMF		
1	01/08/1999	0	M	F	Cirrhosis-Alcoholic cirrhosis	C		23	47	-	√	-/√	-/√/-	√	-
3	21/03/1998	0	F	M	Cholestatic disease-PSC	O		98	44	-	√	√/-	-/√/-	√	-
4	03/12/1999	0	M	F	Cholestatic disease-PSC	C		58	50	-	√	-/√	√/√/-	√	-
7	24/04/1999	0	M	F	Wilson disease	C		141	187	√	√	-/√	√/√/-	√	-
8	17/03/2000	0	M	F	Cholestatic disease-PBC	C		48	66	√	√	-/√	-/√/-	√	-
11	21/08/2000	0	M	F	Cirrhosis-Chronic HCV	C		20	11	√	√	√/-	-/√/-	√	-
12	24/06/2000	0	F	M	Cirrhosis-Chronic HCV	C		58	114	√	√	√/-	-/√/-	√	-
14	07/07/2000	0	M	F	Cirrhosis-chronic HBV	C		42	53	√	√	√/-	-/√/-	√	-
15	15/09/1999	0	M	F	Cirrhosis-Chronic HBV	C		55	68	-	√	-/√	√/√/-	√	-
16	09/11/1999	0	M	F	Cirrhosis-Chronic HBV	C		47	85	√	√	-/√	√/√/-	√	-
20	30/11/2000	0	M	F	Cholestatic disease-PSC	C		81	88	-	√	√/-	-/√/-	√	-
22	13/12/2000	0	M	F	Cholestatic disease-PSC	C		62	63	-	√	√/-	-/√/-	√	
30	14/06/2000	0	M	F	Cirrhosis-Chronic HBV	C		41	72	√	√	√/-	-/√/-	√	-
2	06/11/1999	AR (d36)	M	M	Cirrhosis-Alcoholic cirrhosis	C		120	159	√	√	-/√	√/√/-	√	-
5	23/12/1998	AR (d7)	M	F	Cirrhosis-Alcoholic cirrhosis	C		41	66	-	√	-/√	-/√/-	√	√
6	05/12/1998	AR (d9)	F	M	Cholestatic disease-PSC	C		62	71	√	√	-/√	√/√/-	√	-
13	31/08/2001	AR (m7)	M	M	Cryptogenic cirrhosis	C		25	61	-	√	√/-	-/√/-	√	-
17	21/10/2001	AR (d9)	F	F	Cirrhosis-chronic AIH	C		24	33	√	√	√/-	-/√/-	√	-
18	23/08/2001	AR (d11)	F	M	Cryptogenic cirrhosis	C		16	37	-	√	√/-	-/√/-	√	-

19	19/11/2002	AR (d7)	F	M	AHF	A		381	667	√	√	√/-	-/√/-	√	√
23	23/02/1999	AR (d6)	F	F	AHF- HBV	A		1747	373	-	√	-/√	-/√/-	√	√
24	13/05/1999	AR (d5)	M	M	Cholestatic disease-PSC	C		75	195	√	√	-/√	√/√/-	√	√
29	29/02/2000	AR (d10)	M	F	Cirrhosis-chronic AIH	C		24	49	√	√	√/-	-/√/-	-	√
9	03/11/2000	CR (d12)	M	F	Cirrhosis-Chronic HBV	C		62	88	-	√	√/-	-/√/-	√	-
10	19/01/2001	CR (m3)	F	F	AHF- HBV	A		1306	680	√	√	√/-	-/√/-	-	-
21	23/11/2000	re-LTX (d8)	F	M	AHF	A	Budd-Chiari syndrome	1518	1606	-	√	√/-	-/√/-	√	-
25	23/11/2000	re-LTX (d2)	M	F	HCC and HBV-related cirrhosis	T	Primary non function	81	113	-	√	-/-	-/√/-	√	-
26	04/12/1998	re-LTX (d29)	F	M	Cholestatic disease-PSC	C	Biliary Complications	52	91	√	√	-/√	√/√/-	√	-
27	26/10/2001	re-LTX (d16)	F	M	Cirrhosis-chronic AIH	C	Ischemic biliary damage	32	36	√	√	√/-	-/√/-	√	-
28	15/02/2001	re-LTX (d5)	F	M	Cholestatic disease-PBC	C	Hepatic artery thrombosis	67	79	-	√	√/-	-/√/-	√	-

* 0= no rejection; AR= acute rejection; CR= chronic rejection; re-LTX= re-transplantation of the liver. For each patient with AR or CR, the time of rejection is indicated in days (d) or months (m). For patients with a re-LTX the time between first and second LTX is indicated in days (d).
§ C= chronic; A= acute; O= other; T= tumor
PBC = primary biliary cirrhosis; PSC = primary sclerosing cholangitis; AHF = Acute Hepatic Failure; HCV = Hepatitis C Virus; HBV = Hepatitis B Virus; AIH = auto-immune Hepatitis; HCC = Hepatocellular Carcinoma

Post-LTX levels of HLA-G in serum are higher in patients with acute rejection

As shown in Figure 2A, HLA-G serum levels gradually decreased from 1 month after LTX. Patients with or without episodes of acute rejection did not differ in terms of pre-LTX HLA-G levels. Conversely, despite the large variations among individuals, patients with acute rejection were characterized by higher HLA-G serum levels in the time intervals between 6 and 10 days (p=0.005) and between 11 and 15 days post-LTX (p=0.03) (Figure 2B). These time intervals correspond to the period in which most (12 out of 13) acute rejection episodes occurred in this cohort (Table 1). Importantly, during the interval 6-10 days, patients with acute rejection had a 40% relative increase of their HLA-G serum levels compared to their per-LTX levels, whereas patients without acute rejection showed a 50% relative decrease. During the time interval 11-15 days, HLA-G levels in patients with acute rejection showed a 20% decrease in their HLA-G levels compared to pre-LTX values, while patients without acute rejection had a relative decrease of 70%. The pre-LTX and the average post-LTX levels did not differ significantly between patients of different gender (p=0.98 for pre-LTX and p=0.95 for post-LTX levels, calculated with the Mann Whitney test).

Next, we focused on the HLA-G expression of patients with an episode of rejection. In these patients, we could not observe a clear pattern of HLA-G expression during the episode of acute rejection (Figure 3A). In some of these patients the HLA-G serum levels showed a variable increase around the time of diagnosis of acute rejection while in others the levels were stable or even decreasing. Only two patients in our cohort experienced chronic rejection and their serum HLA-G levels showed discordant patters after LTX and during rejection (Figure 3B). Altogether these data suggest that, during the time interval in which most acute rejections occurred, patients with an acute rejection have higher levels of HLA-G than patients without rejection. Nevertheless, we could not find a consistent pattern of HLA-G expression during episodes of rejection.

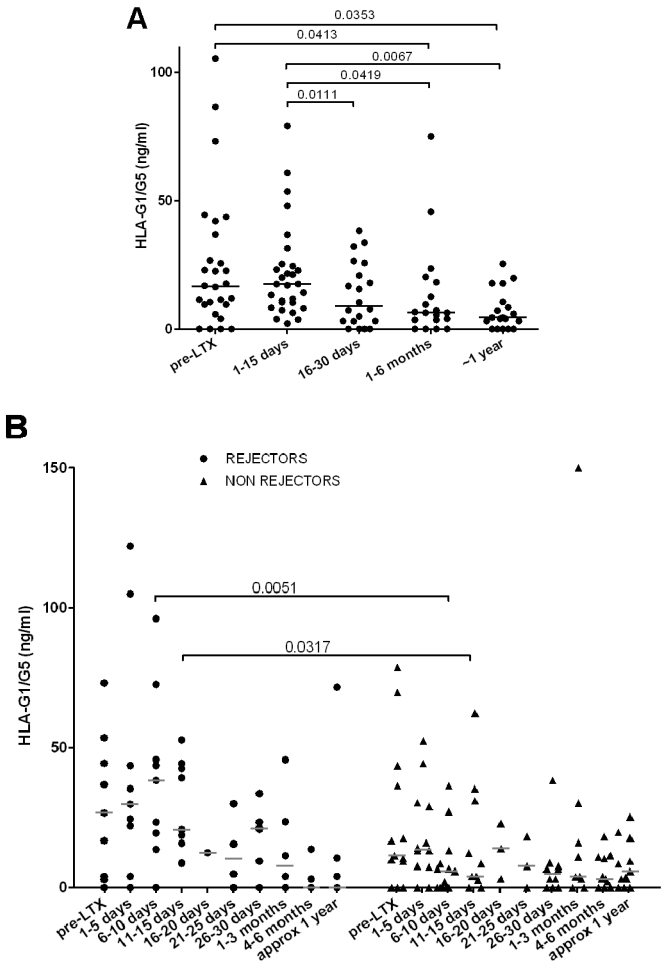

Figure 2. Comparison of the variations in HLA-G serum levels between LTX patients without rejection and with an acute rejection. (A) Graphs showing the variation of HLA-G serum levels during the specified time intervals in the whole cohort of patients (irrespectively of their underlying disease and LTX outcome). As to avoid adding confounders to the analysis, the data from patients that underwent re-LTX were included in this graph only until the day of re-LTX. Statistically significant differences between HLA-G levels in different time intervals, calculated by the Wilcoxon signed rank test, are indicated. **(B)** Pre-LTX and the average post-LTX serum levels of HLA-G during the indicated time intervals are here separated for patients with and without an episode of acute rejection. Statistical significance indicates differences between patients with an acute rejection (black dots) and patients without any rejection (black triangles) for their levels of HLA-G1/G5. Statistically significant differences were calculated by the Mann-Whitney test.

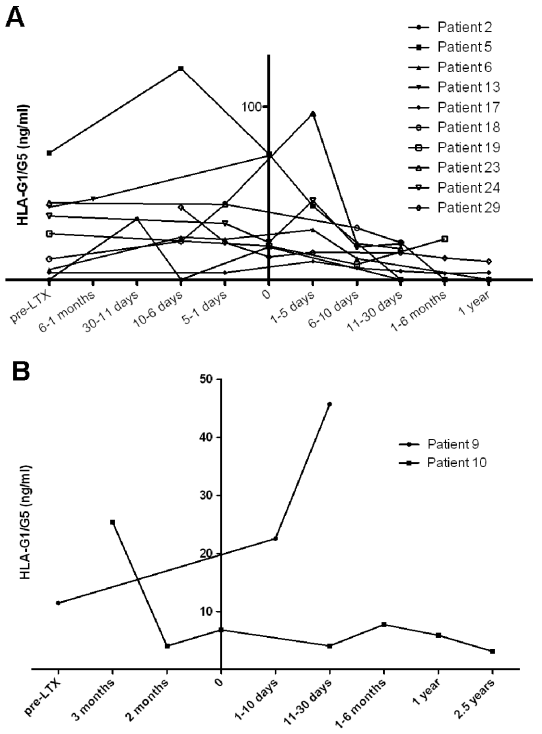

Figure 3. Comparison of the variations in HLA-G serum levels within patients with an acute or chronic rejection. (A) Graphs depict the serum levels of HLA-G1/G5 in LTX patients with an episode of acute rejection. The time-point "0" corresponds to the day of diagnosis of acute rejection while time-points on the left side of the Y-axis indicate HLA-G levels before rejection and the ones on the right side refer to the post-rejection values. **(B)** The same type of graphs is here depicting the HLA-G values for LTX-patients with chronic rejection.

HLA-G expression in PBMC

To analyze whether HLA-G is expressed in circulating leukocytes, and to investigate its possible correlation with outcome after LTX we measured by flow cytometry both the extracellular and intracellular levels of HLA-G in the main subsets of leukocytes from peripheral blood (T cells, NK cells, NKT cells, B cells, $CD14^+CD16^+$ and $CD14^+CD16^-$ monocytes). These measurements were performed on blood samples from five healthy controls, five LTX patients with acute rejection (at different time points) and four patients defined "operationally tolerant". Extracellular membrane-bound HLA-G levels of HLA-G were generally low in all groups and there was no significant difference between the two groups of patients or between patients and healthy controls (Figure 4). Intracellular HLA-G levels were higher in tolerant LTX patients but this difference reached statistical significance only in $CD14^+CD16^+$

monocytes of operationally tolerant patients compared to patients with acute rejection (Figure 4).

In LTX patients who experienced acute rejection, we did not find any clear and common pattern of variation of HLA-G expression (both at the extracellular and intracellular levels) in relation to the time of diagnosis of acute rejection (Tables 2A and 2B).

Taken together, these preliminary data in a small number of patients suggest that HLA-G expression in circulating leukocytes does not change during acute rejection in LTX patients, and that intracellular HLA-G expression may be elevated in $C14^{+}CD16^{+}$ monocytes in operationally tolerant patients.

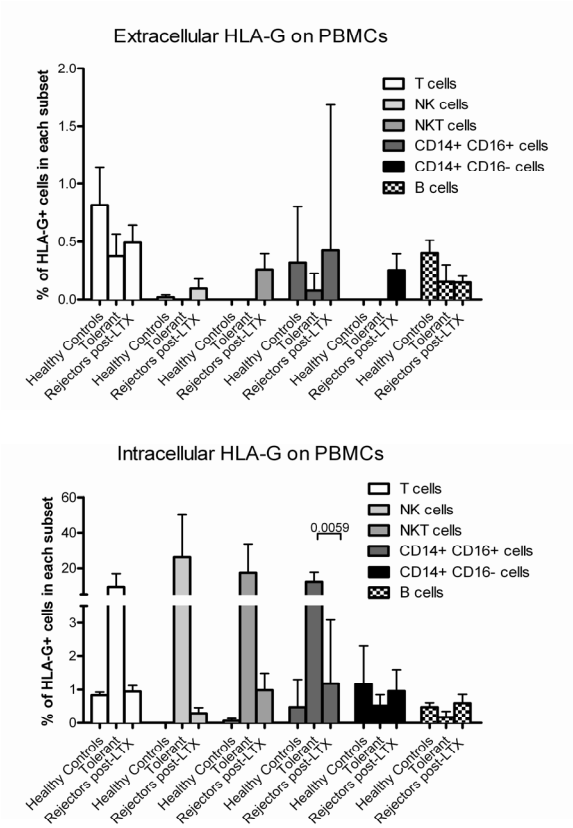

Figure 4. Extracellular and intracellular HLA-G in peripheral blood leukocytes. Graphs are showing the percentage of leukocytes with extracellular (upper panel) and intracellular (lower panel) expression of HLA-G in healthy controls, tolerant LTX patients and LTX patients with acute rejection. Graphs represent mean percentages of $HLA\text{-}G^{+}$ cells ± SEM in the indicate leukocyte subsets of 5 healthy controls, 4 tolerant LTX patients and 5 patients with acute rejection sampled at several time points post-LTX. Statistically significant differences were calculated by the Mann-Whitney test.

HLA-G is released from the donor liver after cold ischemic storage

To test whether HLA-G is released from the liver during cold storage, we measured the levels of soluble HLA-G in 20 samples of liver perfusion fluid (10 from perfusate 1 and 10 from perfusate 2) obtained by flushing liver grafts before LTX. In all the 20 samples of liver perfusion fluid we could detect HLA-G, and its levels in perfusate 1 and 2 were generally similar (data not shown). Importantly, the mean levels of HLA-G1/G5 detected in liver perfusion fluid were at least 9-fold higher than the levels detected in sera from LTX patients (both pre and post-LTX samples) and from healthy controls (Figure 5). This may indicate that the liver is a site of production of HLA-G, and that HLA-G is released during cold ischemic storage.

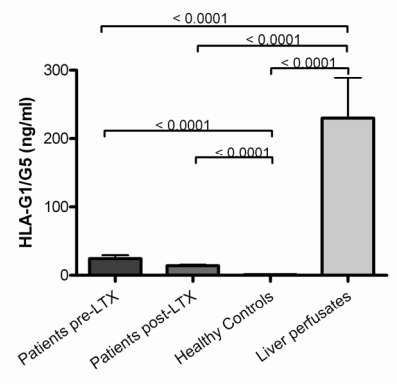

Figure 5. Comparison of HLA-G concentrations in liver perfusion fluid and serum, in LTX patients and healthy controls. Graphs are showing the mean levels ± SEM of soluble HLA-G detectable by ELISA in sera of LTX patients before and after LTX, in sera of healthy controls and in liver perfusion fluid. Statistically significant differences, calculated by the Mann-Whitney test, are indicated in the graph.

Table 2. Extracellular (A) and intracellular (B) HLA-G levels on PBMCs of LTX patients before and after LTX

A

Sample/ patient number§	Days post LTX	T cells	NK cells	NKT cells	CD14+ CD16+ monocytes	CD14+ CD16- monocytes	B cells	Diagnosis Acute Rejection (day)
Pat n.5	-1	0	0	0	0	0	0	
	1	0	0	0	0	0	0	
	7							*
	13	0	0	-	0	0	0	
	20	0	0	0	0	0	0	
	27	0	0	0	0	0	0	
Pat n.19	-1	0.6	0	0	0	0	0	
	6	0.8	0.4	0	0	0	1	
	7							*
	13	1.2	0	0	0	0.2	0.4	
	1year	1.4	0.1	0.2	0.3	0.2	0	
Pat n.23	0	1.5	0	1	0	0	0	
	3	0.9	0	0	0	0.6	0	
	6							*
	7	0.6	1.1	1.6	0	0	0	
	14	0	0	0.4	4.4	0.6	0.4	
	21	0	0	0.2	0.3	0	0	
Pat n.24	**5**							*
	7	0.5	0	0	0	0.5	0.5	
	14	0.5	0	0.7	0	1.8	0.3	
	22	1.4	0	0	0	0	0.2	
	28	0.8	0	0	0	0	0	
Pat n.29	6	0.5	0	0.6	0	0	0	
	10							*
	13	0	0	0	0	0	0	

Grey line= day of diagnosis of acute rejection

B

Sample/ patient number§	Days post LTX	T cells	NK cells	NKT cells	CD14+ CD16+ monocytes	CD14+ CD16- monocytes	B cells	Diagnosis Acute Rejection (day)
Pat n.5	-1	0	0	1.2	0	0	1.4	
	1	0.6	0	0	0	0	0	
	7							*
	13	0	0.2	0	0.4	0	0	
	20	0	0	0	0	0	0.4	
	27	2.3	0	1	5.8	0	0	
Pat n.19	-1	0.9	0	0.2	0	0	0	
	6	1.3	0.8	1.7	0	0.1	1.6	
	7							*
	13	1.2	0	0	0	0.2	2.3	
	1year	1.4	0.1	2	0.3	0.2	0	
Pat n.23	0	2	0	1.1	1.6	1	0	
	3	3.2	0.3	0.5	0	5.5	4.3	
	6							*
	7	0.6	1.1	5.6	0	0	2.8	
	14	1	-	-	4.4	0.7	0.4	
	21	-	-	-	1.2	0	0.9	
Pat n.24	**5**							*
	7	0.7	0	0	0	0.5	0.5	
	14	1.1	0	0.7	0.5	8.5	0.3	
	22	1.4	1.5	1.5	2.6	0	0.2	
	28	0.9	0	0	0	0.5	0	
Pat n.29	6	0.5	-	-	0	0	0	
	10							*
	13	0.7	0	0	0.1	1.8	0	

Grey line= day of diagnosis of acute rejection

DISCUSSION

In this study we showed that pre-LTX levels of HLA-G in serum of LTX patients are significantly higher than in healthy controls but not correlated to a specific liver disease, and that serum HLA-G concentrations decrease after LTX. Second, during the time interval in which most acute rejections occurred (6-15 days), serum HLA-G levels were significantly higher in patients who experienced acute rejection than in patients with no rejection. Despite this difference we could not find a consistent pattern of HLA-G expression in the serum of patients during acute or chronic rejection. Third, we detected substantially higher concentrations of soluble HLA-G in liver perfusion fluid collected from liver grafts before LTX compared to those found in serum, indicating that healthy livers contain HLA-G, which may be released during ischemic storage. Finally, our preliminary data suggest that HLA-G expression in circulating $CD14^{+}CD16^{+}$ monocytes may be increased in operationally tolerant patients compared to patients with acute rejection. By contrast, we found no significant variations of HLA-G expression in PBMC around or during an episode of acute rejection. Overall our data do not support the hypothesis that HLA-G may serve to identify transplanted patients who are likely to accept their allograft, as previously postulated by the group of Carosella [17].

While the tolerogenic role of HLA-G during pregnancy has been largely established, its function in solid organ transplantation is still debated. The main reason of this uncertainty is the fact that the few studies supporting the hypothesis of a tolerogenic function of HLA-G in transplantation are generally not convincing. Most of the recent work in this field has been performed by the group of Carosella. This research group has been supporting the hypothesis of a tolerogenic role for HLA-G in allogeneic organ transplantation with the following arguments: (I) the evidence that in case of multi-organ transplantation the presence of a liver allograft promotes better acceptance of the other grafted organ [25, 26] and that serum HLA-G levels are higher in patients with a combined liver/kidney transplantation than in patients with single liver or kidney transplants [17, 19]; (II) the finding of a positive correlation between the lower incidence of acute rejection (and absence of chronic rejection) and HLA-G expression in the graft of heart-transplanted patients [14, 15]; (III) observations in patients receiving a combined liver/kidney transplantation indicating

that the incidence of acute rejection was negatively correlated with HLA-G expression in liver biopsies ($p<0.05$) and in serum (no p-value reported) [17, 18]; (IV) the presence of soluble HLA-G in serum of 30% of kidney transplanted patients which was negatively correlated with the development of chronic rejection [20]. One of the main limitations of the studies reported above is the low number of patients in whom HLA-G was detected in the transplanted organ [14, 18, 20]. A second limitation is the fact that none of these studies clearly specifies the time points at which the samples (biopsies or serum) were taken [14, 18, 27]: an information that seems to be rather important, given the large variations in HLA-G serum levels detected in our study.

Yet few cohorts of single LTX patients have been analyzed until now. In the cohort of 58 LTX patients included in the study of Creput [18], only 6 patients (10.3%) expressed HLA-G on biliary epithelial cells and no significant correlation was found with incidence of acute rejection. Another study has found increased serum levels of soluble HLA-G in operationally tolerant pediatric and young adult LTX patients compared to patients who experienced acute rejection [21]. These results, however, are hard to compare to our study because of the different age of this cohort, the unspecified single time point of blood collection and, more importantly, the fact that acute rejection within the first year post-LTX was not considered since patients were included in the group of "acute rejectors" only when the episode took place more than one year post-LTX. In other two studies by the group of Carosella [17, 19] a cohort of single LTX patients was also included to measure the serum [17] or plasma [19] levels of HLA-G, but the levels were compared only among different types of transplantation (single LTX, liver-kidney transplantation, single kidney transplantation or healthy controls) and not between patients with/without acute or with chronic rejection. Therefore, although in both cases the results indicated that LTX patients had higher levels of soluble HLA-G than healthy controls and kidney transplanted patients (but lower levels than in combined liver-kidney transplants), we believe that drawing the conclusion that HLA-G is the factor that induces tolerance in liver transplantation is here too premature. Importantly, our results in single LTX recipients suggest the opposite: higher HLA-G serum levels in patients that experienced acute rejection.

Naij and colleagues [19] reported that stably high levels of HLA-G were found in plasma of LTX and liver-kidney transplanted patients but that subjects with single kidney transplantation had HLA-G plasma levels similar to the ones found in healthy

controls. The authors interpreted these findings as a strong suggestion of the specific role of the liver graft in up-regulating HLA-G expression and, thereby, inducing tolerance. However, since our observations indicate that HLA-G levels are already high in patients with end-stage liver disease before LTX (compared with healthy controls) and decrease after LTX, and that higher levels of HLA-G are not correlated with a positive LTX outcome, we cannot confirm stimulation of HLA-G synthesis by the liver graft nor the direct link between the possible hepatic production of HLA-G and its tolerogenic effect.

Interestingly, we observed that fluid collected during perfusion of liver grafts, after cold storage, contained high concentrations of HLA-G. This observation suggesting that donor livers can produce HLA-G after prolonged cold storage needs further confirmation and testing in additional control samples. Our observations that pre-LTX serum HLA-G levels were, irrespectively of the type of liver disease, much higher compared to those of healthy individuals, and that serum HLA-G concentrations decrease after LTX, suggest that HLA-G synthesis in the liver is enhanced during end-stage liver disease.

Another interesting preliminary result in our study regards the higher expression of HLA-G in circulating $CD14^{+}CD16^{+}$ inflammatory monocytes of operationally tolerant LTX patients compared to patients with an acute rejection and to healthy controls. A similar result has been reported before for monocytoid DC from a detailed study analyzing differences in DC-subsets among tolerant LTX patients, patients that maintained immunosuppression and healthy controls [28]. In the abovementioned study the elevated HLA-G expression in tolerant patients was associated with a role of mDC in the immune regulation. For our results, at this early stage we can only hypothesize that HLA-G may be released by inflammatory monocytes as to dampen the immune response both in a paracrine and autocrine fashion (as $CD14^{+}CD16^{+}$ monocytes express high levels of the inhibitory receptor for HLA-G ILT-4 [29]).

Our study is the first in which serum HLA-G concentrations in LTX patients are analyzed longitudinally, including time points before transplantation. Using the most specific antibodies available [30], we observed that serum HLA-G levels are high in patients with end-stage liver disease and decrease after transplantation with a healthy liver. In addition, during the time interval in which most acute rejection episodes occur, serum HLA-G levels are higher in patients with acute rejection compared to non-rejectors. Altogether these preliminary observations suggest that the expression of

HLA-G may be related to a state of inflammation rather than operational tolerance, in accordance to what was proposed by one of the earliest papers of the group of Carosella [31]. In this opinion paper [31] the authors provide an interpretation of the expression of HLA-G in inflammatory diseases by proposing that HLA-G may function as a negative-feedback signal that limits the inflammatory process. The lack of a consistent pattern of HLA-G expression during acute rejection excludes the possibility that, with the current detection techniques, this molecule can be used as a biomarker to identify patients that accept their graft after LTX, as previously proposed by Bastuk and Carosella [1, 16, 17, 32, 33]. Further research and additional laboratory techniques are needed to support our preliminary observations in a larger cohort of LTX patients.

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Chapter 7

Summary and discussion

1. General discussion

The liver is an organ with its own local immune compartment and a peculiar system of blood supply. Due to its anatomical and functional characteristics, which determine its constant exposure to a large antigenic load, the liver needed to evolve a complex system to control unwanted immune responses against harmless antigens. The establishment of this particular environment is also presumably responsible for the relative tolerogenicity of liver grafts. In fact, after clinical LTX, the incidence of chronic rejection is much lower compared to other organ grafts [1, 2]. Moreover, immunosuppression can be safely withdrawn in about 20% of liver transplant recipients, whereas life-long immunosuppression is needed to prevent rejection after transplantation of other organ grafts [3]. Similarly, liver transplants in some rat and mice strains are tolerated without the need of immunosuppression, even across MHC barriers [4-6]. For several decades, these peculiar characteristics have prompted researchers to assess the mechanisms determining immunological tolerance in the liver. Unraveling these mechanisms will help understanding the cellular basis of LTX tolerance and will contribute, in more general terms, to explain the main pathways of peripheral immunological tolerance. These findings may also contribute to the development of novel strategies of tolerance induction in LTX and to the design of new therapies inducing the immune response in liver diseases, such as chronic viral infections (e.g. HCV, HBV), characterized by insufficient intrahepatic responses to the virus.

In general terms, liver-resident lymphocytes are considered key players for the establishment of transplant tolerance. However the contribution of each cell type has not been assessed yet. NK cells possess two important characteristics that, combined, make them uniquely relevant to transplantation: they are able to distinguish allogeneic cells from *self* and they have potent cytolytic effector mechanisms [7]. In addition, NK cells constitute a large percentage, around 40%, of all hepatic lymphocytes. Therefore the role of liver NK cells in LTX is the main focus of this thesis.

2. Characterization of donor hepatic NK cells and their migration patterns into the recipient after LTX

In **chapter 2** we have established the degree of chimerism in peripheral blood of recipients after LTX and compared it to kidney-transplanted patients. In this study we showed that, in addition to T cells, B cells and myeloid DC [8-11], a considerable number of donor NK cells and $CD56^+$ T cells are transferred into the recipient after LTX, but not after kidney transplantation. Donor NK cell chimerism remained detectable for approximately two weeks in the recipients' peripheral blood and expressed a pattern of chemokine receptors and integrins indicating the potential of these donor cells to migrate into spleen, gut-associated lymphoid tissues or bone marrow of the recipient. Further details on the potential displacement of donor NK cells (or their direct precursors) to other sites in the recipient have been reported in chapter 5 of this thesis. Although the precise role of donor leukocyte chimerism after transplantation has not been established yet, it is believed to correlate with the induction of donor-specific tolerance [12-17]. More insights in this mechanism have been reported in chapters 3 and 4.

In addition, in chapter 2 we analyzed the immunophenotypical and functional characteristics of hepatic NK cells in adults and compared them with NK cells from peripheral blood. By this analysis we confirmed that intrahepatic NK cells are physiologically highly activated (the majority of NK cells expressing the early activation marker CD69) and that a large proportion of them belong to the $CD56^{bright}$ subset (around 50% compared to 10% in peripheral blood). A number of additional phenotypical markers were then analyzed and a few differences emerged. Among other surface molecules, hepatic $CD56^{bright}$ NK cells expressed higher levels of CD11a, the α-chain of the integrin LFA-1, highly expressed on all hepatic lymphocytes for adhesion to Kupffer cells and liver sinusoidal endothelial cells [18, 19]. Although $CD56^{bright}$ NK cells from blood are classically considered poorly cytotoxic and mainly cytokine producers, we here demonstrated that hepatic $CD56^{bright}$ NK cells contain considerable amounts of perforin and granzymes and exhibit potent degranulation activity. Compared with their blood counterparts, hepatic NK cells exhibited a two-fold higher cytotoxicity and a similar level of degranulation when co-incubated with K562 target cells. When stimulated by various combinations of stimuli (PMA+ionomycin; IL-12+IL-15; IL-12+IL-18; IL-2+IL-21), blood and liver-derived NK cells

exhibited similar patterns of IFN-γ production and no expression of the immunoregulatory cytokine IL-10 or Th2 cytokines, such as IL-13. These results suggest that with the same levels of degranulation and cytokine production, hepatic NK cells, and especially hepatic $CD56^{bright}$ NK cells, are intrinsically more cytotoxic than blood-derived ones. Altogether these data illustrate the nature of the donor hepatic NK cells transferred by LTX into the recipient and, more generally, the characteristics of intrahepatic NK cells. However, these results do not address the question of the role that donor NK cells may have in LTX in terms of tolerance induction. The *in vivo* effects of transferring these highly cytotoxic donor NK cells are explored in chapter 3, while an LTX rat model illustrates the effects of donor NK-cell depletion on spontaneous LTX tolerance in chapter 4.

3. The role of HLA/KIR genotypes and NK cells in LTX

3.1. Lack of evidence that NK-cell alloreactivity can predict LTX outcome

Chapter 3 illustrates that neither donor/recipient HLA/KIR mismatches nor predictions of NK-cell alloreactivity (in both donor-versus-recipient and recipient-versus-donor direction) are associated with LTX outcome. Our results therefore suggest that donor and recipient NK cells do not play a major role in acute graft rejection or graft and patient survival after LTX. The role of NK cells and of HLA/KIR genotypes in solid organ transplantation [7, 20-26], and especially in liver transplantation [27-31], have been a matter of debate for several years. Interestingly, NK-cell alloreactivity in organ transplantation has always been considered only in the recipient-versus-donor direction while donor-versus-recipient NK-cell alloreactivity has been studied only in the context of hematopoietic stem cell transplantation (HSCT). In HSCT, donor-versus-recipient NK-cell alloreactivity has been shown to prevent graft rejection besides inducing additional beneficial effects for the recipient [32]. Given the universally recognized tolerogenic effects of liver transplants and our previous observations showing transfer of highly cytotoxic donor NK cells after LTX (chapter 2) we hypothesized that, similarly to what observed in HSCT, donor NK cells may have a protective role in LTX. To test this hypothesis we analyzed a cohort of LTX donor/recipient pairs in terms of HLA and KIR genotypes and we determined the predicted effects of donor NK cells on acute rejection, graft and patient survival, by

use of common models describing NK-cell alloreactivity (e.g. "missing self" and "missing ligand"). Our data, however, indicate that donor NK-cell alloreactivity, as predicted by the abovementioned models, does not play a substantial role in LTX outcome. These data are in line with another study performed in a rat LTX model and described in chapter 4.

With regard to the effect of predicted recipient-versus-donor NK-cell alloreactivity in terms of HLA/KIR genotype, very few and contradictory results have been published until now in the field of LTX [28, 31]. We therefore aimed at adding new evidence to the existing data on the effects of recipient NK cells on LTX outcome by analyzing our study cohort in terms of recipient-versus-donor NK-cell alloreactivity. Here again we applied a number of models predicting NK-cell alloreactivity on the basis of donor/recipient HLA/KIR combinations. Our results indicate the absence of a clear effect of recipient NK cells on acute rejection, graft and patient survival.

Overall this chapter illustrates that we found no clear effects of either donor or recipient NK-cell alloreactivity in LTX. However, it is important to consider that several levels of complication add an intrinsic weakness to the interpretation of population studies aimed at correlating HLA/KIR genotypes to clinical outcomes. First, the features of NK-cell biology and the dynamics of KIR-dependent NK-cell alloreactivity are not fully understood [33-43]. Secondly, clinical factors such as different underlying diseases and variations in the immunosuppressive therapy may affect the functional competence of NK cells and may therefore hinder a direct correlation of HLA/KIR genotypes with clinical outcome [7, 25, 26, 44-46]. Lastly, additional factors, such as viral infections, may mask the direct effects of the sole genotype on NK-cell alloreactivity [47-50]. Notwithstanding the existence of the abovementioned limitations in this study, models that predict NK-cell alloreactivity on basis of KIR and HLA genotypes are presently the best approach available, especially since current experimental techniques do not allow direct quantification of alloreactivity of bulk NK cells [32, 51].

3.2. Donor NK cells do not affect acute liver rejection in rats

In **chapter 4** we provide further experimental evidence that donor NK cells do not play a major role in LTX tolerance. By using a well-established rat model of spontaneous LTX tolerance we demonstrated that specific depletion of donor NK cells prior to LTX did not affect the establishment of liver transplant tolerance. For

these experiments we performed liver transplantations from PVG donor rats to DA recipients. Despite being fully MHC-mismatched, a liver transplantation between these two strains does not lead to rejection, whereas other organ allografts (such as skin, heart and kidney) induce acute graft rejection in the same strain combination [52]. If NK cells were the main donor leukocyte subset responsible for liver allograft acceptance, depletion of these cells would lead to hepatic rejection. Specific NK cell depletion was therefore performed in the donors by use of a depleting antibody injected 24 hours prior to transplantation. After the NK-depleted livers of PVG rats were transplanted in DA rats, follow-up was analyzed for a period of more than 100 days. The recipient rats did not show any sign of distress after LTX and survived more than 100 days with functioning grafts, similarly to rats transplanted with non-NK cell depleted liver grafts [53]. Altogether these data support our previous observation that donor liver NK cells do not play an essential role in the induction of LTX tolerance.

4. NK-cell precursors in the liver may generate hepatic NK cells

In **chapter 5** we have tested whether liver-resident NK cells may derive from NK cells (or NK-cell precursors) in peripheral blood or from local hepatic precursors. First, we established the presence of NK-cell precursors in the adult human liver by identifying all the five sequential stages characterizing NK-cell development. These hepatic NK-cell precursors were immunophenotypically analyzed and compared to NK-cell precursors isolated from peripheral blood. While the two initial stages did not differ phenotypically, a few dissimilarities were detected from stage 3 of differentiation. Among other differences, more hepatic stage 3 precursors expressed the α-chain of the IL-7 receptor, CD127. Immunophenotypically similar but functionally diverse from stage 3 NK-cell precursors, Lymphoid Tissue inducer cells (LTi) are also defined as $CD34^{-}$ $CD117^{+}$ $CD94^{-}$ and express CD127 [54]; in addition, they require the expression of the nuclear transcription factor RORC and are characterized by the production of IL-17 and IL-22. Despite their CD127 expression, hepatic stage 3 NK-cell precursors were not LTi as they were $RORC^{neg}$ and did not produce IL-17 or IL-22.

Secondly we found that isolated hepatic $CD34^{+}$ cells generated mature NK cells upon *in vitro* culture in the presence of SCF, FLT3-L, IL-7, IL-15 and IL-21. These NK cells, which developed after 4 weeks of culture, were fully functional as assessed by

cytotoxicity and degranulation assays against the classical K562 target cell line. To answer the question of whether hepatic NK-cell precursors are stably resident in the liver, we then analyzed the number of donor NK cells and NK-cell precursors in recipients after LTX. Our results revealed that early NK-cell precursors in the liver are rapidly replaced by blood-derived ones. Nonetheless, mature donor NK cells were detected in the grafted liver long time after LTX. This observation may indicate that the human liver contains a population of long-lived memory NK cells, as recently demonstrated in mice experiments [55, 56]; alternatively it may indicate that after LTX donor-derived precursors displace to other sites, such as the bone marrow, from where they can replenish the population of donor NK cells in the long term.

In light of our results we propose the following model for NK-cell development in the adult human liver: $CD34^+$ cells, including potential NK-cell precursors, detach periodically from the bone marrow [57-59], recirculate via peripheral blood and relocate to a range of different tissues and peripheral organs, including the liver [57, 58, 60, 61]. Once in the liver, the peculiar local microenvironment can drive their direct differentiation into the so-called "liver-specific NK cells" [62] (Figure 1). These hepatic NK cells are known to reside in the lumen of hepatic sinusoids [63, 64], adhering to Kupffer cells and endothelial cells, and can be harvested by liver perfusion [9, 63, 65, 66]. Similarly to what was recently described in mice models [55, 56], after stimulation these hepatic NK cells may be able to acquire characteristics of immune memory as suggested by the persistence of donor NK cells in the grafted organ long time after LTX.

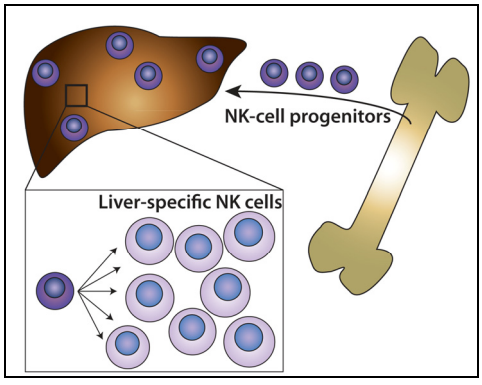

Figure 1. Schematic representation of the model for NK-cell development in the adult human liver. $CD34^+$ NK-cell precursors detach periodically from the bone marrow and reach the liver via peripheral circulation. Once in the liver, these cells can develop under the influence of the local microenvironment into liver-specific NK cells.

5. HLA-G in liver transplantation

In **chapter 6** we report the first results of our study aimed at exploring the role of HLA-G in graft acceptance after LTX. HLA-G is a non-classical HLA class I molecule with unique tissue distribution and immunoregulatory properties [67]. A number of studies have assessed the role of HLA-G during pregnancy [68-73]: HLA-G expression on placental trophoblasts has been linked to maternal immunological tolerance towards the semi-allogeneic fetus. After these initial studies, the expression of HLA-G has been described in other human tissues, especially in the context of solid organ transplantation and in cancer cells (for a critical review of this literature refer to [74]). However, these recent reports and the actual relevance of HLA-G expression in these tissues remain largely controversial for two main reasons. First, the technical limitations linked to the use of anti-HLA-G monoclonal antibodies, which may not only cross-react with other HLA class I molecules but may also be the target of aspecific binding by the Fc-Receptors of macrophages [74]. Second, the fact that our understanding of the biology of HLA-G is still incomplete and cannot provide a clear interpretation of the effects that the binding of HLA-G to different receptors may have on a range of immune cell types. In our study we tried to overcome at least part of these limitations by adopting a multifaceted approach: we selected a cohort of 30 LTX patients and measured their HLA-G expression in different samples (serum, bile, liver perfusion fluid, PBMCs, liver tissue), considering both the HLA-G membrane-bound and soluble forms by using a range of techniques and antibodies. Although the results we report in Chapter 6 do not yet represent the complete study, they constitute a basis for drawing some preliminary conclusions.

Before transplantation, HLA-G serum levels in LTX patients were higher than in healthy controls and decreased after LTX. However, pre-LTX HLA-G levels did not correlate with a specific underlying disease. Among LTX patients, recipients that experienced an episode of acute rejection had higher levels of soluble HLA-G in their serum compared to recipients without rejection. This difference was observed specifically during the time interval in which most acute rejections occurred (6-15 days). Despite this difference we could not observe any consistent pattern of HLA-G expression during acute rejection neither if considering the soluble isoforms of HLA-G (measured by ELISA in serum) nor the membrane-bound forms (measured by flow cytometry on PBMCs). Our findings are in contrast with most of the results reported in literature by the group of Carosella [75-77], although, as discussed in chapter 7, they are

sometimes hard to compare with previous research and may reflect the technical limitations discussed by Apps and colleagues [74]. Nevertheless, our preliminary observations seem to associate HLA-G production to states of inflammation rather than tolerance induction, as proposed in one of the earliest papers by Carosella [78]. Finally, our results suggest that production of HLA-G by the liver seems plausible, since high levels of HLA-G were found in liver graft perfusion fluid from deceased donor livers after cold storage. Overall until now our data do not support the hypothesis of a role for HLA-G in the induction of tolerance after LTX and exclude that HLA-G can be used as a monitoring tool for patients after LTX.

6. Final conclusion

The study described in this thesis represents an attempt to identify possible factors determining the induction of operational tolerance after LTX. Current beliefs propose that the establishment of tolerance is a multifactorial phenomenon in which the combined action of several factors conveys tolerance towards the grafted organ. Among these factors, the transfer of passenger donor leukocytes into the recipient after transplantation and the secretion of specific donor-derived soluble MHC molecules seem to play a relevant role. In this thesis we considered two components as possible inducers of LTX tolerance: donor NK cells (as part of the pool of passenger donor leukocytes) and HLA-G (as soluble HLA molecule). We analyzed different aspects of donor hepatic NK cells as to investigate their contribution to tolerance induction in LTX. Additionally, we laid the foundations for a critical assessment of the role of HLA-G as a tolerogenic molecule in LTX.

Our results suggest that hepatic NK cells in adults derive from hematopoietic $CD34^+$ stem cells that are continuously recruited from peripheral blood (Chapter 5, [79]). Hematopoietic $CD34^+$ stem cells are known to be periodically released from the bone marrow into peripheral blood [57-59]. This continuous release of cells into peripheral blood seems to constitute a mechanism to maintain full occupancy of hematopoietic stem cells niches not only in bone marrow cavities but also in peripheral tissues [57, 58, 61], including the adult liver [60]. Once relocated in the liver, these early progenitors may develop, under the influence of the local microenvironment, into liver-specific NK cells (Chapter 5, [79]). Liver-specific NK cells contain a unique subset of $CD56^{bright}$ NK cells that are physiologically activated and highly cytotoxic (Chapter 2 [80]). These characteristics of hepatic $CD56^{bright}$ NK cells differ from the functional properties attributed to the same subset of NK cells found in blood [81-84] or in other peripheral tissues, such as lymph nodes [85] or uterus [86-88].

After LTX, part of donor hepatic NK cells detach from the grafted organ and are detectable in the recipient circulation for approximately two weeks (Chapter 2 [80]). Besides this portion of migrating cells, donor hepatic NK cells remain in the grafted liver for at least two years after LTX (Chapter 5, [79]). This long-term chimerism can be explained by two scenarios. First, donor hepatic NK cells and their precursors may relocate, after LTX, to specific sites in the recipient (such as bone marrow, gut-

associated lymphoid tissues or spleen, see Chapter 2 [80]) and from there contribute to long-term donor NK-cell chimerism. Alternatively, the donor NK cells found long after LTX within the liver graft may constitute a pool of long-living NK cells similar to the ones recently described in mice liver by the groups of von Andrian, Lanier and Cooper [55, 56, 89-93]. Overall these observations in mice indicate that NK cells activated specifically by a chemical hapten [55] or a virus [56, 91], or non-specifically by a state of lymphopenia [90] or by inflammatory cytokines [89] acquire classical characteristics of immune memory that include an extended lifespan, the ability to self-renew, and the capacity to mount robust and protective recall responses [91]. However, as specific markers for human memory NK cells are not known yet [92], we could not test whether donor hepatic NK cells long after LTX actually represent a pool of NK cells that acquired immune memory.

With regard to the role of these donor-derived NK cells in the induction of allograft tolerance after LTX, we could not find firm evidence that NK cells play a prominent role in tolerance induction (Chapter 3 [94]; Chapter 4 [95]). In fact neither donor nor recipient NK-cell alloreactivity, as predicted by considering donor and recipient HLA/KIR genotypes, was found to correlate with LTX survival or the induction of allograft tolerance (Chapter 3 [94]). Additionally, specific depletion of hepatic donor NK cells prior to LTX in a spontaneously tolerant rat model, did not affect the establishment of tolerance (Chapter 4 [95]). Altogether, based on these results we reject the hypothesis that cytotoxic NK cells from the donor liver play a main role in inhibiting the immune response towards the allogeneic graft by killing alloreactive T cells and APCs from the recipient. Therefore, these results exclude the possibility of exploiting the cytotoxic properties of donor hepatic NK cells for tolerance induction in clinical LTX.

In addition to the study of NK cells, we here presented our initial attempts to assess the role of soluble HLA-G in LTX tolerance. In contrast to what was suggested by few original studies on this topic [67, 76, 77, 96-98], our first results seem to associate HLA-G with states of inflammation rather than tolerance induction (Chapter 6, manuscript in preparation). These results will be further expanded and additional experiments are needed to draw more definite conclusions on this subject.

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Nederlandse samenvatting

De studie die wordt beschreven in dit proefschrift heeft getracht de mogelijke factoren te identificeren die bepalend zijn voor het induceren van operationele tolerantie na levertransplantatie (LTX). Een lever wordt na LTX in het algemeen beter verdragen dan andere organen na transplantatie. Het bereiken van klinische tolerantie lijkt een multifactorieël fenomeen te zijn waarbij verschillende factoren, zoals het overdragen van passante donorleukocyten naar de ontvanger of de secretie van oplosbare MHC moleculen, samen leiden tot het bereiken van tolerantie van het ingebed orgaan. In dit proefschrift beschouwen we twee componenten als mogelijke factoren voor het induceren van LTX tolerantie: donor NK cellen (als deel van de passante donorleukocyten pool) en HLA-G (als oplosbare MHC molecuul).

Wij vonden dat lever NK cellen in volwassenen afkomstig zijn van hematopoëtische $CD34^+$ stamcellen die continu uit het perifeer bloed worden verworven **(hoofdstuk 5)**. Wanneer ze zich herlokaliseren in de lever kunnen deze vroege voorlopers onder de invloed van het lokale micromilieu zich ontwikkelen tot leverspecifieke NK cellen **(hoofdstuk 5)**. Leverspecifieke NK cellen bevatten een unieke subset van $CD56^{bright}$ NK cellen die zowel fysiologisch geactiveerd zijn als hoge cytotoxische activiteit hebben **(hoofdstuk 2)**. Deze karakteristieken van de lever $CD56^{bright}$ NK cellen verschillen van de functionele eigenschappen van dezelfde subset NK cellen die wordt gevonden in bloed of andere perifere weefsels, zoals lymfeklieren en uterus.

Na LTX maken gedeelten van de lever NK cellen zich los van het getransplanteerde orgaan en kunnen gedetecteerd worden in de bloedcirculatie van de ontvanger voor circa twee weken. Dit leidt tot een initiële staat van een kortdurende chimerisme (**hoofdstuk 2**). De overige donor lever NK cellen blijven in de lever voor ten minste twee jaar na LTX (**hoofdstuk 5**). Deze langdurende chimerisme kan verklaard worden door twee scenario's: de donorcellen gaan na LTX zich herlokaliseren in specifieke gebieden in de ontvanger en van daaruit dragen ze bij aan NK cel chimerisme, of deze cellen komen uit een pool van langlevende NK cellen die eerder ook in muizenlever is beschreven.

Betreft de rol van donorafkomstige NK cellen in de inductie van transplantaattolerantie na LTX hebben wij geen sterk bewijs kunnen vinden dat NK cellen daarin een prominente rol spelen (**hoofdstuk 3 en 4**). Voor zowel de donor als ontvanger NK cel alloreactiviteit vonden wij geen correlatie met de overleving van patiënten of de inductie van transplantaattolerantie (**hoofdstuk 3**). Daarnaast vonden wij dat specifieke verwijdering van lever donor NK cellen voorafgaand aan LTX in een spontane tolerante ratmodel geen effect heeft op het bereiken van tolerantie (**hoofdstuk 4**). Al met al, gebaseerd op deze resultaten verwerpen wij de hypothese dat cytotoxische NK cellen van de donorlever een grote rol spelen in het afremmen van de immuunreactie van de ontvanger op het allogene transplantaat. Deze resultaten ondersteunen daarom niet het gebruik van de cytotoxische capaciteit van donor lever NK cellen om tolerantie te induceren in de LTX kliniek.

Aansluitend op de studie van NK cellen beschrijven wij hier onze eerste poging in het identificeren van de rol van oplosbare HLA-G in LTX tolerantie. In tegenstelling tot het beperkte aantal studies die gedaan zijn op dit gebied, lijken onze eerste resultaten te suggereren dat HLA-G meer geassocieerd is met inflammatie dan het induceren van tolerantie (**hoofdstuk 6**).

In conclusie, in dit proefschrift hebben wij de oorsprong en de immunofenotypische en functionele eigenschappen van humane lever NK cellen gekarakteriseerd; hebben wij het bestaan van een kort- en langdurende chimerisme beschreven en de afwezigheid van specifieke tolerogeen effect van donor lever NK cellen na LTX; en hebben wij onze eerste resultaten laten zien dat er geen correlatie is tussen het niveau van HLA-G in serum en tolerantie na LTX.

Riassunto in italiano

Scopo del presente lavoro è stata la ricerca di possibili fattori determinanti tolleranza ai trapianti di fegato. La tolleranza ai trapianti è verosimilmente un fenomeno regolato da molteplici fattori quali, fra gli altri, il trasferimento di leucociti dal donatore al ricevente e il rilascio di molecole MHC solubili. È noto che i trapianti di fegato sono generalmente meglio tollerati rispetto ad altri trapianti d'organo. Abbiamo quindi preso in considerazione due possibili induttori di tolleranza ai trapianti di fegato: le cellule natural killer (NK) del donatore (come parte dei leucociti trasferiti al ricevente) e il complesso maggiore d'istocompatibilità HLA-G (come molecola MHC solubile).

Dai nostri risultati si evince che le cellule NK nel fegato di un individuo adulto derivino da cellule staminali ematopoietiche $CD34^+$ continuamente reclutate dalla circolazione sangiugna (**capitolo 5**). Una volta localizzate nel fegato, queste cellule precursori sono in grado di maturare sotto l'influenza del microambiante locale fino a diventare specifiche NK epatiche (**capitolo 5**). Queste cellule epatiche includono un particolare sottotipo cellulare ($CD56^{bright}$), caratterizzato da un'elevata attivazione fisiologica e da una spiccata capacità citotossica (**capitolo 2**). Tali proprietà appaiono diverse da quelle attribuite al medesimo sottotipo cellulare presente nella circolazione sanguigna o in altri tessuti periferici quali linfonodi e utero.

Subito dopo un trapianto di fegato, parte delle cellule NK del donatore vengono rilasciate dall'organo trapiantato e sono rintracciabili nella circolazione sanguigna per circa due settimane (**capitolo 2**). Ciò determina una condizione temporanea di chimerismo *a breve termine*. Un'altra parte delle cellule NK epatiche del donatore rimane invece confinata nel fegato per almeno due anni dopo il trapianto, configurando una situazione di chimerismo *a lungo termine* (**capitolo 5**). Due ipotesi sono al momento ritenute plausibili per spiegare questo secondo fenomeno. Alcune cellule del donatore, dopo il trapianto, potrebbero essere in grado di migrare verso specifici siti nel ricevente e contribuire quindi, nel tempo, al chimerismo cellulare prolungato. In alternativa, queste cellule del donatore potrebbero rappresentare invece un gruppo specifico di cellule NK con emivita prolungata e memoria

immunologica, simili a quelle recentemente descritte nel fegato di topi in seguito a stimolazione da virus, citochine o apteni.

I risultati del presente studio non hanno fornito alcuna evidenza che le NK del donatore inducano tolleranza ai trapianti di fegato (**capitoli 3 e 4**). Stimando l'alloreattività (la risposta immune indotta da differenze di antigeni tra individui) delle cellule NK, sia del donatore che del ricevente, non sono emerse differenze né in termini di sopravvivenza né di tolleranza al trapianto (**capitolo 3**). Inoltre, in un modello di tolleranza spontanea in ratti, la specifica deplezione di cellule NK epatiche del donatore prima del trapianto di fegato non ha condizionato la successiva accettazione del trapianto (**capitolo 4**).
Sulla base di questi risultati è stata quindi rigettata l'ipotesi sperimentale che cellule citotossiche NK del fegato del donatore abbiano un ruolo prominente nell'inibizione della risposta immunitaria del ricevente nei confronti di trapianto allogenico. Questi risultati sembrerebbero pertanto escludere, al momento, la possibilità di sfruttare le proprietà citotossiche delle cellule NK epatiche del donatore nel tentativo di indurre tolleranza ai trapianti di fegato.

In aggiunta alla sperimentazione appena descritta, in questo lavoro sono stati presentati i risultati preliminari di uno studio condotto per indagare il ruolo della molecola solubile HLA-G nei trapianti di fegato. A differenza di quanto suggerito dai primi studi condotti in questo campo, i nostri risultati sembrano al momento associare HLA-G a stati di infiammazione piuttosto che all'induzione di tolleranza (**capitolo 6**).

In conclusione, in questa tesi abbiamo caratterizzato l'origine e le caratteristiche immunofenotipiche e funzionali delle cellule NK epatiche; abbiamo descritto la formazione di una condizione di chimerismo di breve e lungo termine, e verificato l'apparente assenza di una diretta induzione di tolleranza delle cellule NK epatiche dopo trapianto di fegato. Abbiamo infine fornito le prime evidenze dell'assenza di correlazione tra i livelli postoperatori di HLA-G nel siero e la condizione di tolleranza ai trapianti di fegato.

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makes it sometimes very hard to get used to living in a "traditional and pretty Dutch house, standing on wooden poles" (=a sinking and cracking apartment with little "housemates" running around). But despite all that…I never left. And the only reason was that you two were there. Thanks to Cinthia e Miguel for adding some "Mediterranean spirit" to our great evenings during the Football World Cup (!!) and for your company during this last year. Thanks to Natalia for you company and for giving me some precious tips for finding the source of my thesis cover.

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Argos, September 2011

List of publications

V. Moroso, H.J. Metselaar, S. Mancham *et al.*, Liver grafts contain a unique subset of natural killer cells that are transferred into the recipient after liver transplantation. *Liver Transpl,* 2010. 16: 895-908.

V. Moroso, A. van der Meer, H.W. Tilanus *et al.*, Donor and recipient HLA/KIR genotypes do not predict liver transplantation outcome. *Transpl Int,* 2011.

Y. van Leest, V. Moroso, C. Wang *et al.*, No evidence for involvement of donor NK cells in liver transplant tolerance. *Transpl Immunol,* 2011. 24: 138-139.

V. Moroso, F. Famili, N. Papazian *et al.*, NK cells generate from precursors in the adult human liver. *Europ J of Immunol,* 2011. In press.

V. Moroso and J. Kwekkeboom, When do conventional cells become unconventional? Submitted for publication. Submitted for publication

Curriculum vitae

Viviana Moroso was born in Udine on the 27th of September 1983. In 2002 she started her Bachelor studies in Biotechnology at the Faculty of Medicine of the University of Trieste (Italy), from which she graduated *cum laude* in 2005. At the same University she started her Master degree in Medical Biotechnology. As a student she performed research on the cytokine secretion profiles of mouse endothelial cells under stress conditions at the Department of Experimental and Clinical Pathology under the supervision of Prof. Dr. Aldo Dobrina. For her Master's internship she moved to the Netherlands to perform a research project on the function and regulation of tumor suppressor proteins of the p53-family at the Department of Molecular Biology at the Nijmegen Centre for Molecular Life Sciences (NCMLS), Radboud University of Nijmegen (the Netherlands), under the supervision of Dr. Marion Lohrum. After starting her PhD in January 2008 at the Department of Gastroenterology and Hepatology at the Erasmus Medical Center in Rotterdam (the Netherlands), Viviana obtained *cum laude* her Master's degree in Medical Biotechnology. Her PhD project has been performed under the supervision of her promotor Prof. Dr. Herold J. Metselaar and co-promotor Dr. Jaap Kwekkebbom and the results of her research are presented in this thesis.

Currently, Viviana is training as a scientific and medical writer, editor and translator as to make a job out of her passion: making scientific and medical issues accessible to the general public. She is part of the European Medical Writers Association (EMWA) and the Mediterranean Editors and Translators (MET) and she will soon obtain her first qualification as a medical writer (EMWA Professional Development Programme certificate).

Portfolio

Courses (Erasmus MC):

Introduction to Clinical Research (2008)
Biostatistics for Clinicians (2008)
Regression Analysis for Clinicians (2008)
Survival Analysis for Clinicians (2008)
English Biomedical Writing and Communication (2008)
Basic Introduction Course on SPSS (2010)
Photoshop CS3 Workshop (2010)
Short Introduction Course on Statistics & Survival Analysis for MD's (2010)

Oral presentations at national and international conferences:

Dutch Society of Immunology (NVVI) annual meeting. Noordwijkerhout, the Netherlands, 2008
Dutch Transplantation Society annual meeting. De Eemhof, the Netherlands, 2009
Dutch Association of Gastroenterology (NVGE) meeting. Veldhoven, the Netherlands, 2009
International Liver Transplantation Society (ILTS) annual meeting. New York, USA, 2009
Dutch Transplantation Society annual meeting. Amsterdam, the Netherlands, 2011
The joint International Congress of ILTS, ELITA and LICAGE. Valencia, Spain, 2011

Poster presentations at national and international conferences:

ESOT Basic Science Meeting. Brussels, Belgium, 2009
EMBO Meeting. Amsterdam, the Netherlands, 2009
European Congress of Immunology. Berlin, Germany, 2009
Dutch Society of Immunology (NVVI) annual meeting. Noordwijkerhout, the Netherlands, 2010
Dutch Association of Gastroenterology (NVGE) meeting. Veldhoven, the Netherlands, 2011
Dutch Transplantation Society annual meeting. Amsterdam, the Netherlands, 2011

Other congresses/meetings attended:

EASL Monothematic Conference Immune Mediated Liver Injury. Hamburg, Germany, 2008
Erasmus Liver Day. Rotterdam, the Netherlands, 2008
Symposium & Master Classes on Mucosal Immunology. Rotterdam, the Netherlands, 2008

Scientific awards:

Travel Award for oral presentation at the International Liver Transplantation Congress, New York, 2009

Other activities:

Dutch Organization for Scientific Research (NWO): Talent Day 2010 on scientific communication

Starter's Day by the Society of English-Native-Speaking Editors (SENSE) (2010)

Member of the Mediterranean Editors and Translators (MET)

METmeeting 2011. Barcelona, Spain, 2011; workshops attended:

Getting started in Medical Translation

Study designs in medical research: reporting structures and roles in knowledge-building

Member of the European Medical Writers Association (EMWA)

Pursuing the foundation certificate in Medical Writing offered by EMWA Professional Development Programme; workshops attended (2010/'11):

Editing and proofreading essentials

European regulatory procedures for medical writers

Grant writing: a business opportunity for medical writers?

Summarizing

Sharpen up your writing skills

Why do drugs and medicines have adverse effects?

The patient information leaflet

Targeting your audience

Statistical analysis of binary data

On-line education (eCPD webinars and other webinars, 2010/2011):

Building a website - the Basics

Demonstration of 3 do-it-yourself websites

Overview of CAT tools

Specializing in medical translation

Pricing strategies for translators

Consumer Writing and Patient Education

Essential Marketing Rules for Medical Writer Websites that Win

How to Find, Close, and Keep Medical Writing Business with Online Marketing

Job Hunt Tips from a Recruiter's Notebook - So You Want a Medical Writing Job?