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Intrahepatic and peripheral blood phenotypes of NK and T cells: Differential surface expression of KIR receptors

Short title: Phenotype of liver mononuclear cells

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List of abbreviations: human-liver mononuclear cells (LMCs), Natural killer cells (NK), dendritic cells (DCs), killer cell immunoglobulin-like receptors (KIR), effector T cells (T_E), central memory T cells (T_{CM}), transitional memory T cells (T_{TM}), effector memory T cells (T_{EM}).

Summary

Deep characterization of the frequencies, phenotypes and functionalities of liver and peripheral blood NK, NKT and T cells from healthy individuals is an essential step to further interpret changes in liver diseases. These data indicate that CCR7, a chemokine essential for cell migration through lymphoid organs, is almost absent in liver NK and T cells. $CD56^{bright}$ NK cells, which represent half of liver NK cells, showed lower expression of the inhibitory molecule NKG2A and an increased frequency of the activatory marker NKp44. By contrast, a decrease of CD16 expression with a potential decreased capacity to perform ADCC was the main difference between liver and peripheral blood $CD56^{dim}$ NK cells. Liver T cells with an effector memory or terminally differentiated phenotype showed an increased frequency of MAIT cells, TCR γδ T cells and TCRαβ CD8+ cells, with few naive T cells. Most liver NK and T cells expressed the homing markers CD161 and CD244. Liver T cells revealed a unique expression pattern of KIR receptors, with increased degranulation abilityand higher secretion of IFN-γ. Thus, the liver possesses a large amount of memory and terminally differentiated CD8+ cells with a unique expression pattern of KIR activating receptors that

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have a potent functional capacity as well as a reduced amount of CCR7 that are unable to migrate to regional lymph nodes. These results are consistent with previous studies showing that liver T (and also NK) cells likely remain and die in the liver.

Introduction

The immune response to pathogens, alloantigens and tumours is mainly investigated in peripheral blood. There is limited information concerning the identification and physiological mechanism of immunological participating factors in peripheral tissues and organs. The liver-specific immune response depends on an array of non-parenchymal cells that include liver sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and dendritic cells (DCs), which interact with liver-associated lymphocytes [1]. Previous studies have attempted to characterize the phenotype of non-parenchymal liver cells isolated after enzymatic digestion of liver fragments[2], and several studies [3-5] have analysed the phenotypic and functional characteristics of human-liver mononuclear cells (LMCs). However, extended phenotypic and functional studies may be restricted due to the limited amount of cells available from tissues or organs of patients who underwent surgery for an underlying disease or during liver transplantation. Recently, liver mononuclear cells were isolated during the perfusion of the living donor liver lobe prior to transplantation or through perfusion of livers from deceased donors[6, 7]. The advantage of these approaches is that they avoid tissue destruction and do not require enzymatic treatment. Previous studies[7,8] have demonstrated that liver natural killer (NK) cells, $CD3^{+}CD56^{+}$ and $CD3^{+}CD56^{-}$ T cells, differ in their phenotypes with peripheral blood mononuclear cells but are immersed in a tolerogenic microenvironment[9]. NK cells are innate immune cells that are specialized to eliminate virus-infected cells. These cells can be divided into functionally distinct subsets

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based on their level of CD56 surface expression as primarily cytotoxic $CD56^{dim}$ NK cells and more immunoregulatory cytokine-producing $CD56^{bright}$ NK cells. The functions of both NK cell groups are modulated through inhibitory and activating signals from distinct classes of receptors. Inhibitory receptors include the polymorphic system, killer cell immunoglobulin-like receptors (KIR)[10] and members of the C-type lectin-like receptor CD94/NKG2A family, which recognize HLA-E [11]. Activating receptors include natural cytotoxicity receptors (NKp30, NKp44, and NKp46), NKG2C expressed as a dimer with CD94, signalling lymphocyte activation molecule (SLAM) family receptors[12], lectin-like receptor NKG2D and FcγRIIIa receptor (CD16), which mediates antibody-dependent cytotoxicity [13]. KIR expression on NK cells is largely random and determined based on the KIR gene contents, polymorphisms, and stochastic epigenetic regulation at the promoter level[14,15]. The interactions between KIRs and HLA ligands ensure the maintenance of self-tolerance. Expression of inhibitory receptors is genetically determined, and in the absence of infection, inflammation and other diseases, NK cells primarily receive inhibitory signals. Alternatively, NK cell activation increases the expression of activating receptors[16], enabling NK cells to reach a more responsive state in the context of infection and inflammation.

At least for some KIR receptors, the expression frequencies at the cell surface level are higher in individuals with 2 copies of the respective KIR gene compared to those with only 1 copy, suggesting a gene dose effect[14,17]

These receptors modulate NK cell function for the rapid targeting and destruction of virally infected cells without the need for prior sensitization, making them an important first line of defence against viral pathogens.

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Liver $CD3^+$ cells are characterized by a high frequency of $CD8^+$ cells and subset of NKT cells that are represented by their expression of CD3 and CD56 [2,7]. $CD8^+$ T cells can exist in at least three states of reactivity: naïve $CD8^+$ T cells with low reactivity, activated (effector) $CD8^+$ T cells with high reactivity and memory $CD8^+$ T cells with intermediate reactivity. The overall memory $CD8^+$ T cell compartment is represented by central and memory subsets, which can be recognized based on their phenotype and function [18,19]. However, the state of activation and cell differentiation of liver CD8+ T cells remains controversial. A previous report using liver perfusion described a relatively high level of $CD8^+$ T cells with a naive phenotype, suggesting that in addition to their role as a graveyard for Ag-specific activated $CD8^+$ T cells, naive $CD8^+$ T cells may enter the liver without prior activation[20]. By contrast, other studies have demonstrated that the healthy human liver is a site of intense immunological activity[7] and contains a liver-homing/retention marker-expressing $CD8^+$ T cell pool identified as tissue-resident memory T cells, which are not observed within circulating memory $CD8^+$ T cells [4].

Recently, we showed that expression of KIR genes on T cells is primarily restricted to liver T cells[21], potentially representing highly differentiated T cells.

In the present study, using LMCs isolated from liver perfusion, we performed extensive analyses of NK cells and T-cell subsets to obtain novel insights into the prevalence of various lymphocyte subsets and the putative use of KIR genes by intrahepatic NK and T cells.

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Patients and Methods

Obtaining liver mononuclear cells from healthy liver donors (LMCs)

Liver wedge samples

Liver samples (10-20 grams) were obtained from hepatic transplant donors, collected in sterile physiological solution, stored at 4 °C, and processed within the following 12 hours. After washing with sterile PBS, the samples were mechanically disintegrated and collected in a Petri dish with 10 ml of RPMI 1640 containing 0.5 mg / ml collagenase type II (clostridiopeptidase A), 0.02 mg / ml gentamycin, 2 mmol / l L-Glutamine (Sigma-Aldrich, Darmstadt, Germany) and 10% foetal bovine serum (Natocor, Cordoba, Argentina) and incubated at 37 °C for 30 minutes. The samples were subsequently filtered through a 70 mm metal mesh and washed with 1X PBS. Mononuclear cells were purified through a Ficoll-Hypaque gradient (Sigma-Aldrich, Darmstadt, Germany).

Liver perfusion and cell sample collection

Samples were collected from donor livers according to Kelly et al [6]. Briefly, 23 samples were collected from donor livers during orthotropic liver transplantation: 14 donors at the Austral University Hospital and 9 donors at the liver transplant unit of the Italiano Hospital. During retrieval (at the time of exsanguination), the donor aorta and superior mesenteric vein were flushed with University of Wisconsin (UW) solution (Bristol-Myers Squibb, Uxbridge, UK) or with HTK (histidine-tryptophan-ketoglutarate) solution (Custodiol, NJ, USA). After excision of the organ, the liver was flushed again with UW solution until all blood was removed and the perfused solution appeared clear. At implantation, after completion of the upper inferior cava anastomosis, the livers were flushed with ringer lactate at 4^0C degrees through the portal vein to wash out UW before reperfusion. The

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perfused hepatic fluid was collected from the inferior cava vein, (600–1200 ml). Within the following 12 hours, the samples were sent to the laboratory and centrifuged at 500 g for 5 minutes, and the supernatant was discarded. All pellets were collected washed twice with 1X PBS at 500 g for 5 minutes, and mononuclear cells were subsequently isolated through a Ficoll-Hypaque (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient.

All protocols were approved by the Hospital de Clínicas and Hospital Italiano Institutional Review Board in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. All samples were identified using a transplant procedure number provided by the INCUCAI without the name of the donor. None of the transplant donors were recruited from a vulnerable population, and all donors or next of kin provided written informed consent that was freely given.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained from 5-20 ml of heparinized blood from 35 adult healthy controls and isolated through a Ficoll-Hypaque density gradient.

Monoclonal antibodies and flow cytometry

PBMCs and LMCs were stained with unconjugated antibodies against KIR 3DL1/3DL2 (clone 5.133, courtesy of Dr. M. Colonna), KIR 2DL2/2DS2/2DL3 (clone CHL, courtesy of Dr. S. Ferrini), KIR 2DL1/2DS1 (clone HPMA4), KIR 2DL1/2DS1/2DS3 (clone HP3E4), CD94 (clone 3D9) and NKG2A (clone Z199) courtesy of Dr. M. Bottet and unconjugated isotypes against IgG1, IgG2a, IgG2b, IgM (Becton Dickinson, San Jose, CA, USA). In addition, mouse monoclonal antibodies against 3DL1-FITC, 2DL3-PE (Becton Dickinson, San Jose, CA, USA),

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2DS4-PE, NKG2C-PE (R & D Systems, MN, USA) NKp44-PE, NKp46-PE, 2B4-FITC, CD16-FITC, CD57-APC, CD8-PE/APC, CD4-FITC, CD45RA-PE-Cy7, CD28-PE, CD27-PE-CF594, CCR7-FITC, CD11b-FITC, CD161-APC/PE, HLA-DR-FITC, TCR-γδ-PE, and TCRVα7.2 MAIT-PE (Biolegend, San Diego, CA, USA) and labelled isotypes against IgG1, IgG2a (Biolegend, San Diego, CA, USA) were used. The amount of antibody used was previously determined by a titration experiment performed on PBMCs and LMCs. For the characterization of T cell differentiation, anti-CD27, CD28 and CD45RA were simultaneously labelled, and for NK differentiation analysis, anti-CD27 and CD11b were used in conjunction. Incubation of cells with all of the above-mentioned antibodies was performed at room temperature (RT) for 30 minutes, and subsequently, the cells were washed twice with 1 ml of 1X PBS and centrifuged 5 minutes at 500 g. For unconjugated antibodies, a subsequent incubation with 3 µl of a FITC or PE conjugated anti-mouse immunoglobulin antibody (Dako, Glostrup, Denmark) was performed for 30 minutes at room temperature. After washing, 3 µl of normal mouse serum was added. To analyse the labelling corresponding to NK, NKT and T cells, an incubation was performed with 3 µl of anti-CD3 (Becton Dickinson, San Jose, CA, USA) conjugated to PerCP/FITC and 5 µl of anti-CD56 conjugated to PE/APC (Becton Dickinson, San Jose, CA, USA) or the BV421 antibody (Biolegend, San Diego, CA, USA) for 15 minutes at RT. A suitable fluorochrome combination was performed for each labelling scheme. In the liver samples, the anti-CD45 pan-leukocyte marker conjugated with APC-H7 (Becton Dickinson, San Jose, CA, USA) was also added. After washing again with 1X PBS, cells were fixed with 2% paraformaldehyde and subsequently analysed using a FACSAria II cell sorter (Becton Dickinson, San Jose, CA, USA) flow cytometer. The results were analysed using Flowjo 7.6.2 software (Tree Star, Inc., Ashland, OR, USA). Analyses of a particular population of interest were based on a gate of at least 100,000 events. For KIR analysis, expression of three

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receptors was indirectly inferred: the antibody 3DL1 / 3DL2 showed two clouds with different MFI (mean fluorescence intensity) values, where the lower cloud corresponded to 3DL2 and upper cloud corresponded to 3DL1. As a control method, in all cases, expression of the upper cloud was compared with the monoclonal antibody against 3DL1 and there were no significant differences. Expression of 2DS3 was deduced from the subtraction of expression between 2DL1/2DS1/2DS3 - 2DL1/2DS1. Expression of 2DS1 was deduced from subtraction of expression between 2DL1/2DS1 - 2DL1. Finally, we inferred the expression of 2DL2/2DS2 from subtraction of expression between 2DL2/2DS2/2DL3 - 2DL3. In all cases, the presence of the KIR gene was verified.

We have previously assessed the quality of the results through dead cell staining, demonstrating that dead cell discrimination by forward/side scatter presented excellent correlation with the results obtained using the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, MA, USA), which specifically stains lymphocytes cells (Fig. S1). Thus, dead cells were excluded by FSC/SSC gating.

Functional studies

Mononuclear cells obtained from liver or peripheral blood samples were placed in a 24-well plate at a concentration of $1x10^6$ lymphocytes per well in 1 ml of complete RPMI medium. Three wells were used for each sample according to the following scheme: Two wells were stimulated with PMA (Phorbol 12-Myristate 13-acetate; Sigma-Aldrich, Darmstadt, Germany) at a final concentration of 25 ng/ml and ionomycin (calcium salt of Streptomyces conglobatus; Sigma-Aldrich, Darmstadt, Germany) at a final concentration of 0.5 µg/ml and compared with a not stimulated negative control. An anti-CD107a antibody

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(Becton Dickinson, San Jose, CA, USA) conjugated to FITC was added (20 µl) to the positive and negative controls and 20 µl of the FITC-conjugated IgG1 (Becton Dickinson, San Jose, CA, USA) was included as isotype control . The culture plates were incubated for one hour at 37 °C and 5% CO_2. Subsequently, 1 µl of monensin (BD-Golgi Stop Protein Transport Inhibitor, Becton Dickinson, San Jose, CA, USA) was added to all wells, and the plates were incubated for another five hours. Cells were washed with 1 ml of 1X PBS/A and centrifuged at 500 g for 5 minutes. Cell surface staining was performed on all samples using the following monoclonal antibodies (AtcMo): 3 µl of anti-CD3 PerCP and 5 µl of anti-CD56 APC (Becton Dickinson, San Jose, CA, USA). Pan-Leukocyte anti-CD45 APC-H7 (Becton Dickinson, San Jose, CA, USA) was included in the liver samples. All samples were subsequently incubated for 20 minutes at room temperature and washed twice with 1X PBS/A. Intracytoplasmic labelling was performed for the detection of IFN-γ. The cells were treated with 100 µl of 1X fixation and permeation solution (Cytofix / Cytoperm, Becton Dickinson, San Jose, CA, USA) for 20 minutes at 4 ° C, washed twice with 1 ml of 1X Perm / Wash buffer (Becton Dickinson, San Jose, CA, USA), and centrifuged at 500 g for 5 minutes. Cells were also incubated with 20 µl of anti-IFNγ PE (Becton Dickinson) ,and the isotype control was incubated with 20 µl of the IgG1 PE (Becton Dickinson, San Jose, CA, USA). The tubes were incubated for 30 minutes at 4 ° C, followed by two washes with 1X buffer Perm / Wash. The pellets suspended in 200 µl of 1X PBS/A and fixed with 2% paraformaldehyde were analysed using the FACSAria II flow cytometer (Becton Dickinson, San Jose, CA, USA).

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Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc.). Comparisons between samples (peripheral blood and liver) were performed using the Mann-Whitney U test. The data are presented as the median and IQR (interquartile range). The reported p values are two-tailed, and $p<0.05$ was considered significant.

Results

Comparative analysis of liver and peripheral blood NK and T cell frequencies

In the initial experiments, we analysed the frequencies of NK and T cells in LMCs by comparing the frequencies of these cells in samples obtained from liver homogenate (n=9) and samples obtained from liver perfusion (n=14). We observed no significant differences in the frequencies of NK ($CD56^+CD3^-$), NKT ($CD3^+CD56^+$) and T ($CD3^+$) cells collected using these two techniques. Therefore, because of the low number of cells recovered from liver homogenates, we collected these cell populations from liver perfusion eluates.

Compared with peripheral blood, the liver showed different frequencies of T cells, NKT cells and NK cells. Similar to previous reports[2, 22, 23], we observed more NK cells in the liver. In particular, $CD56^{bright}$ NK cells represented almost 50% of all liver NK cells (14.8% of liver lymphocytes vs. 0.7% in PB lymphocytes, p<0.001), with $CD56^{dim}$ NK cells representing almost the other 50% of NK cells (11.6% vs. 8.3% in PB, p=0.002). Similarly, NKT cells were also highly increased in the liver (13.4 vs.3.8% in PB, p<0.001). As indicated in Fig. 1, NK and NKT populations accounted for approximately 50% of the liver lymphocyte population. The increased frequencies of liver NK and NKT cells occur at the expense of the relatively decreased frequencies of T cells (54.7% in the liver vs. 73.2% in PB, p<0.001).

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Comparative expression of T cell markers in the peripheral blood and liver of healthy individuals

The liver shows a higher frequency of $CD8^+$ cells (66.1% vs. 31.0% in PB, $p<0.001$) and a reduced frequency of $CD4^+$ cells (17.7% vs. 61.5% in PB, $p<0.001$), with an increased frequency of TCRγδ cells (10.8% vs. 2.0% in PB, $p=0.01$)(Fig. 2a). Notably, NKT cells from PB or liver samples showed similar frequencies of CD4, CD8 or TCRγδ T cells (Fig. 2b). These findings indicated that although NKT cells are a heterogeneous population, certain common characteristics are independently maintained in the tissue studied.

In addition, we analysed the invariant receptor (TCR Vα7.2) of MAIT cells (mucosal-associated invariant T-cell antigen). In the liver, 18.4% of T cells expressed TCR-MAIT vs. 5.0% in PB, $p<0.01$. This observation was expected since most MAIT cells are $CD8^+$, consistent with the increased amount of liver $CD8^+$ T cells, indicating that the composition of liver TCRs differs with respect to peripheral blood (Fig. 2a).

T cells markers from liver and peripheral blood samples identified different maturation stages.

The drastically decreased CCR7 expression in liver T cells (11.1% vs. 74.0% in PB $p<0.01$) was used to identify cells that lost their capacity to migrate to lymph nodes. Although the peripheral blood expression of CCR7 in NKT cells was low, its expression in liver NKT cells was even lower (0.7% vs. 6.4% in PB, $p<0.001$). The expression of HLA-DR on CD8 T lymphocytes identified a particular subpopulation with a regulatory capacity[24]. We observed significantly increased expression of HLA-DR in both NKT (15.9% vs. 4.0% in PB, $p=0.05$) and T liver cells (19.7% vs. 3.2% in PB, $p=0.01$) (Figure 3.

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Additionally, liver T cells revealed decreased expression of CD45RA (31.3% vs. 53.7% in PB, $p<0.01$) and the co-stimulatory molecules CD28 (68.7% vs. 94.5% in PB, $p<0.001$) and CD27 (58.0% vs. 89.7% in PB, $p<0.001$). Notably, CD161, a molecule with ascribed homing properties, was increased within liver NKT (83.7% vs. 53.9% in PB, $p<0.001$) and liver T cells (49.5% vs. 17.5% in PB, $p<0.001$) (Fig 3). Expression of CD45RA, CD27, CD28 and CCR7 enabled characterization of different subsets of T cells: naïve, effector cells (T_E), central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}) and terminally differentiated T cells. The strategy used to analyse T cell differentiation is depicted in Figure 4, and the results are summarized in Table 1.

The liver showed a low frequency of $CD45RA^+CD27^+CD28^+CCR7^+$ naive cells (3.2% vs. 43.7% in PB, $p<0.001$). The phenotype $CD45RA^-CD27^+CD28^+CCR7^-$, which represents a transitional stage between T_{CM} and T_{EM} cells, was increased in the liver (38.7% vs. 10.4% in PB, $p<0.05$). Interestingly, the T_{CM} $CD45RA^-CD27^+CD28^+CCR7^+$, which represented 33.7% of PB T cells, was almost undetectable in the liver (3.8%, $p<0.001$). Notably, all T_{EM} cells were increased in the liver: $CD45RA^-CD27^+CD28^-CCR7^-$ (3.3% vs. 0.5% in PB, $p<0.01$); $CD45RA^-CD27^-CD28^+CCR7^-$ (13.8% vs. 2.0% in PB, $p<0.001$); and $CD45RA^-CD27^-CD28^-CCR7^-$ (10.5% vs. 0.6% in PB, $p<0.001$). Finally, terminally differentiated T cells were also increased in the liver: $CD45RA^+CD27^+CD28^-CCR7^-$ (6.2% vs. 2.6% in PB, $p<0.05$) and $CD45RA^+CD27^-CD28^-CCR7^-$ (14.6% vs. 1.5% in PB, $p<0.05$).

Different phenotype of liver and peripheral blood NK cells

NKG2A, one of the strongest NK inhibitory receptors, was detected in 89.4% of $CD56^{bright}$ NK cells in peripheral blood vs. 56.3% of $CD56^{bright}$ NK cells in the liver ($p<0.001$), suggesting a lower activation threshold in the liver (Fig 5). Additionally, liver-$CD56^{bright}$ NK

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cells showed a slight decrease in cell surface expression of their NKG2C-activating counterparts (4.1% vs. 16.6% in PB, $p < 0.05$), while liver $CD56^{dim}$ NK cells showed a significant increase of the activator receptor NKp44 (1.1% vs. 0.1% in PB, $p<0.01$) and decrease of the NK differentiation marker CD57 (8.9% vs. 22.5% in PB, $p<0.01$). Occasionally, it is difficult to differentiate liver $CD56^{bright}$ NK cells from $CD56^{dim}$ NK cells. The comparison between liver and blood $CD56^{bright}$ NK cells showed that CD16 expression was not differentially expressed in both subsets. By contrast, this difference was significant when we analysed $CD56^{dim}$ NK cells obtained from blood or liver samples (31.7% vs. 92.0% in PB, $p<0.001$; Fig 6). Notably, the significantly decreased expression of CD16 on liver $CD56^{dim}$ NK cells supports the concept of the potentially decreased capacity of liver NK cells to perform ADCC In an attempt to further characterize the differences between PB and liver NK cells, we analysed the expression of CD11b and CD27, which identifies distinct stages of human NK cells from different sources.

As shown in Fig 7 most liver and peripheral blood NK cells are $CD11b^{+}$. However, liver $CD56^{bright}$ and $CD56^{dim}$ NK cells showed increased $CD27^{+}$ expression (71.3% vs. 30.0% in PB, $p<0.001$ and 8.4% vs. 4.6%, $p<0.05$, respectively), suggesting that the liver has an increased amount of CD11b and CD27 double positive (DP) NK cells, which are associated with a higher secretory capacity[25]. However, under a nonspecific stimulus, such as PMA-ionomycin, we did not detect differences in the secretory or cytotoxic capacity between PB and liver NK cells (Fig. 9). Additionally, the lowest expression of CD45RA in liver $CD56^{dim}$ NK cells (96.8% vs. 99.6%, $p < 0.001$) may indicate a previous antigenic experience [25].

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A feature of liver CD56^{brigth} NK cells is their dramatic loss of CCR7 (0.4% vs. 65.1% in PB, p<0.001), which may be responsible for their inability to migrate to the regional lymph node, as also recently described[26]. However, a small but significant increase of CD28 was observed in liver CD56^{bright} NK cells (6.8% vs. 1.1% in PB, p=0.001). HLA-DR expression, also increased in liver CD56^{dim} NK cells (5.8% vs. 1.1% in PB, p<0.05), may also be associated with their activated state.

Similar to liver NKT and T cells, the CD161 receptor showed high expression in liver CD56^{bright} NK cells (95.2% vs. 40.3% in PB, p<0.001) and CD56^{dim} NK cells: 85.8% vs. 69.5%, p<0.01 (Fig 7, respectively. The functional role of CD161 is not clear since this protein has been described as both an activation marker and homing marker and may also be expressed in cells with IL-17 secretion capacity. The high expression of CD161 observed in liver NK, NKT and T cells may reflect its homing capacity [27].

Expression of killer cell immunoglobulin-like receptors (KIR) in peripheral blood and liver NK, NKT and T cells

We next compared expression of KIR genes in peripheral blood (n=35), liver eluates (n=7) and a few samples of MNCs obtained from liver biopsies (the number of cells per sample enabled the analysis of only a few KIRs). The strategy used to analyse expression of KIRs is depicted in Figure 8, and the results are summarized in Table 2 In peripheral blood, expression of the KIR genes in NK, NKT and T cells showed the following decreasing order CD56^{dim}> NKT> CD56^{bright}> T cells. In the liver, KIR expression was as follows: CD56^{dim}> NKT> T> CD56^{bright} cells (Table 2. In peripheral blood, the expression of KIR receptors on CD56^{bright} NK cells never exceeded 10%, but liver CD56^{bright} NK cells showed increased expression of KIR3DL2 (12.9% vs. 4.1% in PB, p=0.001) and KIR2DL3 (8.2% vs. 1.7%, p<0.01). As expected,

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expression of KIR genes was higher in $CD56^{dim}$ cells, with KIR2DS4 showing the highest expression in PB $CD56^{dim}$ cells (44.1% vs. 22.2% in the liver, $p<0.01$). Similar comparative analyses between liver and peripheral blood NKT revealed increased frequencies in the liver NKT cells of KIR2DL3 (15.9% vs. 3.1%, $p<0.01$) and KIR2DS1 (2.0% vs. 0.0%, $p<0.01$). Changes were obvious when we compared KIR expression on liver and peripheral blood T cells. Additionally, KIR expression on peripheral blood T cells was almost negligible, as most KIRs were detected in liver T cells: KIR2DL3 (12.9% vs.0.2%, $p<0.001$); KIR3DL2 (8.6 vs. 1.6%, $p<0.001$); KIR2DS1 (1.0% vs. 0.0%, $p<0.01$); KIR2DS3 (3.1% vs. 0.5%, $p<0.05$); and KIR2DS4 (11.3% vs. 0.4%, $p<0.001$). A trend towards significance was also observed in KIR2DL2/2DS2. Interestingly, three of these increased KIR genes are activators, and KIR2DS4 together with the weaker inhibitory KIR2DL3 gene showed the highest expression (Table 2).

Comparative analysis of the cytotoxic and secretor capacity of peripheral blood and liver T and NK cells from healthy subjects

The cytotoxic and secretory capacity of liver and PB T cells were measured by flow cytometry on PMA and ionomycin stimulated T cells. Liver $CD3^+$ cells showed an increased frequency of $CD107a^+$ cells as a marker of degranulation (25.0% vs. 4.5% in PB, $p=0.03$), which are associated with the increased frequency of $CD8^+$ T cells, effector memory T cells and TCR γδ effector cells, representing $CD3^+$ cells. Increased IFN-γ secretion was also detected in liver T and NKT cells, but did not reach statistical significance, most likely because of the small number of experiments performed (Fig. 9).

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It was speculated that decreased expression of NKG2A conferred a higher cytotoxic capacity to these cells. This pattern was not reflected in experiments in which peripheral blood and liver $CD56^{bright}$ and $CD56^{dim}$ NK cells showed similar levels of cytotoxicity and IFN-γ secretor capacity (Fig. 9).

Discussion

The present study aimed to characterize and compare the frequencies, phenotypes and functions of NK, NKT and different subsets of T cells in peripheral blood and liver samples. Consistent with previous reports[2, 22] we confirmed the increased frequency of intrahepatic NK cells, particularly $CD56^{bright}$ NK cells, which represent almost half of liver NK cells. These cells, as immediate precursors of $CD56^{dim}$ NK cells, are rare in peripheral blood, but constitute the majority of NK cells in secondary lymphoid tissues. Peripheral blood $CD56^{bright}$ NK cells are abundant cytokine producers, but are only weakly cytotoxic prior to activation [28]. There is limited data regarding the recirculation of natural killer (NK) cells among human organs. Currently, the available data confirmed that human NK cells populate the blood, lymphoid organs, lung, liver, uterus (during pregnancy), and gut, following a NK cell homing pattern that appears to be subset-specific [29]. These data indicate that liver NK cells lack CCR7 expression, which could be necessary to migrate back to the lymph nodes, although these cells are able to interact with liver antigen presenting cells. Together with the loss of CCR7, liver $CD56^{bright}$ NK cells showed increased expression of CD27 and CD28. Most PB NK cells were $CD56^{dim}$ $CD11b^{+}$ $CD27^{-}$ populations that exhibited a cytotoxic capacity, whereas NK cells from cord blood were $CD11b^{+}$ $CD27^{-}$ and $CD11b^{+}$ $CD27^{+}$ populations. The most

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immature $CD11b^{-}$ $CD27^{-}$ NK subset represents the prevalent NK cells present in decidua. Previous studies have reported that $CD11b^{-}CD27^{+}$ and $CD11b^{+}$ $CD27^{+}$ NK cells secrete cytokines [25]. Data from the present and previous studies suggest that once NK cells migrate to the liver, these cells become tissue resident cells with particular features and activities. An previous study showed that some NK cells can be retained in the liver for up to 2 years [30]. A recent study showed that some NK cells are retained for up to 13 years [3], and these long-term resident NK cells represent an $Eomes^{hi}$ population, [3, 31] which are unable to re-enter circulation and are long-lived in the liver. In addition to the differences that we detected between liver and peripheral blood NK cells, the distribution and phenotype of NK cells across different human organs and tissues was markedly different. In the normal intestinal mucosa, NK cells show a phenotype similar to blood $CD56^{bright}$ NK cells [32, 33]. NK cells represent ~10% of lymphocytes present in human normal lung and belong to the $CD56^{dim}$ $CD16^{+}$ subset [34]. As described above, the most immature $CD11b^{-}$ $CD27^{-}$, $CD56^{bright}$ $CD16^{neg}$ subset is observed during the first trimester of pregnancy, representing 50-90% of the lymphoid cells that infiltrate this tissue [35]. Increased expression of HLA-DR in liver $CD56^{dim}$ NK cells could also be associated with the activation state of liver NK cells. At least in mice, activated NK cells expressing MHC class II have an antigenic presentation [36]. Consistently, liver $CD56^{bright}$ NK cells also showed an increased frequency of NKp44, associated with decreased expression of NKG2A, which is one of the strongest NK inhibitory receptors. These results suggest that this subpopulation (or at least part of it) may represent activated NK cells, in coincidence with a previous study showing that liver resident $CD56^{bright}$ NK cells with reduced proinflammatory potential had also enhanced degranulation activity [31]. As previous discussed, it is occasionally difficult to discriminate between $CD56^{bright}$ and $CD56^{dim}$ NK cells in the liver. Although we achieved some discrimination between these two subsets

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based on differences in CD16 expression, we should be cautious with the results obtained in liver CD56^{bright} cells, considering that some contamination with CD56^{dim} cells may occur. Additional to the contamination of CD56^{dim} into CD56^{bright} cells, there can also be a contamination of CD56^{bright} into CD56^{dim} cells.

Differentiation of peripheral blood CD56^{dim} NK cells was reported to involve acquisition of CD57, loss of NKG2A, and gain of KIRs [37]. In the present study, we observed that in comparison with peripheral blood, the NK differentiation marker CD57 is decreased in liver CD56^{dim} NK cells, but the expression of NKG2A and KIR genes (with the exception of KIR2DS4) did not differ between peripheral blood and liver CD56^{dim} NK cells. These results indicate that in contrast to CD56^{bright} NK cells, the maturation state of liver and peripheral blood CD56^{dim} NK cells was similar. The decreased expression of CD57 in liver CD56^{dim} NK cells could indicate less terminally differentiated cells. We cannot exclude that decreased CD57 could be associated with a diminished memory capacity, as previously suggested[38, 39]. In addition, we detected a strong decrease in the differentiation marker CD16, indicating a potential decreased capacity of liver NK cells to perform ADCC. Thus, liver CD56^{bright} NK cells could more likely be associated with activation, whereas the ADCC capacity and maturation stage of CD56^{dim} NK cells may be decreased.

NK cell activation is governed by the complex interplay of activating and inhibitory receptors. As inferred above, increased KIR expression on NK cells is associated with their maturation, and peripheral blood CD56^{bright} NK cells showed low KIR gene expression. However, liver CD56^{bright} NK cells showed a small but significant increase in KIR3DL2 and KIR2DL3 expression. In peripheral blood, expression of KIR genes was higher in CD56^{dim} NK

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cells, with KIR2DS4 showing the highest surface expression. KIR2DS4 also showed the highest expression at the surface of liver $CD56^{dim}$ NK cells .

T cells expressing the semi-invariant T-cell receptor (TCR Vα7.2-Jα33) are highly abundant in humans. Within the liver, these cells represent approximately 20% of T cells. Although this increment is consistent with the increase of $CD8^+$ T cells, liver TCR-MAIT cells showed a four-fold increase compared with PB, whereas the frequency of $CD8^+$ cells (which include MAIT cells) only showed a 2-fold increase, indicating that the TCR repertoire in the liver is different from that in PB. A subset of TCR γδ T cells also increased in the livers of healthy subjects. In contrast, NKT from PB or liver tissues showed similar frequencies of CD4, CD8 or TCRγδ T cells.

A main feature of liver T cells is the low frequency of naive T cells, and most of these cells have been ascribed to the effector memory phenotype $CD45RA^+27^-28^-$ and $CD45RA^-27^-28^-$ T cells, with a greater capacity for degranulation and higher secretion of IFN-γ. CD107a, a degranulation marker, is also considered to be a cytotoxicity marker. However, we should be cautious as previous studies[4,5] have reported that liver-resident NK and T cells have the ability to degranulate but lack cytotoxic mediators.

Expression of the C-type lectin CD161 in the liver was remarkable because in contrast with peripheral blood, CD161 was expressed in most liver NK, NKT and T cells, Within adult circulation, CD161 expression is restricted to T cells with a memory phenotype. Similarly, CD244 (2B4) is expressed by memory-phenotype $CD8^+$ T cells and all natural killer (NK) cells [40]. The increased expression of these two receptors is consistent with the memory phenotype of liver T cells.

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In contrast with the negligible expression in peripheral blood, a unique pattern of expression of KIR genes was observed in liver T cells. Expression of the activating KIR genes KIR2DS1, KIR2DS3, and KIR2DS4 has been associated with weaker expression of the inhibitory KIR2DL3 gene. It was previously reported that KIR expression could be induced after in vitro activation of a substantial fraction of terminally differentiated effector CD8 T cells [41-43]. Additionally genetics largely determine the expression of inhibitory receptors; activation receptors are heavily environmentally influenced [16]. As previously reported [4], liver T cells primarily comprised effector memory and highly differentiated T cells. Thus, differential expression of KIR genes in liver T cells was expected, indicating their activation state. Although NK cells are the main KIR-expressing immune cells, the presence of KIR on CD8 T-cells was reported to enhance their HLA class I-restricted antiviral effect.

We conclude that the liver has a large amount of $CD8^+$ cells with a prevalence of memory and terminally differentiated phenotypes. The unique expression of KIR-activating receptors with a high cytotoxic and functional capacity and decreased amount of CCR7 that are unable to migrate to the regional lymph node support the previously reported concept[3-5] that liver T cells and NK cells are differentiated and activated cells that remain and die in the liver.

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Figure Legends

Figure 1. $CD56^{bright}$ NK cells, $CD56^{dim}$ NK cells, NKT and T cells frequencies detected in peripheral blood and liver. Data are expressed as the median and IQR. ** p <0.01, *** p <0.001, Mann Whitney U-test.

Figure 2. (a) CD4, CD8, TCR-γδ and TCR-MAIT cells frequencies within $CD3^+CD56^+$ NKT cells from PBMCs and LMCs. (b) CD4+, CD8+, TCR-γδ and TCR-MAIT cells frequencies detected within $CD3^+$ in PBMCs and LMCs. Data are expressed as the median and IQR. * p <0.05, ** p <0.01, *** p <0.001, Mann Whitney U-test.

Figure 3. CD45RA, CCR7, CD28, CD27, CD161, and HLA-DR cells frequencies within $CD3^+CD56^+$ NKT cells from PBMCs and LMCs (a) and within $CD3^+$ cells from PBMCs and LMCs. (b) Data are expressed as the median and IQR. * p <0.05, ** p <0.01, *** p <0.001, Mann Whitney U-test.

Figure 4. Dot plots showing the strategy used to analyse the T cell differentiation pattern. (a) Peripheral blood deduced from FSC / SSC. (b) The pan-leukocyte CD45 marker was used to select the liver lymphocyte cloud. Illustrations in the followings dot plots correspond to analysis of peripheral blood. (c) Doublets were discarded. (d) The $CD3^+$ population was selected, and (e) T cells were subdivided into $CD45RA^+$ and CD45RA- populations. (f) The combined presence of CD27 and CD28. The percentage of positive cells was calculated after subtracting the isotype control (g) and referred to the total $CD3^+$ population.

Figure 5. CD94, NKG2A, NKG2C, NKp44, NKp46, 2B4, CD16 and CD57 cells frequencies within $CD56^{bright}$ NK cells from PBMCs and LMCs (a) and within $CD56^{dim}$ cells from PBMCs and LMCs (b). Data are expressed as the median and IQR. * p <0.05, ** p <0.01, *** p <0.001, Mann Whitney U-test.

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Figure 6. (a) Expression of CD16 on peripheral blood $CD56^{bright}$ and $CD56^{dim}$ NK cells, (b) shows the expression of CD16 on liver $CD56^{bright}$ and $CD56^{dim}$ NK cells.

Figure 7. CD45RA, CCR7, CD28, CD27, CD11b, CD161 and HLA-DR cells frequencies within $CD56^{bright}$ cells from PBMCs (a) and within $CD56^{dim}$ NK cells from LMC (b). Data are expressed as the median and IQR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann Whitney U-test.

Figure 8. (a) Dot plots showing the strategy used to analyse the expression of KIRs in peripheral blood (1) and liver $CD45^{+}$ cells (2) deduced from FSC / SSC, Doublets were discarded (3). $CD56^{bright}$, $CD56^{dim}$, NKT and T cell populations were selected in PBMCs (4) and LMCs (5). (6) Illustrate an example of KIR2DL3 expression on total PBMCs and (7) after the subtraction of its isotype control. 8b and 8c show the gating strategy for peripheral blood and liver NK, NKT and T cells. The following figures illustrate the KIR staining pattern of the different antibodies on $CD56^{+}$ $CD3^{-}$ NK cells obtained from a single individual (one sample for peripheral blood and another sample for liver). b1 and c1 illustrate the staining pattern of the anti-KIR3DL1/3DL2 antibody. The antibody showed two clouds with different MFI (mean fluorescence intensity) values, where the lower cloud corresponds to 3DL2 and the upper cloud corresponds to 3DL1. The staining with anti-KIR3DL1 shown in b2 and c2 confirmed that the frequency in the expression of the KIR 3DL1 antibody correlated with the upper cloud detected by the anti-KIR3DL1/3DL2 antibody. The expression of 2DS3 was deduced from subtraction of expression between 2DL1/2DS1/2DS3, as shown in b3 and c3, and frequency of the expression of the anti-2DL1/2DS1 illustrated in b4 and c4. Notably, expression of 2DL1/2DS1 in the liver sample showed a small difference with 2DL1/2DS1/2DS3 because the genotyping of this sample revealed the absence of the 2DS3 gene. b5 and c5 illustrates the expression of 2DL1 in peripheral blood and liver, respectively. b6 and c6 shows the expression of 2DL2/2DS2/2DL3, which can be compared with the expression of 2DL2 / 2DS2 obtained from subtraction of the signal detected using the anti- 2DL3 antibody (b7 and c7). b8 and c8 shows the expression of KIR2DS4. In all cases, the presence of the KIR genes was verified.

Figure 9. Comparative cytotoxic capacity measured as the expression of CD107a (A) and the secretory capacity (B) of liver (n=4) and peripheral blood (n=4) $CD56^{bright}$, $CD56^{dim}$, NKT and total T cells. Figures show the percentage of positive cells. Data are presented as the means ± SEM. * $p<0.05$, Mann-Whitney U test.

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Supplemental Figure Legends

Figure S1. Entirely viable cells were selected from the lymphocyte cloud that had the highest FSC-A and SSC-A values. The majority of the peripheral blood samples had almost no mortality, and only the high intensity FSC-A SSC-A cloud was analysed. As depicted in Figure S1.a1, the mortality was only 0.3%. Liver samples were collected from cadaveric donors: In this sample, live dead staining (LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit staining specifically for lymphocytes cells) confirmed that the highest mortality was observed in the low intensity cloud of lymphocytes because more time elapsed before these samples were processed, and liver samples in the low-intensity lymphocyte cloud showed a higher frequency of dead cells (23.4%, S1.b1). The gate on the viable lymphocyte cloud (high FSC-A and SSC-A intensity) showed a 0.2% mortality rate (S1.b2).

Tables

Table 1. T cell differentiation pattern

	T lymphocytes Peripheral blood n = 7		**T lymphocytes** Liver n = 7		
CCR7-	Median (%)	(range)	Median (%)	(range)	P
45RA- 27- 28-	0.6	(0.1 – 3.8)	10.5	(4.6 - 16.2)	<0.001
45RA+ 27+ 28+	3.1	(1.3 – 5.7)	4.9	(0.9 – 8.0)	n.s.
45RA- 27+ 28+	10.4	(5.5 – 15.2)	38.7	(4.1 – 52.2)	<0.05
45RA- 27- 28+	2.0	(0.7 – 3.0)	13.8	(3.7 – 26.1)	<0.001
45RA- 27+ 28-	0.5	(0.1 – 2.5)	3.3	(0.5 – 8.4)	<0.01
45RA+ 27- 28+	0.1	(0.0 – 0.9)	0.6	(0.4 – 2.0)	n.s.
45RA+ 27+ 28-	2.6	(0.5 – 5.0)	6.2	(2.7 – 8.8)	<0.05
45RA+ 27- 28-	1.5	(0.5 – 13.6)	14.6	(3.1 - 61.9)	<0.05
CCR7+					
45RA- 27- 28-	0.0	(0.0 – 0.1)	0.0	(0.0 – 0.2)	n.s.
45RA+ 27+ 28+	43.7	(29.1 - 67.0)	3.2	(1.5 – 10.7)	<0.001
45RA- 27+ 28+	33.7	(22.4 – 39.3)	3.8	(1.7 – 5.9)	<0.001
45RA- 27- 28+	1.6	(0.5 – 2.6)	0.2	(0.1 – 3.9)	n.s.
45RA- 27+ 28-	0.0	(0.0 – 0.1)	0.0	(0.0 – 0.6)	n.s.
45RA+ 27- 28+	0.0	(0.0 – 0.1)	0.0	(0.0 – 0.2)	n.s.
45RA+ 27+ 28-	0.2	(0.2 – 0.7)	0.2	(0.1 – 1.3)	n.s.
45RA+ 27- 28-	0.0	(0.0 – 0.1)	0.0	(0.0 – 2.6)	n.s.

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Comparison of the T cell differentiation pattern between peripheral blood (n = 7) and liver (n = 7) CD3+ cells. Phenotypes were separated into $CCR7^-$ (upper table) and CCR7+ (lower table) populations. Data are expressed as the median (range) of cells positive for the different T subpopulations according to the combined expression of CD45RA, CD27 and CD28 referred to as the total number of T lymphocytes. Mann Whitney U-test.

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Table 2. Expression of KIR receptors

	NK CD56^{bright}				NK CD56^{dim}				NKT				T					
	P.B.		Liver		P.B.		Liver		P.B.		Liver		P.B.		Liver		P.B.	Liver
	median	(range)	median	(range)	median	(range)	median	(range)	median	(range)	median	(range)	median	(range)	median	(range)	n	n
2DL1	1.7	(0.0-17.3)	3.0	(1.0-20.1)	16.1	(4.1-70.4)	10.5	(5.5-45.1)	3.8	(0.2-30.4)	1.2	(0.3-7.7)	0.4	(0.0-8.8)	1.0	(0.2-3.7)	34	7
2DL1/2DS1	2.5	(0.0-20.7)	1.9	(0.4-33.8)	17.5	(2.9-65.5)	13.2	(8.6-55.3)	1.1	(0.0-26.8)	2.7	(1.2-11.4)	0.0	(0.0-4.1)	1.2***	(0.7-5.2)	35	7
2DL1/2DS1/2DS3	4.0	(0.6-22.5)	5.7	(2.0-27.0)	19.9	(8.9-70.5)	37.1	(9.7-61.1)	2.7	(0.0-35.3)	3.9	(1.1-53.0)	0.2	(0.0-6.1)	3.7***	(0.6-42.6)	35	7
2DL2/2DS2/2DL3	3.7	(0.5-14.2)	7.5	(2.8-9.4)	31.4	(7.5-67.7)	21.0	(12-36.8)	12.8	(0.3-34.2)	10.6	(1.9-75.0)	1.6	(0.0-15.9)	5.7***	(1.4-57.9)	25	7
2DL3	1.7	(0.0-28.8)	8.2**	(1.4-15.9)	11.9	(4.3-44.9)	14.1	(2.6-46.2)	3.1	(0.0-27.8)	15.9**	(2.5-50.4)	0.2	(0.0-15.6)	12.9***	(1.3-36.9)	25	11
3DL1	2.2	(0.0-11.0)	2.1	(0.7-18.4)	16.4	(4.2-59.8)	7.8	(1.7-36.4)	5.9	(0.0-24.7)	1.5	(0.2-15.6)	0.7	(0.0-8.0)	1.2	(0.2-8.1)	31	6
3DL2‡	4.1	(0.0-18.1)	12.9**	(4.4-33.8)	15.3	(4.9-44.3)	31.2	(8.0-58.8)	6.6	(0.2-45.3)	15.6	(3.8-39.1)	1.6	(0.0-12.6)	8.6***	(4.2-36.2)	35	7
2DS1‡	1.2	(0.0-15.4)	0.0	(0.0-13.7)	6.1	(0.7-19.1)	8.7	(2.1-10.2)	0.0	(0.0-1.9)	2.0**	(0.6-6.8)	0.0	(0.0-0.2)	1.0**	(0.1-3.7)	19	5
2DL2/2DS2‡	1.8	(0.0-13.2)	0.7	(0.0-3.9)	20.6	(2.9-35.6)	7.4	(2.0-19.8)	5.9	(0.0-23.1)	5.3	(0.0-24.6)	0.8	(0.0-2.6)	3.1	(0.0-22.9)	22	7
2DS3‡	3.2	(0.0-17.6)	4.7	(0.0-8.0)	14.5	(0.7-53.1)	28.1	(4.0-28.5)	3.6	(0.2-25.6)	2.7	(0.0-44.9)	0.5	(0.0-2.6)	3.1*	(0.1-38.6)	15	4
2DS4	5.7	(0.1-21.1)	7.3	(2.8-17.5)	44.1	(5.9-69.5)	22.2**	(2.1-49.4)	4.2	(1.2-32.5)	12.6	(1.6-60.1)	0.4	(0.0-6.3)	11.3***	(1.2-45.5)	16	12

KIR expression in NK, NKT and T cells in peripheral blood and liver samples obtained from healthy individuals. (‡) Indicate inferred KIRs expression (see text). Data are expressed as the median (range) of a specific marker within NK CD56^{bright}, NK CD56^{dim}, NKT and T populations. * p <0.05, ** p <0.01, *** p <0.001, Mann Whitney U-test.

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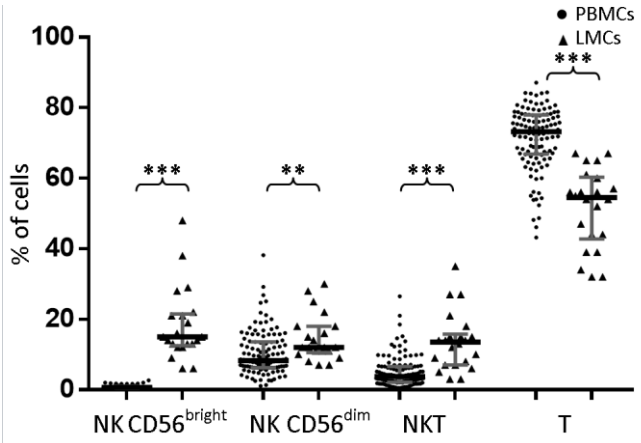

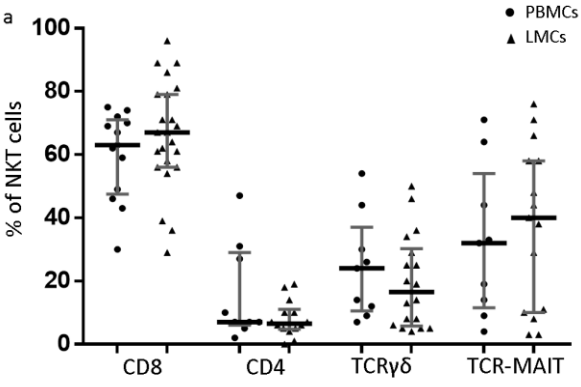

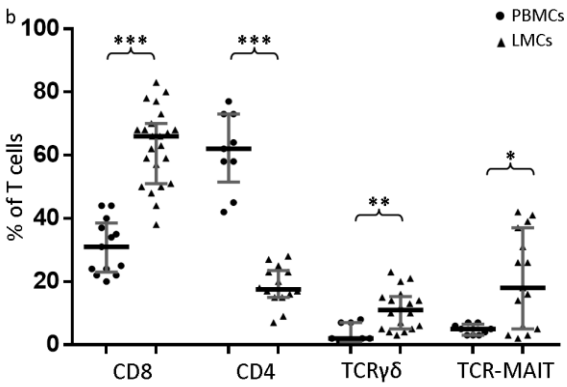

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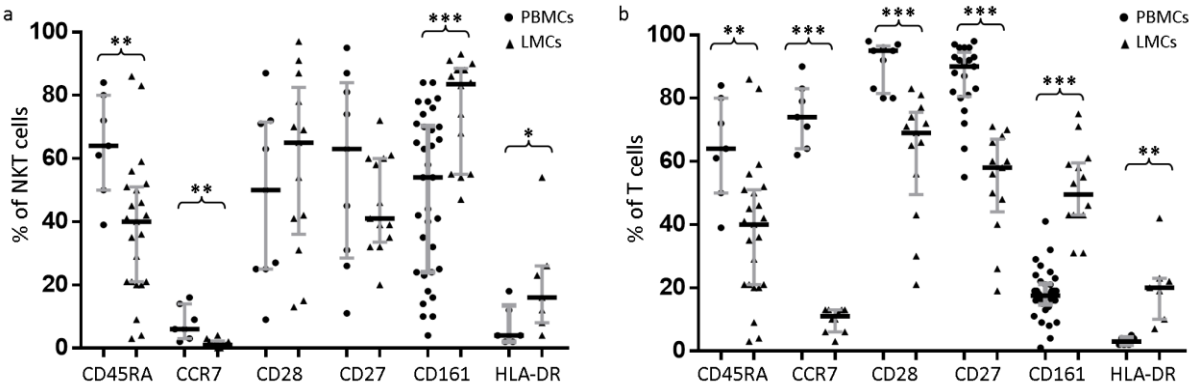

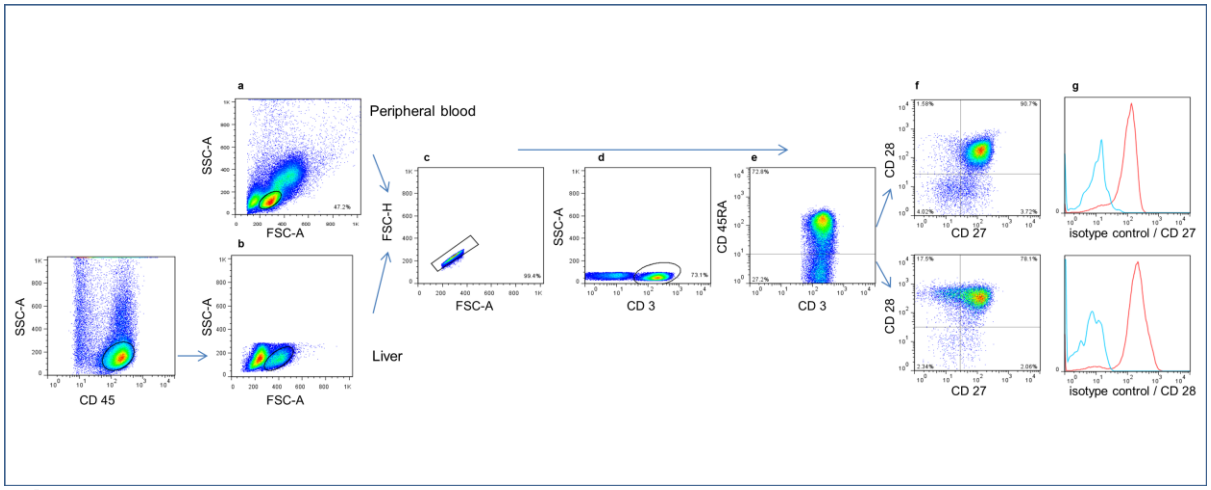

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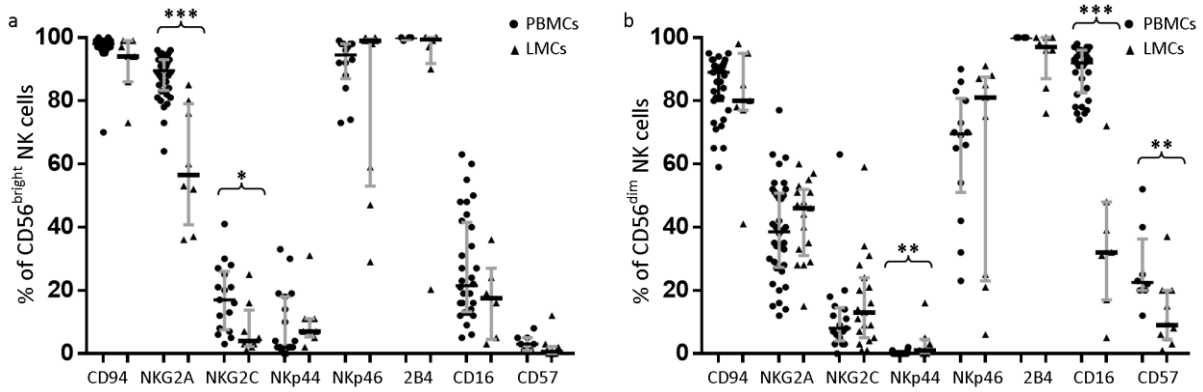

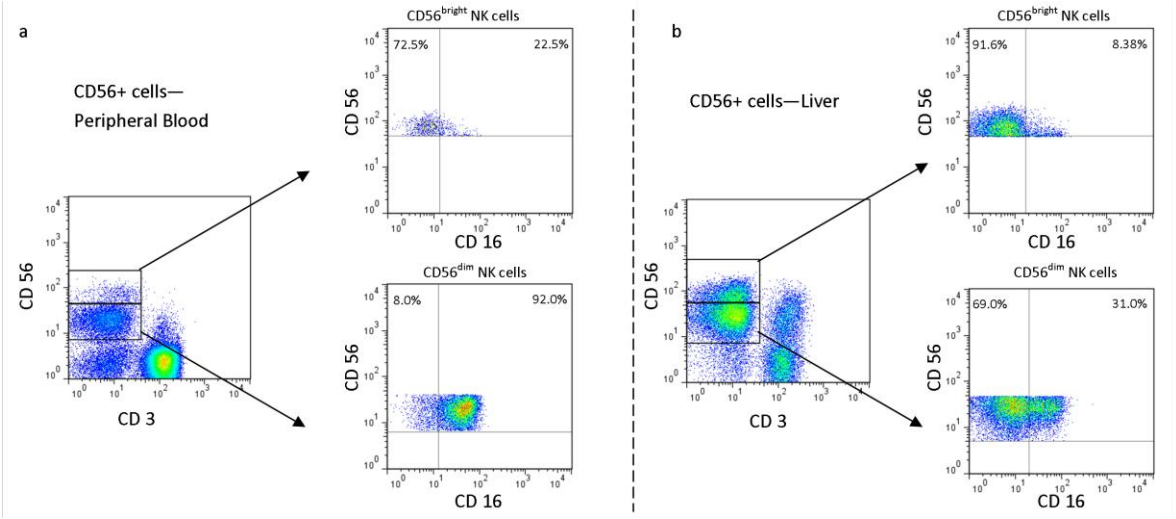

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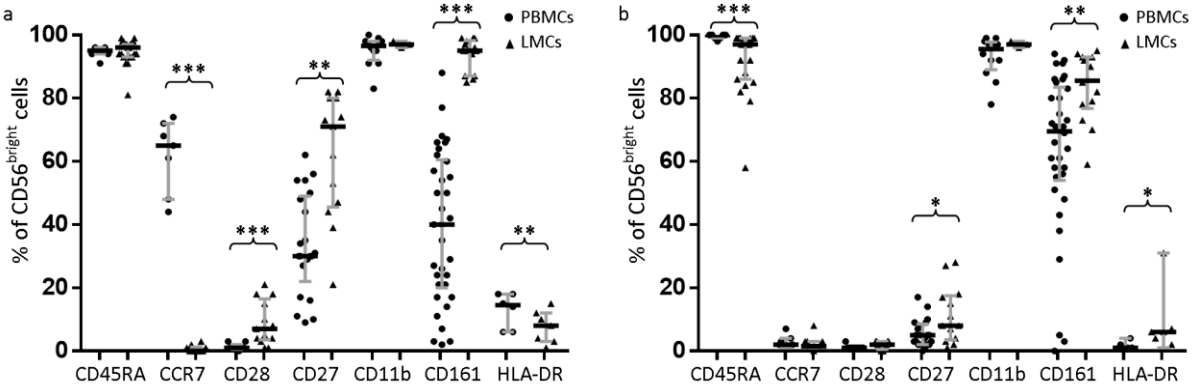

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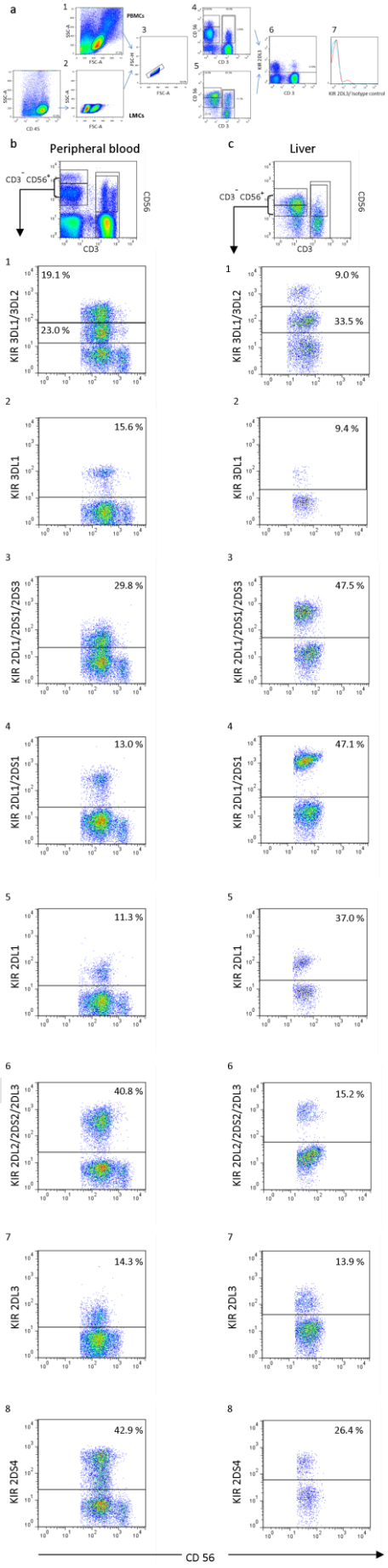

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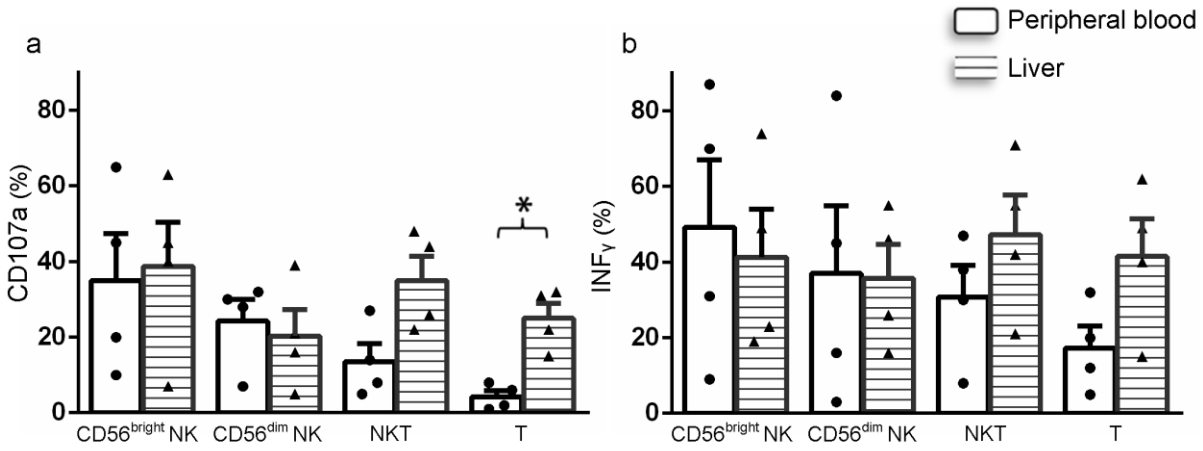

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