Liver Perfusate Natural Killer Cells From Deceased Brain Donors and Association With Acute Cellular Rejection After Liver Transplantation: A Time-to-Rejection Analysis

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Background. The ability to predict which recipients will successfully complete their posttransplant clinical course, which is crucial for liver transplant (LT) programs. The assessment of natural killer (NK) cell subset determined by flow cytometry from a monocentric series of consecutive liver perfusates could help identify risk factors portending adverse LT outcomes. **Methods.** Liver perfusates were collected during the back-table surgical time after the procurement procedures for donors after brain death. Lymphocytic concentrations and phenotypes were matched with donors after brain death characteristics and indications, timing, surgical techniques, outcomes, and biopsy-proven acute cellular rejections (ACRs) in 46 adult recipients who underwent LT between 2010 and 2014 at our institute. Cox regression models were used to study relevant risk factors in order to estimate hazard ratios for episodes of rejection after LT. **Results.** Percentage of NK cells was significantly associated with donor age ($P = 0.05$) and the percentage of NK T cellular subset ($P = 0.001$). The length of follow-up after LT was 41.0 ± 20.9 months, and 11 (23.9%) recipients experienced biopsy-proven ACR. At time-to-rejection proportional regression analysis, a cutoff value of 33.7% was optimal, with a sensitivity of 1, specificity of 0.57, and positive and negative predictive values of 0.42 and 1, respectively. The liver perfusate NK cell subset was strongly associated with biopsy-proven ACR (hazard ratio, 10.7; $P = 0.02$). **Conclusions.** Liver perfusate cytofluorimetric phenotyping may contribute as a targeted preoperative tool to predict the risk of ACR, and as clinical test in translational studies that aim to improve donor allograft procurement and transplant outcomes.

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Liver transplant (LT) is the gold standard therapeutical option for patients suffering from end-stage hepatic disease. The liver microvasculature and hepatic immune microanatomy and physiology are the primary targets of immunologic processes induced in donors after brain death (DBDs), and these events can influence the number of interstitial liver lymphocytes and natural killer (NK) cells.[1] These cell populations play a key role in acute liver cell rejection after LT, and early interaction between donor and recipient immune cells could be crucial for the later development of tolerance, also considering that altered adaptive immune response leads to increased acute and chronic rejection.[2] In addition to the cells involved in adaptive immunity, such as T cells, several innate lymphocytes, such as NK and NK T (NKT) cells, are present in the liver, and play an important role in hepatic physiology, providing unintended benefits for liver grafts by

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modulating immune responses.[1,3] In the transplant setting, significant alterations occur in the hepatic recruitment of T, NK, and NKT cells after LT. An increase in T cells, and a specific decrease in NK and NKT cells, suggest a shift from innate to adaptive hepatic immunity in the liver graft. Furthermore, donor-derived tissue resident memory lymphocytes have been recognized as tolerance inducers for latent viral infections or as potential mediators of alloimmunogenicity.[4] Though NK cells play important roles in both adaptive and innate immunities and may contribute to controlling inflammatory responses in the liver, the exact role of donor NK cells in suppressing and regulating graft rejection has not been clearly defined.[2] Individualized risk estimation of allograft dysfunction can portend adverse graft and patient outcomes by modulating donor quality and recipient factors.[5] Invariably, the evolving tendency to maximize donor-recipient selection requires ameliorating the early prediction of rejection episodes after LT, and planning guidance for targeted immunosuppressive therapy.[6] In this study, we evaluated ex situ LPs from DBDs as a tool for obtaining lymphocyte phenotype in order to identify the potential clinical impact of NK cell fraction variety in a monocentric series of adult recipients after LT.

MATERIALS AND METHODS

Study Population

Perfusates were collected during the back-table surgical time after the procurement procedures for a consecutive series of deceased donor LT for adult patients at Institute for Scientific-Based Care and Research-Mediterranean Institute for Transplantation and Highly Specialized Therapies (ISMETT) from September 2010 to October 2014. The DBDs were all from the region of Sicily, which is an island, for logistical reasons regarding lack of adequate helicopter space.

The following DBD conditions were excluded to render the entire study population homogenous: allergy or autoimmune disease, pregnancy or active breastfeeding, donors requiring systemic immunosuppressive drugs at the time of procurement, history of malignancy and of human immunodeficiency virus positivity, and donors who had previously received an organ transplant. Donor characteristics were collected prospectively from electronic medical records. The following DBD variables were included in these analyses: age, gender, height, weight, body mass index, cause of death, intensive care unit (ICU) length of stay, macrovesicular steatosis, hemodynamic risk factors, including use of amine for more than 6 hours to sustain blood pressure for prolonged hypotension (systolic blood pressure < 60 mm Hg for more than 2 hours), cold ischemia time (CIT), natremia, liver function tests (aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, total bilirubin before aortic cross clamp, viral serologic markers, and history of diabetes or glucose intolerance. Two different multifactorial scoring systems, devised to identify livers at highest risk of early graft dysfunction and failure (Early Graft Loss/Donor Risk Index [EGL-DRI], and DRI were analyzed as continuous variables.[7,8]

Recipients of combined LT, split LT, pediatric LT, living donor LT, and domino LT were excluded. Demographics, etiologies, LT postoperative outcomes in terms of ICU stays, biliary complications, acute cellular rejection (ACR) in the first 9 months after transplantation, and mortality were collected. Liver biopsies obtained within the entire LT postoperative follow-up time, and taken only when clinically indicated, formed the case material for the histology analysis. All needle specimens were previously formalin-fixed, paraffin-embedded, sectioned at 4 μm, and hematoxylin and eosin–stained. Two experienced pathologists in our institute's pathology service interpreted all of the biopsies and dichotomized the following depictions for statistical analyses to score liver allograft biopsies with acute rejection and determine Rejection Activity Index (RAI) according to the Banff International Consensus Document[9]: severe grade of overall inflammation intensity, eosinophilic infiltrate of liver tissue, mild degree of bile duct inflammation/damage, granulomatous lobular inflammation, bile duct loss, bile duct/cholangiolar proliferation (in any portal tract), subendothelial inflammation, presence of fibrosis, lobular disarray or ballooning, necrosis, presence of acidophilic bodies, cholestasis, steatosis, lobular inflammation, portal inflammation, bile duct damage, and venous endothelial inflammation.[10] The study was conducted in accordance with the principles outlined in the Declaration of Helsinki of 1996, and ISMETT's Institutional Research Review Board approved the protocol (protocol number IRRB/14/15).

Liver Perfusate Fraction Analysis

A sample of donor peripheral blood was collected in a BD Vacutainer Blood Collection Tube containing K2 ethylenediaminetetraacetic acid (Becton, Dickinson and Company, Plymouth, UK). All of the graft procurements were performed at the same standard setting of perfusion pressure and temperature, with identical surgical instruments, skilled medical and nursing staffs, and using the same procedure, except for administration of 2 different organ preservation solutions after cross-clamp maneuver: University of Wisconsin (DuPont Pharmaceuticals, Wilmington, DE) or Celsior solution (SangStat Medical Corporation, Fremont, CA). During liver procurement, the aorta was clamped and the liver was flushed in situ via the hepatic artery, with up to 5 L of preservation solution to exsanguinate the deceased donor (in situ fraction). Liver perfusate (LP) was collected via a vacuum-pump suction system, available in the operating room where the graft was excised, directly into an autotransfusion reservoir (ATR) (Fresenius-Kabi, Bad Homburg, DE) using a sterile tubing system connected to a sterile draining system composed of suction tube with a cannula. Before the perfusate was collected, the ATRs were filled with the anticoagulant acid citrate dextrose solution, (Fresenius-Kabi) to a final concentration of 10% anticoagulant citrate dextrose. Liver perfusate procurement was performed under sterile conditions. At the end of the procurement, reservoirs were closed with appropriate sterile lids, and placed near the connectors for suction and drainage tubes. Lids were secured with hose clamps.

After excision, the liver was placed in a sterile Steri-Drape 1003 Isolation Bag (3 M, St. Paul, MN) and transferred to ISMETT on ice in a thermally insulated container to guarantee a constant temperature of about 4°C. Once in the ISMETT operating room, the liver was perfused through the portal vein with a further 1 to 2 L of the preservation solution (ex situ or backtable fraction), as above, and left in the Steri-Drape Isolation Bag until the organ was transplanted.

After the liver was removed from the 3 M bag for engraftment, the residual perfusate in the perfusate Isolation Bag was aspirated into a new ATR. All reservoirs containing LP were kept at 4°C until processing.

Perfusates were serially collected in sterile ATRs in the presence of 10% of anticoagulant citrate dextrose formula-A. The reservoirs were then transferred to the Fondazione Ri. MED's Regenerative Medicine and Biomedical Technology Unit at ISMETT for cell isolation. Lymphocyte phenotypic characterization was determined on the ex situ fraction. Under laminar hood, liver graft perfusates were transferred from the ATR into 250-mL conicals (Corning GmbH HQ, Wiesbaden, Germany), and centrifuged at 2000 rpm, at 10°C, for 10 minutes in the Heraeus Multifuge 4KR DJB (Labcare Ltd, Newport Pagnell, UK). Contaminating erythrocytes were lysed using 10-548E ACK buffer (Lonza, Euroclone, Pero, Italy) for 5 minutes at room temperature. Cells were washed twice with Dulbecco's D8537 phosphate-buffered saline (Sigma-Aldrich s.r.l, Milan, Italy), 2% fetal bovine serum (Sigma-Aldrich), and cell number and vitality were obtained by 17-942E trypan blue exclusion (Lonza). NKT, NK, and T cell concentrations and phenotypes were analyzed.

Flow Cytometry Staining of Lymphocytes and Antibodies

Lymphocytes were isolated from the ex situ fraction by straight red blood cell lysis, without needing separation on a density gradient. This allowed us to standardize the phenotypic analysis, thus minimizing technical variability among samples. In addition, selection of the one last fraction speeded up the phenotypic characterization protocol. Aliquots of 1×10^6 isolated liver-derived cells and peripheral blood mononuclear cells were stained for surface markers with a panel of prediluted fluorochrome-conjugated antihuman monoclonal antibodies: BD Multitest CD3/CD16 + CD56/CD45/CD19 reagent (CD3-FITC (immunoglobulin G [IgG], clone SK7); CD16-PE (IgG1, clone B73.1) and CD56-PE (IgG1, clone NCAM 16.2); CD45-PerCP (IgG1, clone 2D1); CD19-APC [IgG1, clone SJ25C1]), CD3-FITC (IgG2a, clone SK7); CD4-PE-Cy7 (IgG1, clone L200); CD8-APC-Cy7 (IgG1, clone SK1); CD56-AlexaFluor-700 (IgG1, clone B159); antiCD337/NKp30-AlexaFluor-647 (IgG1, clone p30-15); CD335/NKp46-PE-Cy7 (IgG1, clone 9E2/Nkp46); CD314/NKG2D-PerCP-Cy5.5 (IgG1, clone 1D11). These antibodies were purchased from BD Biosciences (Europe, dilution 1:10). CD3-PerCP (IgG2a, clone BW264/56); CD16-APC (IgM, clone VEP13); CD336/NKp44-PE (IgG1, clone 2.29) were purchased from Miltenyi Biotec (Bergisch Gladbach; Germany dilution 1:20). Samples were incubated for 20 minutes at room temperature in the dark, washed once with phosphate-buffered saline/2% fetal bovine serum, and then analyzed by flow cytometry with FACSAria II dual-laser 8-color cytometer (BD Biosciences, Europe). Acquired data were analyzed with FACSDiva software 6.1.3.

Statistical Analysis

Continuous variables are presented as mean and standard deviation, and categorical variables are presented as frequency and percentages; differences between subgroups of patients were tested for statistical significance using the Student *t* test for continuous variables and the χ^2 or Fisher exact test, as appropriate, for categorical ones. Associations of donor characteristics, perfusion solution, and perfusate phenotype with NK cell percentage were evaluated by fitting simple linear regression models. Time to rejection (TTR) was defined as the number of days between LT and occurrence of ACR, and rejection-free survival as the number of days between LT and either ACR or patient's death. Potential effects of each variable on TTR were explored by means of single-variable proportional-hazard Cox regression models. Predictive performance of NK percentage in discriminating patients who experience ACR was evaluated in terms of area under receiver-operating characteristic curve. We then used 3 different methods (Youden index, closest-to-(0, 1) criterion, and Liu's method) and check them for concordance in finding an optimal discriminating cut-point for NK cell percentage, in terms of sensitivity (SEN), specificity (SPE), positive predictive values (PPV), and negative predictive values (NPV).[11] All analyses and graphics were performed in the R statistical computing environment, version 3.4.3.

RESULTS

Ex situ LPs were collected during the pretransplantation time of a series of 46 DBD livers on the bench, with a mean CIT of 478.3 ± 122.7 minutes, and a mean donor age of 52.7 ± 19.1 years (Figure 1). The DBDs were male in 26 (57%) cases, and the main cause of death was a cerebrovascular event in 29 donors. The mean volume of the collected LP (ex situ fraction) was 1196.875 ± 534.62 mL, and the mean cell number was $316 \pm 151.8 \times 10^6$ (Figure 2).

Phenotype analysis of LP fractions and their matched peripheral blood mononuclear cells samples revealed a different lymphocyte composition, with perfusate samples highly enriched in CD45+ lymphocytes (Figure 3), as also observed by others.[12] Phenotypic comparison of in situ and ex situ fractions showed only marginal differences in lymphocyte composition (Figure 4). Red blood cell count of the back-table flush was low, as the cells were washed during the first lavage steps, making this fraction easier to process for cellular extraction. The median percentages of NK, NKT, and T cells were 30.0%, 12.4%, and 26.0%, respectively, based on the entire lymphocytic population obtained by flow cytometry (Figure 3). DBD LPs with a percentage of CD3+ CD56– T lymphocytes below 25% had a percentage of CD8+ T cells as high as 88.9% of total T cells.

Of the 46 adult recipients, hepatitis C–related liver cirrhosis (21 patients, 45.6%) was the most common diagnostic indication for the primary LT, complicated by hepatocellular carcinoma in 11 cases, and followed by hepatitis B-related cirrhosis (9 patients, 19.5%) (Table 1). All transplanted patients with a diagnosis of hepatocellular carcinoma were within the Milan criteria at the time of LT. In 3 (6.5%) patients included in the study population the indication was a first liver retransplant (re-LT) for delayed-graft function. Seven (15.2%) LT recipients had previous portal vein thrombosis, whereas 18 (39.1%) had undergone previous abdominal surgery. All of the recipients were transplanted with classic LT technique, with whole cadaveric allografts, and the same team of surgeons performed all procedures. The entire study population received induction of the immunosuppressive regimen with 20 mg basiliximab, which was administered through IV bolus during anhepatic phase and on the fourth postoperative day.[13] Within 24 hours of the

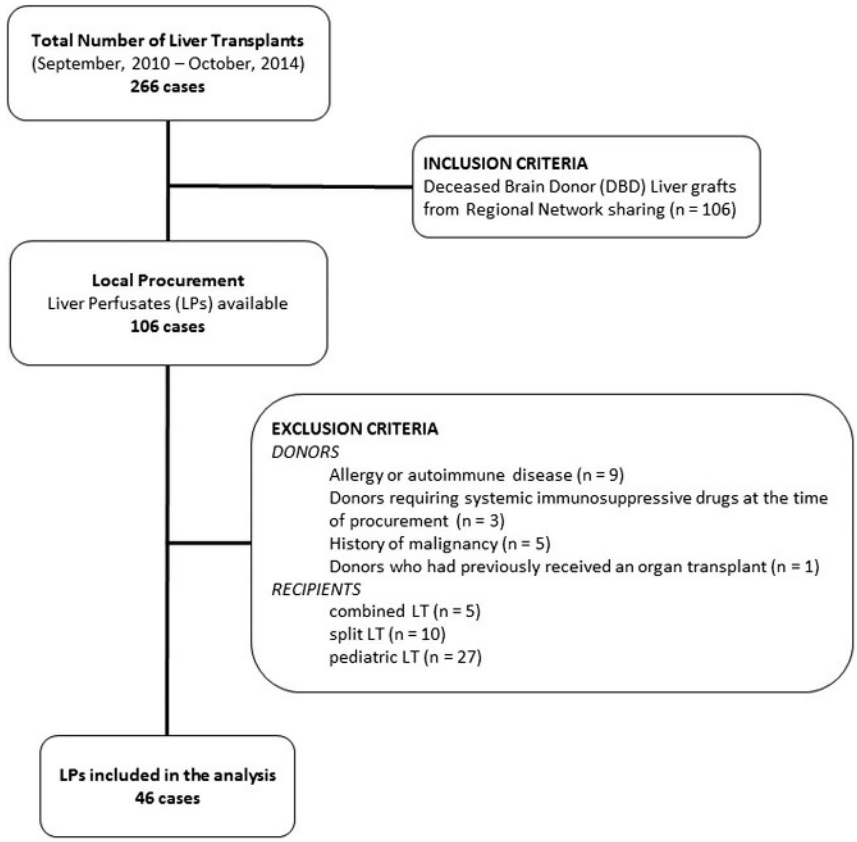

FIGURE 1. Strengthening reporting of observational studies in epidemiology (STROBE) flow diagram. Flow diagram of cases included in, and excluded from, the study. LT, liver transplant.

LT, tacrolimus was administered at 0.15 mg/kg per day by mouth, or through a nasogastric tube, and adjusted to achieve trough levels in the range of 8 to 10 ng/mL. At 30 days posttransplantation, the target trough level was reduced to 5 to 7 ng/mL for the first posttransplant year.[14] The length of follow-up after LT was 41.0 ± 20.9 months, and 21 (45.7%) recipients experienced biliary complications. Patient death occurred in 7 (15.2%) cases. Sepsis-related multiple-organ failure was the most common cause of death (n = 4), and 2 recipients were affected with delayed graft function. None of the recipients experienced graft loss that led to re-LT. Episodes of biopsy-proven ACR occurred in 11 patients in the first 9 months after transplantation, but no patients or grafts were lost because of acute or chronic rejection. In total, 2 biopsies were classified as RAI 1-3, 8 as RAI 4-6, and 1 as RAI 7-9. Steroid treatment of ACR was not indicated for 2 patients due to systemic bacterial infection.[9,10] An HLA compatibility test has been performed in our institute since 2009 only in patients with biopsy-proven ACR (RAI ≥ 6) to target the long-term immunosuppressive regimen. In our study population, HLA compatibility analysis was carried out in only 2 patients, and a mismatch was determined in both.

Estimated rejection cumulative rates at 3 months, 6 months, and 1 year were 9.0%, 11.5%, and 16.5%, respectively, whereas the rejection-free patient survival rates at 3 months, 1 year, and 3 years were 80.4%, 73.9%, and 65.2%, respectively. None of the re-LT patients experienced ACR or died during the follow-up period.

The LP-specific pattern of NK cells was not influenced by the 2 different organ preservation solutions. Neither gender

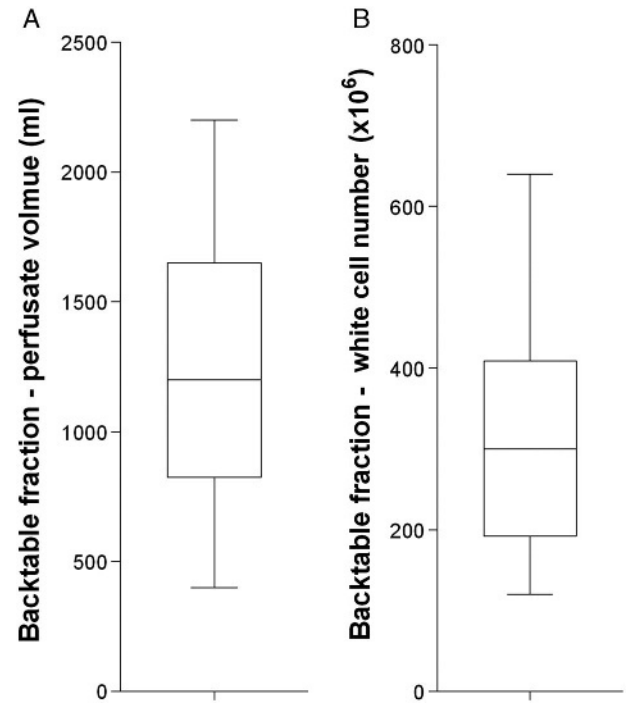

FIGURE 2. Liver perfusate (LP) recovery. Box and whisker plots of volume (milliliter; A) and cell number ($\times 10^6$; B) recovered from the back-table fraction of LPs.

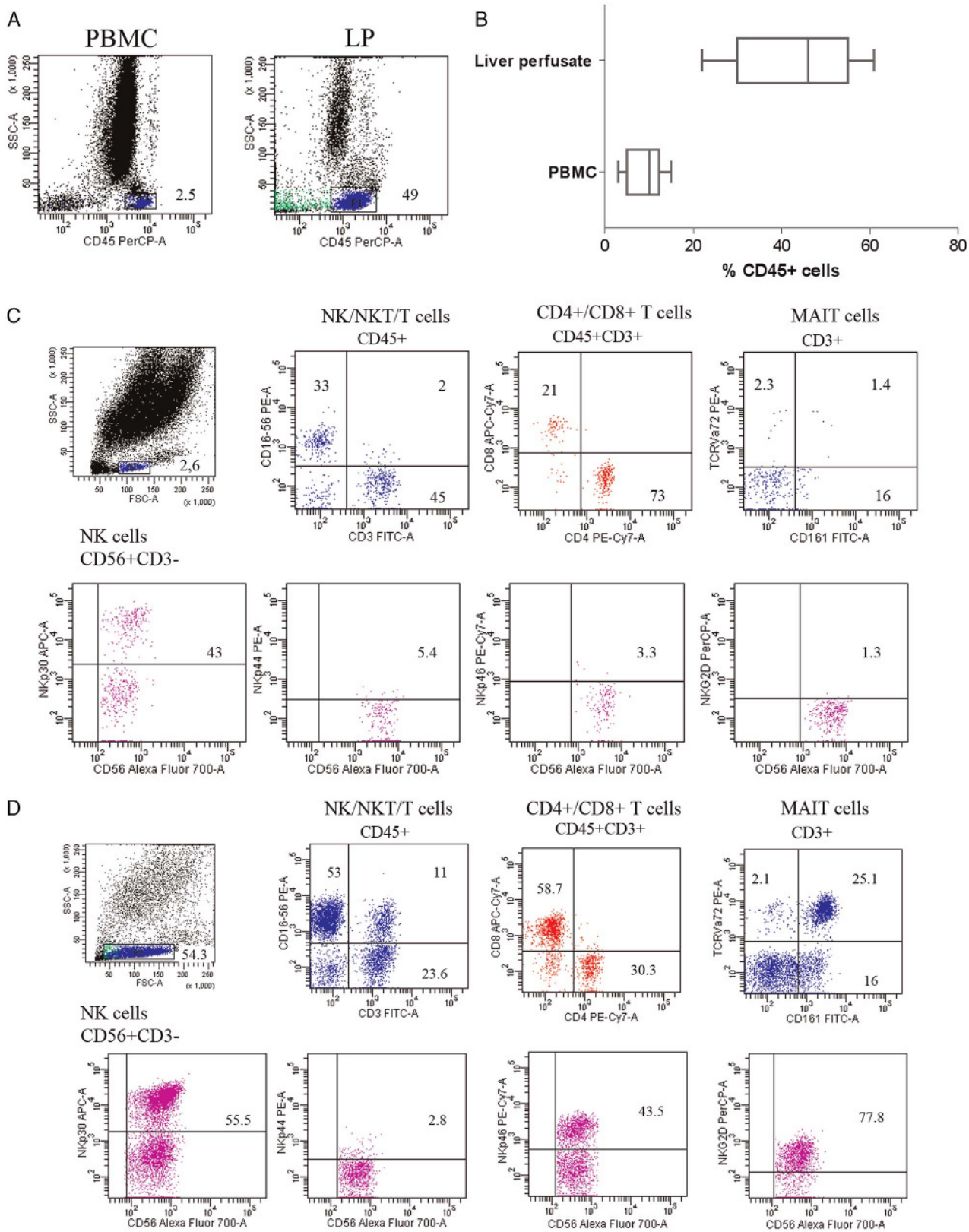

FIGURE 3. Phenotype of peripheral blood mononuclear cells (PBMCs) and matched liver perfusate (LP). Comparison of the abundance of CD45+ leukocytes in PBMC and matched LPs in 1 representative patient (A). Panel B summarizes data obtained from 12 patients. Phenotype characterization of PBMC (C) and LP (D) to identify percentages of infiltrating cellular subpopulations of CD3 T cells (CD4+ and CD8+), natural killer (NK) and natural killer T (NKT) cells. NK cells were also characterized by the expression of common activating markers NKp30, NKp44, NKp46, and NKG2D. Numbers in quadrants represent cell percentages. Multiple color staining and multiple gating analyses were done. (A, C, D) One representative patient is shown.

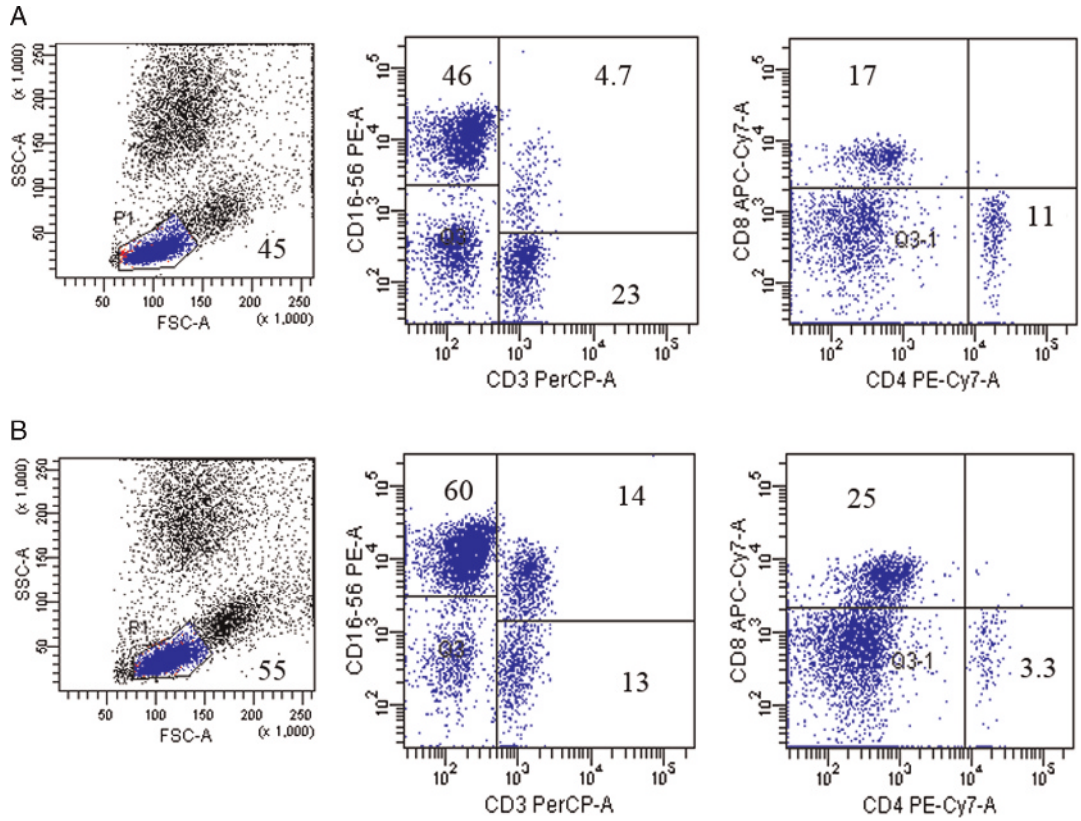

FIGURE 4. Phenotype of on-site and back-table fraction collected during liver procurement and perfusion. Flow cytometry of organ perfusion (on-site fraction) (A) and matched liver perfusate collected on the back-table at the end of procedure (B) for surface markers CD3, CD16-56, CD4, and CD8. Note similar phenotype between the 2 samples. One representative patient is shown.

nor a history of viral infection, diabetes, or glucose intolerance, cardiac arrest, or amine infusion had any correlation with different lymphocyte percentages obtained from DBD LP. Percentage of NK cells was significantly associated with donor age ($P = 0.047$) and the percentage of NKT cellular subset ($P = 0.001$) (Table 2). Regarding recipient outcomes, patients who experienced ACR showed a significantly smaller percentage of NK cell fraction in the LP ($P = 0.015$) (Table 3).

TABLE 1.

List of the distribution of the patients according to diagnosis of HCC and underlying disease

	Total (n = 46)	
	N	%
HCC	19	41
Underlying liver disease		
Chronic viral infection	30	65
Hepatitis B virus infection	9	20
Hepatitis C virus infection	21	46
Alcoholic liver cirrhosis	3	7
Retransplantation (delayed graft function)	3	7
Cryptogenic cirrhosis	2	4
Primary biliary cirrhosis	2	4
Nonalcoholic steatohepatitis	2	4
Cystic fibrosis	1	2
Acute liver failure (mushroom poisoning)	1	2
Hepatic hemangiomatosis	1	2
Hepatic amyloidosis	1	2

HCC, hepatocellular carcinoma.

The Cox proportional regression analysis showed an increased risk for ACR episode of 5.5% for each percentage point of LP NK cell subset (hazard ratio, 1.055; $P = 0.026$). As a preliminary cutoff, we have evaluated the median value of NK cell percentage of LP subset (33.4%), and a significative association was observed between the quote of NK cell under the median value and TTR (hazard ratio, 10.7; $P = 0.02$). No differences were detected in terms of tacrolimus concentration and CIT. The area under receiver-operating characteristic curve for the discriminating ability of NK cells percentage with respect to ACR was 0.743. Furthermore, 3 different statistical methods were used to assess a cutoff value for the prediction of ACR. Both Youden Index and Liu method selected 33.7% (SEN, 1; SPE, 0.57; PPV, 0.42; NPV, 1) as the optimal cutoff value, whereas the closest-to-(0,1) criterion selected 31.7% (SEN, 0.91; SPE, 0.60; PPV, 0.42; NPV, 0.95) of NK cells in the LP. These findings suggest that LP NK percentage subset could be useful to identify those patients that have a very low risk of ACR event. The analysis of postoperative liver biopsy reexaminations did not evidence any association between percentage of NK cellular fraction in the LP and a specific histologic feature of the RAI (Table 4).

DISCUSSION

Hypoxia and reperfusion activate innate mechanisms of inflammation within the graft that underlie stress injuries and can alter the host antigraft adaptive immune response, favoring effector mechanisms over regulation.[3,4] Proinflammatory effector and allospecific recognition properties of recipient NK cells were applied in the context of organ

TABLE 2.

Associations between donor characteristics, perfusion solution and perfusate phenotype with NK cell percentage in LPs

		$CD56^+CD3^-$		
Descriptive statistics	**N (%) or mean ± SD**	**Beta**	**95% CI**	***P***
Donor characteristics				
Males	26 (57)	0.51	−8.14 to 9.15	0.906
Age, y	52.7 ± 19.1	−0.22	−0.44 to 0	0.047
HBcAb	10 (22)	−1.16	−11.55 to 9.23	0.823
CIT, min	478.3 ± 122.7	0.03	−0.01 to 0.07	0.093
BMI, kg/m^2	24.9 ± 3.2	−0.8	−2.14 to 0.53	0.233
Hepatic macrosteatosis (≥10%)	7 (15)	8.97	−2.65 to 20.59	0.127
Diabetes mellitus type II	3 (7)	−8.71	−25.7 to 8.28	0.307
ICU stay, d	4 ± 2.8	−1	−2.56 to 0.57	0.205
DRI	1.9 ± 0.4	−5.06	−17 to 6.87	0.396
EGL-DRI	0.6 ± 2	1.01	−1.27 to 3.29	0.375
Na^+, mEq/L	153.6 ± 12	−0.11	−0.47 to 0.26	0.547
ALT, IU/L	44 ± 49.7	−0.08	−0.16 to 0	0.055
AST, IU/L	54.5 ± 49.4	−0.04	−0.12 to 0.05	0.383
Serum bilirubin, mg/dL	0.9 ± 0.9	2.7	−1.84 to 7.23	0.237
GGT, IU/L	58.1 ± 81.6	−0.03	−0.09 to 0.02	0.228
Amine assumption	36 (78)	−3.54	−13.88 to 6.79	0.493
Artery anomalies	9 (20)	−7.85	−18.39 to 2.69	0.14
Perfusion solutions (University of Wisconsin)	10 (22)	−2.97	−13.33 to 7.38	0.566
LP phenotype, %				
$CD56^+CD3^-$				
$CD56^+CD3^-NKp30^+$	71 ± 21.5	−0.24	−0.63 to 0.15	0.209
$CD56^+CD3^-NKp44^+$	7.3 ± 16.6	0.08	−0.51 to 0.67	0.775
$CD56^+CD3^-NKp46^+$	61 ± 29.9	0.01	−0.29 to 0.31	0.926
$CD56^+CD3^-NKG2D^+$	66.4 ± 34.6	−0.1	−0.31 to 0.12	0.343
$CD56^-CD3^+$				
$CD56^-CD3^+CD8^+$	52.1 ± 16.3	0.02	−0.38 to 0.42	0.913
$CD56^-CD3^+CD4^+$	34.2 ± 18.6	−0.16	−0.5 to 0.19	0.359
$CD56^+CD3^+$	15.3 ± 11.1	−0.57	−0.91 to 0.23	0.001

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; 95% CI, 95% confidence interval; GGT, gamma-glutamyl transferase; HBcAb, hepatitis B core antibody.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; CIT, cold ischemia time; DRI, Donor Risk Index; EGL-DRI, Early Graft Loss–Donor Risk Index; GGT, gamma–glutamyl transferase; HBcAb, hepatitis B core antibody; ICU, intensive care unit; LP, liver perfusate; NK, natural killer; SD, standard deviation.

transplantation as a crucial key player of innate immunity for allograft rejection.[15] Despite this capacity of allorecognition, and although recipient NK cells no doubt play a role in the rejection process,[16-18] it is unclear whether they adopt a tissue-resident phenotype or contribute to tolerance or rejection.[19] The ability of NK cells to exert effector functions without previous antigen-specific sensitization, as well as their antibody-dependent cellular cytotoxicity capacity, their influence on cells without any somatic rearrangement of their receptor genes, and their potential for inflammatory modulation via interferon-γ release make these cells key players in sympathetic activation after brain death.[20] The reduced percentage of NK cells in LP lymphocytic cell fraction analysis from aged donors may be explained by the fact that these liver grafts are more susceptible to sinusoidal milieu injuries and show reduced blood flow and impaired energy metabolism compared with younger liver grafts.[21] In this study, we observe an inverse association between the percentages of NK and NKT cells in the LP. As observed in different experimental models of liver injury, NKT cell numbers decrease while NK cell abundance increases. It has been associated to hepatic ischemia-reperfusion injury after LT and liver resection.[3]

Despite the fact that donor NK cell depletion before LT does not have an effect on graft survival,[22] preclinical research in rats showed donor liver NK cell infusion alone can alleviate liver allograft acute rejection and improve the recipients' survival.[23] In 2011, Moroso et al[24] explored, for the first time, the effect of donor-versus-recipient NK cell alloreactivity on LT outcome, analyzing the effects of HLA/killer cell immunoglobulin-like receptor matching to estimate the effects of donor NK cells. They concluded that, in contrast to what is shown in hematopoietic stem cell transplantation, donor-derived NK cells may not contribute to preventing liver graft rejection, and that recipient-versus-donor NK cell alloreactivity does not affect acute rejection, or graft and patient survival. However, predicting NK cell allograft rejection in LT, based on HLA/killer-cell immunoglobulin-like receptor matching is not considered a suitable model because NK cells can modulate alloreactivity through several immunologic mechanisms.[2] Although studies of immunosuppression withdrawal in humans suggest a role for NK cells in promoting tolerance,[25] a clinical impact of donor NK cells in regulating and suppressing allograft rejection has not been comprehensively reported.[26] In particular, NK cells in LPs have not been investigated in LT for predicting clinical outcomes and, therefore, their potential involvement in rejection of whole liver graft from DBDs remains to be defined. Considering that donor NK cells derived from the grafted liver

TABLE 3.

Baseline characteristics of 46 dead-brain donors, perfusion solutions, perfusate phenotypes, and LT recipients

	Total n (%) or mean ± SD	No rejection, n (%) or mean ± SD	Rejection, n (%) or mean ± SD	*P*
Overall number of patients	46	35	11	
Donor characteristics				
Males	26 (56.5)	20 (57.1)	6 (54.5)	1.000
Age, y	52.7 ± 19.1	50.5 ± 20.1	59.9 ± 14.2	0.155
HBcAb	10 (21.7)	8 (22.9)	2 (18.2)	1.000
CIT, min	478.3 ± 122.7	476.4 ± 133.4	483.8 ± 91.0	0.865
BMI, kg/m^2	24.9 ± 3.2	24.8 ± 3.2	25.2 ± 3.2	0.746
Hepatic macrosteatosis (≥10%)	7 (15.2)	5 (14.3)	2 (18.2)	1.000
Diabetes mellitus type II	3 (6.7)	3 (8.8)	0 (0.0)	0.565
ICU stay, d	4.0 ± 2.8	4.2 ± 2.8	3.2 ± 2.8	0.277
DRI	1.9 ± 0.4	1.9 ± 0.4	1.9 ± 0.3	0.835
EGL-DRI	0.6 ± 2.0	0.6 ± 2.2	0.5 ± 1.5	0.787
Na^+, mEq/L	153.6 ± 12.0	153.5 ± 12.4	154.1 ± 11.1	0.886
ALT, IU/L	44.0 ± 49.7	45.2 ± 54.9	40.2 ± 29.5	0.776
AST, IU/L	54.5 ± 49.4	51.6 ± 43.1	63.2 ± 67.1	0.507
Serum bilirubin, mg/dL	0.9 ± 0.9	1.0 ± 1.0	0.7 ± 0.5	0.437
GGT, IU/L	58.1 ± 81.6	62.8 ± 92.3	44.3 ± 37.1	0.544
Amine assumption	36 (78.3)	25 (71.4)	11 (100.0)	0.088
Artery anomalies	9 (19.6)	6 (17.1)	3 (27.3)	0.664
Perfusion solutions				
University of Wisconsin	10 (21.7)	7 (20.0)	3 (27.3)	0.682
Celsior	36 (78.3)	28 (80)	8 (72.7)	
LP phenotype, %				
$CD56^+CD3-$	32.8 ± 14.3	35.7 ± 15.0	23.8 ± 6.1	0.015
NKp30	71.0 ± 21.5	70.5 ± 24.1	72.3 ± 15.7	0.893
NKp44	7.3 ± 16.6	3.2 ± 2.8	16.5 ± 30.3	0.194
NKp46	61.0 ± 29.9	66.3 ± 30.4	47.9 ± 28.0	0.318
NKG2D	66.4 ± 34.6	66.5 ± 35.2	66.3 ± 36.7	0.992
$CD56-CD3^+$				
$CD8^+$	52.1 ± 16.3	53.2 ± 17.8	49.5 ± 13.1	0.632
$CD4^+$	34.2 ± 18.6	33.2 ± 18.9	36.4 ± 19.0	0.710
$CD56^+CD3^+$	15.5 ± 11.4	14.8 ± 11.1	17.9 ± 12.7	0.435
Recipient characteristics				
Males	34 (73.9)	26 (74.3)	8 (72.7)	1.000
Age at transplant, y	55.1 ± 10.8	54.3 ± 10.1	57.6 ± 13.0	0.408
BMI, kg/m^2	26.4 ± 3.6	26.6 ± 3.6	25.8 ± 3.9	0.616
Serum creatinine, mg/dL	1.3 ± 0.9	1.3 ± 0.9	1.2 ± 0.5	0.806
Serum bilirubin, mg/dL	6.3 ± 9.9	6.9 ± 11.1	4.3 ± 4.3	0.450
Chronic viral infection	30 (65.2)	23 (65.7)	7 (63.6)	1.000
Hepatitis B virus infection	9 (19.6)	9 (25.7)	0 (0)	0.089
Hepatitis C virus infection	21 (45.7)	14 (40.0)	7 (63.6)	0.298
Prior abdominal surgery	18 (39.1)	13 (37.1)	5 (45.5)	0.728
Portal vein thrombosis	7 (15.2)	6 (17.1)	1 (9.1)	1.000
EGL-RRI	3.6 ± 4.8	3.7 ± 5.1	3.3 ± 4.1	0.817
ICU stay, d	8.9 ± 15.2	8.8 ± 15.2	9.3 ± 16.2	0.930
MELD	16.6 ± 8.3	16.0 ± 8.0	18.5 ± 9.1	0.404
MELD-Na	19.2 ± 7.9	19.0 ± 7.6	20.0 ± 9.1	0.731
Length of follow-up, mo	41.0 ± 20.9	38.4 ± 20.7	49.3 ± 20.0	0.130
Biliary complications	21 (45.7)	14 (40.0)	7 (63.6)	0.298
Death	7 (15.2)	6 (17.1)	1 (9.1)	1.000

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DRI, Donor Risk Index; EGL-DRI, Early Graft Loss–Donor Risk Index; EGL-RRI, Early Graft Loss–Renal Risk Index; GGT, gamma–glutamyl transferase; HBcAb, hepatitis B core antibody; ICU, intensive care unit; LP, liver perfusate; LT, liver transplant; MELD, Model for End-Stage Liver Disease; NK, natural killer; SD, standard deviation.

were detected as passenger leukocytes in the periphery of LT recipients during the early postoperative period,[27] donor NK cells may be involved in early interactions between donor and recipient immune cells and in the mechanism underlying late tolerance.[2,28-32] Furthermore, there is accumulating evidence that the donor NK cells have been identified within liver grafts up to 2 years after, and the adult liver could support in situ differentiation of hepatic NK cells from hematopoietic progenitors in the liver.[33] In the transplant setting, immune-regulatory effects of NK cells were recently considered the main

TABLE 4.

Associations between specific histologic features of the RAI and percentage of NK cellular fraction in the LP

	Descriptive statistics	CD56 + CD3-		
	N (%)	Beta	95% CI	*P*
Overall inflammation intensity: severe	8 (73)	−0.18	−0.55 to 0.06	0.197
Eosinophilic infiltrate of liver tissue	10 (91)	−0.26	−1.21 to 0.15	0.361
Bile duct inflammation/damage: mild	4 (36)	0.08	−0.13 to 0.33	0.458
Granulomatous lobular inflammation	1 (9)	−0.03	−0.51 to 0.36	0.882
Bile duct loss	2 (18)	−0.17	−0.68 to 0.12	0.342
Bile duct/cholangiolar proliferation (in any portal tract)	7 (64)	−0.05	−0.28 to 0.17	0.669
Subendothelial inflammation	5 (45)	0.1	−0.11 to 0.34	0.377
Fibrosis	7 (64)	0.1	−0.12 to 0.37	0.399
Lobular disarray/ballooning	4 (36)	0.09	−0.13 to 0.33	0.446
Necrosis	10 (91)	0.15	−0.23 to 0.92	0.521
Acidophilic bodies	7 (64)	0.03	−0.18 to 0.27	0.763
Cholestasis	5 (45)	0.16	−0.06 to 0.44	0.187
Steatosis	7 (64)	−0.06	−0.3 to 0.16	0.594
Lobular inflammation	8 (73)	−0.06	−0.32 to 0.18	0.618

CI, confidence interval; LP, liver perfusion; NK, natural killer; RAI, Rejection Activity Index.

orchestrators of rejection episodes days to weeks after LT.[2,34] According to our present preliminary retrospective single-center study, the percentage of donor-derived NK cell fraction in LP appears to be associated with the biopsy-proven ACR episode event in a group of small but homogenous LT adult recipients. In particular, we found very interesting the association of an NK cell fraction in LP of 34.7% or less, with reduction of TTR. These data suggest the potential posttransplant predictive value of the LP cytofluorimetric examinations and could help embed the belief that the strategies that deplete passenger donor lymphocytes within the liver allograft, (eg, ex vivo normothermic perfusion of the organ after retrieval), might prolong LT survival and influence immunosuppressive therapy management.[34,35]

Despite the fact that several reliable prognostic scores have been generated for early graft loss and liver allograft outcome prediction by fitting donor risk factors and recipient characteristic indicators, none of them used any characterization of lymphocytes and/or cytokines released from the donor liver allograft after secondary (ex situ) preservation flush to associate with transplant outcomes.[3,4,36] To the best of our knowledge, this retrospective study of 46 DBDs is the first to assess the association between the percentage of lymphocytes isolated from LP and DBD characteristics, which are traditional risk factors for suboptimal functioning after LT[7,8,12-14] and LT recipient outcomes.[37] We believe that ex situ LP fraction could be representative of the nonparenchymal liver cell content and might offer an advantage in terms of technical manipulation for isolation of infiltrating cells. In our DBD cohort, this fraction was, in fact, washed of most of the red blood cell component, rendering the LP less dense and easier to handle than the fractions collected at the beginning of donor graft perfusion (in situ fraction).

Cytofluorimetric LP fraction analysis could offer a targeted method of leveling the differences of the liver sinusoidal milieu, hormonal deregulation, and hemodynamic microcirculation disturbance, guiding decisions on accepting or declining high-risk organs. A simple lysis of erythrocytes, as opposed to density gradient centrifugation or even leukapheresis, is required for optimal isolation of viable cells; it is also faster, less expensive, and more reproducible.[38] Though the phenotypic characterization of lymphocytes infiltrating the liver from biopsy samples has been described, the technique requires dissociation of the cellular content from tissue by digestion with collagenase IV and DNase I, which can alter key features of the infiltrate in terms of phenotype and function. Only a limited number of cells can be obtained from small liver biopsies, greatly reducing opportunities for downstream applications, and the small liver fragment obtainable in a biopsy does not represent the whole organ.[39] We propose using the ex situ fraction as a tool for obtaining the hepatic lymphocyte phenotype and determining whether NK cell percentages can vary among DBD characteristics. Liver perfusate phenotyping can be obtained by flow cytometry analysis very quickly, and, theoretically, it is possible to know the results before the implantation of the organ. These results of the laboratory analysis might help to manage the change of immunosuppressive regimen when the patients arrive on the ICU after LT. Furthermore, it would be interesting to potentially manipulate the liver before transplantation to boost LP lymphocyte content given that sinusoidal endothelial cells, for example, have been found to directly relate to donor organ quality, with potential translational relevance and impact on surgical decision making for adjuncts in organ preservation after cold storage and machine perfusion device usage.[40,41]

Our analysis has never been applied to deceased donor organ procurement research, but the study has several limitations. First, there was a significant selection bias for donor inclusion criteria. We excluded living liver donor candidates, DBDs who could undergo split graft procurements, and donors after circulatory death. As a result, donors who could not present pathophysiological changes occurring after and/or during brain death were not included in the study group. However, regarding the recipients, paradoxically, a possible confounding effect due to the inclusion of 3 re-LT patients was not evident because they experienced no episodes of ACRs and survived the follow-up period. Therefore, we could not collect data on other DBD parameters, including liver biopsies, nutritional status, levels of cardiovascular, and/or endocrine changes, sarcopenia, inflammation, and

immune activation, due to the retrospective nature of the study's design. We might infer from the literature that DBD characteristics could influence the homing potential of NK cells, thus suggesting that nonspecific inflammatory signals related to brain death may alter adaptive immunity.[42,43]

CONCLUSIONS

Findings from our exploratory study suggest the possibility of including cytofluorimetric LP analysis to better discriminate patients who have less probability to experience an ACR event. However, further prospective longitudinal cohort studies with less potential sources of bias are necessary to confirm our findings, and hypothesis-driven validation studies are required before using the method described in our study in clinical practice.

Although the cutoff analysis is very explorative, if widely validated as a clinical biomarker to target allograft damage in DBDs, it might help direct the decision making process for stratifying graft preservation, and indirectly improve the post-LT outcomes of patients receiving suboptimal livers, which represent an ideal environment for evaluating the cost-effectiveness of graft procurement management practices and interventions.[1,2]

Liver perfusate cytofluorimetric phenotyping may contribute as a targeted preoperative tool to predict the risk of ACR episode, and as clinical test in translational studies that aim to improve donor allograft procurement and transplant outcomes.

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