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Distribution of intrahepatic T, NK and CD3$^+$CD56$^+$NKT cells alters after liver transplantation: Shift from innate to adaptive immunity?

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ABSTRACT

Background: The liver is an immunological organ containing a large number of T, NK and NKT cells, but little is known about intrahepatic immunity after LTx. Here, we investigated whether the distribution of T, NK and CD3$^+$CD56$^+$NKT cells is altered in transplanted livers under different circumstances.
Methods: Core biopsies of transplanted livers were stained with antibodies against CD3 and CD56. Several cell populations including T (CD3$^+$CD56$^-$), NK (CD3$^-$CD56$^+$) and NKT cells (CD3$^+$CD56$^+$) were studied by fluorescence microscopy. Cell numbers were analyzed in relation to the time interval after LTx, immunosuppressive therapy and stage of acute graft rejection (measured with the rejection activity index: RAI) compared to tumor free liver tissue from patients after liver resection due to metastatic disease as control.
Results: Recruitment of CD3$^+$CD56$^+$NKT cells revealed a significant decrease during high RAI scores in comparison to low and middle RAI scores (RAI 7–9: 0.03 ± 0.01/HPF vs. RAI 4–6: 0.1 ± 0.005/HPF). CD3$^+$CD56$^+$NKT cells were also lower during immunosuppressive therapy with tacrolimus (0.03 ± 0.01/HPF) than with cyclosporine (0.1 ± 0.003/HPF), cyclosporine/MMF (0.1 ± 0.003/HPF) or sirolimus (0.1 ± 0.01/HPF) treatment. Intrahepatic T cell numbers increased significantly 50 days after LTx compared to control liver tissue (4.5 ± 0.2/HPF vs. 1.9 ± 0.1/HPF). In contrast, NK cells (0.3 ± 0.004/HPF) were significantly fewer in all biopsies after LTx compared to the control (0.7 ± 0.04/HPF).
Conclusions: These data indicate significant alterations in the hepatic recruitment of T, NK and CD3$^+$CD56$^+$NKT cells after LTx. The increase in T cells and the decrease in NK and CD3$^+$CD56$^+$NKT cells suggest a shift from innate to adaptive hepatic immunity in the liver graft.

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1. Introduction

Although improved immunosuppressive strategies have significantly reduced the risk of acute rejection after solid organ transplantation, the development of chronic and acute rejection with restricted graft survival still remains a serious problem in transplantation medicine [1]. Rejection is mediated by cells of the innate and adaptive immune systems that recognize the allograft as non-self [2,3]. T cells as part of the adaptive immune system recognize genetically different major histocompatibility complex (MHC) molecules using two different pathways. Either the T cell receptor recognizes intact donor MHC molecules on antigen presenting cells that are 'passengers' in the transplant, or host-type antigen-presenting-cells process and present donor MHC molecules in the context of self to T cells [4]. Natural killer (NK) cells are part of the rapidly-acting innate immune response, recognizing targets in a manner that is not MHC-restricted and does not require prior sensitization. However, NK cells can also lyse healthy target cells that express non-self MHC molecules. This alloreactive NK cell response results from the mechanisms by which NK cells discriminate healthy from unhealthy autologous cells [5,6].

The yet to be clarified linkage between innate and adaptive immunity is an emerging concept in the field of transplantation research. NKT cells represent an interesting subpopulation of lymphocytes in this specific area. They have a T cell antigen receptor (TCR) that is restricted in diversity and share properties with cells of the innate immune system. NKT cells recognize lipid antigens presented by the MHC class I-related antigen CD1d [7,8]. Moreover, NKT cells are enriched within the liver. They represent approximately 20% of intrahepatic lymphocytes in humans, which is the highest NKT-cell/T-cell ratio found among different organ systems [9–11]. However, these cells do not represent a unique population. So far, three types of NKT cells have been described. First, there are the invariant Vα14-NKT cells (iVα14-NKT), also called type I

Abbreviations: LTx, liver transplantation; NK cells, natural killer cells; NKT cells, natural killer T cells; MMF, mycophenolate mofetil; RAI, Banff rejection activity index; HPF, high power field.

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NKT cells or iNKT cells. Second, a population of CD1d-reactive NKT cells that express diverse non-Vα14TCRs, referred to as type II NKT cells or non-invariant, have been classified. A third category is called NKT-like cells, which are CD1d-independent and also express diverse TCRs [12]. Another frequently used surrogate marker for human NKT cells is CD56 in combination with CD3 [10,13]. Regarding the role of NKT cells in transplantation, it has been shown in several animal models that they are required for the induction of tolerance after solid organ allografting [14,15]; also, hepatic reperfusion injury is initiated by the CD1d-dependent activation of NKT cells [16].

The aim of this study was to look for the distribution of intrahepatic T, NK and NKT cells after LTx, and in the clinical setting of graft dysfunction. In addition to routine histopathological evaluation, liver core biopsies were studied by immunohistochemistry for $CD3^+/CD56^+$ (NKT) cells, $CD3^+/CD56^-$ (T) cells and $CD3^-/CD56^+$ (NK) cells. Findings were correlated to the pathological diagnosis and other clinical parameters. We show that NK cell numbers decrease after LTx and that the time period after LTx influences T cell distribution in the liver. Furthermore, our data demonstrate a significant reduction in $CD56^+$NKT cells in case of severe acute rejection and immunosuppressive therapy with tacrolimus.

2. Objective

Our objective was to study the intrahepatic recruitment of T, NK and $CD3^+CD56^+$NKT cells after LTx.

3. Patients and methods

3.1. Patients

After LTx patients with graft dysfunction were screened for acute rejection and intrahepatic cell distribution in histological specimens. In total, liver core biopsies of 69 patients who had undergone liver transplantation for chronic or acute liver failure were included. Tumor free liver tissue of ten patients after liver resection due to metastatic disease served as a non-transplanted control group. Written informed consent has been obtained by all patients.

3.2. Liver biopsy

Percutaneous liver core biopsy was performed by Menghini technique using Hepafix (Braun, Melsungen, Germany) [17,18]. First, the liver was localized by ultrasonography and the site of puncture was anesthetized with 2% Mepivacain hydrochloride. Biopsies were carried out transcostally and the obtained liver specimen was assessed for adequate size. Tissue was fixed in formalin and sent for routine histological examination. All core biopsies had a length of at least 1.5 cm, a diameter from 1.2 to 1.8 mm, and in each case more than 10 portal fields per biopsy could be found.

3.3. Immunofluorescence

Standard H&E staining for histological examination was performed and a histopathological diagnosis was made. CMV infection was assessed by in situ hybridization. Liver specimens that revealed rejection were divided according to the Banff rejection activity index (RAI) [19]. In total, 9 biopsies could be classified as RAI 1–3, 19 as RAI 4–6 and 5 as RAI 7–9. Subsequently, expression of CD3 and CD56 was detected by double-immunofluorescence. For this, two-micrometer sections were cut from formalin-fixed, paraffin wax-embedded tissue samples. Immunofluorescence staining of dewaxed sections was performed with microwave-based antigen retrieval using 10 mM citrate buffer (pH 7.2) (microwave oven for 35 min at 250 W). Each specimen was first incubated with rabbit anti-human CD3 (clone:SP7, NeoMarkers, Fremont, USA) and mouse anti-human CD56 (clone:123 C3.D5, ImmunoLogic, Duiven, Netherlands), followed by labeling with two secondary antibodies: Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse, respectively (both Invitrogen, Carlsbad, USA). Finally, nuclear staining with DAPI (4′,6-Diamidin-2-phenylindol, Vectashield mounting medium with Dapi, Vector laboratories, Burlingame, USA) was carried out. Cell subpopulations were analyzed by fluorescence microscopy using different filters for CD3 (red), CD56 (green) and double-labeled cells (yellow) with a 400x magnification. In each case 10 high-power fields (HPF) were counted by two independent and blinded observers. As the size of a liver core biopsy is limited, most often the whole tissue was screened to count approximately 10 HPF; all existing portal fields were included. Antibodies were tested on sections of palatine tonsils for positive controls.

3.4. Statistics

Histology and cell count data are presented as the mean value ±SEM. Statistical analyses were performed using either a Student's *t*-test or the Mann–Whitney-*U*-test. Multivariate influences including donor age, underlying liver disease, time period after LTx, immunosuppressive therapy, histological findings and RAI score were evaluated with stepwise linear regression analysis (SPSS 17.0 for windows, LEAD technologies). Differences were considered significant at $P<0.05$.

4. Results

4.1. Characteristics of the study population

Over a period of 3 years 156 liver transplantations were performed and screened for graft dysfunction by serological examinations. Finally 69 patients received a biopsy and were included in the study (Table 1). Time from LTx to biopsy varied from 6 to 341 days. Underlying diagnoses were mostly alcohol-induced liver disease (n = 20) followed by chronic hepatitis C infection (n = 13) and non-alcohol steatohepatitis (n = 10). Immunosuppression was initiated with Basilixmab on days 0 (20 mg i.v.) and 4 (20 mg i.v.) after transplantation. In addition, all except patients with a viral hepatitis received prednisolone, starting with 1 mg/kg body weight and reduced by 5 mg every other day. Immunosuppression was established with cyclosporine A (CyA) (n = 31), CyA and mycophenolate mofetil (MMF) (n = 24), tacrolimus (n = 9) or sirolimus (n = 5) and was

Table 1
Baseline characteristics and histological biopsy findings of 69 patients who received liver biopsy after LTx.

Number of patients (male)	69 (44)
Mean recipient age (range)	51 (20–67)
Mean donor age (range)	49 (15–76)
Mean days between LTx and biopsy (range)	52 (6–34 1)
Underlying liver disease:[a]	
Alcoholic liver disease	20
Non-alcoholic steatohepatitis	10
Chronic HCV Infection	13
Chronic HBV infection	5
Hepatocellular carcinoma	9
Primary biliary cirrhosis	5
Primary sclerosing cholangitis	4
Secondary sclerosing cholangitis	3
Autoimmune hepatitis	2
Budd Chiari syndrome	4
Cholangiodysplastic pseudochirrosis	1
Cryptogenic cirrhosis	2
Immunosupression:	
Cyclosporine A	31
Cyclosporine A + Mycophenolate Mofetil	24
Tacrolimus	9
Sirolimus	5
Biopsy findings:	
Acute rejection	33
Cholangitis	18
Cholestasis	7
CMV infection	3
Portal hepatitis	4
Others	4

[a] Difference to 69 total patients because of multiple diagnosis in some patients.

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