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Rapid exchange of large numbers of donor- and host leukocytes after human liver transplantation

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Abstract After liver transplantation, the release of donor leukocytes into the host and the uptake of host leukocytes by the graft is one of the earliest immunologic interactions between donor and host. Using three-color flow cytometry, these interactions were investigated in eight patients from 5 min-24 h after receiving HLA unmatched liver grafts. Five minutes after reperfusion, 5.0% ± 1.4% of all blood leukocytes in the host were of donor origin, decreasing to 1.1% ± 0.8% after 24 h. Donor granulocytes preferentially disappeared from the host circulation, whereas no differences were found between NK-cells and various B- and T cell subpopulations. Furthermore, host granulocytes were preferentially retained in the donor liver. Thus, despite extensive pre-operative perfusion, more than 10^9 donor leukocytes quickly leave the liver graft while host granulocytes preferentially accumulate there. A better understanding of the molecular mechanisms mediating these early interactions might help to develop new strategies for diagnosis and therapy of liver graft rejection.

Keywords Leukocyte trafficking · Leukocyte subsets · Liver transplantation · Reperfusion damage

Abbreviations *FFP* Fresh frozen plasma · *mAb* Monoclonal antibodies · *NK* Natural killer cells · *PE* Phycoerythrin · *RBC* Packed red blood cells

Introduction

The liver contains about 5×10^9 lymphocytes, which is half of the number of lymphocytes contained in the whole blood [16, 28]. Within the liver, the lymphocytes are found in distinct compartments such as the sinusoids, the parenchyma, and the periportal field [5, 11]. Their adhesion molecule expression pattern [5], their interaction with the extracellular matrix [23], and their proliferation rate [11] differs, depending on the compartment they are in. In addition to lymphocytes, natural killer cells (NK) and granulocytes are also found to adhere to the endothelium of the liver [8]. Therefore, it is not surprising that after liver transplantation, donor leukocytes leave the liver and are found in the circulation of the host. Donor derived leukocytes can be found

Table 1 Patient characteristics, clinical course and outcome

ID	Sex	Age	Hepatic disease[a]	Reperfusion Damage[b]	Rejection[c]	Outcome[d]
1	m	63	Fulminant Hep.	±	+	DC
2	f	34	Hep. B, Cirr.	–	+	DC
3	m	38	Hep. C, Cirr.	–	+	D (208)
4	f	57	PBC	+	*	D (14)
5	f	53	PBC	±	–	DC
6	m	48	HCC	+ + +	+ +	D (40)
7	f	63	SSC, SBC	–	–	DC
8	f	40	Hep. B, Cirr.	+	–	DC

[a] Hepatic disease: *Hep.* hepatitis, *Cirr.* cirrhosis, *PBC* primary biliary cirrhosis, *HCC* hepatocellular carcinoma, *SSC* secondary sclerosing cholangitis, *SBC* secondary biliary cirrhosis

[b] Reperfusion damage score. Aspartate aminotransferase postoperatively: –0–500 U/l, + 500–1000 U/l, + + 1000–2000 U/l, + + + 2000–4000 U/l, + + + + > 4000 U/l

[c] Rejection score: -none, + bilirubin ↑, + + bilirubin and AST/ALT ↑, + + + rejection steroid resistant, *AST* aspartate aminotransferase, ALT alanine aminotransferase, *not determined because of early death

[d] Outcome: *DC* discharged home, *D* death, *in parentheses* days after transplantation, Cause of death: *patient 3* liver failure, sepsis, pneumonia, *patient 4* renal failure, sepsis, *Patient 6* liver and renal failure, sepsis

in the blood, lymph nodes, and skin of the host for many years, if not for the whole life, after undergoing transplantation [19, 20]. In addition, recent evidence suggests that donor cells play an important role in the regulation of allograft survival [10, 14, 21, 24]. For example, in a rat model of heart allograft transplantation, removal of donor leukocytes immediately after transplantation led to graft rejection, whereas removal of donor leukocytes 18 days after transplantation had no effect on graft survival [10].

Since very little is known about the early release of donor granulocytes, donor NK-cells, and donor lymphocytes (including their subpopulations) in humans, the present study analyzed the appearance of donor leukocytes in the host circulation from 5 min-24 h after liver transplantation by three color flow cytometry in eight patients. In addition, the early interaction of host leukocytes with the donor liver tissue was studied. Thus, the present study provides data which help to better understand which role donor and host leukocyte subsets play for reperfusion damage, post-reperfusion syndrome, and graft survival immediately after liver transplantation.

Materials and methods

Patients

Eight adult patients (5 women and 3 men) liver grafted for end stage cirrhosis ($n = 6$), carcinoma ($n = 1$) or fulminant hepatitis ($n = 1$) were investigated (Table 1). The mean age of the patients was 50 ± 11 years. All patients in this study had received AB0 blood group-identical but human leukocyte antigen (HLA)-unmatched liver grafts. Based on this HLA mismatch, the leukocyte origin (donor or host) was detected by monoclonal antibodies (mAb) against polymorphic epitopes of HLA class I antigens. The study was approved by the ethics committee of the Medical School Hannover.

Operation technique and anesthesia

Prior to transplantation, the donors received not more than 2 units of packed red blood cells (RBC), and the graft was perfused with and stored at 4 °C in cold preservation fluid. This perfusion was done during organ harvesting through the hepatic artery and in four cases also through the portal vein with 5–8 l of University of Wisconsin (UW) solution or 8–18 l of HTK solution (Köhler Chemie, Alsbach, Germany). Liver transplantation was carried out orthotopically with replacement of the retrohepatic vena cava by standard surgical technique. In brief, the host liver was mobilized and devascularized by clamping the hepatic artery and the portal vein. The infra- and suprahepatic vena cava were clamped and removed with the diseased organ. In four cases, veno-venous bypass was used to shunt the venous blood from the lower extremities and the gut to the axillary vein. During the anhepatic phase, the donor organ was placed in the abdominal cavity and the vessels were anastomozed, beginning with the supra- and infrahepatic vena cava, followed by the portal vein. Finally, the hepatic artery anastomosis was performed. Prior to re-establishing systemic circulation through the graft, the liver was flushed with approximately 400 ml of portal venous blood, and this reperfusion blood was collected at the hepatocaval anastomosis. The anesthesiological management for liver transplantation has been described in detail elsewhere [2, 3]. Anesthesia was induced with thiopental, fentanyl and midazolam. As neuromuscular blocking agents, succinylcholin and pancuronium were used. Anesthesia was continued as balanced anesthesia, including thiopental, midazolam, 0.5%–0.8% isofluran and fentanyl. RBC and fresh frozen plasma (FFP) were given as needed. The patients received an average of 10 units of RBC (range 2–18) and an average of 16 units of FFP (range 2–23). For fluid management, patients received NaCl 0.9%, HES 200/0.5 6% and Glucose 5%. Cefotaxime and metronidazole were given as perioperative antibiotic prophylaxis for 48 h. Other drugs used were calcium gluconate, potassium chloride, nitrates,

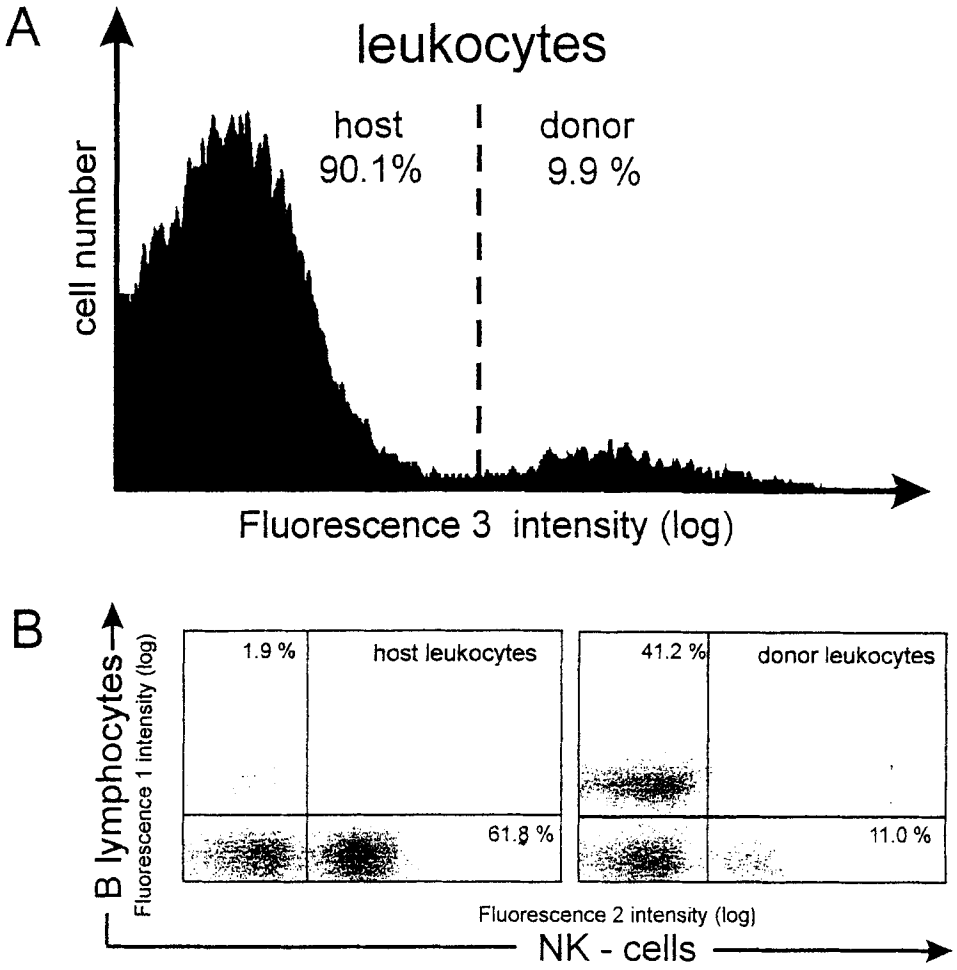

Fig. 1 A, B Three-color flow cytometry analyzing the blood lymphocyte subsets among donor and host leukocytes. **A** FACS histogram showing the separation of donor and host leukocytes by a monoclonal antibody against a HLA epitope of the donor which is different from that of the host (fluorescence 3). **B** By gating on either donor or host cells the lymphocytes were further differentiated with monoclonal antibodies directed against B-cells (fluorescence 1) and NK-cells (fluorescence 2). Note the different subset composition of donor and host leukocytes

furosemide, sodium bicarbonate, lidocaine, dopamine, epinephrine, norepinephrine and orciprenaline. Prior to reperfusion, all patients received a single dose of 500 mg methylprednisolone.

Specimen collection

The baseline leukocyte subset composition of the host was determined in a blood sample taken from a central venous catheter after induction of anesthesia. Further samples were taken in the late anhepatic stage (5 min prior to reperfusion), and 5 min and 90 min after reperfusion. An additional sample was obtained 24 h after transplantation. A sample of the reperfusion blood released from the liver graft during the flushing with portal venous blood was taken prior to opening the caval anastomosis.

Detection of donor- and host leukocytes by three-color flow cytometry

Blood samples were collected in plastic syringes prefilled with heparin. Leukocyte numbers were determined, and a differential blood cell count was performed on blood smears. Red blood cells were removed by incubation with lysing reagent (8.3 g NH4CL; 0.1 g EDTA; 1.0 g KHCO3 ad 1 l Aqua dest). After centrifugation, the pellet was re-suspended with 1% PBS, incubated with human serum, further stained with monoclonal antibodies, and prepared for three-color flow cytometry. To distinguish between donor- and host cells, the specimens were incubated with a biotin-conjugated antibody against polymorphic epitopes of HLA class I antigens as described elsewhere [15] and detected by SA-Red 670 fluorescence 3 (Gibco, Eggenstein, Germany). Clones HB122 (mouse IgG_{2a} specific for HLA A3), HB54 (mouse IgG_1 specific for HLA A2,B17), and HB59 (mouse IgG_1, specific for HLA B7,B40) were obtained from American Type Culture Collection, Rockville Md., USA. The HLA class I antigen pattern for the donor was analyzed routinely as a part of the pre-transplantation work-up. The antigenetic pattern of the host cells was determined by all three antibodies. To exclude cross-reactivity, the three antibodies were tested against donor- and recipient leukocytes and only antibodies recognizing either donor- or host cells were employed. Lymphocytes, granulocytes, and monocytes were identified according to their forward and sideward scatter characteristics in flow cytometric analysis. Lymphocyte subpopulations were determined with FITC- and phycoerythrin (PE)-labeled antibodies (fluorescence 1 and 2, respectively) and analyzed flow cytometrically (FACScan, Becton Dickinson, Erembodegen Aalst, Belgium) as described previously [30, 31]. The following subsets were identified using monoclonal antibodies: NK-cells (CD56-PE), B-lymphocytes (CD20-FITC), T-lymphocytes (CD3-PE), helper T cells (CD4-FITC), cytotoxic T cells (CD8-FITC), and among both T cells subsets naive T cells (CD45RA-PE) and memory T cells (CD45RO-PE). The antibod-

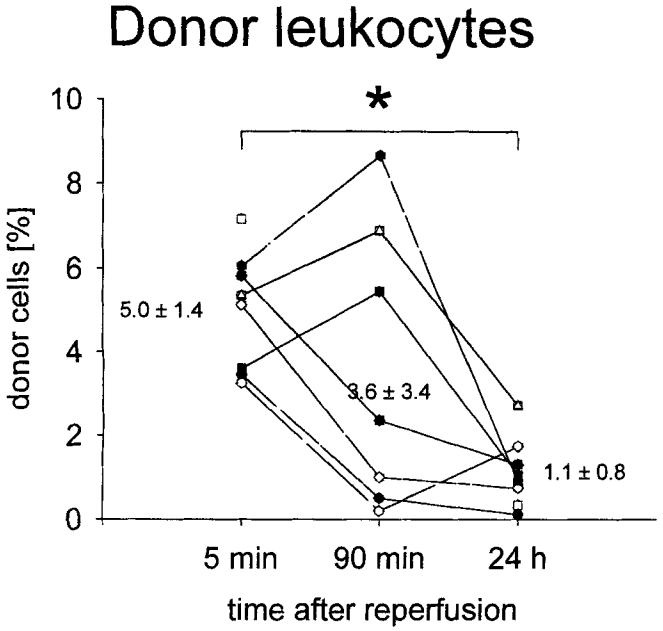

Fig. 2 Number of donor leukocytes as a percentage of all leukocytes in the host circulation at 5 min, 90 min and 24 h after graft reperfusion. The values for individual patients are connected. Indicated are means and standard deviations. The *horizontal bracket with asterisk indicates* a significant difference between the 5 min and 24 h value ($P < 0.05$, Wilcoxon-matched-pairs signed-rank test)

ies were purchased either from Becton Dickinson or Camon (Wiesbaden, Germany).

Data analysis

All data are reported as mean and standard deviation. Means of two time points were compared with the Wilcoxon-matched-pairs signed-rank test using SPSS for Windows 6.0.1. (SPSS Inc., Chicago, Il., USA). A P-value < 0.05 was considered to be significant.

Results

Large numbers of donor leukocytes enter the host circulation early after liver transplantation

With antibodies against HLA I antigens it was possible to distinguish between donor and host leukocytes (Figure 1 a), and to determine their phenotype (Figure 1 b). At 5 min after reperfusion, 5.0 % of all leukocytes found in the blood were donor derived (Figure 2). Given a total number of about 10×10^9 blood leukocytes, this means that within the first 5 min, 5×10^8 donor leukocytes were found in the circulation. In the course of 24 h this number decreased to about 1 % (Figure 2).

Donor granulocytes preferentially disappear from the host circulation

The subset composition of donor leukocytes in the liver reperfusion-blood prior to opening the hepatocaval anastomosis (i.e. the source of donor leukocytes) and in the peripheral blood of the host 5 min after opening the anastomosis was determined (Figure 3). Thereby it was possible to analyze whether donor leukocytes interact with host tissues. The leukocyte composition of the reperfusion blood consisted of granulocytes (20 %), NK-cells (40 %) and lymphocytes (45 %). Five minutes after release into the host blood, the donor leukocyte composition had changed. The number of granulocytes was significantly decreased, no change was observed for the number of NK-cells, and the number of lymphocytes increased (Figure 3). Further analyses of the lymphocyte subsets showed that 5 min after release into the host circulation the donor T/B-cell ratio had decreased significantly (Figure 3). In contrast, neither the donor $CD4^+/CD8^+$ T-cell ratio (Figure 3) nor the donor naive to memory ratio ($CD45RA^+/CD45RO^+$) among both $CD4^+$ and $CD8^+$ T-cells changed (data not shown).

Host granulocytes, but not lymphocytes are preferentially retained in the donor liver. The effect of the liver graft on the host leukocyte was analyzed by comparing the composition of host leukocytes in the blood with that of the reperfusion blood, i.e. after its passage through the donor liver (Figure 4). The percentage of host granulocytes significantly decreased, whereas that of host NK-cells and lymphocytes increased. This indicates that host granulocytes were preferentially retained in the graft. Further analyses of lymphocyte subpopulations revealed no changes with regard to both the T/B and CD4/CD8 ratios (Figure 4). In addition, no changes of the naive to memory ratio ($CD45RO^+/CD45RA^+$ ratio) was observed both among $CD4^+$ and $CD8^+$ T-cells (data not shown). Since the number of host lymphocyte subsets, and especially the $CD4^+/CD8^+$ T-cell ratio was not different when pre-transplantation, post-transplantation, and post-operation values were compared (data not shown); the drug regimen applied intra-operatively did not considerably influence the leukocyte numbers in the host circulation, as has been reported for other operative procedures [29].

Discussion

Using antibodies directed against HLA epitopes that differ between donor and host, it was possible to clearly identify donor cells in the host blood and host cells in the reperfusion blood, i.e. after passage through the transplanted liver.

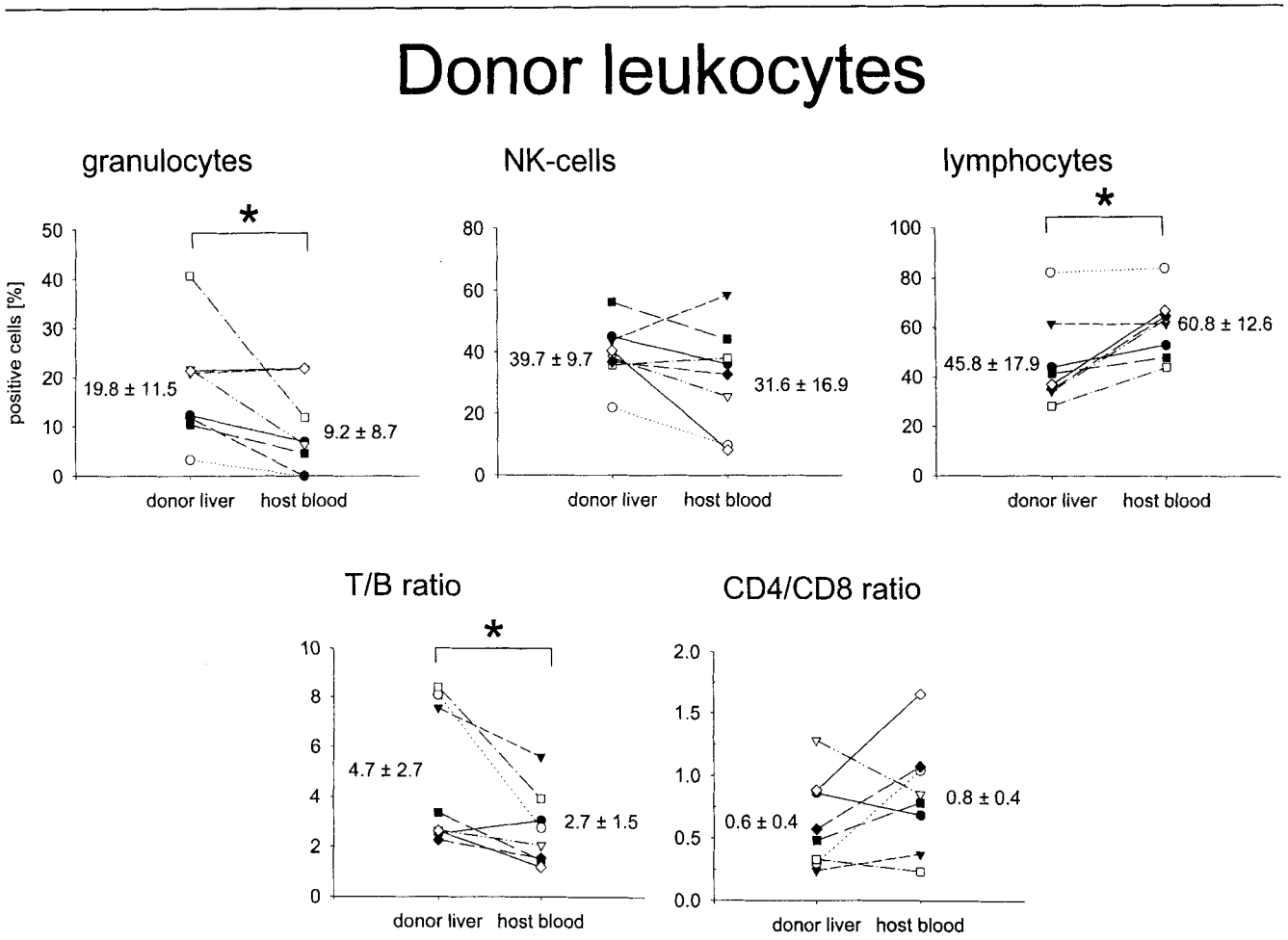

Fig. 3 Subset composition of donor leukocytes in reperfusion blood (donor liver) and host circulation at 5 min after reperfusion (host blood). For granulocytes, NK-cells, and lymphocytes, the percentage among all donor leukocytes is given. The lymphocytes are further characterized by the T/B-cell and the $CD4^+/CD8^+$ T cell ratio. Each point represents one patient. Indicated are means and standard deviations. *Horizontal brackets with asterisks* indicate significant differences between the two leukocyte sources ($P < 0.05$, Wilcoxon-matched-pairs signed-rank test)

Despite pre-operative perfusion of the liver, many donor leukocytes enter the host circulation

Although the graft had been flushed with up to 18 l of preservation solution during organ harvesting, donor leukocytes still comprise about 5% of all leukocytes found in the host circulation 5 min after liver transplantation. Given 5000 leukocytes per µl blood and a blood volume of about 5 l, this means that there are at least 1000 million donor leukocytes in the circulation. Since leukocyte-depleted RBC contain approximately 5–10 million leukocytes per unit blood [1], this shows that the transplanted liver releases about 100 times more donor leukocytes into the host than a single blood unit. This is still an under-representation of the leukocyte number actually released by the transplanted liver. For example, for lymphocytes it is known that 5 min after injection into animals, about 35% of the radioactively-labeled lymphocytes have already left the blood and are found in the lung (25%) and the spleen (10%; [18]). In addition, 90 min after transplantation, still about 5% of donor leukocytes are present in the host circulation, indicating continuos release of donor leukocytes from the transplanted liver into the host circulation. Thus, it is not surprising that it is very difficult to detect donor leukocytes after transfusion of about 10 units of leukocyte depleted RBC since the number of transferred cells is too low to be detected in the circulation [15].

Not only lymphocytes but also granulocytes and NK-cells are transferred by the liver graft

The main leukocyte population in the liver reperfusion blood are lymphocytes, which is in agreement with previously published data [8]. However, the present study shows that also NK-cells (40% of donor leukocytes) and

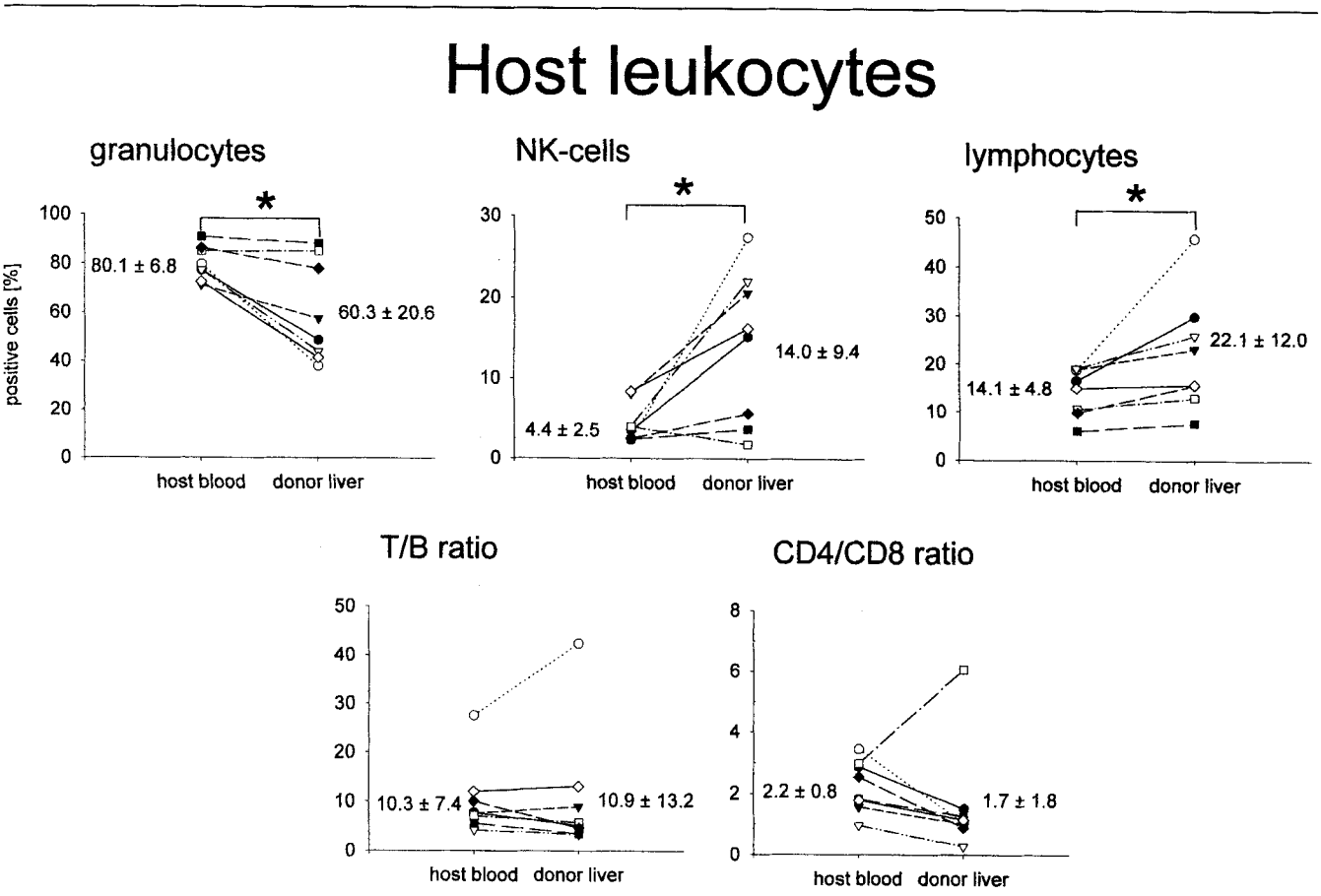

Fig. 4 Subset composition of host leukocytes in the host circulation before reperfusion (host blood) and in reperfusion blood (donor liver). For granulocytes, NK-cells, and lymphocytes the percentage among all host leukocytes is given. The lymphocytes are further characterized by the T/B-cell and the $CD4^+/CD8^+$ T-cell ratio. Each point represents one patient. Indicated are means and standard deviations. *Horizontal brackets with asterisks* indicate significant differences between the two leukocyte sources ($P < 0.05$, Wilcoxon-matched-pairs signed-rank test)

granulocytes (20% of donor leukocytes) leave the liver. This study demonstrates that the number of all three donor leukocyte subsets considerably declines in the host blood within the first 24 h after liver transplantation. This shows, for example, that the donor lymphocytes observed in the host circulation days or weeks after liver or small bowel transplantation [6, 15] are not representative of the actual number of lymphocytes initially transferred into the host circulation by the graft. The decrease of granulocytes from 20% among donor leukocytes in the reperfusion blood (i. e. when leaving the transplanted liver) to 10% of donor leukocytes 5 min after entering the host circulation clearly shows that donor granulocytes, in contrast to donor lymphocytes and donor NK- cells, preferentially disappear from the host blood. So far it is not known whether the donor granulocytes are rapidly destroyed in the host or if they quickly marginate to the wall of small venules [9] in order to enter non-lymphoid and lymphoid organs [22]. Independent of the cause, however, the present study shows that the donor leukocytes reveal a differential pattern of leaving the host circulation. The lymphocytes and NK-cells remain longer in the circulation than the granulocytes, and are therefore available for delivery to the tissues for longer periods. The differential presence of donor leukocytes in the host circulation might have clinical consequences. For example, the granulocytes released from the liver graft might influence the circulatory instability observed after liver transplantation which is associated with high levels of neutrophil and macrophage activation [27]. In addition, it is well known that injection of donor lymphocytes [17, 32] and the release of donor lymphocytes from the graft [10] can enhance graft survival in experimental animal models. Further studies should analyze the role of the longer persistence of donor B cells in the host blood compared to donor T cells, as indicated by the decline of the T/B-cell ratio. In addition, although the disappearance pattern from the host blood is comparable for donor CD4- and CD8-T cells and the naive- and memory population among them, further analysis of, for example, activation markers on these populations might reveal important differences.

Host granulocytes are retained in the donor liver

When leukocyte subset composition prior to liver reperfusion is compared with the host leukocyte subset composition in the reperfusion blood (after donor liver passage), a significant decrease of host granulocytes is observed, showing that host granulocytes are retained in the donor liver. This is in accordance with a recent study reporting sequestration of granulocytes and an increase in hydrogen peroxide production after liver transplantation [13]. It is well known that granulocytes play an important role in reperfusion damage after liver transplantation [4]. Granulocytes marginate to the endothelium of the liver sinuses [25] and produce, for example, oxygen-free radicals. Treatment regimens reducing the adherence of granulocytes and the production of toxic substances are able to reduce the reperfusion damage [7, 12]. However, when radiolabeled granulocytes were injected into patients after liver transplantation, no correlation was found between the presence of granulocytes in the liver and the clinical and biochemical evidence of tissue injury [33]. In this study, the number of radiolabeled granulocytes was determined only 12–24 h after transplantation. Therefore, analyzing the early retention of host granulocytes in the donor liver as performed in the present study but comprising more patients, may still be a promising approach. It is technically easy to perform and might be of value in the early prediction of reperfusion damage after liver transplantation.

Conclusion

The present study investigated donor- and host leukocyte subsets in the early phase after liver transplantation and provided data on early cellular interactions of donor cells with the host tissues, and host cells with the liver graft. This seems to be a promising approach, since a recent study showed that after liver transplantation the expression of adhesion molecules on circulating leukocytes correlates with the reperfusion damage [26]. However, it remained unclear whether the activated leukocytes were of donor or host origin. In our study we found that large numbers of donor leukocytes are released from the liver graft very early after transplantation, and that mainly host granulocytes are retained in the liver graft. Future research should investigate how this cell trafficking can be modified to therapeutically influence reperfusion damage and possibly graft survival.

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