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Wheeler, Kristina
Campo-Fernandez, Beatriz
Davila, Alejandra
et al.

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Apheresis of Deceased Donors as a New Source of Mobilized Peripheral Blood Hematopoietic Stem Cells for Transplant Tolerance

Rebecca A. Sosa, PhD[1,2], **Thomas Mone, MS**[3], **Bita V. Naini, MD**[1], **Donald B. Kohn, MD**[4,5,6], **Elaine F. Reed, PhD**[1,2], **Kristina Wheeler, BSN**[3], **Beatriz Campo-Fernandez, PhD**[4], **Alejandra Davila, BS**[4], **Donald J. Chaffin, MD**[7], **Joseph DiNorcia, MD**[8], **Fady M. Kaldas, MD**[8], **Aaron Cohen, MS**[3], **Erik L. Lum, MD**[9], **Jeffrey L. Veale, MD**[10], **Neil M. Kogut, MD**[10]

[1]Dept of Pathology and Lab Medicine, UCLA, Los Angeles, CA

[2]UCLA Immunogenetics Center, Los Angeles, CA

[3]OneLegacy, Los Angeles, CA

[4]Department of Microbiology, Immunology & Molecular Genetics, UCLA, Los Angeles, CA

[5]Dept of Pediatrics, Los Angeles, CA

[6]Dept of Molecular & Medical Pharmacology, Los Angeles, CA

[7]LifeStream Blood Bank, San Bernardino, CA

[8]Dept of Surgery, Los Angeles, CA

[9]Dept of Medicine, UCLA, Los Angeles, CA

[10]Dept of Urology, UCLA, Los Angeles, CA

Abstract

Background: Solid organ transplantation is the therapy of choice for many patients with end-stage organ failure; however, recipients must remain on lifelong immunosuppression, leaving them susceptible to infections and cancer. The study of transplant tolerance to prolong graft survival in the absence of immunosuppression has been restricted to recipients of living donor allografts; however, deceased donors significantly outnumber living donors. Mobilization of hematopoietic stem cells (HSCs) from the bone marrow to peripheral blood could allow PB-HSCs to be used to

Correspondence: Rebecca A. Sosa, Department of Pathology and Laboratory Medicine, 1000 Veteran Ave. Los Angeles, CA 90095 United States, Tel: 310-206-6204, Fax: 310-206-3216, rsosa@mednet.ucla.edu, Twitter: @cuzimbec.

Authorship:

RAS, TM, DBK, EFR, AC, ELL, JLV, and NMK designed research study. RAS, BVN, BCF, AD, DJC, JD, and FMK conducted the experiments and/or acquired the data. RAS, BVN, BCF, AD, DJC, AC, ELL, JLV, and NMK analyzed the data. RAS and NMK wrote the manuscript, and TM, BVN, DBK, EFR, KW, BCF, AD, DJC, JD, FMK, AC, ELL, and JLV critically reviewed the manuscript.

Disclosure:

Thomas Mone, Jeffrey L. Veale, and Erik L. Lum have an ownership stake in 34Plus. Neil M. Kogut is on the Board of Directors of 34Plus. Thomas Mone, Jeffrey L. Veale, and Neil M. Kogut have applied for a patent based on technology described in this manuscript. Donald B. Kohn is a member of the Scientific Advisory Boards of Orchard Therapeutics and Allogene Therapeutics. The Regents of the University of California have licensed intellectual property to Orchard Therapeutics based on technology which Dr. Kohn invented. Rebecca A. Sosa, Bita V. Naini, Alejandra Davila, Bea Campo-Fernandez, Joseph DiNorcia, Fady M. Kaldas, Donald J. Chaffin, Elaine F. Reed, Kristina Wheeler, Aaron Cohen report no conflict of interest.

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induce tolerance in deceased donor kidney recipients; however, a major concern is the well-known concomitant mobilization of immune cells into the liver.

Methods: We mobilized HSCs to the peripheral blood using a protocol of 2 doses of GCSF and 1 dose of plerixafor, followed by the collection of mobilized cells via apheresis in 3 deceased donors. The physiological, laboratory, and radiographic parameters were monitored throughout the procedure. Longitudinal biopsies were performed to assess the potential for ectopic liver mobilization.

Results: The use of both agents led to successful mobilization of peripheral blood CD34+ cells, demonstrating the potential for use in transplant tolerance protocols. Increased immune cell trafficking into the liver was not observed, and apheresis of mobilized cells resulted in a uniform decrease in all liver leukocyte subsets.

Conclusions: HSCs can be mobilized and collected from the peripheral blood of brain-dead donors. This new approach may facilitate the dissemination of immune tolerance trials beyond living donor kidney transplantation to deceased donor transplantation, without sacrificing the transplantability of the liver.

INTRODUCTION

For the past 75 years, researchers in the field of transplantation have yearned for immune tolerance to become the norm rather than the exception.[1,2] The twin goals of prolonging graft survival and freedom from immunosuppression are especially important in view of the emerging Covid-19 pandemic.[3-6] The best-studied approach to induce immune tolerance is the combination of kidney and hematopoietic stem cell transplantation from the same living donor. There have been multiple reports of prolonged graft survival following the withdrawal of immunosuppression using this technique.[7-10] However, immunosuppression-free tolerance protocols have been restricted to living donors, whereas the majority of solid organ transplants rely on deceased donors.

Immune tolerance research requires a reliable source of hematopoietic stem cells (HSCs). Investigators at several academic centers have developed the ability to collect large numbers of HSCs from the bone marrow of deceased donors. This approach involves resection of a large portion of the donor spine, followed by a complex process to isolate stem cells from the crushed vertebrae[11,12] There are significant drawbacks to this technique, in that it is invasive and disfiguring, requires reconstruction of the spine for viewing the donor at a funeral home, necessitates additional surgical time in the operating room, there is a risk of contamination, and the process for isolating the HSCs is restricted to only a few highly specialized laboratories.

Mobilization of HSCs from the bone marrow to the peripheral blood could allow PB-HSCs to be obtained from deceased donors[12] and be used to induce tolerance in kidney recipients; however, a major concern is the well-known concomitant mobilization of immune cells into other organs, such as the liver, lungs, heart and kidney.[13,14] In addition to their capacity for rapid maturation into functional inflammatory myeloid or lymphoid mediators, such as neutrophils, macrophages, NK cells, or lymphocytes, mobilized CD34+ cells are also capable of differentiating into vascular cells.[15] Here, we focused on the liver, based on our

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intent to develop this procedure in donors of abdominal organs only as a first step. As 95% of transplanted livers are obtained from a limited pool of deceased donors, the field cannot afford any further reduction in available organs.

We show that mobilization with 2 doses of GCSF and 1 dose of plerixafor, followed by immediate collection of mobilized cells via apheresis, does not result in the infiltration of immune cells into the liver, allowing the donor liver to remain transplantable. This strategy has the potential to extend the utility of tolerance induction to include deceased donors, which is a major advancement in the field of transplantation.

MATERIALS AND METHODS

Study Design, Cohort Characteristics and Sample Collection

Deceased donors with stable organ function and adequate blood counts with 1 or more contraindications to organ donation were considered as candidates for this study. Three donors were identified between November 2019 and February 2021. The 3 donors registered their donation decisions through the California Department of Motor Vehicles and Donate Life, California, including authorization for research. Since the research took place in the United States and involved deceased donors, and no cells/tissues/organs were given to living subjects, a waiver for IRB approval was obtained.[16,17] Brain death was declared when the donors were inpatients at local hospitals according to California State requirements, which included examinations and declarations by 2 separate physicians. Subsequently, the donors were transferred to the transplant recovery center of OneLegacy, an organ procurement organization (OPO) in the greater Los Angeles area. All deceased donors at this OPO receive some type of hemodynamic support due to brain death, including central venous catheters (CVCs) for the administration of medications and evaluating pressure to maintain hemodynamic stability and the 3 donors in this series underwent these interventions. No additional hemodynamic support was required.

Table 1 describes the 3 deceased donor cases with respect to demographics, cause of death, contraindication to organ donation, and the interval between the declaration of brain death and the start of apheresis. Physiological, laboratory, and radiographic parameters were closely monitored and remained stable in all 3 cases. The representative values are listed in Table 2.

The standardized mobilization regimen is shown in Figure 1. Briefly, plerixafor 0.24 mg/kg subcutaneous was administered 8-10 hours before apheresis. The first dose of G-CSF (10 μg/kg) was administered at 38 and 39 h, and the second dose 13.5 and 15 h before apheresis. CD34 cell enumeration from total nucleated cells (TNCs) in peripheral blood was performed using the UCLA Immune Assessment Core. Peripheral blood HSCs (PB-HSCs) were collected using a continuous mononuclear cell procedure on a Spectra Optia Apheresis System (Terumo BCT, Inc. Lakewood, Colorado). The total blood volume (24 L) was processed within an 8 h maximum collection time. CD34 cell counting of apheresis products was performed at the University of California Los Angeles Human Gene and Cell Therapy Facility. Automated cell processing was performed using the Miltenyi CliniMACS Prodigy

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device (Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany). The cells from Case 3 were not processed due to technical problems.

Two liver biopsies were obtained from each of the 3 donors, with the first prior to administration of G-CSF and plerixafor and the second after administration of these agents and completion of apheresis (Figure 1). A second biopsy was performed approximately 48 h after the first dose of G-CSF, 24 h after the second dose of G-CSF, and 19 h after a single dose of plerixafor. The specimens were analyzed and graded for inflammation, necrosis, sinusoidal congestion, hepatocellular ballooning, steatosis, and cholestasis, according to a histopathological grading system developed for the assessment of ischemia-reperfusion injury after liver transplantation.[18]

Immunohistochemical Staining of Liver Biopsies

Immunohistochemistry (IHC) staining of leukocyte subsets was performed as previously described.[19] Briefly, Formalin-fixed, paraffin-embedded biopsy sections were stained with primary antibodies against CD45, CD31, CD68, MPO, LYSO, CD56, CD3, CD4, or CD8, detected using horseradish peroxidase (Agilent, Santa Clara, CA, USA), visualized with diaminobenzidine, and counterstained with hematoxylin. Whole-stained slides were converted to high-resolution digital bright-field images using an Aperio ScanScope AT high-throughput scanning system (Leica Biosystems, Buffalo Grove, IL). Images were then acquired using Aperio ImageScope software (Leica Biosystems), and analysis was performed using Tissue Studio software (Definiens).

Statistical Analysis

Two-way analysis of variance (ANOVA) with Sidak's multiple comparisons test was used to determine differences between time and other parameters. Statistical significance was set at $P<0.05$.

RESULTS

Peripheral blood samples, apheresis samples, and liver biopsies were obtained before and after administration of mobilization agents and apheresis (Figure 1). Table 1 describes the 3 deceased donor cases with respect to demographics, cause of death, contraindication to organ donation, and the interval between the declaration of brain death and the start of apheresis. Physiological, laboratory, and radiographic parameters were closely monitored in all 3 cases. They remained stable, except for acute kidney injury in case 2, which preceded the administration of mobilizing agents (data not shown). The vital signs, arterial blood gas results, blood counts and chemistries, and whole-body CT scans for Case 1 are shown in Table 2.

First, we confirmed sufficient mobilization and collection of PB-HSCs (Figure 2). The cell collection and processing results are presented in Table 3. The resulting CD34-enriched cells in all cases except the third had high purity and excellent viability. CD34+ cells from the first 2 runs grew distinct hematopoietic progenitor colonies, confirming that the recovered cells retained their clonogenic potential and were capable of expansion and differentiation. The yield in case 2, mobilized with G-CSF and plerixafor, was higher than anticipated,

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exceeding the capacity of the Miltenyi device and resulting in significant cell loss. In case 2, 110 ml of bone marrow was obtained from the ilia following mobilization and apheresis. CD34 cells comprised 0.29% of the total nucleated cells in the bone marrow, suggesting that the majority of the HSCs were successfully mobilized by the regimen used.

We then investigated liver biopsies for severity of inflammation and necrosis, the 2 principal histopathological scoring parameters for the evaluation of ischemia-reperfusion injury (IRI) in liver transplant recipients,[18] to determine the impact of the mobilization procedure and subsequent apheresis on the inflammatory status of the liver, which could affect its transplantable immune status (Figure 3). As expected for a donor organ that did not undergo the normal process of procurement and transport that a liver being prepared for transplant would, we saw no evidence of hepatocellular necrosis on biopsies from the premobilization (PRE) or postapheresis (POST) time points (Figure 3A, 3B). The second component of the IRI score is the severity of the inflammatory infiltrates. A slight increase in neutrophilic inflammation was noted in the postapheresis biopsies, which was not significant and did not reach pathological severity (Figure 3B, dashed line). Similarly, other histopathological features commonly observed in liver transplant recipients with IRI complications, including hepatocellular ballooning, biliary cholestasis, sinusoidal congestion, and steatosis, were also unaffected by these procedures. Moderate large-droplet macrovesicular steatosis of similar severity (~25% of the parenchyma) was present in both PRE and POST biopsies (Figure 3B).

To further investigate the effect of PB-HSC mobilization on the inflammatory milieu found in the liver, immunohistochemical (IHC) staining for leukocyte subsets was performed on the liver biopsy specimens (Figure 3C). IHC showed a nearly uniform average decrease in all positively stained cell types; however, this change was not significant between PRE and POST biopsies when quantified (Figure 3C). We observed a slight increase in CD56+ NK cells and a slight decrease in CD4+ T cells in the POST biopsies when compared to the PRE time point; however, it should be noted that the number of these cell types is already very low compared to other immune cell subsets in the liver, regardless of the timing of biopsy tissue collection.

DISCUSSION

Transplantation researchers, clinicians, and patients have long desired immune tolerance to become the norm rather than exception.[1,2] The Covid-19 pandemic has dramatically illustrated the risk of fatal infections in transplant recipients with standard immunosuppression, creating heightened awareness of the need to develop and expand immunosuppression-free approaches.[3,4,6] Here, we show that following a protocol of 2 doses of G-CSF[20-22] followed by a single dose of plerixafor[23-25] in deceased donors is effective for mobilizing sufficient numbers of CD34+ HSCs known to induce tolerance in kidney recipients[26,27] and should not affect the transplantability of the deceased donor liver. Our results demonstrate the potential for this process to be of use in transplant tolerance protocols.

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This series represents the first reported experience of HSC mobilization and apheresis in the peripheral blood of deceased donors. The mobilizing agents plerixafor and G-CSF have long been used in healthy allogeneic HSC donors and have a proven safety and efficacy track record.[20,21,23,24,28] Therefore, it is not surprising that the physiological, laboratory, and radiographic parameters in these 3 cases remained stable throughout this process, suggesting that it could be performed safely in deceased donors without jeopardizing organ recovery. In addition, large-volume apheresis is easily accomplished because the duration of the procedure is not limited by donor discomfort or inconvenience.

To be acceptable to donor families, it is critical that mobilization and apheresis processes do not impede organ donation. The standard mobilization regimen utilizing G-CSF daily for 5 days would delay the process of organ recovery, since the average interval from the declaration of brain death to organ donation is only 67 hours. For this reason, plerixafor, with a short onset of action of 4-10 hours[22] was used as the primary mobilizing agent. There is some experience in using plerixafor as a single agent for mobilization of healthy allogeneic donors.[23,25] However, a single dose of plerixafor is unlikely to mobilize a sufficient quantity of CD34+ cells to meet the higher requirements of some immune-tolerance protocols.[10] This caveat is borne out by the results of the present study. The total initial CD34+ cell count in case 2 mobilized with 2 daily doses of G-CSF and a single dose of plerixafor was approximately 15 times greater than that in case 1 using single-agent plerixafor alone. The cell yield exceeded the capacity of the Miltenyi CliniMACS Prodigy device, resulting in a significant cell loss. The number of HSCs collected in Case 2 likely would have been sufficient for most living-donor immune tolerance protocols if technical problems not occurred in the processing of the cells.[8,10] It is possible that a second dose of Plerixafor and the selection of deceased donors who do not have an excluding co-morbidity for organ donation may result in higher yield of CD34 cells.

The recovered PB-HSCs demonstrated excellent viability and sufficient clonogenicity, indicating their potential to expand and differentiate. However, it is recognized that plerixafor and G-CSF may mobilize a variety of immune cells in addition to HSCs,[29,30] and even the intended CD34+ progenitors have an undesirable capacity to mature ectopically. Because immune cells traffic to the liver,[31] and our initial intent is to develop this procedure in donors of abdominal organs only as a first step, we obtained liver biopsy specimens before and after mobilization and apheresis to investigate the potential for PB-HSCs to become activated and extravasate into the liver parenchyma by signals received while being mobilized from the bone marrow. We utilized our clinical scoring system for liver IRI[18] to investigate potential changes in the livers of mobilized deceased donors, as these are the same histopathological parameters that we evaluate for deceased donor livers destined for transplantation. In future studies, it will be critical to compare these parameters when evaluating livers from mobilized donors for placement into a recipient. We found no additional signs of injury or inflammation in the livers of deceased donors, suggesting that the mobilization of CD34+ cells from the bone marrow into the peripheral blood was not overtly damaging to the liver.

Although the liver is a tolerant organ, increased immune infiltration of donor cells into the liver prior to transplantation can cause insurmountable problems for the recipient,

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particularly when combined with injury due to procurement and transport. Mobilized CD34+ cells possess the capacity for rapid maturation into functional inflammatory myeloid or lymphoid mediators, such as neutrophils, macrophages, NK cells, or lymphocytes, as well as differentiation into vascular cells, either of which could promote allograft injury or rejection.[15] In the liver, additional risk lies in mobilizing cells that could potentiate graft vs. host disease (GVHD). Importantly, a nearly uniform average decrease was observed in most leukocyte subsets, as evaluated by IHC. It is reasonable to speculate that this decrease was mediated by the apheresis procedure, particularly when considering the reduced immune presence in the sinusoids, raising the possibility that this may be an effective way to avoid liver infiltration by mobilized or other immune cell subsets. It is also conceivable that this approach might ameliorate the risks of graft rejection and/or GVHD; however, these effects require further study in the transplant setting.

This approach may ultimately facilitate the dissemination of immune tolerance trials beyond living donor kidney transplantation to deceased donor transplantation. As most transplant procedures are from deceased donors, recipients of other solid organs as well as vascularized composite allografts would therefore become candidates for immune tolerance trials. Here, we have focused on the liver with the intent to develop this procedure in recipients of abdominal organs as a first step towards this important goal. Additional studies including bronchoalveolar lavage, lung and heart biopsies will need to be done on research-only deceased donors before considering use of thoracic organs from donors who have undergone mobilization and apheresis using the process described in this report. Before this research advances into transplantation and infusion into living human transplant candidates, the need for IRB approval and appropriate consent will need to be considered with the relevant stakeholders – transplant physicians/surgeons, the OPO community, and patient advocacy groups. Further studies are needed to confirm that immune activation (via transplant-specific processes, such as IRI) does not increase in liver and other allografts obtained from diseased donors following mobilization and apheresis.

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Abbreviations:

G-CSF	granulocyte colony stimulating factor
HSCs	hematopoietic stem cells
IHC	immunohistochemistry
IRI	ischemia-reperfusion injury

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PB-HSCs	peripheral blood hematopoietic stem cells

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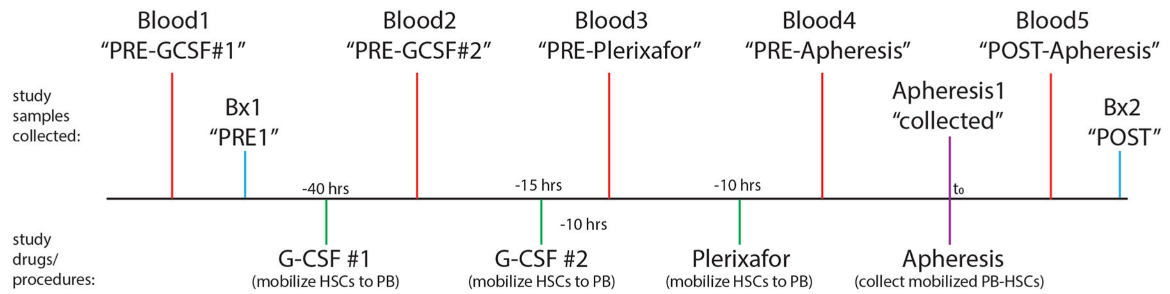

Figure 1. Experimental Design.

Donor peripheral blood, apheresis product, and liver biopsies were obtained according to a standarized protocol at key timepoints before and after mobilization of peripheral blood HSCs (PB-HSCs) using 2 doses of G-CSF followed by one dose of Plerixafor, as well as before and after apheresis procedure to collect mobilized PB-HSCs.

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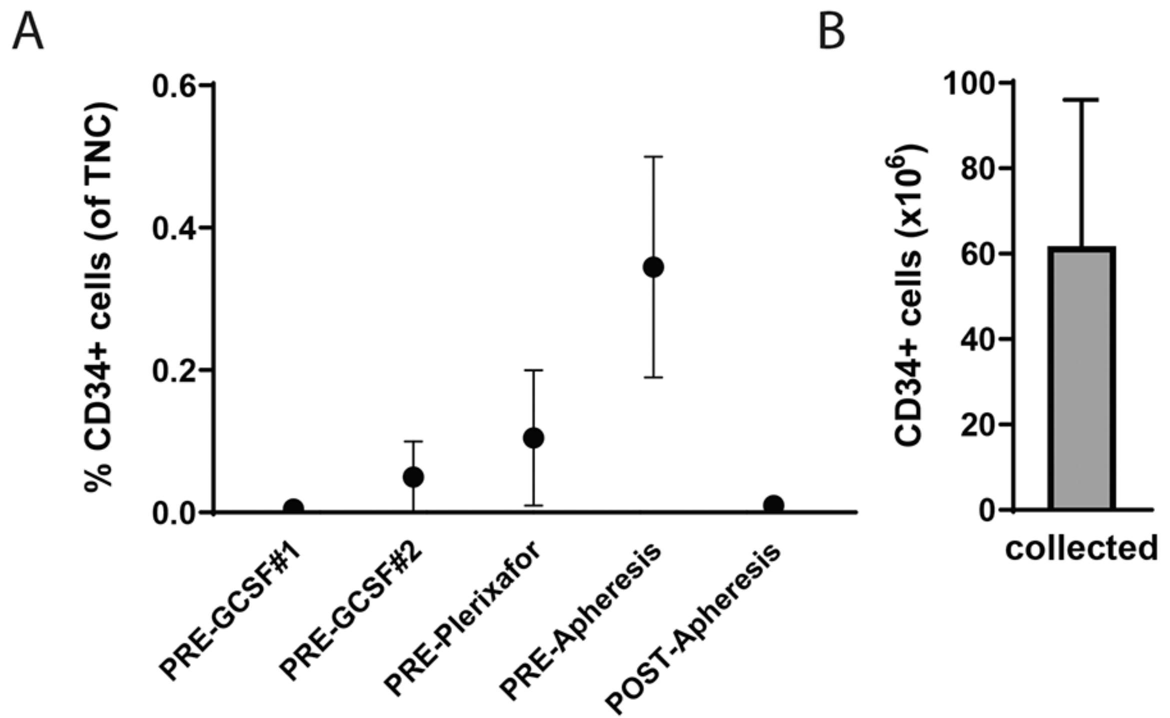

Figure 2. Mobilized CD34+ cells can be collected in suitable numbers by apheresis.

(**A**) Percent of CD34+ cells detected by flow cytometry of peripheral blood products (left panel), or (**B**) absolute number of CD34+ cells in apheresis product (x10^6). $n = 2$

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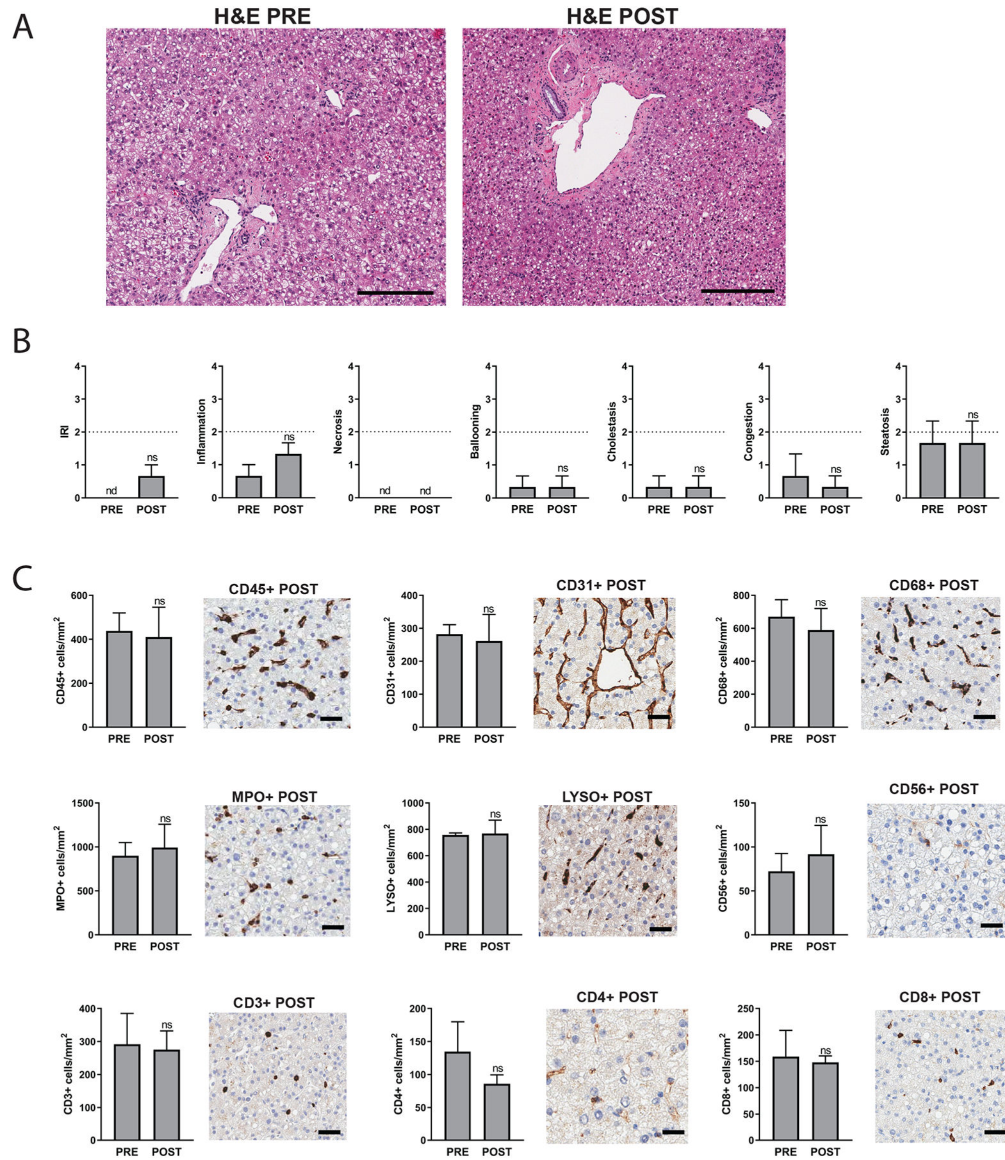

Figure 3. Histopathological features and cellular milieu are similar before mobilization of PB-HSCs and after collection by apheresis.

(**A**) Representative images from H&E staining of liver biopsies ($n = 3$). scale bars = 200 um (**B**) Scores for ischemia-reperfusion injury (IRI) include evaluation of inflammation and necrosis, as well as 4 additional histopathological features of cholestasis, steatosis, congestion, and/or ballooning, which did not reach a severity known to correlate to IRI in transplanted organs (dotted lines). $n = 3$ (**C**) Numbers and representative images (scale bars = 40 um) of immune cells obtained premobilization (PRE) and postapheresis (POST) are shown for CD45+, CD31+, CD68+, MPO+, LYSO+, CD56+, CD3+, CD4+, and CD8+ cells. Data are presented as bar graphs representing mean cells per mm^2 for n=3 deceased donors with error bars representing standard error of the mean (SEM). nd = not detected; ns = not significant; $*P < 0.05$.

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Table 1.

Deceased Donor Clinical Demographics

Case	Age	Gender	Cause of death	Contraindication to organ donation	Interval between declaration of brain death and apheresis start
1	68	Male	CVA w/ IC hemorrhage	Malignancy	58 hours
2	61	female	Respiratory failure/drug abuse	Abdominal mass suspicious for cancer	52 hours
3	71	female	Intracranial hemorrhage	Metastatic cancer	52 hours

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Table 2.

Deceased Donor Physiologic, Laboratory and Radiographic parameters

Data Point	Before Plerixafor 8/6 12:08	After Plerixafor 8/7 01:04	Before Apheresis 8/7 06:00	After Apheresis 8/7 16:15
Vital Signs				
Heart Rate	97	79	63	70
Temperature	36.2	36.3	36.2	36
BP (MAP)	117/88 (101)	118/83 (92)	120/88 (99)	124/80 (95)
Urine output (mL/hr)	48	250	450	80 ml/hr
Arterial Blood Gas				
pH	7.36	7.55	7.53	7.35
PaCO2 (mmHg)	45.3	26.7	27.2	63.3
PaO2 (mmHg)	111	201	215	557
HCO3 (mEq/L)	25.9	25.6	25.3	31.5
BE	0.1	1.3	0.9	7.6
SaO2 (%)	98.20%	99.90%	99.60%	99.80%
FiO2 (%)	30%	47%	47%	47%
Complete Blood Count				
WBC (K/mcL)	8.9	11.5	15.5	13.3
RBC (M/mcL)	2.42	2.53	2.73	2.25
Hgb (g/dL)	7.4	7.8	8.4	6.9
Hct (%)	22.6	24	26	22
Platelets (K/mcL)	187	198	191	77
Chemistry Panel				
BUN (mg/dL)	9	13	12	10
Creatinine (mg/dL)	0.83	0.9	0.8	0.7
T. Bili (mg/dL)	0.4	0.8	0.5	0.4
AST (U/L)	31	38	47	37
ALT (U/L)	20	22	23	19
Alk Phos (U/L)	61	64	69	54
T. Protein (g/dL)	4.9	5.3	5.6	4.9
Albumin (g/dL)	2.4	3.1	3	2.9
PT	20.2	17.1	17.3	19.3
PTT	41.1	40	41	39
INR	1.8	1.5	1.5	1.7
CPK	528	not done	not done	958
CK-MB (ng/dL)	3.5	not done	not done	13.9
Troponin I (ng/mL)	0.01	not done	not done	0.02
Amylase (U/L)	221	not done	228	228
Lipase (U/L)	27	not done	15	15

Data Point	Before Plerixafor 8/6 12:08	After Plerixafor 8/7 01:04	Before Apheresis 8/7 06:00	After Apheresis 8/7 16:15
Radiologic Studies	**Before Plerixafor 8/6 23:30**		**After Apheresis 8/7 19:20**	
CT CAP (chest, abd, pelvis)	**Lungs:** No pneumo, patchy right basilar infiltrate, likely atelectasis **Heart:** Normal size, no effusion **Pancreas:** Normal **Spleen:** Normal **Liver:** No evidence of mass **Kidneys:** no hydronephrosis, stones, kidneys symmetric & smooth		**Lungs:** No pneumo, patchy right basilar infiltrate, likely atelectasis, minimal atelectasis in left base **Heart:** Normal size, no effusion **Pancreas:** Normal **Spleen:** Normal **Liver:** No evidence of mass **Kidneys:** no hydronephrosis, stones, kidneys symmetric & smooth	

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Table 3.

CD34+ cell characterization

	Case 1	Case 2
Initial TNC	1.49×10^{10}	1.55×10^{11}
Initial CD34%	0.87%	1.3%
Total initial CD34 cells	1.29×10^{8}	*2.01×10^{9}
Total CD34s recovered	1.16×10^{8}	2.52×10^{8}
Purity (CD34%)	95%	89%
Viability	95%	97%
Yield	90%	*13%
Clonogenicity	43.5%	58%

*The cell capacity limit of 6×10^{8} total CD34s was exceeded for the Miltenyi CliniMACS Prodigy equipment in Case 2, as the yield with combined G-CSF and plerixafor was much greater than expected. The cells from Case 3 were not processed owing to technical problems.