Received: 28 March 2024 | Accepted: 6 August 2024

DOI: 10.1097/LVT.0000000000000461

ORIGINAL ARTICLE

Hypothermic oxygenated machine perfusion influences the immunogenicity of donor livers in humans

Marwa Elgosbi[1] | **Ada Sera Kurt**[1] | **Maria-Carlota Londoño**[1,2] | **Aranzazu Caballero-Marcos**[1] | **Tiong Yeng Lim**[1] | **Juan J. Lozano**[3] | **Mona Dave**[4] | **Nigel Heaton**[1,5] | **Alberto Sánchez-Fueyo**[1] | **Miriam Cortes-Cerisuelo**[1,5]

[1]Department of Inflammation Biology, Institute of Liver Studies, School of Immunology and Microbial Sciences at King's College London University and Hospital, UK

[2]Liver Unit, Hospital Clínic Barcelona, Institut de Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelonna, Centro de Investigación Biomédica en Red Enfermedades Hepáticas y Digestivas (CIBEREHD), European Reference Network on Rare Liver Disorders (ERN-Liver), Barcelona, Spain

[3]Bioinformatic Platform, Biomedical Research Center in Hepatic and Digestive Diseases (CIBEREHD), Carlos III Health Institute, Barcelona, Spain

[4]Clinical Perfusion Service, Institute of Liver Studies, King's College Hospital, London, UK

[5]Department of Liver Transplant Surgery, Institute of Liver Studies, King's College Hospital, London, UK

Correspondence
Miriam Cortes-Cerisuelo, Department of Inflammation Biology, Institute of Liver Studies, School of Immunology and Microbial Sciences, King's College London University and King's College Hospital, Denmark Hill, London, SE5 9RS, UK.
Email: m.cortescerisuelo@nhs.net

Abstract

Hypothermic oxygenated machine perfusion (HOPE) is an organ preservation strategy shown to reduce ischemia-reperfusion injury (IRI)-related complications following liver transplantation. In animal models, HOPE can also decrease alloimmune responses after transplantation, but this remains to be evaluated in humans. Our study, involving 27 patients undergoing liver transplantation enrolled in 2 randomized controlled trials comparing static cold storage with HOPE (14 HOPE-treated and 13 static cold storage–treated), delves into the impact of HOPE on the molecular profile of liver allografts and on the immune responses elicited after transplantation. Following HOPE treatment, fewer intrahepatic immune cells were observed in liver perfusates compared to static cold storage. Analysis of liver tissue transcriptome at reperfusion revealed an effect of HOPE on the reactive oxygen species pathway. Two weeks after transplantation, HOPE recipients exhibited increased circulating CD4+FOXP3+CD127lo regulatory T cells ($p < 0.01$), which corresponded to a higher frequency of donor-specific regulatory T cells ($p < 0.01$) and was followed by reduced alloreactivity index of CD8+ T cells 3 months after transplant. Our study provides novel mechanistic insight into the capacity of HOPE to influence liver ischemia-reperfusion injury and to modulate effector and regulatory donor-specific T-cell responses after transplantation. These findings, which confirm observations made in animal models, help explain the decreased rejection rates reported in patients receiving HOPE-treated allografts.

Abbreviations: ACR, acute cellular rejection; APC, antigen-presenting cells; DBD, donors after brain death; DCD, donors after circulatory death; D-HOPE, dual HOPE; FoxP3, forkhead box P3; HOPE, hypothermic oxygenated machine perfusion; IRI, ischemia-reperfusion injury; LMC, liver mononuclear cells; LT, liver transplantation; SCS, static cold storage; TLR, toll-like receptor; Tregs, regulatory T cells.

Marwa Elgosbi and Ada Sera Kurt shared the first authorship.

Alberto Sánchez-Fueyo and Miriam Cortes-Cerisuelo shared senior authorship

Supplemental Digital Content is available for this article. Direct URL citations are provided in the HTML and PDF versions of this article on the journal's website, www.ltxjournal.com.

SEE EDITORIAL ON PAGE 262

INTRODUCTION

The need to employ an ever-growing number of extended criteria donors to increase organ utilization rates and reduce waitlist mortality has resulted in a resurface of ex situ and in situ machine perfusion technologies to preserve donor livers before transplantation. Among the ex situ technologies, normothermic machine perfusion and hypothermic oxygenated machine perfusion (HOPE) have become the most widely used in the clinical setting. To date, 5 randomized controlled trials comparing the outcomes of end-ischemic HOPE with static cold storage (SCS) have been published.[1–5] Altogether, these trials have shown a significant benefit of HOPE in improving patient outcomes and decreasing the rate of complications after liver transplantation (LT) when using either donors after circulatory death (DCD) or donors after brain death (DBD). DCD livers that were perfused for 2 hours with dual HOPE (D-HOPE) had significantly less incidence of symptomatic nonanastomotic biliary strictures following transplantation.[1] In conventional DBD LT, HOPE was shown to reduce the risk of severe liver-related complications and graft failure.[2] Similarly, extended criteria donor DBD perfused with HOPE exhibited a lower peak of serum ALT following reperfusion, reduced incidence of severe post-LT complications, and shorter hospital stay.[3]

Mechanistically, the clinical benefits of HOPE in LT have been attributed to decreased mitochondrial injury at the time of reperfusion as a consequence of increased cellular energy storage and reduced accumulation of succinate. This resulted in reduced release of radical oxygen species, proinflammatory cytokines, and nuclear danger–associated molecular patterns, ultimately decreasing the magnitude of the ischemia-reperfusion injury (IRI) response.[6]

In experimental LT in rodents, it has also been reported that HOPE reduces the activation of the innate and adaptive immune system, as shown by lower expression of CD40 on KCs and of CD80 on liver endothelial cells, reduced frequencies of activated CD4+ T cells (CD154+ and CD28+ T cells), and lower levels of proinflammatory IL-2 and IL-17.[7] These immunological effects resulted in striking outcomes in an allogeneic rat LT model in which full-dose tacrolimus is needed to ensure long-term engraftment, while low-dose tacrolimus prevents early acute cellular rejection (ACR) but not the development of allograft fibrosis by week 4. In this model, treating the donor livers with HOPE for 1 hour before implantation eliminated the need to administer tacrolimus to prevent lethal ACR. In addition, the combination of HOPE-treated livers and low-dose tacrolimus administration resulted in long-term engraftment.[8]

Many of the clinical studies published in the last 5 years observed a trend toward a reduced incidence of ACR whenever HOPE is employed. This includes a lower incidence of ACR in both HOPE and D-HOPE–treated livers as compared to the SCS group.[3,9,10] However, little has been explored about the effect of HOPE on the immune system of the recipients undergoing LT.[11] Based on these observations, we hypothesized that treating human livers with HOPE before transplantation would reduce the magnitude of the alloimmune response in the recipient. In the current study, we investigated for the first time in humans the effects of HOPE on the molecular profile of the liver allografts and on the immune responses elicited in the recipient following transplantation.

METHODS

Enrollment of recipients of transplant and management of donor livers

Patients undergoing LT at King's College Hospital between November 2018 and July 2019, who had been enrolled in 2 randomized controlled trials comparing SCS with HOPE (*HOPE* and *D-HOPE* trials, employing either donor after brain death [DBD] or DCD, respectively), consented to participate in our observational study. As previously described,[1,2] patient randomization took place after the donor liver had been retrieved and accepted for transplantation. The donor livers that were randomized to the experimental group were perfused on arrival to the transplant center for the duration of a minimum of 1 hour before implantation as per the trial protocols.[1,2] Participation in the observational study involved sequential collection of biological specimens from donors and recipients, as described below. Acute cellular rejection was diagnosed on biopsy as per Banff criteria[12]; it was considered late ACR when occurring at least 6 months after LT.[13] The model for early allograft function score was used to define early allograft dysfunction.[14] Functional warm ischemia time for DCD was defined as the time from oxygen saturation $<70\%$ or systolic blood pressure less <50 mm Hg to aortic perfusion. The immunosuppression protocol was based on tacrolimus and steroids. Basiliximab was administered on days 1 and 5 after transplant in selected patients as part of the renal sparing protocol. Mycophenolate mofetil was added in those patients with persistent renal impairment 1 week after the surgery. Patients were followed up for 5 years. The study was approved by the Institutional Review Board of King's College Hospital (REC18/SC/0330) and adhered to the Declaration of Helsinki and the Declaration of Istanbul.

Sequential biological specimen collection

Isolation of liver mononuclear cells from liver perfusates

Baseline perfusates from the donor liver were obtained after perfusing the liver with 1 L of Belzer UW

(University of Wisconsin) solution for the livers in the control group and Belzer UW MPS for the livers in the study group during the bench work on arrival to the transplant center. Perfusates were also obtained before starting the implantation by rapidly flashing the livers with 1 L of cold HTK solution (Custodiol-HTK; Essential Pharmaceuticals). All perfusates were processed within 6 hours by centrifugation to reduce the volume down to 30 mL. Liver mononuclear cells (LMCs) were then isolated by density-gradient centrifugation (Lymphoprep; GE) and cryopreserved in liquid nitrogen.

Liver biopsies

Paired liver samples were collected using a Tru-cut needle at 2 different time points: (1) at the time of arrival to King's College Hospital and before initiating HOPE (pre-reperfusion); and (2) 90–120 minutes after graft implantation and reperfusion (post-reperfusion sample) (Figure 3A). Liver tissue samples were kept in RNAlater reagent for 24 hours and subsequently cryopreserved at −80°C.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells from the recipients of transplant were collected on the day of the transplant before the surgery (baseline), and on postoperative week 2 and month 3 after LT. Peripheral blood mononuclear cells were isolated employing a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech, Ltd.) and cryopreserved in liquid nitrogen.

Characterization of markers correlating with donor alloreactivity

We quantified donor-specific T-cell alloimmune responses by flow cytometry before transplantation (baseline) and on posttransplant week 2 and month 3, employing both intracellular cytokine staining and an activation-induced marker assay previously described by our group,[15] which allows simultaneous enumeration of antigen-specific effector and regulatory T cells (Tregs). The alloreactivity index was calculated as a ratio of donor-specific T-cell response to third-party T-cell response in both assays. Details of both assays are thoroughly described in Supplemental Data, http://links.lww.com/LVT/A640.

Flow cytometric enumeration of LMCs

Cryopreserved flushed-out effluent LMCs isolated from the donor liver perfusates were thawed rapidly in a 37°C water bath. Cells were then diluted in prewarmed complete RPMI media (RPMI [Thermo Fisher Scientific] with 10% FBS [Thermo Fisher Scientific] and 5% penicillin-streptomycin and L-Glutamine [Thermo Fisher Scientific]), washed and centrifuged, and the pellet was resuspended in a working solution of live/dead viability dye, prepared by diluting stock 1:1000 in PBS. The sample was mixed using a vortex (Vortex Genie) and incubated for 10 minutes on ice in the dark. At this stage, the cells were washed with similar centrifugation settings with the usage of staining buffer, which included PBS (Thermo Fisher Scientific) with 2.5% bovine serum albumin (Thermo Fisher Scientific). The pellet was resuspended in 100 µL of master mix, consisting of staining buffer with antibodies according to each panel described in Supplemental Tables S1 and S2, http://links.lww.com/LVT/A640. Samples were incubated for 30 minutes at 4°C in the dark. Following that, samples were washed as before, and the cell pellet was resuspended in 300 µL fixation buffer (BioLegend). Finally, samples were acquired using flow cytometry. The flow cytometry panels used for those assays are described in Supplemental Tables S3 and S4, http://links.lww.com/LVT/A640.

Liver tissue RNASeq gene expression experiments

Gene expression analyses were performed on sequential liver biopsies collected from 16 DBD donors (HOPE: n = 8; SCS: n = 8). For total RNA extraction, cryopreserved liver tissue samples were homogenized in TRIzol reagent (Invitrogen) using bead-beating technology (Tissuelyser, Qiagen) in 1.5 mL reaction tubes (Eppendorf). Total RNA was then extracted following the manufacturer's guidelines. The concentration, purity, and quality of the RNA were measured and assessed using Nanodrop (ThermoScientific). RNA libraries were prepared and amplified using TruSeq stranded mRNA sample preparation with the corresponding kit (Illumina Inc). High-quality total RNA was sequenced using Illumina NovaSeq 6000 as the sequencing platform with read length at PE-150 and 50 million read pair sample data output. STAR program[16] against Homo Sapiens genome hg38 was used for mapping the reads, followed by the quantification of genes and transcripts with the RSEM[17] program using GENCODE v26 reference annotation.[18] We used the quantile method and limma-voom transformation[19] to normalize the non-biological variability. Differential expression between different groups was assessed using moderated *t*-statistics.[20] In addition, we performed Gene Set Enrichment Analysis using the Gene Set Enrichment Analysis function in clusterProfiler package using previously computed *t*-statistic.[21] We employed the

pathway databases available from the Molecular Signatures Database: Hallmark, Gene Ontology and Canonical Pathways. The RNA-Seq data set included a total of 14,847 genes that were assessed with a cutoff for the significance of 1.5-fold change and of p value < 0.01.

Statistical analysis

Data were analyzed with IBM SPSS Statistics version 24 (IBM Corporation) and GraphPad Prism 9.0 for Mac (GraphPad). Continuous variables were expressed as median and interquartile range unless stated otherwise. Categorical variables were expressed as frequencies and proportions (%). Categorical variables were compared using the Fisher exact test and Pearson chi-square test and continuous variables with a Mann-Whitney U test. Statistical significance was assumed at p value < 0.05. For experiments where a nonparametric test was required to analyze repeated measures and considering the missing values, significance was determined by the Friedman test, and p values represent pair `wise comparisons of adjusted p values using the Wilcoxon test. Statistical significance was shown at p value < 0.05.

RESULTS

Clinical characteristics of enrolled patients

Twenty-seven patients were included in the study, 14 of whom received a liver treated with HOPE and the other 13 were preserved on SCS only (Table 1). No differences between the 2 groups of patients were identified in terms of age, MELD score, cold ischemia time, Model for Early Allograft Function score, median of tacrolimus during the first 2 weeks, induction with basiliximab, addition of mycophenolate mofetil before discharge, and graft weight. The functional WIT for DCD livers was < 30 minutes. Patients who received a liver preserved in SCS only showed a significantly higher incidence of early ACR (ie, within the first month after LT), as compared to those who received livers treated with HOPE (46% vs. 7%, $p = 0.03$). No episodes of primary non-function, ischemic cholangiopathy, or late ACR were diagnosed. The median follow-up was 4.6 years. In the SCS group, 1 patient died secondary to malignancy within 1 year. In the machine perfusion group, 2 patients died of cardiac arrest and malignancy 2 and 3 years after LT, respectively, and 2 additional patients underwent retransplantation within 3 years of the first transplant (for late HAT and de novo HCC, respectively).

Recipients transplanted with HOPE-treated livers exhibited increased frequencies of circulating Tregs with preferential expansion of donor-specific alloreactive clones

To explore if the use of HOPE influenced the immunogenicity of the donor livers and, as a result, the characteristics of the recipients' alloimmune responses, we enumerated the number of circulating T-ell subsets and evaluated their expression of markers correlating with alloantigen specificity before and after transplantation (gating strategy; Figure 1A). Total numbers of circulating CD4+ and CD8+ T cells remained stable throughout the study (the only significant change was a decrease in the proportion of CD4+ cells from baseline to month 3 in the SCS group; Figure 1B). In contrast, as compared to recipients transplanted with livers preserved in SCS, recipients grafted with HOPE-treated livers exhibited a significantly increased number of circulating CD4+Foxp3+CD127lo Tregs (Figure 1C) at week 2 after transplant. We cultured recipient peripheral blood mononuclear cell samples with either donor or third-party allogeneic cells and performed staining for intracellular cytokines and surface membrane activation markers to thoroughly characterize the changes in donor-specific T-cell responses, expressing the results as an alloreactivity index. The results of the activation-induced marker assay revealed that the 2 groups of samples (SCS and HOPE) differed in the alloreactivity index for CD8+ T cells (increased in the SCS group between week 2 and month 3), and for Tregs (increased between baseline and week 2 in the HOPE group) (Figure 1D). A similar trend was observed when we quantified the number of Granzyme B-secreting CD8+ T cells (Supplemental Figure S1, http://links.lww.com/LVT/A640). These results indicate that the increase in CD4+Foxp3+CD127lo cells observed 2 weeks post-transplantation in recipients of HOPE-treated livers can be attributed to the preferential expansion of alloreactive Treg clones. This finding could explain the blunted expansion of alloreactive CD8+ T cells observed in the HOPE group thereafter as compared to recipients who received SCS livers.

Perfusates from HOPE-treated donor livers displayed lower number of intrahepatic immune cells compared to SCS

To determine if HOPE treatment had an influence in the number and characteristics of intrahepatic immune cells, we conducted additional flow cytometry experiments on the effluent cells recovered upon flushing from perfusates of 9 DBD-HOPE and 9 DBD-SCS. The number of effluent LMCs recovered from livers preserved in SCS did not change between the 2 time points

TABLE 1 Study population

	MP (n = 14)	SCS (n = 13)	p
Age	52 (31.0–63.5)	50 (43.0–61.5)	0.33
Sex (male), n (%)	7 (50.0)	8 (61.5)	0.70
MELD	15 (8–21)	17 (9–21)	0.34
Time on the waiting list (d)	87 (13.7–176.0)	52 (13–150)	0.33
DBD/DCD, n (%)	11(78.6)/3 (21.4)	10 (76.9)/3(23.1)	0.99
MP duration (min)	121 (110–154)		
CIT	556 (523–648)	552 (513–636)	0.56
MEAF	5.5 (3.6–8.3)	5.4 (4.6–6.2)	0.62
AST peak	1199 (737–1698)	1480 (467–2071)	0.57
Basiliximab induction, n (%)	4 (28.6)	5 (38.5)	0.69
Mycophenolate mofetil, n (%)	1 (15)	2 (14.2)	0.59
Acute cellular rejection, n (%)	1 (7.1)	6 (46.2)	0.03
Diagnosis	PSC-4 ARLD-1 Retransplantation 2 AIH 1 HCV 1 HCC 2 NASH + HCC 1 Polycystic liver disease 1 Cryptogenic cirrhosis 1	PSC 3 ALD 6 PBC 2 MASLD 1 Polycystic liver disease 1	
Levels of tacrolimus (μg/L)[a]	5.1	5.5	0.50
Graft weight (gm)	1601 (1268–1917)	1649 (1295–2031)	0.70
Days in ICU	2 (2–3)	1 (1–2.3)	0.34
Days in hospital	10.5 (10–14)	11 (10–15)	0.58

Note: Variables are expressed as a median and interquartile range, and categorical variables as absolute numbers and percentages.
[a]Median tacrolimus levels of tacrolimus during the first 2 weeks after liver transplantation.
Abbreviations: AIH, autoimmune hepatitis; ALD, alcoholic liver diseases; ARLD, alcohol-related liver disease; CIT, cold ischemia time; DBD, donor after brain death; DCD, donor after circulatory death; MASLD, metabolic dysfunction–associated steatotic liver disease; MEAF, model for early allograft function; MP, machine perfusion; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

investigated. In contrast, in livers that were perfused using HOPE, we observed a significant reduction in the perfusate LMC numbers following HOPE treatment (Figure 2A). We next investigated if this reduction in the total number of LMCs was associated with specific changes in the repertoire of immune cell subsets (gating strategy, Supplemental Figure S2, http://links.lww.com/LVT/A640). As previously reported, LMCs contained a lower frequency of CD4+ cells (around 20%) than CD8+ cells (40%–55%),[22] but this was similar for SCS and HOPE-treated livers (Figure 2B). Similarly, the proportion of Tregs (which was very low as compared to what could be seen in the blood), as well as the proportions of B-cell subsets, monocytes, and myeloid dendritic cells, were comparable in both treatment groups (Figures 2C–E), with the only statistically significant difference being a reduction in the proportion of plasmacytoid DCs in the HOPE treatment group (Figure 2D).

Transcriptional impact of SCS and HOPE in the reperfusion response

To understand the impact of HOPE on the IRI response at a transcriptional level, we compared the whole-genome RNASeq profiling of liver tissue specimens collected from DBD donors before and after reperfusion in the recipient. In HOPE-treated livers, we detected 951 genes that were upregulated after reperfusion with p value < 0.01. The top significant upregulated genes were associated with cellular response to heat (eg, *IER5*, *MAPAKAPK2, DNAJC2, HSPA1B, JUND,* and *FOSB*) and hypoxia (eg, *HIF1A* and *SLC9A1*). In addition, we observed overexpression of genes involved in acute inflammatory responses (eg, *IL18RAP, TLR2, IFNGR1, NFKB1, CXCL3, IL1B*, and *IL6ST*), T-helper cell differentiation (eg, *RIPK2, IL27RA, BCL3*, and *IL4R*), and neutrophil homeostasis and angiogenesis. On the other hand, 59 genes were significantly

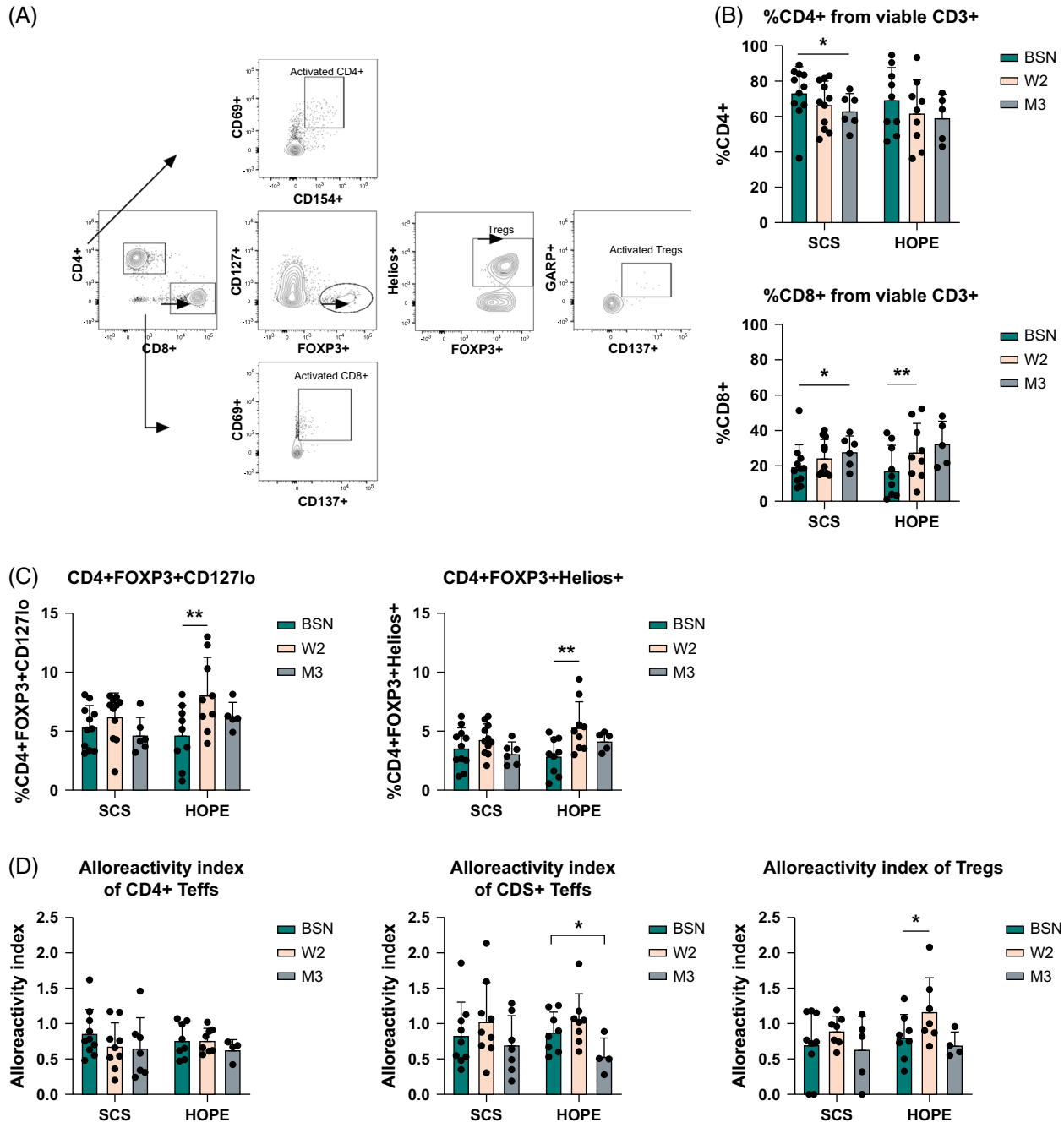

FIGURE 1 HOPE-treated livers exhibited an increase in the frequency of circulating Tregs following the preferential expansion of donor-specific Treg clones 2 weeks after transplantation. (A) Gating strategy used for identifying alloantigen-specific CD4+, CD8+, and Tregs following 16-hour culture with donor or third-party PBMCs in the activation-induced marker assay. (B) Quantification of CD4+ and CD8+ cells from viable CD3+ cells. (C) Percentage of CD4+CD127loFOXP3+, CD4+FOXP3+HELIOS+, from frozen patient PBMCs at the time points of pretransplantation baseline (BSN), week 2 following transplantation (W2), and month 3 following transplantation (M3). (D) Stimulation assay was performed to identify antigen-specific CD4+ Teffs (characterized as CD69+CD154+CD4+ cells), CD8+ Teffs (characterized as CD137+CD69+CD8+ cells), and Helios+ Tregs (characterized as CD137+GARP+FOXP3+CD4+ cells) following 18-hour culture with SEB, donor alloantigens or third P alloantigens. The alloreactivity index was calculated as a ratio of donor-specific T-cell response to third-party T-cell response. (B–D) Each data point corresponds to an individual patient and represents 1 experiment. At baseline, HOPE (n = 9) and SCS (n = 11); at week 2, HOPE (n = 9) and SCS (n = 11), and at month 3, HOPE (n = 5) and SCS (n = 6) and significance was determined by the Friedman test and *p* values represent pairwise comparisons of adjusted *p* values using the Wilcoxon test. The values are shown as the mean ± SD. *$p < 0.05$, **$p < 0.01$. Abbreviations: HOPE, hypothermic oxygenated machine perfusion; PBMC, peripheral blood mononuclear cell; SCS, static cold storage; Tregs, regulatory T cells.

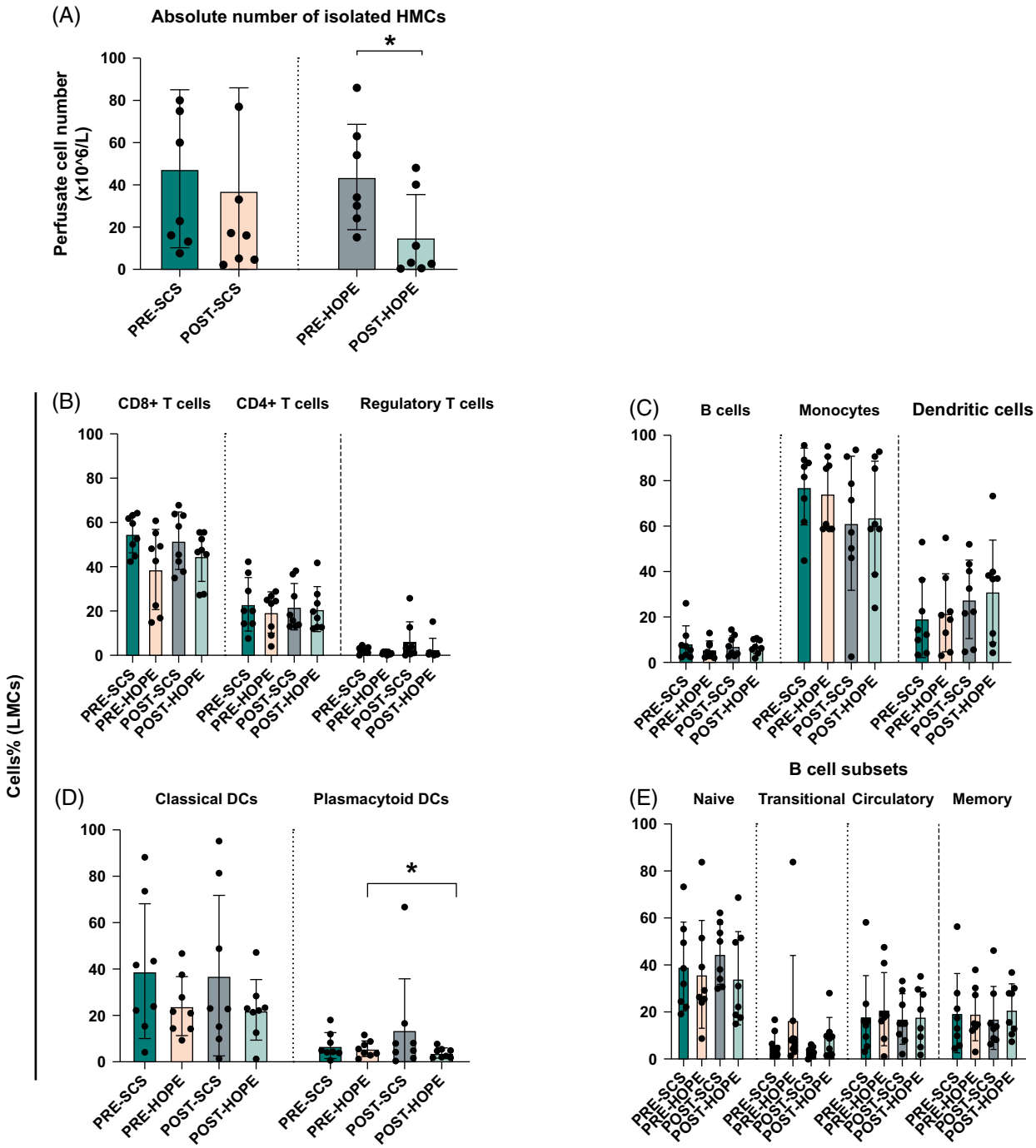

FIGURE 2 HOPE reduced the frequency of intrahepatic cells after machine perfusion without any impact on innate and adaptive cells. (A) Number of flushed-out LMCs isolated from per liter perfusate of HOPE-treated and SCS-treated livers at the arrival of the hospital (before SCS and HOPE) and at the end of preservation (after HOPE and SCS). (B) Expression of CD4+, CD8+ and Tregs across the different groups. (C) B cells, monocytes, and dendritic cells frequencies in pre- and post-SCS and HOPE perfusates. (D) Frequency of 2 different DC subsets before and after SCS and HOPE. (E) Frequency of the different B-cell subsets before and after preservations. (A–E) Each data point corresponds to an individual patient and represents 1 experiment. HOPE livers (n = 8) and SCS livers (n = 8). Groups were compared using 2-way ANOVA with Sidak's multiple comparisons test. *$p < 0.05$. Abbreviations: DC, dendritic cell; HOPE, hypothermic oxygenated machine perfusion; LMC, liver mononuclear cell; SCS, static cold storage.

downregulated following reperfusion with p value < 0.01, and these included *GIMAP7* (involved in inducing oxidative stress and apoptosis), the adenosine receptor *ADORA3*, and the cytotoxic cell-specific *GZMA*. The transcriptional changes observed following the reperfusion of SCS-preserved livers included 781 upregulated genes, most of which are known to be involved in the cellular response to oxidative stress (eg,

RHOB, IL18RAP, MMP9, HSPAIB, EGR1, PANX1, GATA6, and *MYC*), leukocyte migration (eg, *CCL3, CCL2, CCL20*, and *GPR183*), and macrophage differentiation and activation. On the contrary, SCS-treated liver showed downregulation of 515 genes, 40 of which were related to the positive regulation of metabolic processes (eg, *DDB1*, *PRKAB2*, and *ATP2B*) (Figures 3B, C).

To better elucidate the molecular pathways associated with these transcriptional changes, we performed a Gene Set Enrichment Analysis with a cutoff for significance of $<10\%$ false discovery rate. We identified 24 pathways overrepresented at the time of reperfusion, both in HOPE-treated and SCS-preserved livers. These included pathways previously described in ischemia-reperfusion response, such as "inflammatory response," "complement," "apoptosis," "TNF-alpha signaling," "hypoxia," and "interferon-gamma response regulated immune responses" (Figure 4A). Of note, all these proinflammatory pathways exhibited a higher normalized enrichment score in SCS than in HOPE-treated livers. Furthermore, the "radical oxygen species pathway," which contained *KLF2, TNFAIP3*, and *HMOX1* genes, was only significantly enriched in SCS-preserved livers (Figures 4B, C).

DISCUSSION

End-ischemic HOPE is rapidly being adopted as a standard procedure in transplant centers worldwide for its simplicity and the possibility of safely extending the preservation time, but especially for the reported advantages in decreasing IRI-related complications after transplantation.[1,2,22] In contrast to the scarcity of immunological studies conducted in humans, multiple studies conducted in rodent models indicate that HOPE treatment exerts beneficial effects on post-LT alloimmune responses. Thus, in addition to the report by Schlegel et al[6] already described, Lauschke and colleagues showed in a rat model of DCD LT that, as compared to DCD livers preserved with SCS, HOPE decreased the expression of intercellular adhesion molecule-1 and MHC class II on reperfusion. The authors also observed a reduced proportion of CD80/CD86 APCs when compared to those treated with tacrolimus.[11] Altogether, this could have resulted in decreased liver allograft immunogenicity by reducing direct alloantigen presentation. Similar beneficial effects have been described in a kidney transplant rodent model by Kron et al.[9] In this report, kidney recipients who received no immunosuppressive treatment and

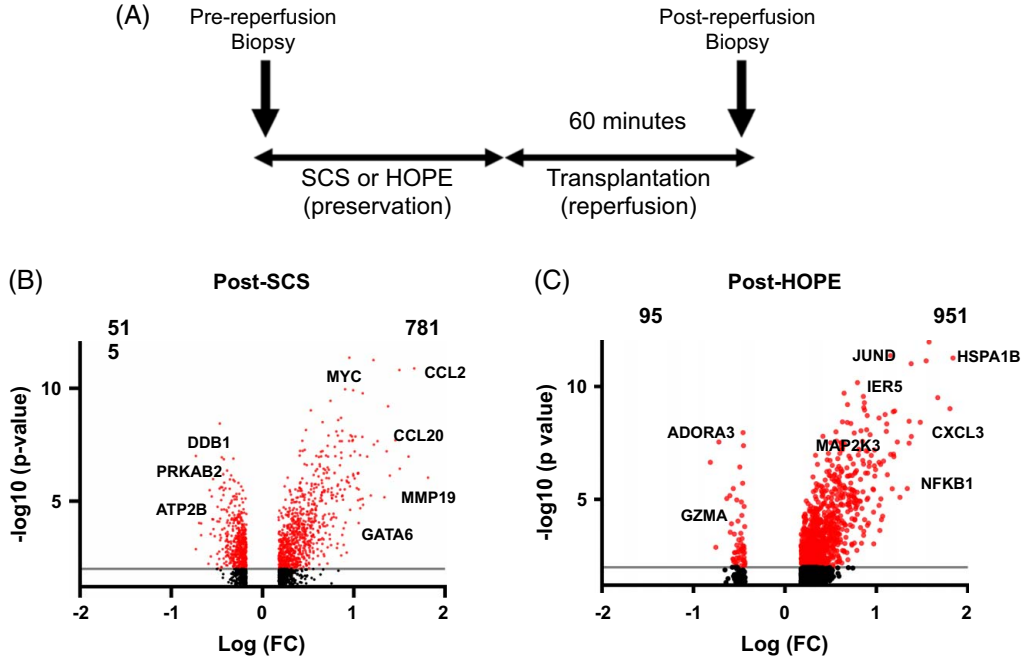

FIGURE 3 Gene expression profiles following RNA sequencing (RNA-seq) of HOPE-treated and SCS-treated liver grafts. (A) Diagram showing the timing of liver biopsies. (B, C) Volcano plots showing the changes in expression (upregulation and downregulation) of all genes analyzed by RNA-seq in SCS-treated and HOPE-treated livers. The red area denotes significantly changed genes; the number of those genes was displayed, and examples were displayed. Gene expression analyses were performed on sequential liver biopsies collected from 16 DBD donors (HOPE: n = 8; SCS: n = 8). Significance was decided by a cutoff of p value <0.01. Abbreviations: ADORA3, adenosine A3 receptor; ATP2B, ATPases 2B; CCL2, chemokine (C-C motif) ligand 2; CCL20, CC chemokine ligand 2; CXCL3, C-X-C motif chemokine ligand 3; DDB1, damage specific DNA binding protein1; GATA6, GATA binding protein 6; GZMA, granzyme A; HOPE, hypothermic oxygenated perfusion; HSPA1B, heat shock protein family A member 1B; IER5, immediate early response 5; JUND, Jun proto-oncogene; MAP2K3, mitogen-activated protein kinase 3; MMP19, matrix metallopeptidase 19; MYC, myelocytomatosis oncogene; NFKB1, nuclear factor kappa B subunit 1; PRKAK2, protein kinase AMP-activated non-catalytic subunit beta 2; SCS, static cold storage.

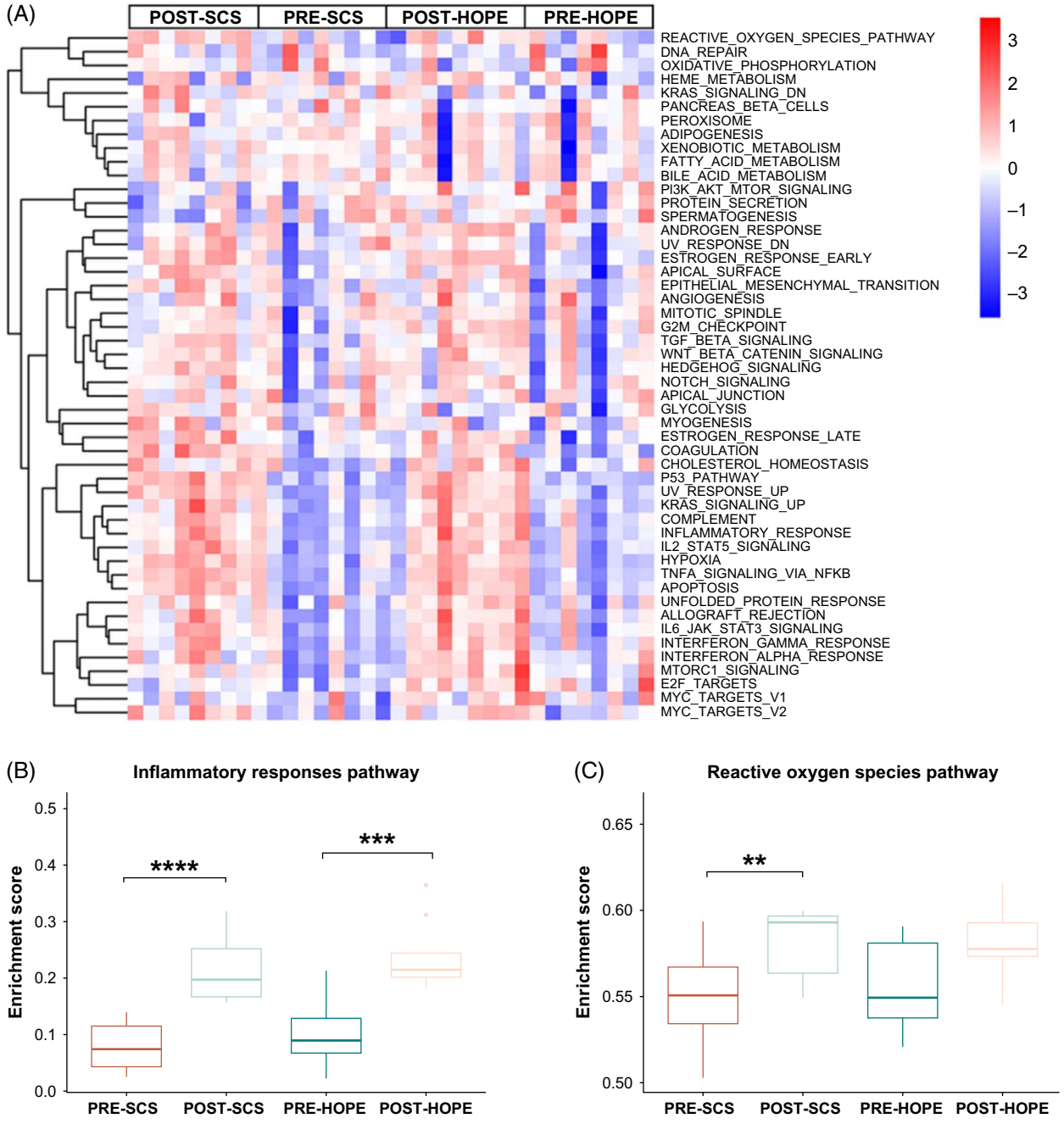

FIGURE 4 HOPE upregulated inflammatory pathways and limited reactive oxygen species pathways. We analyzed the gene data using GSEA canonical pathway analysis using a cutoff for the significance of p value < 0.01. We focused on longitudinal analysis in the 2 groups (HOPE: n = 8 and SCS: n = 8) by looking at the expressions of 50 different pathways. (A) Heatmap of 50 gene sets, showing the upregulated and downregulated pathways for HOPE and SSC liver biopsies taken before reperfusion and after reperfusion. (B) Boxplot illustrating the enrichment score for the inflammatory response in pre- and post-HOPE and SCS livers with the p values. (C) Boxplot showing the enrichment score for reactive oxygen specious pathways for pre- and post- HOPE and SCS livers with the p values. Significance between groups was defined by 2-way ANOVA with the Sidak multiple comparisons test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Abbreviations: GSEA, Gene Set Enrichment Analysis; HOPE, hypothermic oxygenated machine perfusion; SCS, static cold storage.

were grafted with a HOPE-treated allograft exhibited lower numbers of circulating and infiltrating CD4+ and CD8+ T cells, reduced incidence of acute rejection, lower Banff scores, and minor complement activation. These immunological effects were associated with reduced IRI, as demonstrated by a decrease in the release of markers of oxidative injury and cytokines.

In the current study, for the first time in humans, we describe the immunoregulatory effect of HOPE-treated livers following LT and provide novel translational observations that are in keeping with the previously reported findings observed in animal models. Thus, we demonstrate that the peripheral T-cell compartment of recipients of transplant reacts differently to liver allografts that have been

treated with HOPE or just preserved in SCS. Early after LT, recipients grafted with HOPE-treated livers exhibited an increase in the number of circulating Tregs and in the proportion of them exhibiting alloantigen-specificity. The use of HOPE-treated livers was also associated with a selective reduction of donor-specific CD8+ T cells 3 months after LT. On note, these observations could not be explained by differences between the SCS and HOPE groups in the levels of calcineurin inhibitors after LT, or in the use of basiliximab induction, both of which are known to have a negative impact on the number of circulating Tregs[10,23,24] and constitute a common clinical confounder in immunological studies in LT.

Boosting the number and function of suppressive immune cells, such as regulatory CD4+CD25+FoxP3+ T cells (Tregs), has been described as one of the central mechanisms of hepatic peripheral immune tolerance to enhance the tolerogenic microenvironment in the liver during homeostasis and transplantation.[25,26] CD4+CD25+Foxp3+ Tregs can suppress a multiplicity of different immune effector mechanisms. Of note, they require an antigen-specific stimulation through their T-cell receptor to be activated, but once activated, they suppress all cell types present in their microenvironment (bystander suppression).[27] In experimental murine models of LT, if Tregs are eliminated at the time of transplantation, the spontaneous acceptance of the liver allograft is abrogated.[28,29] Furthermore, in rodent LT models, it has been shown that the administration of donor-specific Tregs together with short-term tacrolimus can induce permanent engraftment and allograft tolerance.[30,31] Demirkiran et al[32] showed significant lower frequencies of Treg levels after LT in patients who experience episodes of ACR. Han et al[33] recently published the kinetics of Tregs early after LT comparing rejectors versus not rejectors, showing a significant lower frequency of Treg on day 7 after liver transplant in biopsy-proven and suspected ACR when compared to nonrejectors. More recently, it has been described that human LT per se results in a selective reduction in the circulation of Treg clones recognizing donor alloantigens,[34] although the mechanistic explanation for this observation remains unclear. On the other hand, there is also abundant literature linking the number of circulating allospecific cytotoxic T cells with the development of rejection following LT.[35]

In addition to the impact on donor-specific effectors and Tregs, another effect of HOPE that could have immunomodulatory consequences is the reduction in the number of effluent immune cells detected in the liver perfusates obtained before LT, which likely reflects a "wash-out" phenomenon. We have previously described that the immune cell composition of the liver allograft perfusates constitute a good representation of what is observed within the liver tissue, particularly what regards the liver T-cell compartment.[22] Passenger antigen-presenting cells (APC) contained in the allograft are a fundamental mechanism of ACR through the direct pathway of allorecognition.[36] Multiple experimental animal model studies have described a causal link between donor liver APCs and immunological outcomes, although rather than the absolute number, the phenotype of the APCs (immunogenic vs. regulatory phenotype) appears to be key.[37,38] In keeping with this concept, the use of hypothermic machine perfusion with a nitrogenated, rather than oxygenated, perfusate failed to exert immunomodulatory effects despite providing a similar "wash-out" of the tissue-resident immune cells.[8]

To further understand the mechanisms through which HOPE could modulate the recipient's immune responses, we characterized the IRI response by performing transcriptional profiling. To our knowledge, we describe, for the first time, the gene expression analysis of human donor livers treated with HOPE. Due to the sample size, we decided to focus on DBD livers only. At the post-reperfusion stage, genes involved in cell proliferation, cell cycle, metabolism, protein synthesis, antiapoptotic, and induced type 1 interferon pathways were upregulated with HOPE. Minor et al,[39] in a porcine animal model of allogeneic LT, described a tendency toward a reduction in TNF-a expression during hypothermia and further reduced after continuous oxygenated rewarming; however, Caspase 3 activity was reduced after HOPE and continuous oxygenated rewarming. Interestingly, the authors did not observe an upregulation in the expression of intercellular adhesion molecule-1 nor TLR4 in the SCS versus HOPE and continuous oxygenated rewarming. Of note, although biological pathways associated with inflammatory responses, TNF-a signaling, hypoxia, interferon-gamma response, and allograft rejection were upregulated both after HOPE and after DBD SCS, the radical oxygen species pathway was only enriched in DBD samples following SCS. This confirms the key mechanism of action through which HOPE has been shown to ameliorate IRI.[6]

We acknowledge there are several limitations in the interpretation of our findings. First is the fact that our study evaluates a small cohort of liver recipient patients, with a very heterogenous disease etiology leading to transplantation. This may limit the interpretation of the study results on the immunomodulatory role of HOPE on posttransplant cellular immune responses. Second, we cannot definitely establish whether our findings (ie, the association between HOPE treatment and changes in the balance between donor-specific Tregs and effector T cells) are the cause or a consequence of the decreased incidence of ACR. The third limitation involves the lack of DCD liver biopsies in our transcriptional profiling studies. This is particularly relevant given the well-documented beneficial effect of HOPE on IRI-related complications and rejection following DCD transplantation and is a topic that will need to be investigated in future studies.[40] Finally,

while our demonstration that HOPE reduces the number of effluent leukocytes that can be obtained from liver perfusates, which has been reported in both normothermic machine perfusion and HOPE before as well[41,42] constitutes a potential mechanism of action, the immunogenic profile of these cells remains to be investigated further.

In conclusion, despite the limitations presented, we believe our study has significant clinical implications, given that it provides the rationale for conducting future studies employing HOPE as a component of novel therapeutic strategies aiming at reducing the dose of immunosuppression after LT.

DATA AVAILABILITY STATEMENT

The bulk-RNA sequencing data is published in the GEO and the accession number is GSE276531.

ACKNOWLEDGEMENTS

The authors express their appreciation to Mr. Ralph Smith and the King's Hospital Charity for their generous funding and contribution to this research. The authors also extend their appreciation to all the patients who participated in this study from the HOPE and D-HOPE trials.

FUNDING INFORMATION

This work was supported by the King's College Hospital Charity.

CONFLICTS OF INTEREST

Maria-Carlota Londoño consults for and is on the speakers' bureau for Advanz, Ipsen, and GSK. She consults for Falk. She is on the speakers' bureau for Cymabay and Gilead. Nigel Heaton is on the speakers' bureau for Sirtex. The remaining authors have no conflicts to report.

ORCID

Marwa Elgosbi https://orcid.org/0009–0001–4585–2210

REFERENCES

1. van Rijn R, Schurink IJ, de Vries Y, van den Berg AP, Cortes Cerisuelo M, Darwish Murad S, et al. Hypothermic machine perfusion in liver transplantation—A randomized trial. N Engl J Med. 2021;384:1391–401.
2. Schlegel A, Mueller M, Muller X, Eden J, Panconesi R, von Felten S, et al. A multicenter randomized-controlled trial of hypothermic oxygenated perfusion (HOPE) for human liver grafts before transplantation. J Hepatol. 2023;78:783–93.
3. Czigany Z, Pratschke J, Froněk J, Guba M, Schöning W, Raptis DA, et al. Hypothermic oxygenated machine perfusion reduces early allograft injury and improves post-transplant outcomes in extended criteria donation liver transplantation from donation after brain death: Results from a multicenter randomized controlled trial (HOPE ECD-DBD. Ann Surg. 2021;274:705–12.
4. Ravaioli M, Germinario G, Dajti G, Sessa M, Vasuri F, Siniscalchi A, et al. Hypothermic oxygenated perfusion in extended criteria donor liver transplantation—A randomized clinical trial. Am J Transplant. 2022;22:2401–8.
5. Brüggenwirth IMA, Lantinga VA, Lascaris B, Thorne AM, Meerdink M, de Kleine RH, et al. Prolonged hypothermic machine perfusion enables daytime liver transplantation—An IDEAL stage 2 prospective clinical trial. EClinicalMedicine. 2024; 68:102411.
6. Schlegel A, de Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol. 2013;58:278–86.
7. Schlegel A, Graf R, Clavien PA, Dutkowski P. Hypothermic oxygenated perfusion (HOPE) protects from biliary injury in a rodent model of DCD liver transplantation. J Hepatol. 2013;59: 984–91.
8. Schlegel A, Kron P, Graf R, Clavien PA, Dutkowski P. Hypothermic Oxygenated Perfusion (HOPE) downregulates the immune response in a rat model of liver transplantation. Ann Surg. 2014;260:931–7
9. Kron P, Schlegel A, Muller X, Gaspert A, Clavien PA, Dutkowski P. Hypothermic oxygenated perfusion: A simple and effective method to modulate the immune response in kidney transplantation. Transplantation. 2019;103:e128–36.
10. Welzl K, Kern G, Mayer G, Weinberger B, Säemann MD, Sturm G, et al. Effect of different immunosuppressive drugs on immune cells from young and old healthy persons. Gerontology. 2014;60:229–38.
11. Lauschke H, Olschewski P, Tolba R, Schulz S, Minor T. Oxygenated machine perfusion mitigates surface antigen expression and improves preservation of predamaged donor livers. Cryobiology. 2003;46:53–60.
12. Demetris AJ, Bellamy C, Hübscher SG, O'Leary J, Randhawa PS, Feng S, et al. 2016 Comprehensive update of the Banff Working Group on liver allograft pathology: Introduction of antibody-mediated rejection. Am J Transplant. 2016;16: 2816–35.
13. Uemura T, Ikegami T, Sanchez EQ, Jennings LW, Narasimhan G, McKenna GJ, et al. Late acute rejection after liver transplantation impacts patient survival. Clin Transplant. 2008;22:316–23.
14. Pareja E, Cortes M, Hervás D, Mir J, Valdivieso A, Castell JV, et al. A score model for the continuous grading of early allograft dysfunction severity. Liver Transpl. 2015;21:38–46.
15. Lim TY, Perpiñán E, Londoño MC, Miquel R, Ruiz P, Kurt AS, et al. Low dose interleukin-2 selectively expands circulating regulatory T cells but fails to promote liver allograft tolerance in humans. J Hepatol. 2023;78:153–64.
16. Dobin A, Gingeras TR. Mapping RNA-seq reads with STAR. Curr Protoc Bioinform. 2015;51:11.14.1–19.
17. Li B, Dewey CN. RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinform. 2011;12:323.
18. Frankish A, Diekhans M, Ferreira AM, Johnson R, Jungreis I, Loveland J, et al. GENCODE reference annotation for the human and mouse genomes. Nucleic Acids Res. 2019;47(D1):D766–73.
19. Law CW, Chen Y, Shi W, Smyth GK. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 2014;15:R29.
20. GK S. LIMMA: Linear Models for Microarray Data, Springer; 2005.
21. Yu G, Wang LG, Han Y, He QY. clusterProfiler: An R package for comparing biological themes among gene clusters. OMICS. 2012;16:284–7.
22. Xystrakis E, Yuksel M, Lin F, Huang X, Pop OT, Quaglia A, et al. Impact of donation mode on the proportion and function of T lymphocytes in the liver. PLoS One. 2015;10:e0139791.
23. Akimova T, Kamath BM, Goebel JW, Meyers KEC, Rand EB, Hawkins A, et al. Differing effects of rapamycin or calcineurin

inhibitor on T-regulatory cells in pediatric liver and kidney transplant recipients. Am J Transplant. 2012;12:3449–61.
24. Krystufkova E, Sekerkova A, Striz I, Brabcova I, Girmanova E, Viklicky O. Regulatory T cells in kidney transplant recipients: The effect of induction immunosuppression therapy. Nephrol Dial Transplant. 2012;27:2576–82.
25. Zheng XX, Sanchez-Fueyo A, Domenig C, Strom TB. The balance of deletion and regulation in allograft tolerance. Immunol Rev. 2003;196:75–84.
26. Fan Z, Spencer JA, Lu Y, Pitsillides CM, Singh G, Kim P, et al. In vivo tracking of 'color-coded' effector, natural and induced regulatory T cells in the allograft response. Nat Med. 2010;16:718–22.
27. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med. 1998;188:287–96.
28. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K, Ohkura N. Regulatory T cells and human disease. Annu Rev Immunol. 2020;38:541–66.
29. Li W, Kuhr CS, Zheng XX, Carper K, Thomson AW, Reyes JD, et al. New insights into mechanisms of spontaneous liver transplant tolerance: The role of Foxp3-expressing CD25+CD4 + regulatory T cells. Am J Transplant. 2008;8:1639–51.
30. Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H. Both CD4(+)CD25(+) and CD4(+)CD25(-) regulatory cells mediate dominant transplantation tolerance. J Immunol. 2002;168:5558–65.
31. Pu LY, Wang XH, Zhang F, Li XC, Yao AH, Yu Y, et al. Adoptive transfusion of ex vivo donor alloantigen-stimulated CD4(+)CD25(+) regulatory T cells ameliorates rejection of DA-to-Lewis rat liver transplantation. Surgery. 2007;142:67–73.
32. Demirkiran A, Kok A, Kwekkeboom J, Kusters JG, Metselaar HJ, Tilanus HW, et al. Low circulating regulatory T-cell levels after acute rejection in liver transplantation. Liver Transpl. 2006;12:277–84.
33. Han JW, Joo DJ, Kim JH, Rha MS, Koh JY, Park HJ, et al. Early reduction of regulatory T cells is associated with acute rejection in liver transplantation under tacrolimus-based immunosuppression with basiliximab induction. Am J Transplant. 2020; 20:2058–69.
34. Tang Q, Leung J, Peng Y, Sanchez-Fueyo A, Lozano JJ, Lam A, et al. Selective decrease of donor-reactive T. Sci Transl Med. 2022;14:eabo2628.
35. Ashokkumar C, Soltys K, Mazariegos G, Bond G, Higgs BW, Ningappa M, et al. Predicting cellular rejection with a cell-based assay: Preclinical evaluation in children. Transplantation. 2017; 101:131–40.
36. Lechler R, Ng WF, Steinman RM. Dendritic cells in transplantation—Friend or foe? Immunity. 2001;14:357–68.
37. Ueta H, Xu XD, Yu B, Kitazawa Y, Yu E, Hara Y, et al. Suppression of liver transplant rejection by anti-donor MHC antibodies via depletion of donor immunogenic dendritic cells. Int Immunol. 2021;33:261–72.
38. Nakano R, Tran LM, Geller DA, Macedo C, Metes DM, Thomson AW. Dendritic cell-mediated regulation of liver ischemia-reperfusion injury and liver transplant rejection. Front Immunol. 2021;12:705465.
39. Minor T, Efferz P, Fox M, Wohlschlaeger J, Lüer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant. 2013;13:1450–60.
40. Maspero M, Ali K, Cazzaniga B, Yilmaz S, Raj R, Liu Q, et al. Acute rejection after liver transplantation with machine perfusion versus static cold storage: A systematic review and meta-analysis. Hepatology. 2023;78:835–46.
41. Lurje I, Uluk D, Hammerich L, Pratschke J, Tacke F, Lurje G. Comparing hypothermic oxygenated and normothermic liver machine perfusion: Translation matters. J Hepatol. 2024;80: e163–5.
42. Hautz T, Salcher S, Fodor M, Sturm G, Ebner S, Mair A, et al. Immune cell dynamics deconvoluted by single-cell RNA sequencing in normothermic machine perfusion of the liver. Nat Commun. 2023;14:2285.

How to cite this article: Elgosbi M, Kurt AS, Londoño M, Caballero-Marcos A, Lim TY, Lozano JJ, et al. Hypothermic oxygenated machine perfusion influences the immunogenicity of donor livers in humans. Liver Transpl. 2025;31:311–322. https://doi.org/10.1097/LVT.0000000000000461