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Normothermic Machine Perfusion (NMP) Inhibits Proinflammatory Responses in the Liver and Promotes Regeneration

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Liver transplantation (LT) is a successful treatment for patients with liver failure. However, organ shortage results in over 11% of patients losing their chance of a transplant attributed to liver decompensation (LD) and death. Ischemia/reperfusion injury (IRI) following conventional cold storage (CS) is a major cause of injury leading to graft loss after LT. Normothermic machine perfusion (NMP), a method of organ preservation, provides oxygen and nutrition during preservation and allows aerobic metabolism. NMP has recently been shown to enable improved organ utilization and posttransplant outcomes following a phase I and a phase III randomized trial. The aim of the present study is to assess the impact of NMP on reducing IRI and to define the underlying mechanisms. We transplanted and compared 12 NMP with 27 CS-preserved livers by performing gene microarray, immunoprofiling of hepatic lymphocytes, and immunochemistry staining of liver tissues for assessing necrosis, platelet deposition, and neutrophil infiltration, and the status of steatosis after NMP or CS prereperfusion and postreperfusion. Recipients receiving NMP grafts showed significantly lower peak aspartate aminotransferase (AST) levels than those receiving CS grafts. NMP altered gene-expression profiles of liver tissue from proinflammation to prohealing and regeneration. NMP also reduced the number of interferon gamma (IFN-γ) and interleukin (IL)-17–producing T cells and enlarged the $CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ regulatory T cell (Treg) pool. NMP liver tissues showed less necrosis and apoptosis in the parenchyma and fewer neutrophil infiltration compared to CS liver tissues. *Conclusion:* Reduced IRI in NMP recipients was the consequence of the combination of inhibiting inflammation and promoting graft regeneration. (Hepatology 2019;70:682-695).

Liver transplantation (LT) is the only treatment for patients with end-stage acute and chronic liver failure. It is a successful treatment with a 5-year patient and graft survival rate exceeding 80% (NHS BT report 2017). However, organ shortage remains a global problem that results in removal of over 11% of patients placed on the waiting list because of death or worsening of their condition.[1]

The rate of organ donations has increased over the last 10 years. However, this increase is mainly limited to high-risk, so-called marginal donors, which largely comprise donors deceased after circulatory death

Abbreviations: ALF, acute liver failure; ALP, alkaline phosphatase; AST, aspartate transaminase; CS, cold storage; Ct, cycle threshold; CXCL, chemokine (C-X-C motif) ligand; Cy, cyanine; DBD, donation after brain death; DCD, donation after cardiocirculatory death; FDR, false discovery rate; HMCs, hepatic mononuclear cells; ICU, intensive care unit; IFN-γ, interferon gamma; IHC, immunohistochemical; IL, interleukin; INR, international normalized ratio; IRI, ischemia-reperfusion injury; LT, liver transplantation; NK, natural killer; NKT, natural killer T cell; NMP, normothermic machine perfusion; MPO, myeloperoxidase; PD1, programmed cell death protein 1; POD, paracetamol overdose; TGF-β, transforming growth factor beta; Th, T helper; THBD, thrombomodulin; Treg, regulatory T cell.

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(DCD),[2] older donors, and fatty livers.[3] There is evidence that marginal organs have greater vulnerability to ischemia reperfusion injury (IRI), an inevitable process of organ transplantation.[4]

A number of experimental and clinical studies have shown that reperfusion of allografts is associated with organ inflammation characterized by the release of inflammatory mediators, up-regulation of adhesion molecules, immune cell infiltration, and increased cell death.[5-7] This damage affects short- and long-term organ outcome by contributing to development of primary nonfunction and ischemic cholangiopathy, respectively.[8]

Normothermic machine perfusion (NMP) is a technology that allows preservation and transport of organs from donor hospital to recipient hospital at body temperature while providing the organ with oxygen and nutrients.[9] NMP has been introduced into clinical practice with the aim of minimizing IRI and its effects on the liver graft, enabling successful preservation and functional testing of marginal livers, improving the logistics of transplantation by allowing prolonged preservation from 12 to 24 hours, and ultimately increasing the number of livers successfully transplanted. A phase I clinical trial using an NMP device (*metra*; OrganOx Ltd, Oxford, UK) demonstrated safety of the technology.[10] The NMP device also led to a reduction in the levels of liver enzyme aspartate aminotransferase (AST) in recipients, which is used as a surrogate marker of liver damage.[10] Reduction in allograft damage and improved organ utilization of NMP over cold storage (CS) has been further confirmed in a randomized, controlled, multicenter trial including seven LT centers from the UK and Europe (Consortium for Organ Preservation in Europe; www.cope-eu.org), in which a total of 137 NMP and 133 CS grafts were enrolled,[11] with 120 NMP and 100 CS livers ultimately transplanted.

The current investigation aims to assess the impact of NMP preservation on degree of liver allograft IRI compared to conventional CS and explore the mechanisms underpinning any observed differences.

Materials and Methods

DONORS AND RECIPIENTS

In 2013, we carried out 14 LTs using donation after brain death (DBD; n = 12) and DCD (n = 2) grafts preserved with NMP, as part of a phase I clinical trial that recruited a total of 20 patients.[10] In the current analysis, we included only the 12 DBD grafts and excluded the DCD transplants because of the small number. These livers were matched with 27 CS DBD grafts that were transplanted in the same period. Donors and recipients in both groups were matched according to age, intensive therapy unit (ITU) stay,

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Potential conflict of interest: Dr. Friend owns stocks in and is employed by OrganOx LTD. Dr. Heaton is on the speakers' bureau for Astellas and Novartis. Dr. Coussios owns stocks in, is employed by, and consults for OrganOx LTD.

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infections, inotropic support, and donor liver steatosis. Recipients were also matching for their original liver diseases, with a similar proportion of alcoholic-related liver diseases, autoimmune liver diseases (autoimmune hepatitis [AIH], primary sclerosing cholangitis [PSC], and primary biliary cholangitis [PBC]), hepatitis C virus (HCV) infection, hepatocellular carcinoma (HCC), inherited hepatic cholestasis, and drugs-induced acute liver failure (ALF; paracetamol overdose [POD] and non-POD); they also presented with a similar severity of liver disease, represented by Model for End-Stage Liver Disease (MELD) scores assessed at time of listing for LT. Their demographic and clinical data are summarized in Tables 1 and 2 (Table 1 for donors and Table 2 for recipients, respectively). This study was approved by the Ethics Committees of St Thomas's Hospital (REC Reference: 09/H0802/100), and Dulwich Ethics Committee Approvals were also obtained from National Health Service Blood and Transplant, National Research Ethics Committee, and the Medicines and Healthcare Products Regulatory Authority. The trial was registered with the International Standard Registered Clinical/Social Study Number (14355416)

TABLE 1. Characteristics of Liver Donors and Grafts in the NMP and CS Groups

Parameters	NMP	CS	*P* Value
No.	12	27	
Donor sex: M/F (% of male)	8/4 (66)	13/14 (48)	NS
Donor age, median (range years)	60 (41-85)	54 (26-82)	NS
Donor BMI: mean ± SD	25.4 ± 2.6	28.1 ± 5.4	NS
ICU stay: days (median and range)	2 (1-13)	2 (1-15)	NS
Inotropic support: yes/no	9/3	20/7	NS
Steatosis status and score: no. (%)			
1 = nonsteatosis	6 (50)	12 (44)	
2 = mild	6 (50)	13 (48)	NS
3 = moderate	0	1 (4)	
4 = severe	0	1 (4)	
Infection status: yes/no/unknown	5/6/1	7/19/1	NS
Cold ischemia time: mean ± SD minute	N/A	571 ± 160	

Abbreviations: N/A, not applicable; NS, not significant.

LIVER PERFUSATE AND ISOLATION OF HEPATIC MONONUCLEAR CELLS

We previously showed that intrahepatic lymphocytes (hepatic mononuclear cells; HMCs) obtained through liver perfusion are representative of liver-resident lymphocytes.[12] Donor livers were perfused at the end of NMP or CS with preservation solution as described.[10] In brief, in NMP the perfusion was stopped at the end of preservation and the organ was cooled by rapid flashing of 2 L of cold HTK solution (Custodiol-HTK; Essential Pharmaceuticals, Ewing, NJ).[10] An additional 1 L was used to further flush the grafts, and the clear perfusate was collected for mononuclear cell extraction. In CS livers, grafts were perfused with 1 L of UW solution at the end of preservation and the perfusate was similarly collected. All perfusates were processed within 6 hours by centrifugation to reduce the volume. The final volume of 30 mL of HMC-rich solution was used to isolate HMCs by density-gradient centrifugation (Lymphoprep; GE, Sweden).[12] HMCs isolated after NMP (8 of the 12 cases) were compared to those after CS (20 of 27 grafts).

PHENOTYPIC AND FUNCTIONAL ANALYSIS OF HMCs USING FLOW CYTOMETRY

HMCs were stained for 20 minutes at 4°C using monoclonal antibodies (mAbs) specific for CD3/PE (phycoerythrin)/Cy (cyanine)7, CD4/APC (allophycocyanine)/Cy7, CD8/peridinin-chlorophyll-protein complex/Cy5.5, CD19-APC, CD25-PE, CD127/fluorescein isothiocyanate (FITC; Cambridge Bioscience, Cambridge, UK), and forkhead box protein O3 (FOXP3)/FITC (eBioscience, San Diego, USA). For intracellular cytokine detection, HMCs were stimulated with phorbol 12-myristate-13-acetate/ionomycin/brefeldin (eBioscience) for 4 hours, fixed (Cytofix/CytoPerm eBiosciences), then stained using mAbs specific for interleukin (IL)-2, IL-10, IL-17, IL-22, IL-23, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α; BioLegend, San Diego, CA). 7-Aminoactinomycin D was used to exclude dead cells from the analysis. Cells were acquired on a BD Canto II (BD Bioscience, San Jose, CA). Analysis was performed using FlowJo software (Tree Star Inc., Ashland, USA).

TABLE 2. Characteristics of Recipients in the NMP and CS Groups

Parameters	NMP	CS	*P* Value
N.	12	27	
Recipient sex: M/F (% of male)	6/6 (50)	17/10 (63)	NS
Recipient age: median (range, years)	54 (38-62)	50 (17-70)	NS
Recipient MELD score at the time of listing for transplant: median (range)	15 (9-27)	16 (2-26)	NS
Cause of liver function failure: no. (%)			NS
1. Alcohol-related liver disease	4 (33)	9 (33)	
2. Autoimmune liver diseases (PBC, PSC, and AIH)	4 (33)	8 (30)	
3. HCV	1 (8)	2 (7)	
4. HCC	1 (8)	3 (11)	
5. Inherited hepatic cholestasis	1 (8)	3 (11)	
6. ALF: POD	0 (0)	1 (4)	
7. ALF: nonparacetamol drug induced	1 (8)	1 (4)	
ICU stay days: median (range)	3 (1-8)	5 (1-28)	NS
Acute rejection (biopsy proven; 2-week posttransplant)	0/12	1/27	NS
Graft survival at 1 year	11/11*	27/27	NS
Recipient survival at 1 year	11/12*	27/27	
Peak AST within 7 days (IU/L) median (range)	371 (162-1,709)	924 (162-8,029)	*<0.01*
Peak total bilirubin within 7 days (μMol/L) median (range)	118 (35-298)	98 (20-576)	NS
Total bilirubin on day 7 (μMol/L) median (range)	44 (9 -211)	30 (6-258)	NS
Peak ALP within 7 days (U/L) median (range)	247 (88-568)	233 (135-4,160)	NS
ALP on day 7 (U/L) median (range)	213 (79-568)	205 (78-661)	NS
Peak INR within 7 days median (range)	1.6 (1.40-2.74)	2.03 (1.43-2.90)	*0.07*
INR on day 7 median (range)	1.05 (0.89-1.22)	1.02 (0.86-1.43)	NS
30-day mortality (%)	0 (0)	0 (0)	NS

*One recipient died from recurrent alcoholic liver disease at month 8; this case was excluded from the calculation of graft survival at 1 year, because this death was not related to graft quality.

LIVER BIOPSIES

Tru-cut liver biopsies were performed on NMP and CS grafts at the end of preservation (prereperfusion) and 60 minutes following reperfusion (postreperfusion; Fig. 1A). A 2- to 3-mm portion of the needle biopsy liver cylinder was immediately snap-frozen in liquid nitrogen and transferred to an −80°C freezer. The remaining cylinder was formalin fixed and paraffin embedded.

LIVER TISSUE RNA EXTRACTION AND PROCESSING

For total RNA extraction, cryopreserved liver tissue samples were homogenized in TRIzol reagent (Invitrogen) using a pestle and nuclease-free 1.5-mL reaction tubes (Ambion Inc, Foster City, CA). Total RNA was then extracted following the manufacturer's guidelines, and quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

LIVER TISSUE MICROARRAY GENE EXPRESSION ANALYSIS

Microarray data were analyzed using Illumina microarrays: Expression data were computed using BeadStudio data analysis software (Illumina Inc, San Diego, CA) and subsequently processed utilizing quantile normalization using the Lumi bioconductor package. Next, a step of conservative probe-filtering was used to excluding those probes with a detection *P* value >0.05 in all samples, which resulted in the selection of a total of 27,084 probes from the original set of 29,377. Differential expression was assessed by using linear models and empirical Bayes moderated t-statistics. Linear Models for Microarray Analysis (LIMMA) R-package software was used for analysis of gene expression microarray data. Group comparisons and determinations of false discovery rates (FDRs; computation using Benjamini-Hochberg procedure) were performed.[13] All liver tissue microarray data discussed in this publication have been deposited

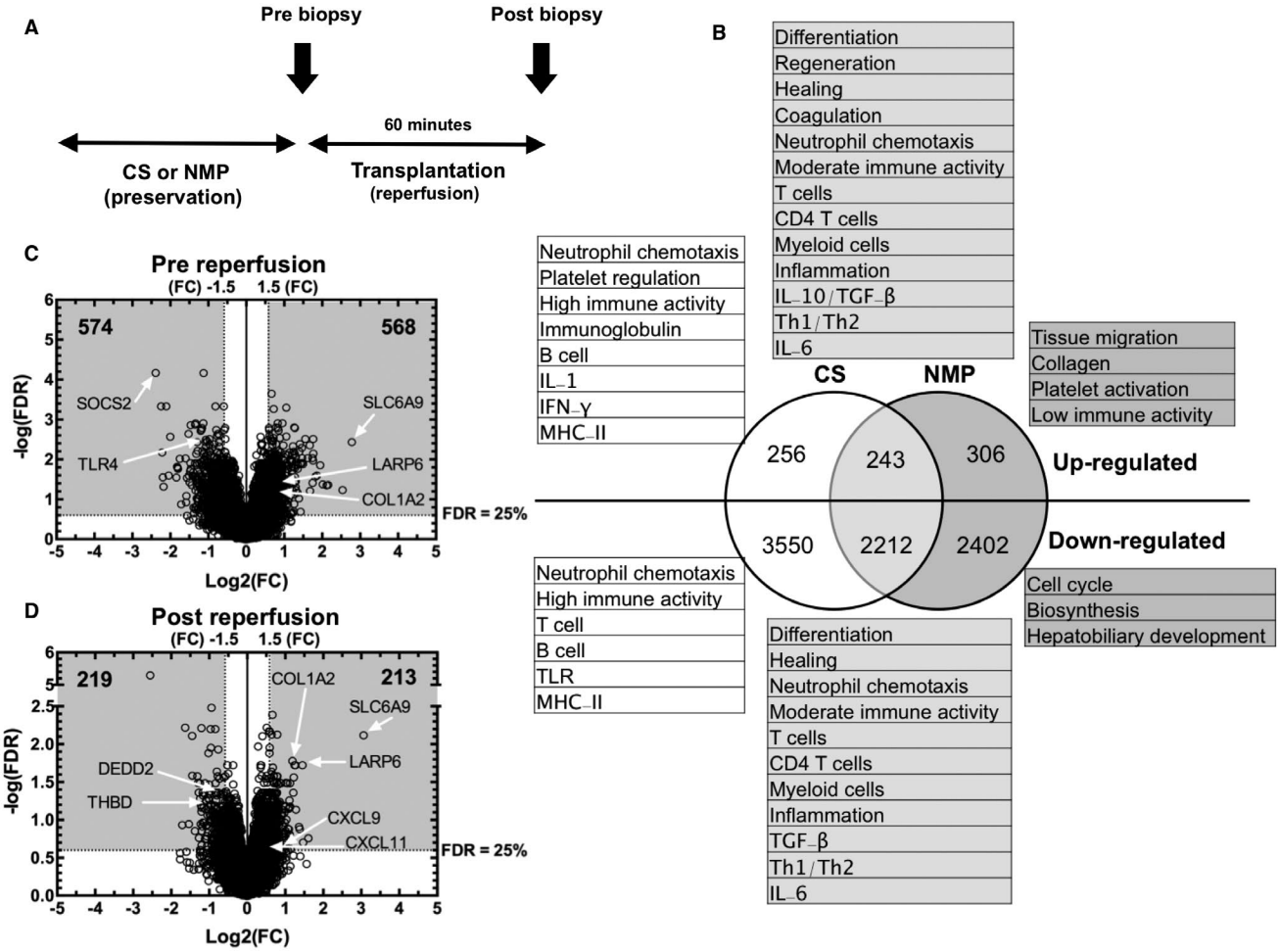

FIG. 1. Microarray showing gene expression profiles of NMP liver grafts compared to CS grafts indicating reduced inflammation and increased regeneration. (A) Schematic diagram showing the timing of liver-tissue biopsies; the first time point was after a period of preservation by either CS or NMP right before reperfusion in the donor (prebiopsy), and the second time point was 60 minutes after reperfusion (postbiopsy). (B) Venn diagram showing the changes in gene expression (up- and down-regulated) in postreperfusion relative to prereperfusion biopsies, as analyzed by the R/Bioconductor package GO stats. In both NMP and CS, numbers of genes significantly altered were displayed along with a list of the functional categories most represented by these genes. (C,D) Volcano plots showing the changes in expression (up and down) of all the genes obtained by microarray analysis, in NMP liver grafts relative to CS liver grafts at the prereperfusion stage (C) and postreperfusion stage (D). Gray area denotes significantly changed genes, the number of those genes was displayed, and examples were displayed with arrowed labels. Significance was decided by a cutoff of 1.5-fold change and of <25% FDR. Abbreviations: COL1A2, collagen type I alpha 2 chain; DEDD2, death effector domain-containing 2; FC, fold change; LARP6, La ribonucleoprotein domain family member 6; MHC-II, major histocompatibility complex class II; TLR, Toll-like receptor.

in NCBI's Gene Expression Omnibus (GSE112713). To further explore the functional relationships between groups, we dissected the molecular pathways contained in the microarray differential gene expression data set utilizing both gene set enrichment analysis (GSEA) using canonical pathways C2 gene-set collection.[14]

qPCR ON LIVER TISSUE

qPCR was conducted using the ABI 7900 Sequence Detection System and TaqMan LDA microfluidic plates (Applied Biosystems, Foster City, CA). DNA was removed from total RNA preparations using Turbo DNA-free DNAse treatment (Ambion), and RNA was

then reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To quantify transcript levels, target gene cycle threshold (Ct) values were normalized to the housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase) to generate ΔCt values. Eighty-eight genes were analyzed in total.

IMMUNOHISTOCHEMISTRY STAINING AND HISTOLOGICAL IMAGING

Prereperfusion and postreperfusion biopsies were fixed in 10% buffered formalin, paraffin embedded, then cut into 4-μm-thick sections fixed to microscope slides. Tissue sections were stained with routine hematoxylin and eosin (H&E), as well as immunohistochemical (IHC) staining for myeloperoxidase (MPO), using a fully automated IHC Leica BOND-MAX immunostainer (Leica Biosystems, Newcastle, UK), and each biopsy was scored using standard criteria (see Supporting Information S1). The signal was detected with a Leica Bond Polymer Refine Detection kit (Novocastra, Newcastle, UK). The antibodies used were obtained from Abcam Plc (Cambridge, UK).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA). Data are presented as mean values ± SEM, and statistical significance was determined using the Mann-Whitney U test when comparing two unpaired/unmatched groups; the Kruskal-Wallis test when comparing multiple unpaired/unmatched groups with post-hoc Dunns multiple comparison tests; and the two-way analysis of variance (ANOVA; ordinary) with Sidak's multiple comparisons test when comparing multiple unmatched groups with more than one grouping variable. All tests carried out were nonparametric. Statistical significance was considered when: $^{*}P \leq 0.05$.

Results

CLINICAL DATA SHOWED REDUCED IRI

Consistent with the clinical data derived from two clinical trials,[10,11] peak AST within 7 days was significantly lower in the NMP group compared to the CS group ($P < 0.01$; Table 2). Additionally, peak international normalized ratio (INR) within 7 days was lower in NMP group compared to CS group ($P = 0.07$). The other biochemical postoperative parameters that include alkaline phosphatase (ALP) and total bilirubin together with post-transplant clinical parameters, such as the days of ITU stay, the rates of acute rejection, and 1-year graft and recipient survival, which were comparable between the two groups. The data are shown in Tables 1 and 2.

NMP UP-REGULATED TISSUE REGENARATION GENES AND DOWN-REGULATED PRO-INFLAMMATORY GENES

In order to gain a global picture of how NMP protects hepatocytes and maintains allograft function, we assessed the gene expression profiles of NMP (n = 11) and CS (n = 11) liver biopsies taken after preservation (prereperfusion) and after transplantation (postreperfusion) by using microarray analysis (Fig. 1A).

A total of 19,416 genes were assessed with a cut-off for significance of 1.5-fold change and of <25% FDR. We assessed the gene expression changes that occurred from prereperfusion to postreperfusion for both NMP and CS liver tissues; we also assessed the unique genes changed in each cohort and the changes common to both. For both NMP and CS, there were more genes down-regulated (CS, 3,550; NMP, 2,402; both, 2,212) than up-regulated post-reperfusion (CS, 256; NMP, 306; both, 243). Among those 306 up-regulated genes in NMP, there are genes that relate to tissue regeneration and platelet function, but not many genes which relate to immune cell functions. In contrast, there was a high representation of immune-related genes within the 256 up-regulated in CS, in particular proinflammatory cytokines and genes involved in humoral immunity, neutrophil chemotaxis, and platelet function. A similar functional representation was noted for down-regulated genes in the 2,402 NMP genes and the 3,550 CS genes. The genes that commonly changed in both NMP and CS contained a moderate number of immune-related genes; both pro- and anti-inflammatory, neutrophil- and coagulation-related genes, and those involved in tissue regeneration (Fig. 1B).

We then investigated the differences in gene expression between NMP and CS at the time of prereperfusion and postreperfusion. The differences were greater at the prereperfusion stage (568 up- and 574 down-regulated genes in NMP relative to CS) compared to those in postreperfusion stage (213 up- and 219 down-regulated genes in NMP relative to CS; Fig. 1C,D). To investigate what biological pathways are altered because of these changes in gene expression, we analyzed the gene data using GSEA canonical pathway analysis using a cut-off for significance of <25% FDR. NMP showed a down-regulation of 123 pathways prereperfusion and 74 pathways postreperfusion relative to CS (see Supporting Information S2 and S3). Pathways down-regulated in prereperfusion included "allograft rejection," "graft versus host disease," seven platelet/coagulation pathways, and 16 immune pathways, including TH1TH2, IFNG signaling, programmed cell death protein 1 (PD1) signaling, IL-2, IL-12, IL-6, and C-C chemokine receptor type 5. Pathways down-regulated in postreperfusion included three platelet/coagulation pathways and 12 immune pathways, including IL-17, neutrophils, IFNFLAM, B-cell antigen receptor, integrin 2, leukocyte transendothelial migration, and PD1 signaling.

In order to validate our findings from the microarray analysis, we performed qPCR on 88 genes that were either up- or down-regulated in NMP livers relative to CS livers, including genes involved in stress response, growth and regeneration, apoptosis, metabolism, and immune responses.

We also investigated a number of IRI-related genes described by us.[5] The majority of genes showed a clear differential expression between livers that were maintained by NMP and those stored using CS. A number of genes involved in response to stress and cell death or apoptosis were up-regulated in CS, but not in NMP, livers postreperfusion (Fig. 2A), examples of which include the genes for thrombomodulin (THBD) and IFN-γ (IFNG; Fig. 2B). NMP livers showed a significant up-regulation of genes involved in immune-trafficking compared to CS in both prereperfusion and postreperfusion stages; such genes included chemokine (C-X-C motif) ligand (CXCL)9, CXCL10, and CXCL11 (Fig. 2B).

$CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ REGULATORY T CELLS WERE MORE ABUNDANT IN NMP GRAFTS

We have previously shown that intrahepatic lymphocytes obtained through liver perfusion are representative of liver-resident lymphocytes.[12] We assessed the impact of NMP on the proportions of HMCs compared to CS using flow cytometric analysis of liver perfusates collected after each respective preservation (prereperfusion; Fig. 1A).

Viability of HMC from both NMP and CS liver grafts was constantly higher than 95%. Approximately 9 to 50 × 10^6 living HMCs were isolated from NMP liver grafts (n = 8) and 40 to 60 × 10^6 from CS grafts (n = 21).

The proportion of hepatic $CD3^{neg}CD19^{pos}$ B cells, $CD3^{pos}CD4^{pos}$ T cells, and $CD3^{pos}CD56^{pos}$ natural killer (NK) T (NKT)-like cells were comparable among NMP and CS grafts. The proportion of $CD3^{neg}CD56^{pos}$ NK cells tended to be higher in CS than in NMP grafts (P = 0.08; Fig. 3A,B). By contrast, the proportion of $CD3^{pos}CD8^{pos}$ T cells (41.97% ± 16.03 vs. 18.6% ± 8.2; P = 0.0005; Fig. 3B) and $CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ regulatory T cells (Tregs; 4.36% ± 3.27 vs. 1.9% ± 1.8; P = 0.0156) were significantly higher in NMP than in CS liver grafts (Fig. 3C).

NMP GRAFTS CONTAINED FEWER PROINFLAMMATORY CYTOKINE-PRODUCING T CELLS THAN CS GRAFTS

Intrahepatic T cells are a rich source of T helper (Th) and regulatory cytokines; chemical mediators capable of promoting inflammation, cellular regeneration, fibrosis, or immune tolerance. Using flow cytometry, we next assessed the effect of NMP on Th1 (IL-2, IL-17, and IFN-γ), Th2 (IL-4), and Treg (IL-10 and transforming growth factor beta [TGF-β]) cytokine production by intrahepatic $CD4^{pos}$ and $CD8^{pos}$ T cells (Fig. 3D,E). NMP had significantly lower proportions of $CD4^{pos}$ T cells producing IL-4 (P < 0.05), IL-2 (P < 0.001), IFN-γ (P < 0.05), and IL-17 (P < 0.0001). There was no change in production of the regulatory cytokines, IL-10 (Fig. 3E) or TGF-β (data not shown). Furthermore, NMP

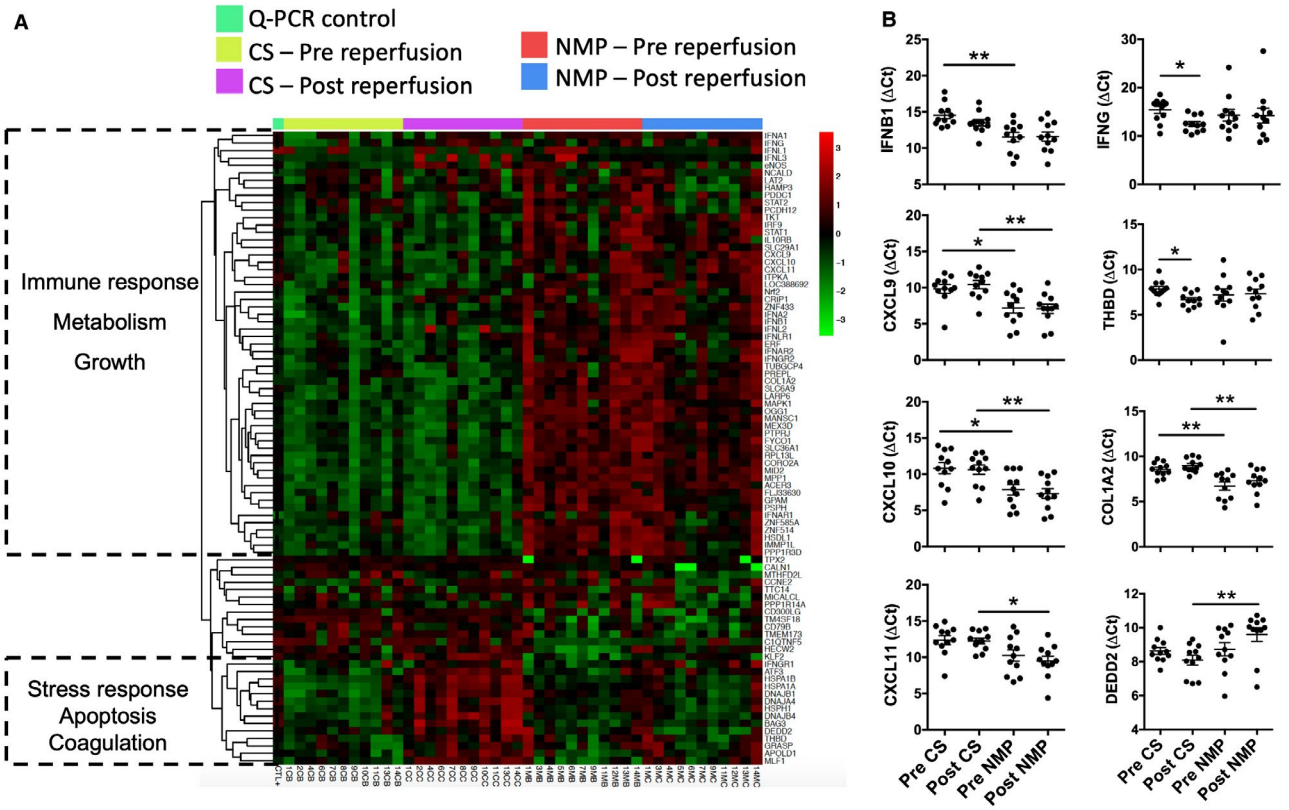

FIG. 2. qPCR confirming gene expression findings by microarray. (A) Heatmap showing expression levels of 88 genes, tested by qPCR from NMP and CS liver biopsies taken prereperfusion and postreperfusion (11 cases per group), as well as a qPCR assay control. Red denotes up-regulated expression, and green is down-regulated expression; genes grouped by function were outlined by dashed lines. (B) ΔCt values (inversely proportional to gene levels) for eight genes in liver biopsies from: pre-CS and pre-NMP (prereperfusion), post-CS and post-NMP (postreperfusion); groups were compared using the Kruskal-Wallis test (*$P \leq 0.05$ and **$P \leq 0.01$). Abbreviations: COL1A2, collagen type I alpha 2 chain; DEDD2, death effector domain-containing 2.

significantly decreased the proportions of $CD8^{pos}$ T cells producing IFN-γ ($P < 0.001$), while modest reductions in IL-17 were also observed. There were no changes in IL-2 production by $CD8^{pos}$ T cells after NMP (Fig. 3E).

DONOR INTENSIVE CARE UNIT STAY INFLUENCED T-CELL FUNCTION IN NMP GRAFTS

We then sought the associations between HMC function and allograft functions. Among HMCs collected after NMP, there was a positive correlation between days of donor intensive care unit (ICU) stay and frequency of IL-17–producing $CD4^{pos}$ T cells ($R^2 = 0.69$; $P = 0.01$; Fig. 3F). There was no correlation between donor infection status, body mass index (BMI), and/or fatty liver score and HMC functions.

COLD ISCHEMIA TIME WAS THE MAIN FACTOR INFLUENCING ALLOGRAFT FUNCTION

In the setting of CS, there was a positive correlation between peak aspartate aminotransferase (AST) levels and duration of cold ischemia ($R^2 = 0.34$; $P = 0.0071$), and a negative correlation between IL-10 producing $CD4^{pos}$ T cells and duration of cold ischemia ($R^2 = 0.56$; $P = 0.01$; Fig. 3F).

Although we did not observe a correlation between frequency of IFN-γ–producing $CD8^{pos}$ T cells and peak AST levels as reported by us in 2015 for CS grafts, after combining the CS and NMP cases, there was a positive correlation between the frequency of IFN-γ–producing $CD8^{pos}$ T cells and peak AST levels ($R^2 = 0.4$; $P < 0.05$; data not shown). We did not observe any correlations

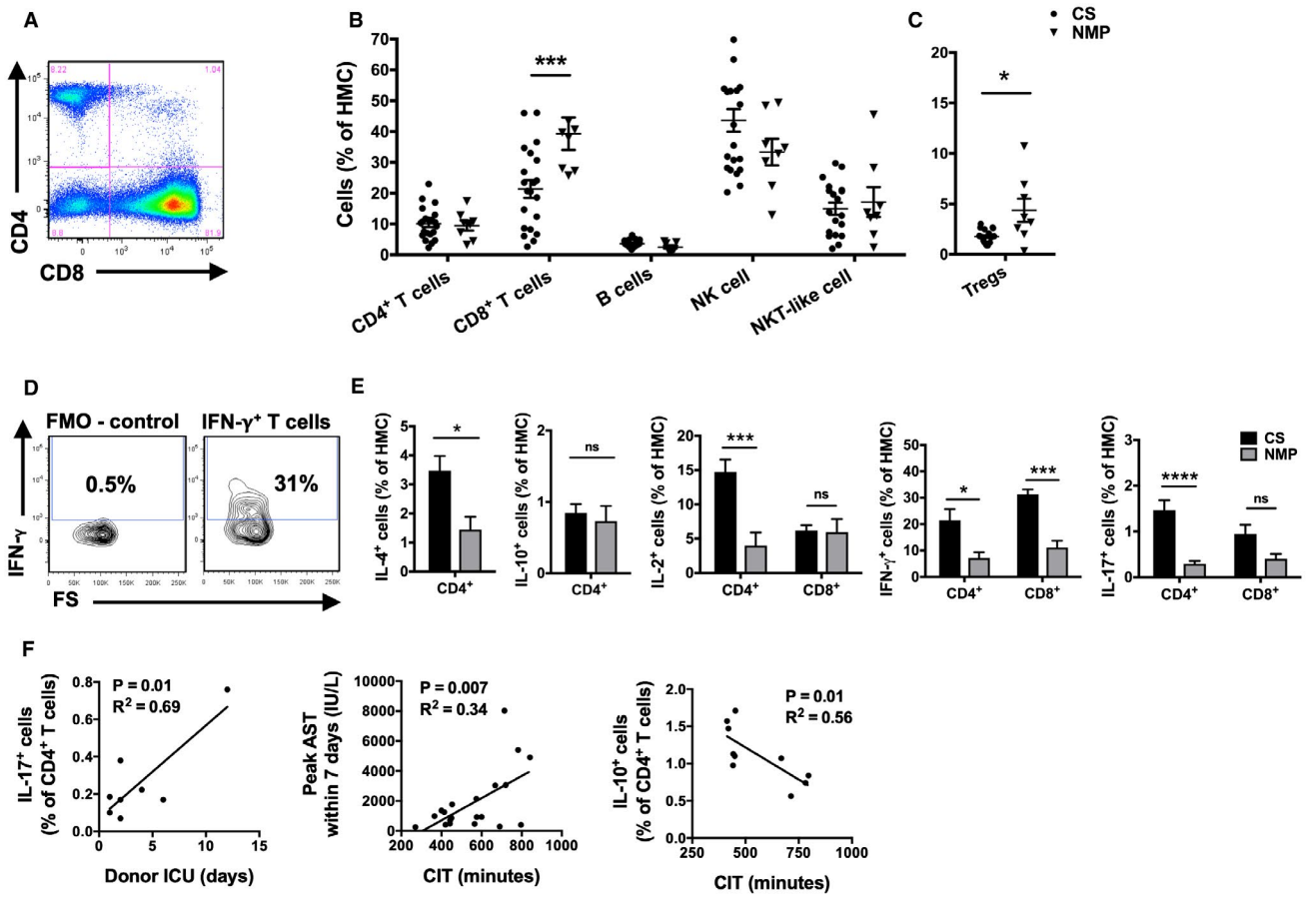

FIG. 3. Hepatic lymphocytes from NMP liver grafts exhibiting an anti-inflammatory phenotype. (A) Representative flow cytometry density plot showing T-cell markers of CD4 and CD8 expression on T cells from HMCs. Frequencies of $CD4^{pos}$ T, $CD8^{pos}$ T, B, NK, NKT-like cells (B), and Tregs (C) within HMCs from NMP and CS livers prereperfusion. (D) Representative flow cytometry contour plot showing IFN-γ expression by T cell from HMCs (right) and fluorescence-minus-one (FMO) control with HMCs stained with all antibodies except anti-IFN-γ. (E) Frequencies of IL-4–, IL-10–, IL-2–, IFN-γ–, and IL-17–producing $CD4^{pos}$ and $CD8^{pos}$ T cells within HMCs from NMP and CS livers. (F) Linear regression analysis from left to right showing the positive correlation between the percentages of IL-17–producing $CD4^{pos}$ T cells in NMP livers and the days of donor staying in ICU; a positive correlation between peak levels of recipient aspartate aminotransferase (AST) and cold ischemic time (CIT); and a negative correlation between percentages of IL-10–producing $CD4^{pos}$ T cells in CS livers and CIT. Groups were compared using two-way ANOVA with Sidak's multiple comparisons test (*$P \leq 0.05$ and ***$P \leq 0.001$). Abbreviation: FS, forward scatter.

with donor parameters, including age, BMI, days of ICU stay, infection status, and fatty liver score.

REDUCED NEUTROPHIL INFILTRATION AND CELL DEATH FOLLOWING NMP

By performing tissue staining on biopsies collected prereperfusion and postreperfusion, we sought evidence to support the hypothesis that reduced IRI is the consequence of NMP (H&E staining not shown), given that both prereperfusion and postreperfusion biopsies showed fewer apoptotic/necrotic cells in NMP compared to CS, although apoptosis/necrosis was increased in postreperfusion biopsies in both NMP and CS conditions compared to prereperfusion (Figs. 4A,B and 5A).

Number of neutrophil clusters tended to be lower in prereperfusion compared with postreperfusion biopsies in both NMP and CS (Fig. 5B). However, NMP livers showed lower numbers of clusters compared to CS livers in both prereperfusion and postreperfusion stages (Fig. 5B).

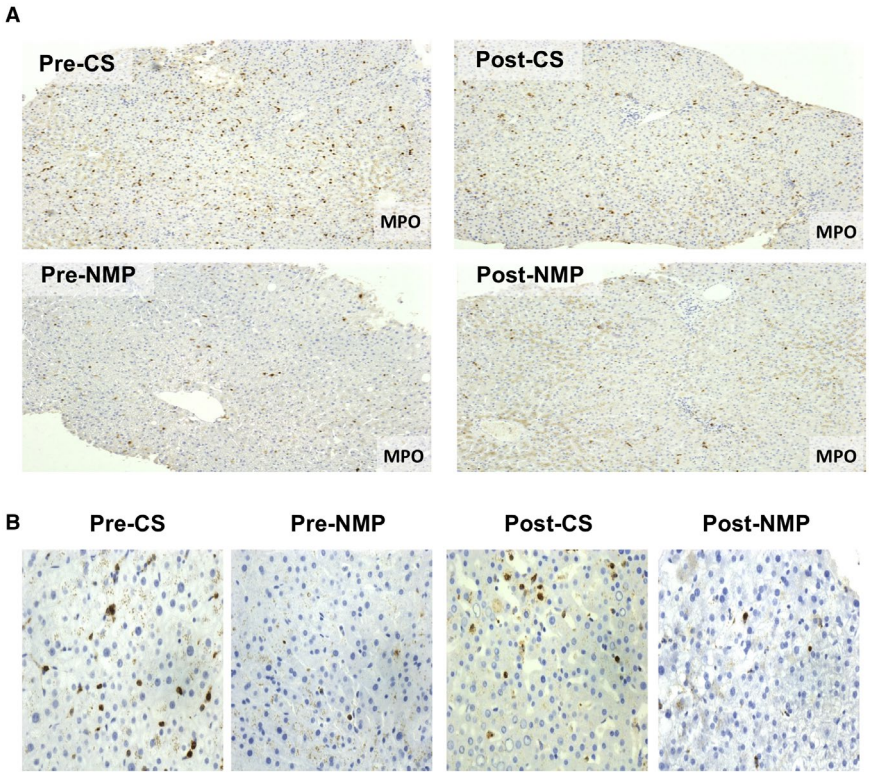

FIG. 4. Reduced immune cell infiltrates in NMP liver tissue. Representative micrographs of MPO (A) stained biopsies from NMP and CS liver grafts at prereperfusion and postreperfusion stages, showing more disseminated immune cell infiltrate in CS compared to NMP livers, which is identified as neutrophils by MPO IHC staining. Neutrophil infiltration in hepatic lobules was reduced in NMP liver grafts. Representative micrographs showing MPO staining in hepatic lobules (B) of biopsies from NMP and CS liver grafts at prereperfusion and postreperfusion stages. High-power fields were randomly selected for each case from the lobular region.

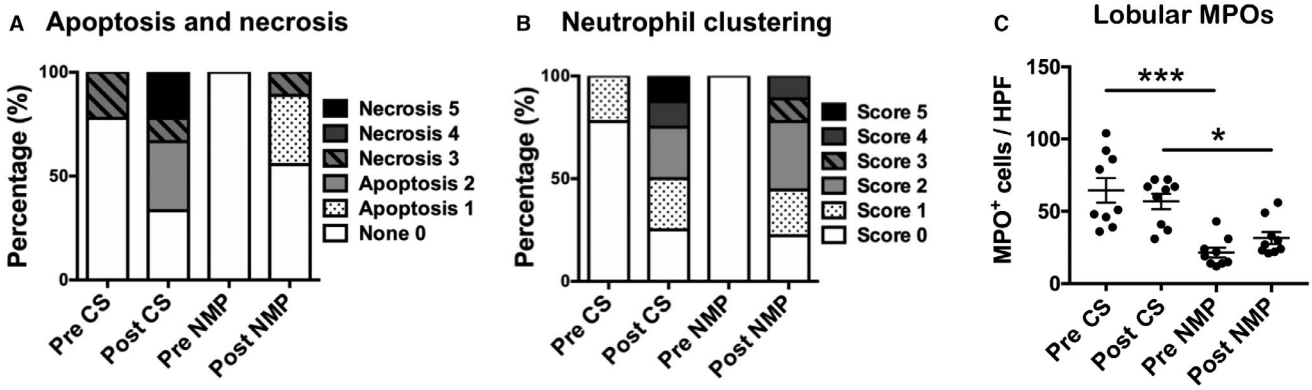

FIG. 5. IRI was reduced in NMP liver grafts compared to CS. (A) Percentage of cases with cell death, apoptosis (score 1 or 2), and necrosis (score 3, 4, or 5) from NMP and CS liver grafts at both prereperfusion and postreperfusion stages. (B) Percentage of cases with absence (score 0) or presence of neutrophil clustering (score 1-5) from NMP and CS grafts at both prereperfusion and postreperfusion stages. (C) Proportions of MPO^{pos} cells in the hepatic lobule from NMP and CS grafts at both prereperfusion and postreperfusion stages. Groups were compared using the Kruskal-Wallis test (*$P \leq 0.05$; ***$P \leq 0.001$). Abbreviation: HPF, high-power field.

We also evaluated neutrophil infiltrate by MPO single-epitope staining. The NMP group had significantly fewer MPO^{pos} cells in liver lobules, in both prereperfusion (P = 0.0002) and postreperfusion (P = 0.036) reperfusion biopsies compared to CS allografts, although reduction of MPO^{pos} cell staining was more pronounced at the prereperfusion stage (Fig. 5C).

STEATOSIS STATUS DID NOT CHANGE AFTER NMP

Finally, we assessed impact of NMP on fatty content of the liver grafts. We did not observe significant differences in terms of steatosis, both small and large droplet (data not shown), between prereperfusion and postreperfusion biopsies.

Discussion

NMP has emerged as an effective tool for liver graft preservation because it has the ability of avoiding cold ischemia and reducing IRI sequelae, as shown in a phase I clinical trial[10] and a recent randomized multicentre controlled clinical trial.[11] It is assumed that the beneficial effect of NMP is the consequence of a combination of factors, including perfusion at body temperature, oxygenation, and addition of nutrients and antioxidants, which maintain the liver as closely as possible to physiological conditions. In the current study, we showed that the beneficial effect of NMP was derived from the combination of the following: maintaining liver metabolism, inhibiting inflammation, and reducing cell death.

We have confirmed that NMP did have an impact on graft quality by reducing IRI sequelae in liver grafts, in the form of lower peak AST levels in the first postoperative week compared to those receiving CS grafts. AST is a well-defined surrogate marker for long-term graft function and survival.[15] This is consistent with the previously reported data,[10,11] confirming that NMP is indeed able to reduce the degree of IRI. Although the impact of NMP on 1-year graft and patient survival was comparable between NMP and CS groups, a longer period of follow-up would be more desirable.

By collecting liver tissues at two key time points, prereperfusion (post-NMP) and postreperfusion, we assessed gene expressing patterns in three ways: (1) differences occurred from prereperfusion to postreperfusion for both NMP and CS liver tissues; (2) differences between NMP and CS at prereperfusion and postreperfusion; (3) the unique genes changed in each cohort and the changes common to both.

When NMP and CS were assessed separately in terms of the gene expression changes that occurred postreperfusion relative to prereperfusion, NMP liver showed significant gene activity in areas of growth, metabolism, and tissue repair, whereas CS livers showed more immune-related gene activity postreperfusion. In order to directly compare NMP to CS, the differences in gene-expression profiles were assessed in both prereperfusion and postreperfusion biopsies. NMP showed the greatest difference from CS at the prereperfusion stage, in particular, the down-regulation of pathways such as allograft rejection, graft versus host disease, and platelet/coagulation; we believe that the alterations in these pathways possibly contributed to the beneficial effect of NMP to minimize IRI. It is worth pointing out that some of the pathways down-regulated in NMP have been defined previously as being involved in inflammation in the setting of transplantation,[16] such as IFNG signaling,[17] IL-2,[18] IL-6, and IL-12.[19] In combination with PD1 signaling,[20] down-regulation of these pathways could suppress initiation of IRI and reduce cell apoptosis. More important, PD1 signaling was continuously down-regulated postreperfusion, along with several others with known roles in IRI, including platelet/coagulation,[21] neutrophils,[22] and IL-17.[23]

In addition, both NMP and CS promoted up-regulation of pathways, including differentiation, regeneration, and healing. Taking together the fact that NMP down-regulated inflammation, the overall impact of NMP should favor preservation of grafts in good quantity by minimizing inflammatory and cell death events associated with IRI.

While confirming the alterations of 88 key genes following NMP by qPCR, we further evaluated expression levels of individual genes (Fig. 2B). Of these genes, the CXC chemokine family genes, CXCL9, CXCL10, and CXCL11, known to elicit their chemotactic functions by interacting with the chemokine receptor, CXCR3,[24] changed their expression in synchrony. Their levels were significantly higher in NMP tissues than in CS at both time points,

although CXCL11's level was higher at postreperfusion only. It is known that IFN-γ induces expression of CXCL9[25]; its up-regulation was not hindered by reduced levels of IFN-γ in NMP grafts. The CXC family was recently shown in the mouse to have a role in converting CD4 T effectors into CD4/CD8 double-negative Tregs and their migration to the liver[26]; hence, this pathway might contribute to reduced IRI.

Next, we tested the hypothesis that inhibited gene expression defined by microarray could be linked to their translation status, that is, cytokine secretion by donor-derived T cells to inhibit inflammation. We collected hepatic lymphocytes at the end of NMP, the same time point when prereperfusion liver biopsies were taken. Therefore, we were able to evaluate the impact of NMP directly on donor hepatic lymphocytes, the major player in graft rejection and tolerance induction[27] by comparing with lymphocytes collected from CS grafts. We observed reduced numbers of proinflammatory cytokines IFN-γ and IL-17 producing $CD4^{pos}$ and $CD8^{pos}$ T cells, which are known to be involved in the process of IRI.[28,29] In conjunction with reduced AST levels in recipients, it is logical to speculate that down-regulated pathways IFNG signaling and IL-17 contribute to the overall impact of NMP in reducing IRI. The other possibility is that the altered ratio of Tregs to T effectors provides a counterbalance for the tissue-damaging effects of IL-17 producing T effectors in NMP grafts. This assumption is based on an unexpected finding that $CD4^{pos}CD25^{high}CD127^{neg}$ $FOXP3^{pos}$ Tregs pool was enlarged following NMP. In NMP liver grafts, Tregs were approximately 2.3-folds compared to those in CS livers, indicating that preservation using NMP was able to retain or even increase the Treg number. It is well recognized that Tregs could reduce IRI.[30] In view of the fact that the liver contains approximately 10^{10} lymphocytes, of which two thirds are T cells, and donor derived lymphocytes are constantly released into the recipient's circulation and could still be detected even 30 years after engraftment,[31] donor-derived Tregs are likely to be involved in the process of induction of graft acceptance. Comparing the attempt of generating *ex vivo* Tregs from recipients pre-LT, with target number of 10×10^6/kg recipient body weight (The ONE study UK Treg trial: https://clinicaltrials.gov/ct2/ show/NCT02129881), donor-derived Tregs outnumber those derived from the recipient. Once the outcome of this trial is released, further comparison of the efficacy of Tregs derived from either donors or recipients can be carried out.

Regarding the immunoregulatory cytokine, IL-10, we observed a negative correlation between frequency of IL-10–producing $CD4^{pos}$ T cells and cold ischemia time, indicating that CS could influence HMC immunoregulatory function in contrast with NMP, which has the potential to enhance production of IL-10 through an enlarged Treg pool.

The other important finding is that down-regulated genes in neutrophils pathways did lead to a significant reduction in neutrophil infiltration in NMP grafts. This reduction was observed in both prereperfusion and postreperfusion liver tissues. Given the important role played by platelets on IRI and inflammation,[32] we believe that the reduction in platelet/coagulation gene expression may have contributed to the reduced IRI in NMP recipients, and we intend to investigate this further in future studies.

Attenuation of neutrophil infiltration in prereperfusion biopsies in NMP livers is of interest. Leucocyte infiltration in DBD donors is well documented given that results of systemic inflammatory response associate with brain death.[33] It is possible that NMP reduces neutrophil levels by the fact that there is circulation during the preservation time and possibly to an anti-inflammatory hepatic environment, discussed earlier. More work is necessary to elucidate neutrophil movements during NMP and its impact on graft outcome.

Research in recent years has provided rich data to elucidate the interaction between platelets and neutrophils,[34] in the context of LT, by forming complex platelets and neutrophils jointly drive IRI in the liver.[35] Therefore, reducing neutrophil infiltration will lead to controlling the formation of this complex and ameliorating IRI.

One of the key goals of NMP is to improve the quality of marginal liver grafts, which are currently labeled as unusable and rejected by all LT centers because of their high fat content (>60%)[36]; we also assessed whether the fat content could be reduced by NMP. However, our investigation was restricted by the fact that all the liver grafts included in the current study were usable and the majority of them contain very low to no fatty content (Table 1). No change in fat content was noted following NMP. Recently, Liu et al.[37] assessed lipid metabolism and function of discarded human livers with steatosis (up to 33.3%) after 24-hour NMP. When the period of

NMP finished, fatty content was almost unchanged, indicating that NMP is unlikely to impact on liver fat content when it is at an acceptable level and only a high proportion of fat (i.e., >60%) should be treated by NMP and assessed.

In conclusion, the data of the current report have provided mechanistic support to the clinical application of NMP. By minimizing inflammation, cell death, and promoting liver regeneration and healing, NMP might be an ideal tool to rescue those currently discarded marginal allografts, including DCD, and potentially to increase the donor pool.

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