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Original Article

Ischemia-free liver transplantation improves the prognosis of recipients using functionally marginal liver grafts

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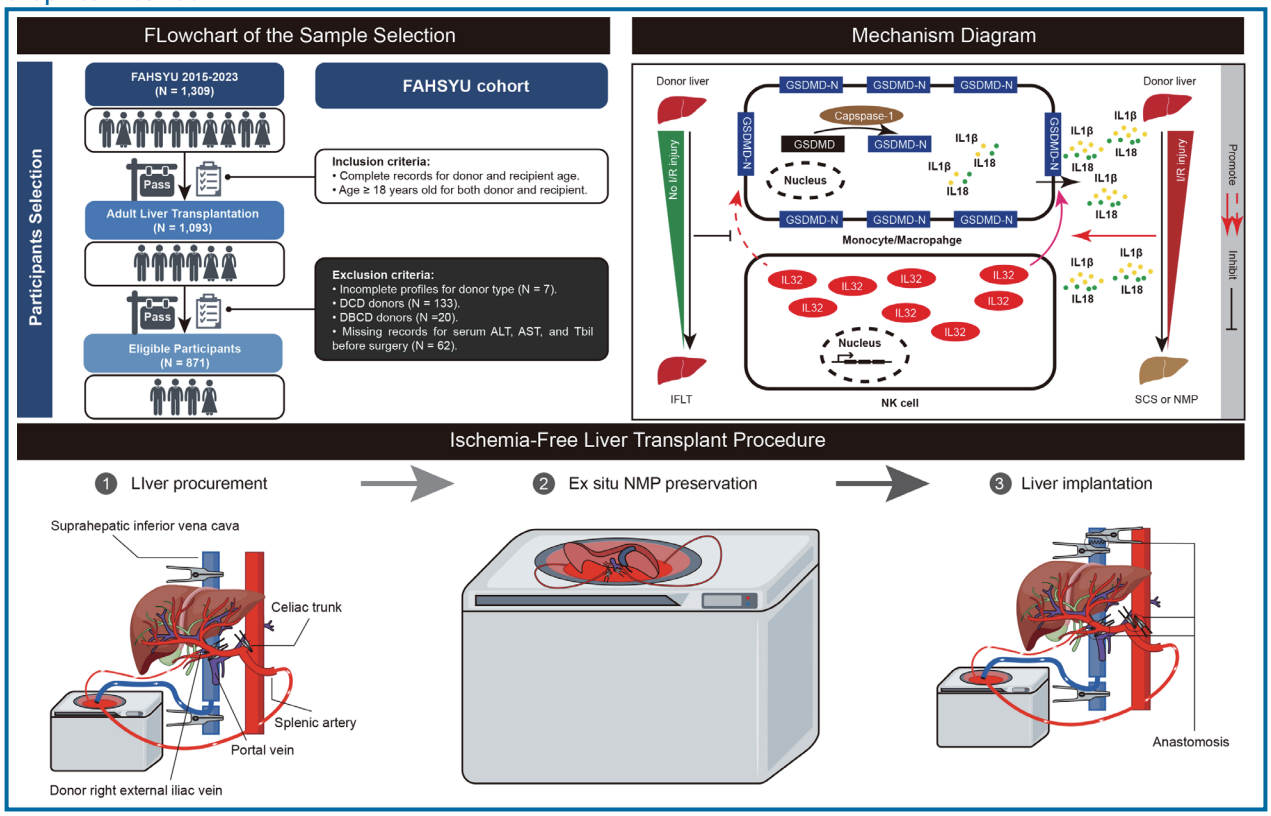
Graphical Abstract



Study Highlights

- It was challenging to balance the benefits and drawbacks of marginal livers in liver transplantation. Functionally marginal liver grafts were associated with worse prognosis than other marginal livers. Ischemia-free liver transplantation can significantly alleviate liver injury via inhibiting the infiltration of NK cells and pyroptosis level, which contributed to a better clinical benefit. This provided us a novel direction when addressing the marginal liver issue.

Background/Aims: The shortage of donor liver hinders the development of liver transplantation. This study aimed to clarify the poor outcomes of functionally marginal liver grafts (FMLs) and provide evidence for the improvement of ischemia-free liver transplantation (IFLT) after FML transplantation.

Methods: Propensity score matching was used to control for confounding factors. The outcomes of the control group and FML group were compared to demonstrate the negative impact of FMLs on liver transplantation patients. We compared the clinical improvements of the different surgical types. To elucidate the underlying mechanism, we conducted bioinformatic analysis based on transcriptome and single-cell profiles.

Results: FMLs had a significantly greater hazard ratio (HR: 1.969, P=0.018) than did other marginal livers. A worse 90-day survival (Mortality: 12.3% vs. 5.0%, P=0.007) was observed in patients who underwent FML transplantation. Patients who received FMLs had a significant improvement in overall survival after IFLT (Mortality: 10.4% vs 31.3%, P=0.006). Pyroptosis and inflammation were inhibited in patients who underwent IFLT. The infiltration of natural killer cells was lower in liver grafts from these patients. Bulk transcriptome profiles revealed a positive relationship between IL-32 and Caspase 1 (R=0.73, P=0.01) and between IL-32 and Gasdermin D (R=0.84, P=0.0012).

Conclusions: FML is a more important negative prognostic parameter than other marginal liver parameters. IFLT might ameliorate liver injury in FMLs by inhibiting the infiltration of NK cells, consequently leading to the abortion of IL-32, which drives pyroptosis in monocytes and macrophages. **(Clin Mol Hepatol 2024;30:421-435)**

Keywords: Marginal liver grafts; Liver transplantation; Ischemia-free liver transplantation; Static cold storage; Normothermic machine perfusion; Transplantation immunology

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Abbreviations:

FMLs, functionally marginal liver grafts; IFLT, ischemia-free liver transplantation; PSM, propensity score matching; SCS, static cold storage; NMP, normothermic machine perfusion; HMP, hypothermic machine perfusion; EAD, early allograft dysfunction; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Tbil, total bilirubin; NK Cells, natural killer cells; IL-32, interleukin 32; CASP1, caspase 1; IL1B, interleukin 1 beta; IL18, interleukin 18; GSDMD, gasdermin D; ECDs, extended criteria donors; ICU, intensive care unit; BMI, body mass index; PNF, primary nonfunction; EAD, early allograft disfunction; ITBLs, ischemic-type biliary lesions; GGT, gamma-glutamyl transpeptidase; HCC, hepatocellular carcinoma; DCD, donation after cardiac death; DBCD, donation after brain death followed by circulatory death; HBV, hepatic B virus; MELD Score, model for end-stage liver disease score; AKI, acute kidney injury; GSVA, gene set variation analysis; CLT, conventional liver transplantation; EP, end of preservation; PR, post-graft revascularization; SNPs, single nucleotide polymorphisms; GWAS, genome-wide association studies; scRNAseq, single-cell RNA-sequencing; PP, pre-procurement; ssGSEA, single-sample gene set enrichment analysis; IL-27, interleukin 27; I/R, ischemia-reperfusion; IL-2RB, interleukin 2 receptor subunit beta; TNF-α, tumor necrosis factor-alpha

INTRODUCTION

Liver transplantation is a life-saving treatment for patients with end-stage liver disease, such as cirrhosis, liver failure, and hepatocellular carcinoma (HCC). An elevated burden of liver disease leads to an increased demand for liver transplantation. In 2021, 34,944 liver transplantations were performed globally.[1] However, organ shortage poses an obstacle to the development of liver transplantation. The mortality rate of patients on the waiting list has continued to increase since 2009.[2] Therefore, surgeons and clinical researchers have attempted to extend this donor pool.

In the liver transplantation field, extended-criteria donors act as "double-edged swords." According to a questionnaire survey from 35 different centers, extended criteria donors (ECDs) are defined as steatosis, age up to 80 years, serum sodium >165 mmol/L, serum alanine aminotransferase (ALT) >105 U/L, intensive care unit (ICU) stay with ventilation >7 days, body mass index (BMI) >30, serum aspartate transaminase (AST) >90 U/L, and total bilirubin (Tbil) >3 mg/dL.[3] With donations after cardiac death, advanced age, and hepatic B virus (HBV)-infected patients assumed to be eligible donors, the number of liver transplantations increases annually.[4] However, recipients with ECD livers have a greater incidence of primary nonfunction (PNF), early allograft dysfunction (EAD), and ischemic-type biliary lesions.[5] Donors with hypernatremia, advanced age, or steatosis have been shown to have increased mortality due to liver transplantation.[6,7] However, the prognosis of donors with high serum ALT, AST, and Tbil levels has not yet been clarified. Accordingly, functionally marginal liver grafts (FMLs) are defined as those with ALT >105 U/L, AST >90 U/L, or Tbil >3 mg/dL. Therefore, two questions need to be answered: What are the consequences of FML usage, and how can we alleviate liver injury during the FML transplantation process?

In 2018, a novel and promising technique called ischemia-free liver transplantation (IFLT) was introduced at the First Affiliated Hospital of Sun Yat-sen University.[8] Recently, a randomized controlled trial of IFLT demonstrated that patients could gain greater clinical benefits from IFLT than from conventional liver transplantation. More specifically, there were lower incidences of EAD, postreperfusion syndrome, and non-anastomotic biliary strictures.[9] Moreover, several studies have widened the application of IFLT. Compared with conventional liver transplantation, IFLT reduced postoperative peak AST, gamma-glutamyl transpeptidase, and creatine levels, and steatotic patients achieved better survival with a lower occurrence of EAD (IFLT: 0%, conventional liver transplantation: 60%).[10] Based on transcriptome and metabolome profiles, IFLT significantly abrogated graft ischemia-reperfusion injury and suppressed inflammation.[11] In addition, the incidence of recurrence was low in patients with HCC who underwent IFLT.[12] All these results suggested that IFLT could function as a novel and promising surgery for the transplantation of FMLs, but this needs to be further examined. Here, we investigated the impact of FMLs on liver transplantation outcomes and clarified the effect of IFLT on the prognosis of FMLs from both clinical and molecular perspectives.

MATERIALS AND METHODS

Population

From January 1, 2015, to October 1, 2023, 1,309 patients underwent liver transplantation at the Organ Transplantation Center at the First Affiliated Hospital of Sun Yat-sen University. The grafts during machine perfusion were discarded if the parameters of viability did not reach the VITTAL criteria (lactate ≤2.5 mmol/L and two or more of bile production, pH ≥7.30, glucose metabolism, hepatic arterial flow ≥150 mL/min and portal vein flow ≥500 mL/min, or homogeneous perfusion).[13] All adult liver transplants with both donor and recipient ages ≥18 years (n=1,093) were included in this study. The data of 871 eligible participants were acquired by excluding donations after cardiac death; donation after brain death followed by circulatory death; and individuals with missing information about donor type, serum ALT, AST, and Tbil. We defined FMLs as ALT >105 U/L, AST >90 U/L, or Tbil >3 mg/dL, according to a previous review.[7] Ultimately, 353 FMLs were identified. More specifically, 279, 24, and 50 FML donors underwent surgery using different preservation techniques, such as static cold storage (SCS), normothermic machine perfusion (NMP), and IFLT, respectively.

This study involving humans was approved by the Institutional Ethics Committee for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen Univer-

sity. The studies were conducted in accordance with local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement for written informed consent for participation from the participants or their legal guardians/next of kin due to the retrospective, minimal-risk nature of the study.

Data collection

For the donors, the clinical parameters of sex, age, BMI, diabetes status, hypertension status, HBV infection status, and serum concentrations of sodium, potassium, haemoglobin, Tbil, ALT, AST, creatinine, and steatosis status were collected before the operation. For recipients, clinical characteristics, including sex, age, BMI, model for end-stage liver disease score, diagnosis (decompensated cirrhosis, liver failure, or liver tumor), operation time, blood loss, respiratory support time, ICU stay time, reintubation ratio, duration of resumed diet, PNF, EAD, acute kidney injury (AKI), thrombosis ratio (in the hepatic artery, portal vein, or postcaval vein), biliary fistula, biliary stricture, wound infection, and pulmonary infection, were acquired from our hospital management system. In addition, overall and 90-day survival information was recorded through postoperative follow-up. The ALT, AST, and Tbil levels of the recipients were measured daily, and the data were collected seven days after liver transplantation.

Bioinformatic analysis

Using gene set variation analysis (GSVA), we evaluated the activity of different cell death pathways (PANoptosis, ferroptosis, proptosis, pyroptosis, autophagy, necroptosis, and apoptosis) to determine the mechanism related to liver injury using previously published transcriptome data from six conventional liver transplantation (CLT) and six IFLT samples.[11] The gene lists for PANoptosis and Cuproptosis were acquired from two published reviews,[14,15] whereas other gene lists were downloaded from the Molecular Signatures Database (MSigDB).[16,17] Liver biopsy tissues were collected at the end of preservation (EP), and resting samples were acquired at post-graft revascularization (PR). To determine the gene pattern associated with liver injury, we extracted single nucleotide polymorphisms (SNPs) related to serum ALT, AST, and Tbil levels in both European and Asian populations. With the "twoSampleMR" package,[18] we downloaded the corresponding genome-wide association studies (GWAS) data from the IEU Open GWAS project (https://gwas.mrcieu.ac.uk/).[18] Detailed information regarding these data were provided in Supplementary Table 6. SNPs with a *P*-value <1e-5 were identified as significant liver injury-related SNPs. We then used the g:Profiler tool to map the SNPs to gene names (https://biit.cs.ut.ee/gprofiler/snpense).[19] The corresponding genes were defined as liver injury genes. We further conducted a differential gene expression analysis using previously described transcriptome data. Liver injury-related genes with |logFC| >1 and false discovery rate <0.05 were identified, and the corresponding expression patterns were shown in the heatmap. In addition, we performed enrichment analysis for both upregulated and downregulated genes using the online enrichment tool Metascape (https://metascape.org/gp/index.html#/main/step1).[20]

To further investigate the relationship between liver injury and the immune system, we reanalyzed single-cell RNA-sequencing (scRNA-seq) data that included liver tissue samples at pre-procurement (PP), EP, and PR. We extracted EP and PR samples for further analysis to ensure consistency with the transcriptome data. The "Single R" package[21] and the online tool CellMaker (http://xteam.xbio.top/CellMarker/)[22] were utilized to define cell types. The expression of the previously described significant liver injury-related genes was detected in different cell types. Using single-sample gene set enrichment analysis (ssGSEA), we used the top 10 DEGs to estimate the proportions of different immune cells in the transcriptome data. The Wilcoxon test was used to compare cell proportions between the SCS and IFLT groups. In addition, we carried out correlation analysis among different immune cells with the package "corrplot."

Statistical analysis

All analyses were performed using R version 4.2.0. Graphs were drawn using both R 4.2.0 and GraphPad Prism software. The package 'gtsummary' was used to compare different groups. We evaluated the outcomes of these groups using a K–M plot. Propensity score matching (PSM) analyses were performed to control for bias. Categorical variables were presented as the frequencies (per-

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centages) and were compared using Pearson's chi-square test and Fisher's exact test. Continuous variables with a normal distribution were presented as the means (standard deviation, SD) and were analysed using a t test and repeated measures analysis of variance. Continuous variables with a non-normal distribution were presented as the medians (interquartile ranges, IQRs) and were analysed using the Wilcoxon test. Statistical significance was set at $P<0.05$.

RESULTS

FMLs contribute to poor prognosis for LT patients

In total, 871 participants were enrolled in this study. The parameters used to define the extended criteria for donor livers were presented in Supplementary Table 1. Multivariate Cox regression analysis was used to compare the impact of different ECD parameters (FMLs, donor liver steatosis, donor BMI, donor serum sodium levels, and donor age) on postoperative 90-day survival. Compared with the other factors, FMLs had a significantly greater hazard ratio (HR: 1.969, $P=0.018$) (Fig. 1A). The baseline characteristics of the normal and FML groups before and after PSM were shown in Supplementary Table 2. The age and BMI of the FML group were lower than those of the control group. There were 32.8% steatotic livers in the FML donors. FML donors had higher serum levels of sodium, ALT, AST, and Tbil. Considering the differences at baseline, we performed PSM analysis to control for confounders: age, BMI, serum sodium level, serum creatine level, liver steatosis status of donors, and primary diagnosis of cirrhosis (Supplementary Table 2). Recipients who received FMLs exhibited a longer ICU stay (40.8 hours vs. 35.8 hours, $P=0.294$); resumed diet time (114 hours vs. 108 hours, $P=0.277$); and had a greater probability of reintubation (11.4% vs. 5.1%, $P=0.076$), PNF (6.6% vs. 3.7%, $P=0.174$), and pulmonary infection (14.6% vs. 7.8%, $P=0.023$) (Table 1). The percentage of deceased patients within 90 days increased from 5.0% to 12.3% ($P=0.007$), and the overall death ratio increased from 23.3% to 30.1% ($P=0.105$). Similarly, a poor survival plot was generated for both overall and 90-day

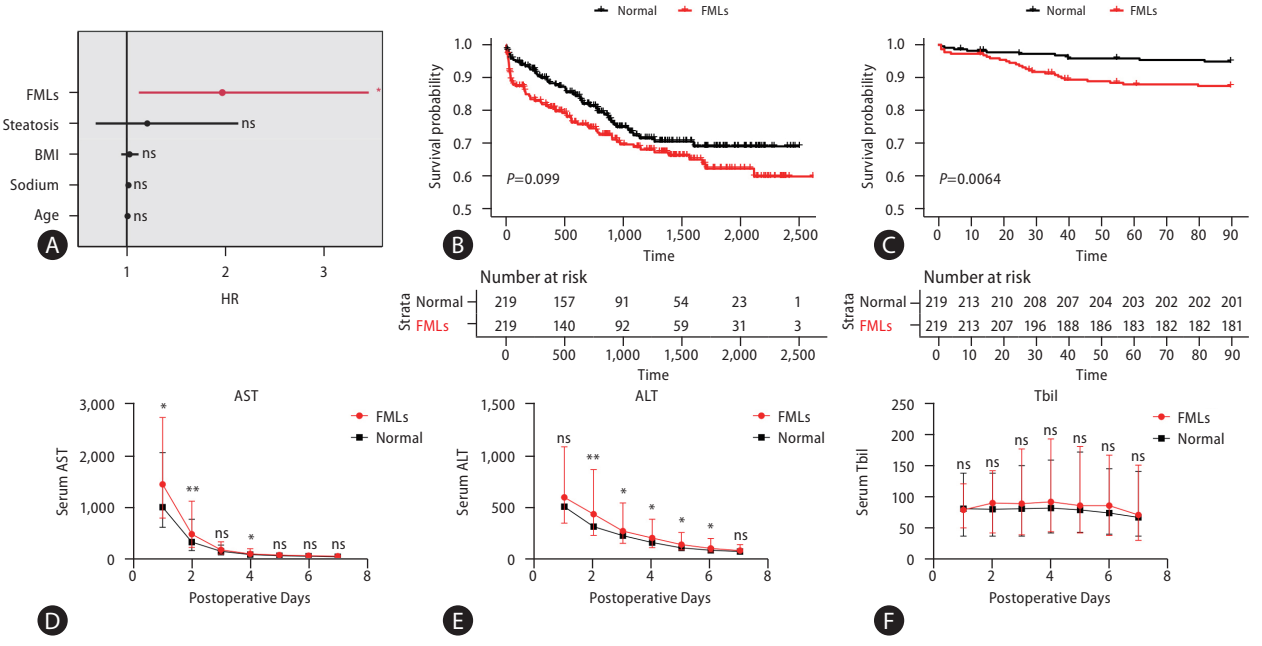

Figure 1. FMLs lead to poor outcomes in liver transplantation patients. (A) The forest plot for multivariate Cox regression analysis including ECD factors, FMLs, steatosis, BMI, serum sodium, and age. K-M plot of overall survival (B) and postoperative 90-day survival (C) between normal controls and FML patients. The serum AST (D), ALT (E), and Tbil (F) levels of patients who received normal livers and FMLs seven days after the operation. FMLs, functionally marginal liver grafts; ECDs, extended criteria donors; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Tbil, total bilirubin. *$P<0.05$, **$P<0.01$.

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Table 1. Different characteristics of prognosis between FMLs and normal

Characteristic	Before PSM			After PSM (1:1)		
	Normal (n=436)	FMLs (n=279)	*P*-value	Normal (n=219)	FMLs (n= 219)	*P*-value
Outcomes						
Respiratory support time (h)‡	18.0 (12.7, 43.3)	17.0 (11.9, 44.5)	0.330	17.0 (12.0, 35.0)	17.0 (11.5, 41.0)	0.900
ICU stay time (h)‡	39.0 (23.0, 91.9)	42.5 (23.0, 89.0)	0.659	35.8 (22.0, 82.0)	40.8 (22.0, 87.9)	0.294
Reintubation (%)*	13.0 (5.9)	16.0 (10.9)	0.086	6.0 (5.1)	14.0 (11.4)	0.076
Resume diet (h)‡	109.5 (82.6, 149.4)	114.0 (85.0, 164.3)	0.412	108.0 (86.0, 137.0)	114.0 (86.0, 173.0)	0.277
HA thrombosis (%)*	16.0 (4.1)	13.0 (5.3)	0.486	5.0 (2.6)	11.0 (5.6)	0.128
PV thrombosis (%)*	9.0 (2.3)	4.0 (1.6)	0.559	4.0 (2.1)	4.0 (2.1)	>0.999
PC thrombosis (%)*	4.0 (1.0)	4.0 (1.6)	0.494	1.0 (0.5)	2.0 (1.0)	>0.999
Biliary fistula (%)*	2.0 (0.5)	5.0 (2.0)	0.115	2.0 (1.0)	2.0 (1.0)	>0.999
Biliary stricture (%)*	13.0 (3.3)	9.0 (3.6)	0.830	7.0 (3.6)	9.0 (4.6)	0.624
PNF (%)*	23.0 (5.4)	15.0 (5.5)	0.930	8.0 (3.7)	14.0 (6.6)	0.174
EAD (%)*	197.0 (45.2)	114.0 (41.2)	0.290	89.0 (40.6)	87.0 (40.1)	0.907
AKI (%)*	19.0 (4.8)	14.0 (5.7)	0.637	11.0 (5.6)	11.0 (5.6)	0.999
Pulmonary infection (%)*	41.0 (9.4)	41.0 (14.7)	0.030	17.0 (7.8)	32.0 (14.6)	0.023
Wound infection (%)*	6.0 (1.4)	5.0 (1.8)	0.758	2.0 (0.9)	11.0 (5.6)	>0.999
Overall mortality (%)*	117.0 (26.9)	80.0 (28.7)	0.604	51.0 (23.3)	66.0 (30.1)	0.105
90-day mortality (%)*	38.0 (8.7)	34.0 (12.2)	0.132	11.0 (5.0)	27.0 (12.3)	0.007

Adjusted variables: donor age, body mass index, serum sodium level, serum creatine level, liver steatosis, and recipient liver cirrhosis. *Categorical variables were exhibited as frequencies (percentages). ‡Continuous variables with non-normal distribution were exhibited as median (interquartile range).
FMLs, functionally marginal liver grafts; PSM, propensity score matching; ICU, intensive care unit; HA, hepatic artery; PV, portal vein; PC, postcaval vein; PNF, primary non function; EAD, early allograft disfunction; AKI, acute kidney injury.

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survival (Fig. 1B and C). The serum levels of ALT, AST, and Tbil of the recipients after the operation were significantly greater in the FML group (Fig. 1D–F).

NMP is insufficient for improving the outcome of FML patients

The baseline parameters of FMLs who underwent NMP and SCS were compared in Supplementary Table 3. There were clear increases in donor age, BMI, ratio of liver decompensated cirrhosis, and liver failure in the NMP group. Therefore, we conducted PSM analysis based on these factors, and the corresponding results were presented in Supplementary Table 4. Patients in the NMP group had a lower AKI ratio (0.0% vs. 4.7%, *P*=0.533) and better overall and 90-day survival rates. The percentage of deceased patients within 90 days decreased from 14.6% to 12.5% (*P*>0.999), whereas the overall percentage of deceased patients decreased from 22.9% to 12.5% (*P*=0.359). However, the respiratory support time (19.5 hours vs. 16.0 hours, *P*=0.388), ICU stay time (57.5 hours vs. 53.5 hours, *P*=0.400), resumed diet time (232 hours vs. 114 hours, *P*=0.076), reintubation ratio (33.3% vs. 23.8%, *P*=0.633), pulmonary infection rate (12.5% vs. 10.4%, *P*>0.999), and EAD rate (69.6% vs. 39.1%, *P*=0.017) were greater in the NMP group. In addition, there was no significant difference in the K–M plot between the NMP and SCS groups (Supplementary Fig. 1A and B). The improvements in ALT and AST levels after surgery were not significant (Supplementary Fig. 1C and D). The serum Tbil levels were greater in the NMP group (Supplementary Fig. 1E).

IFLT significantly improves the prognosis of FML patients

After conducting PSM analysis to adjust for confounders, creatine, and steatosis status (Supplementary Table 5), we compared the effects of SCS and IFLT on the survival of LT patients (Table 2). There were no differences in the baseline characteristics between the two groups. There was a shorter ICU stay (35.3 hours vs. 38.8 hours, *P*=0.151); shorter resumed diet time (86.0 hours vs. 102.1 hours, *P*=0.143); and lower prevalence of PNF (0.0% vs. 6.7%, *P*=0.092), EAD (29.2% vs. 40.6%, *P*=0.179), AKI (5.4% vs. 5.8%, *P*>0.999), reintubation (6.9% vs. 15.8%, *P*=0.320), and pulmonary infection (6.3% vs. 15.6%, *P*=0.109) in IFLT patients. IFLT significantly increased the 90-day survival rate from 88.5% to 95.8% (*P*=0.220) and reduced the overall mortality rate from 31.3% to 10.4% (*P*=0.006). Similarly, the K–M plot revealed a better prognosis for patients who received IFLT (Fig. 2A and B). Postoperative serum ALT, AST, and Tbil levels were distinctly lower in the IFLT group than in the SCS group (Fig. 2C–E).

IFLT alleviates liver injury through anti-cell death and anti-inflammatory effects

Using GSVA, we investigated the effect of IFLT on liver injury from a microscopic perspective. Pyroptosis, autophagy, and necroptosis were inhibited in IFLT patients (Fig. 3A). Pyroptosis was the most significantly altered pathway in the IFLT and SCS groups. The expression patterns of liver injury-related genes were presented in Figure 3B and C. Most of these genes, including the inflammatory factors interleukin 27 (IL-27) and IL-32, were immune-associated and were significantly downregulated in patients who underwent IFLT. Through enrichment analysis, we demonstrated that most inflammation-related pathways, including cytokine signalling in the immune system, signalling by interleukins, and the Fc gamma R-mediated phagocytosis pathway, were downregulated in patients who received IFLT (Fig. 3E).

IFLT constructs a microenvironment characterized by low NK cell infiltration

The strong correlation between IFLT and immune reactions led us to investigate the microenvironmental characteristics of patients undergoing IFLT and SCS. The immune cell types of patients with SCS in the ER and PR stages were annotated into nine clusters: hepatocytes, smooth muscle cells, endothelial cells, monocytes, macrophages, NK cells, T cells, B cells, and erythroblasts (Fig. 4A). Using the ssGSEA method, we estimated cell types in patients with IFLT and SCS. NK cells, T cells, and monocytes tended to infiltrate together (Fig. 4B). There was a significantly lower infiltration of NK cells in IFLT patients than in SCS patients, whereas the change in other cell types did not reach significance (Fig. 4C). In addition, the expression of four classical pyroptosis-related genes (CASP1, GSDMD,

Table 2. Different characteristics between IFLT and SCS after PSM

Characteristic	Before PSM			After PSM (2:1)		
	SCS (n=279)	IFLT (n=50)	*P*-value	SCS (n=96)	IFLT (n=48)	*P*-value
Outcomes						
Respiratory support time (h)‡	17.0 (11.9, 44.5)	16.0 (12.3, 34.8)	0.847	16.0 (11.0, 40.1)	16.0 (12.0, 34.3)	0.904
ICU stay time (h)‡	42.5 (23.0, 89.0)	35.3 (18.3, 58.5)	0.037	38.8 (21.4, 88.3)	35.3 (18.8, 57.5)	0.151
Reintubation (%)*	16.0 (10.9)	2.0 (6.7)	0.742	9.0 (15.8)	2.0 (6.9)	0.320
Resume diet (h)‡	114.0 (85.0, 164.3)	86.0 (64.0, 121.5)	0.029	102.1 (82.9, 169.3)	86.0 (64.0, 121.5)	0.143
HA thrombosis (%)*	13.0 (5.3)	1.0 (2.6)	0.701	6.0 (6.9)	1.0 (2.7)	0.673
PV thrombosis (%)*	4.0 (1.6)	1.0 (2.6)	0.525	2.0 (2.3)	1.0 (2.7)	>0.999
PC thrombosis (%)*	4.0 (1.6)	0.0 (0.0)	>0.999	3.0 (3.4)	0.0 (0.0)	0.554
Biliary fistula (%)*	5.0 (2.0)	0.0 (0.0)	>0.999	0.0 (0.0)	0.0 (0.0)	NA
Biliary stricture (%)*	9.0 (3.6)	2.0 (5.1)	0.650	4.0 (4.5)	2.0 (5.4)	>0.999
PNF (%)*	15.0 (5.5)	1.0 (2.0)	0.482	6.0 (6.7)	0.0 (0.0)	0.092
EAD (%)*	114.0 (41.2)	15.0 (30.0)	0.137	39.0 (40.6)	14.0 (29.2)	0.179
AKI (%)*	14.0 (5.7)	2.0 (5.1)	0.999	5.0 (5.8)	2.0 (5.4)	0.999
Pulmonary infection (%)*	41.0 (14.7)	3.0 (6.0)	0.096	15.0 (15.6)	3.0 (6.3)	0.109
Wound infection (%)*	5.0 (1.8)	0.0 (0.0)	>0.999	3.0 (3.1)	0.0 (0.0)	0.551
Overall mortality (%)*	80.0 (28.7)	6.0 (12.0)	0.013	30.0 (31.3)	5.0 (10.4)	0.006
90-day mortality (%)*	34.0 (12.2)	3.0 (6.0)	0.202	11.0 (11.5)	2.0 (4.2)	0.220

Adjusted variables: donor liver steatosis and serum creatine level. *Categorical variables were exhibited as frequencies (percentages). ‡Continuous variables with non-normal distribution were exhibited as median (interquartile range).

IFLT, ischemia-free liver transplantation; SCS, static cold storage; PSM, propensity score matching; ICU, intensive care unit; HA, hepatic artery; PV, portal vein; PC, postcaval vein; PNF, primary nonfunction; EAD, early allograft disfunction; AKI, acute kidney injury.

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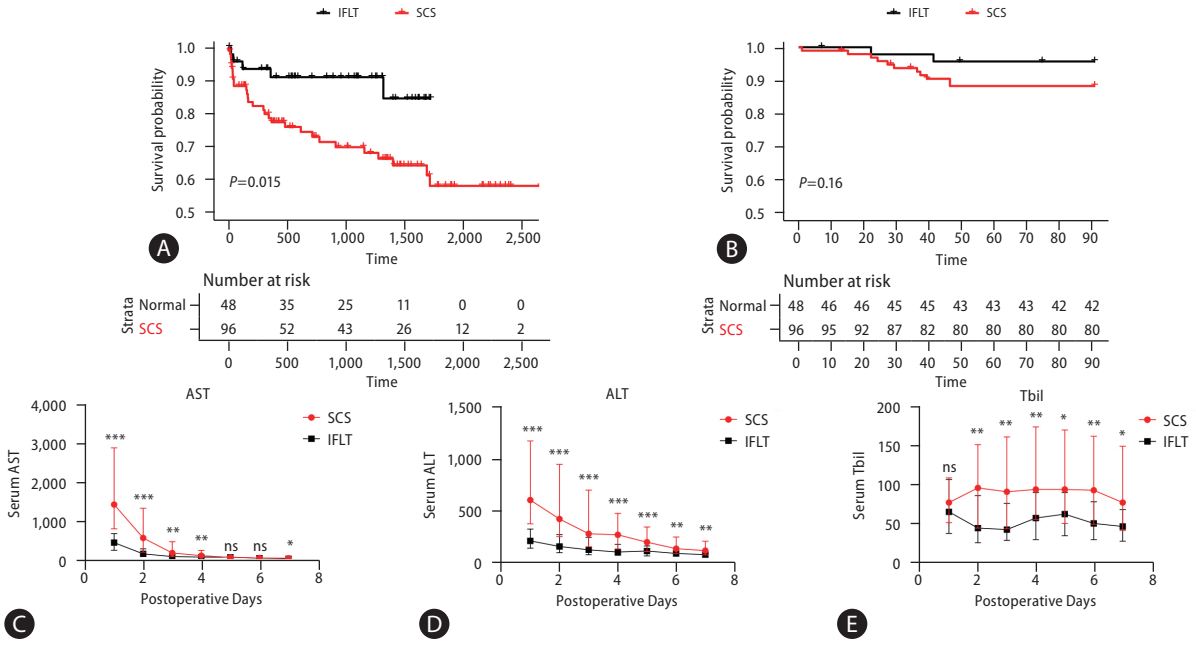

Figure 2. IFLT significantly improves the outcome of patients who receive FMLs. K-M plot of overall survival (A) and postoperative 90-day survival (B) of FML patients. The serum AST (C), ALT (D), and Tbil (E) levels of patients who received normal livers and FMLs seven days after the operation. IFLT, ischemia-free liver transplantation; FMLs, functionally marginal liver grafts; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Tbil, total bilirubin. **P*<0.05, ***P*<0.01, ****P*<0.001.

IL1B, and IL-18) in different cells was determined from the scRNA-seq data. IL-32 was significantly upregulated in NK and T cells, whereas CASP1, IL1B, and IL-18 levels were elevated in monocytes and macrophages (Fig. 4D). Furthermore, the correlations between the expression of IL-32 and genes in the classical pyroptosis pathway (CASP1, GSDMD, IL1B, and IL-18) were evaluated using bulk RNA-seq data. A significant positive relationship was observed between the expression of CASP1, GSDMD, and IL-32 (Fig. 4E).

DISCUSSION

The large imbalance between the waiting list and organ pool presents an urgent need to expand the donor pool.[2] ECDs are considered promising donor resources. Based on the definition of ECDs in 35 organ transplant centers,[3] we innovatively summarized a novel type of ECD called FMLs (a detailed definition is provided in the Methods section). In this cross-sectional study, we clarified the negative effect of FMLs on the prognosis of LT patients, which was not influenced by the primary disease of the recipients. The impact of FMLs on postoperative 90-day survival was greater than that of other ECD parameters, including steatosis, BMI, serum sodium, and age. In contrast to normal donors, patients who received FMLs were more likely to develop PNF and pulmonary infections. This led to more frequent medical interventions during the postoperative period, including longer ICU stays, resumption of diet, and a greater risk of reintubation. These patients spent more time recovering and had poorer long-term outcomes. Therefore, more care should be given to the utilization of FMLs to expand the liver donor pool. It is also important to find a method to alleviate liver injury caused by FMLs.

The results showed that both SCS and NMP preservation were insufficient to improve liver function in FMLs, and comparable outcomes were observed in recipients. Although conventional SCS can significantly reduce metabolism, ROS accumulation leads to severe reperfusion injury.[23] In recent years, clinical research has focused on a new organ preservation method, NMP, which is characteristic of livers perfused with oxygenated blood. Although NMP avoids the cooling process and reduces graft injury by 50%,[24] there is still an ischemia-reperfusion (I/R) process during the operation. Another kind of machine perfu-

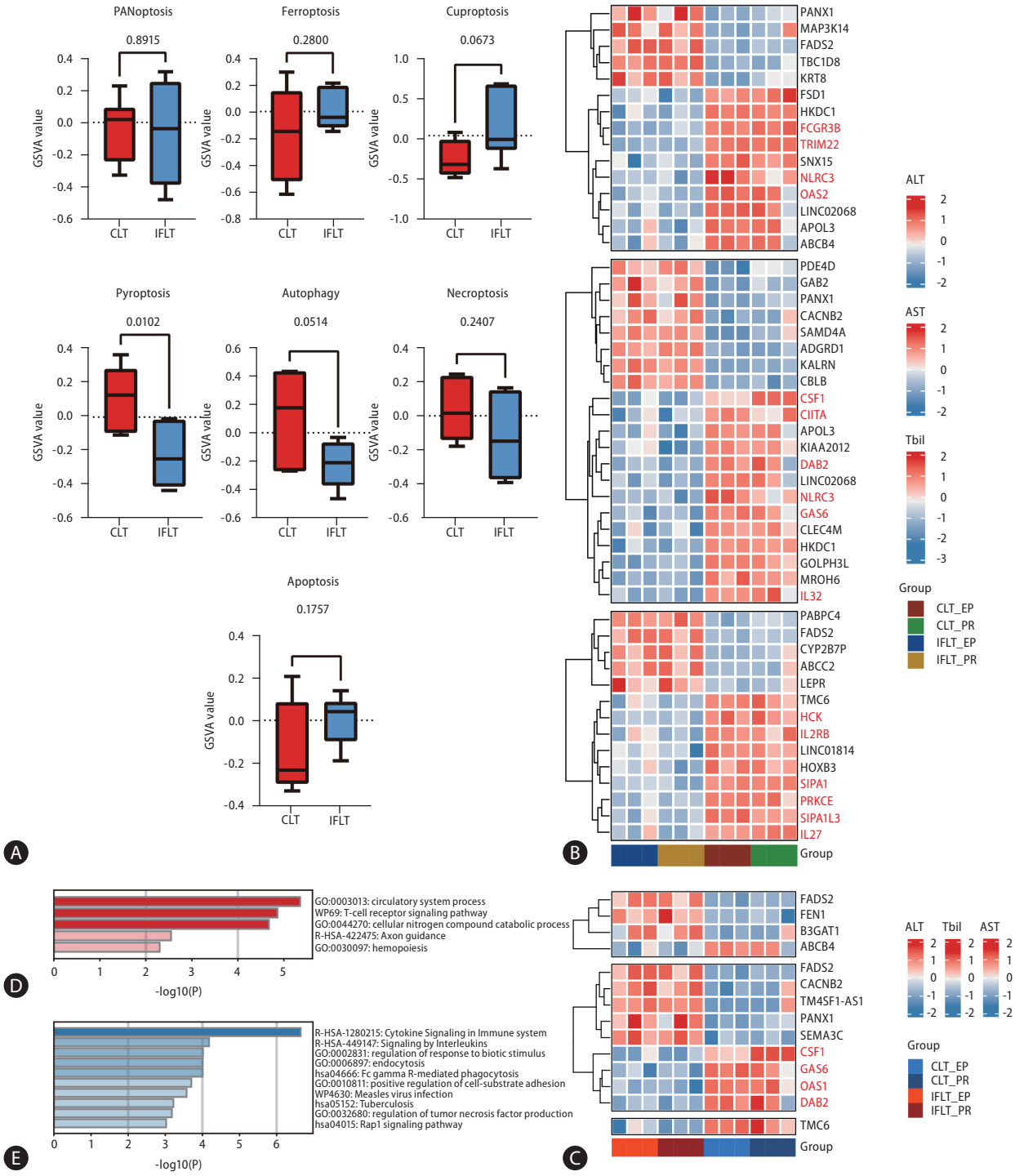

Figure 3. IFLT alleviates liver injury by suppressing cell death and inflammation. (A) GSVA scores of different cell death pathways between the CLT (underwent SCS) and IFLT groups. For both the European (B) and Asian (C) populations, the expression patterns of genes significantly related to liver injury among tissues from CLT and IFLT patients are depicted. The enrichment analysis for upregulated (D) and downregulated (E) liver injury-related genes in IFLT subjects. IFLT, ischemia-free liver transplantation; GSVA, gene set variation analysis; CLT, conventional liver transplantation; SCS, static cold storage.

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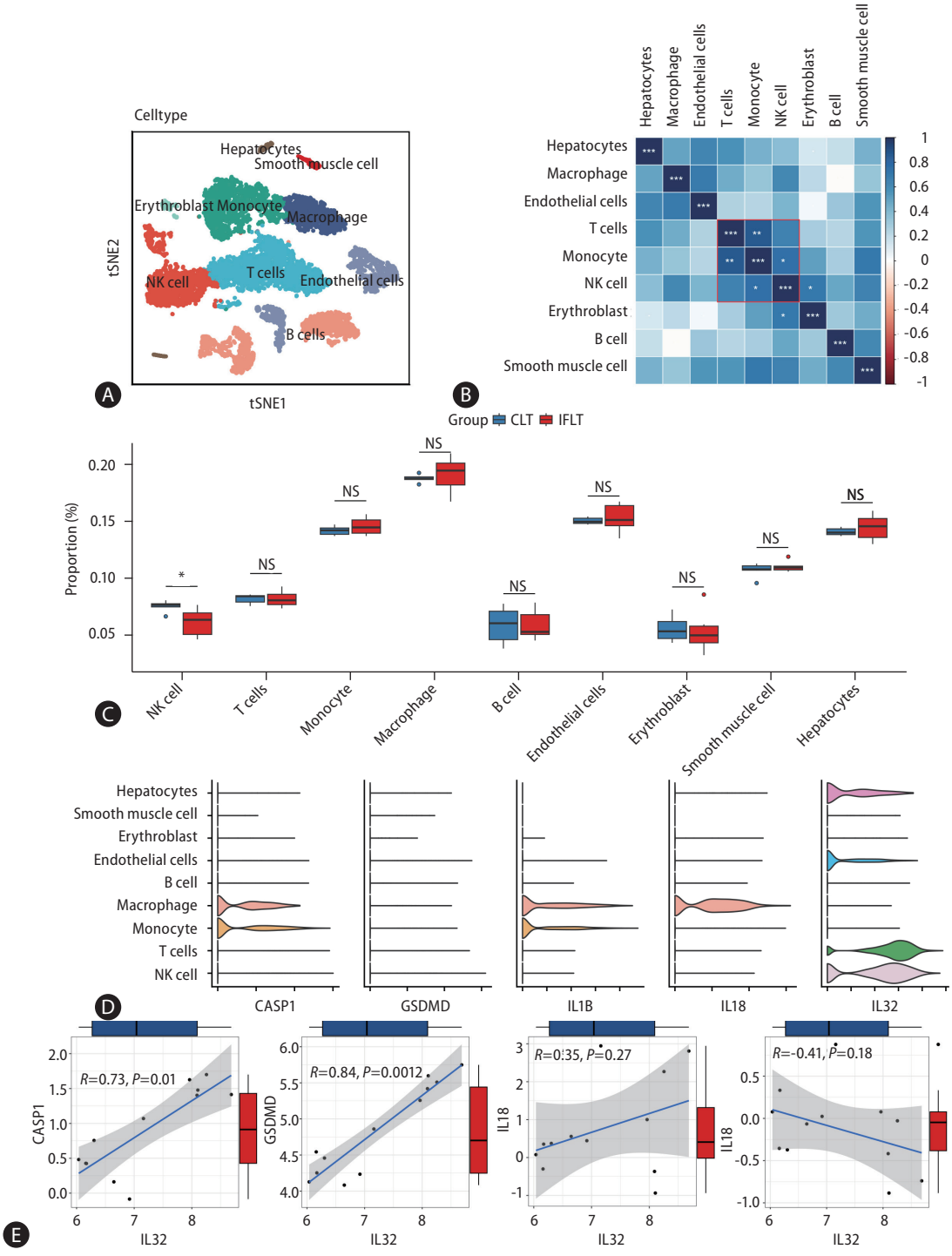

Figure 4. IFLTs induce an immune microenvironment with low infiltration of NK cells. (A) The annotation of cell types in the scRNA-seq profiles. (B) The co-infiltration of immune cells among patients who underwent liver transplantation. (C) The proportions of different immune cells measured by ssGSEA between the CLT (underwent SCS) and IFLT groups. (D) The expression patterns of four classical pyroptosis genes (CASP1, GSDMD, IL1B, and IL-18) and IL-32 are shown among different immune cells. (E) The correlation between the expression of IL-32 and that of four classical pyroptosis genes according to bulk RNA-seq profiles from the CLT and IFLT groups. IFLT, ischemia-free liver transplantation; scRNA-seq, single-cell RNA-sequencing; ssGSEA, single-sample gene set enrichment analysis; CLT, conventional liver transplantation; SCS, static cold storage; CASP1, caspase 1; IL1B, interleukin 1 beta; GSDMD, gasdermin D; IL1B, interleukin 1 beta; IL18, interleukin 18. *P<0.05, **P<0.01.

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sion, HMP (hypothermic machine perfusion), combines static cold storage and machine perfusion. Preclinical research revealed that HMP could restore mitochondrial function, inhibit the immune response, and alleviate liver injury.[25-27] Several single- or multicenter clinical trials have demonstrated that HMP reduces the incidence of hepatobiliary preservation injury, nonanastomotic biliary strictures and EAD.[28-31] However, these preservation methods cannot address the interruption of blood flow. Recently, a novel type of liver transplantation surgery, IFLT, has shown significant clinical benefits. In contrast to traditional surgery (SCS and NMP), IFLT can reduce I/R injury without disrupting the blood cycle during organ procurement, preservation, and implantation.[9] In this cohort study, different organ preservation methods were compared among recipients with FMLs. The protective effect of NMP on the short- and long-term outcomes of FMLs could not be verified in our study. The incidence of EAD was significantly greater in the NMP group than in the SCS group. Surprisingly, patients with FMLs who underwent IFLT had a shorter ICU stay, resumed diet, and were less likely to be reintubated, which indicated a lower cost of IFLT than SCS. In addition, the liver function factors ALT, AST, and Tbil decreased rapidly, which was consistent with the lower incidence of complications during the early postoperative period, including PNF, EAD, AKI, and pulmonary infection. Therefore, IFLT can significantly reduce the mortality rate in patients undergoing liver transplantation. The results indicate that IFLT can assist with the utilization of FMLs and reduce the risk to an acceptable level.

Mechanistically, pyroptosis was significantly inhibited in IFLT. Pyroptosis is originally called caspase-1-dependent programmed cell death and induces the secretion of IL-1b and IL-8.[32-34] Many studies have revealed that pyroptosis-induced inflammation accounts for I/R injury in liver transplantation.[33,35-37] Therefore, considering the proinflammatory role of pyroptosis, we investigated the effect of IFLT on FML inflammation. Most of the downregulated liver injury-related genes were proinflammatory genes, such as IL-32, IL-27, and interleukin 2 receptor subunit beta (IL-2RB). Specifically, IFLT significantly reduced NK cell infiltration. The function of NK cells in liver transplantation remains controversial owing to conflicting clinical and experimental results.[38] However, accumulating evidence suggests that NK cells are involved in the development of I/R injury during conventional liver transplantation.[39] The depletion of NK cells can protect the liver from I/R injury, as evidenced by decreased ALT and AST levels.[40] The presence of NK cells in the liver perfusate is strongly associated with acute cellular rejection.[41] Therefore, we concluded that IFLT alleviates liver injury by reversing pyroptosis-induced inflammation and inhibiting NK cell infiltration.

Additionally, IL-32 was significantly upregulated in the NK cells of SCS patients who underwent I/R injury. IL-32 is originally cloned in human NK cells and is a proinflammatory factor that contains three isoforms: IL-32a, IL-32b, and IL-32g.[42] IL-32g is a proinflammatory mediator that promotes the expression of IL-1b, IL-18, and tumor necrosis factor-alpha (TNF-a).[43,44] The immune cell patterns of CASP1, IL-1b, and IL-18 were remarkable in monocytes and macrophages. There was a positive relationship between IL-32, CASP1, and GSDMD in both SCS and IFLT patients. Thus, we can infer that IFLT alleviates liver injury in FMLs by inhibiting the infiltration of NK cells, thereby leading to the abortion of IL-32-induced pyroptosis and decreased secretion of downstream inflammatory factors (IL-1b and IL-18) in monocytes and macrophages.

In summary, clinical data analysis reveals that FMLs lead to poorer outcomes in LT patients than other ECDs (steatosis, advanced age, advanced BMI, and hypernatremia) in terms of postoperative complications and median survival. Transplant surgeons should be prudent regarding the utilization of this type of marginal liver. Surprisingly, we demonstrate that IFLT significantly improves the survival of patients who underwent FMLs. Furthermore, IFLT ameliorates liver injury in FMLs by inhibiting the infiltration of NK cells, leading to the abortion of IL-32-driven pyroptosis and the release of downstream inflammatory factors (IL-1β and IL-18) in monocytes and macrophages (Fig. 1B).

Limitations

Our study has three main limitations. First, the small number of patients in the NMP and IFLT groups weakened the statistical results. Larger population studies are required to address this issue (Supplementary Table 7). Second, the mechanism underlying the improvement of IFLT was revealed using bioinformatic analysis. Although a pyroptosis-immune network was constructed, more detailed experiments should be conducted to elucidate the mecha-

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nism of liver injury in the SCS and NMP groups as compared with IFLT. Third, some low-quality grafts tended to be evaluated under machine perfusion, which could have led to an underestimation of the protective effect of NMP in our study.

Authors' contribution

Conceptualization: S.W., M.C., and W.J; Writing the original draft: S.W., X.L., and Y.T; Date collection: Y.L., M.Z., Z.X., Y.G., and Y.D; Statistical analyses: S.W. and X.L; Bioinformatics: S.W; Review & editing: Q.Z., Z.G., D.W., and X.H. Founding: X.H., W.J., and M.C.

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Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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