

Recipient Toll-like Receptor 4
Determines the Outcome of Ischemia-
Reperfusion Injury in Steatotic
Liver Transplantation in Mice

レシピエントの Toll-like receptor 4
はマウス脂肪肝グラフト移植における
虚血再灌流障害の予後を決定する

田 中 康 介

Recipient Toll-like Receptor 4 Determines the Outcome of Ischemia-Reperfusion Injury in Steatotic Liver Transplantation in Mice

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K.T. conducted the animal transplant model and basic assay, and wrote the main body of the manuscript. Y.U. and K.K. designed the main part of the research. K.K., H.H., and K.N. supervised the basic assay and interpretation of the results. H.K., Y.K., K.S., and M.I. interpreted the results critically. H.K and S.K. supervised the technical aspect of the transplantation model. T.K., H.T., T.W., and E.H. supervised the writing of the manuscript. All authors critically reviewed the manuscript and accepted the final version of the manuscript.

Abbreviation

ALT, alanine aminotransferase

ANOVA, analysis of variance

BMDMs, bone marrow-derived macrophages

CDAHFD, choline-deficient L-amino acid-defined high-fat diet

CIT, cold ischemic time

CXCL1, C-X-C motif chemokine ligand 1

CXCL2, C-X-C motif chemokine ligand 2

H&E, hematoxylin and eosin

HMGB1, high mobility group box 1

IL-6, interleukin 6

Ischemia-reperfusion injury, IRI

LPS, lipopolysaccharide

LT, liver transplantation

MASLD, Metabolic dysfunction-associated steatotic liver disease

ORO, Oil-Red O

SEM, standard error of the mean

TLR4, toll-like receptor 4

Western blotting, WB

Keywords: Steatotic liver grafts; liver transplantation; ischemia-reperfusion injury; Toll-like receptor 4

ABSTRACT

Toll-like Receptor 4 (TLR4) plays a crucial role in ischemia/reperfusion injury (IRI) after liver transplantation (LT). However, the role of TLR4 in the context of steatotic grafts remains unclear. In this study, we developed a mouse model to explore IRI mechanisms in steatotic LT using TLR4 knockout mice as recipients. We successfully transplanted steatotic grafts with approximately 35% macrosteatosis and 5 hours of cold storage. Compared to normal LT, steatotic LT resulted in significantly higher serum level of alanine aminotransferase (ALT) and High Mobility Group Box 1 (HMGB1), higher transcriptional expression of inflammatory markers (C-X-C motif chemokine ligand 2, caspase-1, and caspase-11), and increased infiltration of CD11b-positive cells, correlating with lower survival. Serum HMGB1 and cleaved caspase-3 activation peaked earlier than serum ALT, with cold-stored steatotic grafts releasing more HMGB1. Notably, TLR4 knockout recipients demonstrated improved survival, attenuated inflammatory response, and reduced apoptosis. These findings suggest that

TLR4 deficiency in recipients ameliorates IRI in steatotic LT, highlighting the importance of recipient immune modulation in mitigating steatotic graft injury.

1 Introduction

Liver transplantation (LT) has been widely accepted as the treatment for end stage liver failure and hepatobiliary malignancies. However, many candidates dropped off from the waiting list due to the donor shortage, and a significant number of liver graft are discarded due to moderate to severe macrosteatosis ¹. The transplantation of moderately steatotic liver grafts (>30% of macrosteatosis) has traditionally been avoided due to their association with severe ischemia-reperfusion injury (IRI) and the risk of early allograft dysfunction or primary non-function ²⁻⁴.

Recent clinical efforts have explored the feasibility of using steatotic liver grafts, with some studies demonstrating successful long-term transplantation outcomes under careful recipient selection and perioperative management ⁵⁻⁷. Overcoming post-reperfusion injury (hyperacute phase reaction) is essential for the long-term survival of steatotic liver grafts ⁸. Emerging technologies such as machine perfusion have also shown promise in assessing the viability of steatotic grafts ⁹. To further advance these clinical advancements, it is crucial to understand the mechanisms of IRI using orthotopic steatotic LT model, yet the mechanisms remain poorly understood at present. It would be because there has been a little published model of steatotic LT in mice so far.

Macrophage is recognized as a pivotal mediator of sterile inflammation in LT^{10 11}. In the context of IRI, Toll-like receptor 4 (TLR4) signaling is crucial for activating innate immunity¹²⁻¹⁶, facilitating interactions with T cells¹⁷, and promoting the differentiation of monocytes into inflammatory macrophages¹⁸. High mobility group box 1 (HMGB1) is one of ligands that engages TLR4¹⁹. In the context of prolonged cold-preserved LT, increased amounts of HMGB1 were released from injured grafts²⁰. Similarly, in the context of steatotic LT, the activation of immune cells, including macrophages, is hypothesized to play a critical role of severe IRI^{21,22}. Clinical retrospective studies have suggested that steatotic grafts may release more HMGB1 than normal grafts²³. Experimental rat model of steatotic LT has indicated that machine perfusion may mitigate HMGB1 release from steatotic grafts and reduce sterile inflammation after transplantation²⁴. However, the precise mechanism of HMGB1 release from steatotic liver grafts and the HMGB1/TLR4 interaction in this context remain poorly understood.

First, we have established a mouse model of steatotic LT, which has rarely been published so far. Second, we hypothesize that steatotic grafts release higher amounts of HMGB1 than normal grafts and that the TLR4-mediated inflammation is more activated in steatotic LT. Finally, we aim to explore the mechanism of severe IRI in steatotic LT using TLR4 knock out mice.

2 Materials and methods

2.1 Animal

The present study was approved by the Kyoto University Animal Research Committee (Med Kyo 24174 and Med Kyo 24516). C57BL/6NJ male mice (10–13 weeks old, 25–30 g) were purchased from OrientalBioService, Inc. (Kyoto, Japan). TLR4 congenic knockout (B6.129-Tlr4^{tm1Aki}/Obs) mice were purchased as frozen embryos from OrientalBioService, Inc. (Kyoto, Japan) and were approved by the Kyoto University Recombinant DNA Experiment Safety Committee (Approval No. 240018). All animals were maintained under specific-pathogen-free conditions and provided food ad libitum. All experimental procedures were conducted in compliance with Kyoto University's Regulations on Animal Experimentation and Institutional Animal Care and Use Committee policies.

2.2 High-fat diet

Steatotic liver were induced to donor mice by feeding them a choline-deficient L-amino acid-defined high-fat diet (CDAHFD, Cat# A06071302)²⁵ purchased from Research Diets Inc. (New Jersey, United States) ad libitum.

2.3 Analysis of liver grafts after static cold storage

Donor mice were anesthetized with 2.5% isoflurane for liver graft extraction. The University of Wisconsin solution (4°C) was perfused via the portal vein, and the liver graft was stored

at 4°C. After 5 hours of cold storage, 2 ml of normal saline was flushed through the portal vein, and the effluent from the inferior vena cava was collected as “liver flush.” The liver tissue was paired analyzed.

2.4 Orthotopic liver transplant procedure

The majority of the methods followed a previously published model²⁶⁻²⁸. The extracted liver graft was stored in University of Wisconsin solution at 4°C for either 1 hour or 5 hours prior to transplantation into a syngeneic healthy recipient mouse. The liver graft was orthotopically transplanted into an anesthetized recipient mouse using a cuff method²⁷ and a continuous suture of the suprahepatic inferior vena cava. The bile duct was reconstructed using a plastic stent. The hepatic artery was not reconstructed. Anhepatic time (approximately equal to warm ischemic time) was set as 19 minutes in all experimental protocols in the present study. The 5-hour cold storage period is based on a surgical technique perspective and is unrelated to the analysis timepoint following reperfusion.

2.5 Liver function and metabolic test

Serum levels of alanine aminotransferase (ALT), triglycerol and total cholesterol were determined using a standard spectrophotometric method performed by Nihon Rinsho, Inc. (Kyoto, Japan).

2.6 Quantitative reverse transcription polymerase chain reaction analysis

RNA was extracted with a RNeasy® Protect Mini Kit (QIAGEN). A total of 500ng of extracted RNA was reverse transcribed into cDNA. Quantitative reverse transcription polymerase chain reaction was performed using StepOnePlus (Applied Biosystems). Forward and reverse sequences of primer genes were listed in supplementary Table 1. Target gene expressions were normalized by their ratios to the housekeeping GAPDH gene using $\Delta \Delta Ct$ method.

2.7 Western blotting (WB)

Liver samples were dissolved in radio-immunoprecipitation assay buffer (Thermo Fisher Scientific) and proteins were extracted. The protein concentration was measured using a BCA Assay Kit (Thermo Fisher Scientific) and standardized to 2 $\mu\text{g}/\mu\text{l}$ with Laemmli sample buffer (Sigma-Aldrich). Serum samples (1 μl) were mixed with phosphate-buffered saline and Laemmli sample buffer to a final volume of 10 μl . Equal amounts of protein lysate (20 μg for liver samples, 10 μl for serum samples) were electrophoresed. The antibodies used in this study are listed in Supplementary Table 2. The relative values were normalized to beta-actin. Membranes blotted with serum samples were stained with Ponceau-S staining solution to visualize and ensure equal protein loading.

2.8 Histopathology

Formalin-fixed liver sections (5 μ m thick) were stained with hematoxylin and eosin (H&E). Frozen sections were sliced into 7 μ m-thick sections and stained with Oil-Red O (ORO) by Kyoto Institute of Nutrition & Pathology, Inc. (Kyoto, Japan). The percentage of ORO-positive area was calculated by ImageJ (ver. 1.53t).

2.9 Immunohistochemistry

Formalin-fixed liver sections (5 μ m thick) were deparaffinized and rehydrated. Antigen retrieval was performed using a citrate buffer or a TE buffer and heated by autoclave (121°C, 15 mins). Endogenous peroxidase was inactivated by 3% hydrogen peroxide. Secondary antibodies were conjugated by horseradish peroxidase and 3,3'-Diaminobenzidine was used as a chromogenic substrate. Mayer's hematoxylin was used as a counter-staining. The number of stain-positive cells in high power field was counted as the average of 10 fields of view.

2.10 Immunofluorescence

For formalin-fixed liver sections, the same protocol was followed up to the primary antibody reaction described above. For frozen sections, the inactivation of endogenous peroxidase was omitted. Immunofluorescent signals were visualized with Alexa Fluor Antibodies

(Invitrogen, Thermo Fisher Scientific). Hepatic Ly6g⁺ cells were scored semiquantitatively by counting cells in 10 high power field/section (×400).

2.11 Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of HMGB1 was measured with ELISA kits (Shino test) according to the manufacturer's protocol.

2.12 Statistical analysis

All statistical analyses were performed using GraphPad Prism (Version 9.5.1, GraphPad Software, San Diego, CA, USA). Categorical data were compared using Fisher's exact test. Continuous data were presented as the mean and standard error of the mean, compared using a Student's t-test. The comparisons among multiple groups were assessed using one-way analysis of variance (ANOVA) with Turkey test. A survival curve was described using Kaplan-Meier method and significance was determined by a log-rank test. All *p*-values were unpaired and two-sided, and *p* < 0.05 was considered to be statistically significant.

3 Results

3.1 Induction of moderate steatosis in donor mice

To establish a steatotic LT model, we first needed to induce steatosis in the donor mice. Donor mice were fed CDAHFD ad libitum for 2, 3, or 4 days to achieve approximately 30% macrosteatosis, which is consistent with the clinical threshold for discarding steatotic grafts (Figure 1A). Normal liver donors were fed a control diet ad libitum. Serum ALT levels were significantly higher in the steatotic liver donor group compared to the normal liver donor group, with a notable elevation observed after 4 days of feeding compared to 3 days (Figure 1B). There were no significant differences in serum triglyceride and total cholesterol levels across the different periods of CDAHFD feeding (Figures 1C and 1D). ORO staining demonstrated that steatotic liver donors had a higher percentage of ORO-positive area compared to normal liver donors (Figure 1E). Clinically relevant macrosteatosis was achieved with 3-day feeding ($35.1 \pm 2.9\%$). H&E and ORO staining results for each donor are shown (Figure 1F), indicating that macrosteatosis was predominantly observed in the portal area.

3.2 Establishment of steatotic liver transplant model in mice

We aimed to transplant the steatotic grafts with clinically relevant macrosteatosis into syngeneic recipient mice and determine the most appropriate experimental conditions by varying the duration of CDAHFD feeding and the cold ischemic time (CIT) to either 1 hour or 5 hours (Figure 2A). As displayed in Figure 2B, Gray dots are visible on the surface of liver grafts with 3 days of CDAHFD feeding (the first image). These dots turned more clearly visible

after procurement (the second image). Just after reperfusion, steatotic grafts tended to look white compared to normal grafts (the third image), and this condition persisted until skin closure (the fourth image). Serum ALT levels under various conditions are displayed in Figure 2C. In the setting of 1-hour CIT, there were no significant differences between normal and steatotic grafts. However, with setting of 5-hour CIT, serum ALT levels were significantly higher in steatotic LT (3 days of CDAHFD feeding) compared to normal LT. When steatotic grafts made by 4 days of CDAHFD feeding were stored for 5 hours, no recipient mice could survive even for 6 hours. When setting CIT as 1 hour, recipient survival correlated with the period of CDAHFD feeding (Figure 2D). When donor mice were fed CDAHFD for 5 days, no recipient mice could survive for 6 hours. When setting CIT as 5 hours, recipient survival was significantly worse in steatotic LT (3 days of CDAHFD feeding) compared to normal LT (Figure 2E). From these results, the experimental model of steatotic LT was established with the setting of 3 days of CDAHFD feeding (approximately 35% macrosteatosis) and 5 hours of CIT. This model allowed all recipient mice to survive for more than 6 hours after reperfusion.

3.3 Steatotic liver grafts exacerbate ischemia-reperfusion injury via activation of inflammatory cytokines and infiltrative immune cells

An overview of the transplant model in the present study is displayed in Figure 3A. No massive necrosis, congestion, or thrombosis was observed either after cold storage or reperfusion (Figure 3B). The counts of CD11b-positive cells were higher in steatotic LT at 6

hours after reperfusion (9.4 ± 0.97 vs 17.8 ± 1.79 , $p < 0.001$), while Ly6g-positive cells were comparable (8.4 ± 0.92 vs 11.0 ± 0.92 , $p = 0.09$) (Figure 3C/3D). Steatotic livers exhibited inflammatory patterns similar to normal livers prior to cold storage, aligning with clinical observations of liver grafts without pre-existing inflammation (Figure 4B). However, at 6 hours after reperfusion, steatotic LT increased transcriptional expressions of inflammatory cytokine (interleukin-6, IL-6), chemokines (C-X-C motif chemokine ligand 2, CXCL2) and pyroptosis-related proteins (caspase-1 and caspase-11) (Figure 4B). The expression of anti-inflammatory cytokine (interleukin-10) was also upregulated. At 1 hour after reperfusion, steatotic LT increased transcriptional expressions of tumor necrosis factor alpha and caspase-1. These data indicate steatotic liver grafts induces inflammatory responses after cold ischemia/reperfusion insult. WB analysis revealed significant activation of cleaved caspase-1 and cleaved gasdermin D at 1 hour post-reperfusion in steatotic LT (Supplementary Figure 1). However, these activations did not correlate with serum interleukin-18 levels. At 6 hours post-reperfusion, while cleaved caspase-11 was upregulated in parallel with its transcriptional activation, neither caspase-1 nor gasdermin D showed upregulation.

3.4 Serum HMGB1 and apoptosis peak at 1 hour after reperfusion, and HMGB1 translocation mainly occur in sinusoid cells

We next analyzed the time-dependent trend of serum HMGB1 and ALT post-transplant. While serum ALT increased gradually after reperfusion (at 1 hour, 705 ± 115 vs 927 ± 164 U/L,

$p = 0.30$; at 6 hours, 1378 ± 118 vs 2835 ± 304 U/L, $p = 0.004$), serum HMGB1 peaked at 1 hour after reperfusion (39.7 ± 2.64 vs 75.4 ± 21.14 ng/ml, $p = 0.15$) and kept significantly higher in steatotic LT than normal LT at 6 hours after reperfusion (31.5 ± 4.47 vs 44.5 ± 2.97 ng/ml, $p = 0.04$) (Figure 4A). The increase in serum ALT from 1 to 6 hours after reperfusion was pronounced in steatotic LT. WB analysis revealed that cleaved caspase 3 in liver tissue also peaked at 1 hour after reperfusion and kept higher in steatotic LT than normal LT. At 6 hours after reperfusion, cytoplasmic HMGB1 was scarcely detectable via immunofluorescence in normal LT, whereas it prominently localized in stabilin-2-positive liver sinusoidal endothelial cells in steatotic LT (Figure 4C).

3.5 Cold-preserved steatotic liver grafts release greater amount of HMGB1 compared to normal liver grafts

Since acute-phase HMGB1 surge was observed in steatotic LT, we examined whether cold-preserved steatotic grafts release more HMGB1. The experimental protocol is outlined in Figure 5A. Liver flush analysis aims to evaluate damage-associated molecular patterns from the graft immediately after reperfusion. This analysis revealed significantly elevated levels of ALT and HMGB1 in steatotic grafts as compared to normal grafts (Figure 5B), while HMGB1 protein levels were comparable in liver tissue (Figure 5C). Thus, steatotic grafts released more HMGB1 than normal grafts during cold storage.

3.6 TLR4 deficiency in recipients ameliorates IRI of steatotic grafts and improves post-transplant survival

Having the enhanced inflammatory response in transplanted steatotic grafts (Figure 3/4) and the increased release of HMGB1 from these grafts (Figure 5), we hypothesized that recipient TLR4, a receptor of HMGB1¹⁹, is crucial in mitigating IRI in steatotic LT. We employed TLR4 knockout mice as recipients to investigate the role of recipient TLR4 in the context of steatotic LT. Compared to steatotic LT into wild-type recipients, steatotic LT into TLR4 knockout recipients displayed significantly improved post-transplant survival which is superior to normal LT into wild-type recipients (Figure 6A). TLR4 deletion in steatotic LT recipients also resulted in reduced serum ALT and HMGB1 levels after reperfusion especially at 6 hours after reperfusion (ALT, 3036 ± 337 vs 1796 ± 232 U/L, $p = 0.026$; HMGB1, 52.8 ± 6.07 vs 7.6 ± 2.17 ng/ml, $p = 0.0001$) (Figure 6B). Histology demonstrated no massive necrosis and congestion (Figure 6C). In the context of steatotic LT, at six hours after reperfusion, transcriptional expressions of C-X-C motif chemokine ligand 1 (CXCL1), CXCL2, caspase-11, Interleukin-1 β and interleukin-6 were significantly lower in TLR4 knockout recipients compared to in wild-type recipients (Figure 6D). In contrast, no significant reduction in transcription was observed at 1 hour post-reperfusion. The counts of CD11b-positive and Ly6g-positive cells also significantly decreased in TLR4 knockout recipients compared to in wild-type recipients (Figure 6E). Moreover, at 1 hour after reperfusion, the ablation of recipient TLR4 abolished the elevations of hepatic cleaved caspase-3 (Figure 6F). The data

indicate that recipient TLR4 plays a crucial role in regulating inflammatory responses and enhancing the survival of steatotic liver grafts. On the other hand, the upregulation of pyroptosis-associated proteins induced by steatotic grafts was not ameliorated in TLR4 knockout recipients (Supplementary Figure 2).

4 Discussion

The prevalence of metabolic dysfunction-associated steatotic liver disease (MASLD) is increasing, now affecting approximately a quarter of the general population ²⁹. Consequently, the proportion of steatotic liver grafts in the donor pool is high, and many grafts are discarded due to steatosis ¹. The efficient use of steatotic liver grafts remains a critical area of research in liver transplantation. However, the progress has been limited due to the lack of established mouse models for studying steatotic liver grafts. In our study, we developed a clinically relevant mouse liver transplantation model with macrosteatosis (approximately 30%) in liver grafts, which underwent 5-hour static cold storage before transplantation. This model suggested that one of the mechanisms exacerbating IRI involves the greater release of HMGB1 from steatotic grafts. Furthermore, we observed that the deletion of TLR4 in the recipient alleviated IRI and improved survival in steatotic LT. The present study suggests that recipient TLR4 may play a central role as a mediator of IRI in steatotic LT, highlighting TLR4 as a potential target for therapeutic intervention.

Although rat models using steatotic liver grafts have been extensively reported, such models in mice are scarce. In previous studies using *ob/ob* mice, even with CIT of only 1 hour, early post-transplant mortality and histological signs of primary non-function were observed ³⁰. Although one recent study reported a long-term high-fat diet-induced mouse model, details were lacking ³¹. Limitations of these models include poor post-transplant survival rates and the lengthy preparation required. On the other hand, our model offers a more practical approach, inducing MASLD in donor mice with short-term feeding and enabling the evaluation of post-transplant events, even after 5 hours of cold ischemia. Moreover, significant necrosis was not observed in the graft after reperfusion, making this model worthwhile for further study. However, there are challenges that remain. Studies in rats have shown that the etiology of steatotic liver plays a significant role in post-transplant survival ³². Although there was no significant difference in inflammation-related genes between normal and steatotic livers at the sham stage in our study, further research is needed to clarify whether the short-term dietary induction of steatotic liver in mice truly mimics human steatotic LT.

A previous large cohort study proposed prolonged cold preservation and moderate-to-severe macrosteatosis as risks of early allograft dysfunction in IRI grafts ^{2,4}. Prolonged cold preservation graft-related IRI has been extensively studied, and bone marrow-derived macrophages (BMDMs) are considered key mediators of sterile inflammation after LT ¹⁰. Similar mechanisms, such as the activation of colony stimulating factor 3-positive Kupffer

cells, have been suggested in liver IRI caused by steatotic liver grafts ²², indicating the importance of controlling BMDMs in this process. In this study, we found that in the steatotic liver graft model, the transcriptional expression of CXCL2, caspase-1 and caspase-11 significantly increased at 6 hours post-transplant (Figure 4B) and the number of CD11b-positive cells increased (Figure 3C). These also suggest that the enhancement of TLR4-associated inflammation cascades via BMDMs also plays a central role even in the context of steatotic grafts.

Both steatotic and prolonged cold-preserved liver grafts are categorized as marginal, but their IRI responses show notable differences. Prolonged cold-preserved grafts often exhibit extensive necrosis and congestion after reperfusion (Supplementary Figure 3). However, in this study, steatotic liver transplants displayed neither marked necrosis nor significant ALT elevation yet had substantially lower survival rates. This discrepancy may stem from an early peak in HMGB1 release, potentially triggering excessive activation of BMDMs and contributing to IRI. Supporting this hypothesis, we observed that serum HMGB1 levels in steatotic transplants peaked before ALT (Figure 4A), contrasting with the concurrent increases in HMGB1 and ALT typically seen in prolonged cold-preserved grafts ²⁰. In the context of prolonged cold-preserved grafts, sustained HMGB1 elevation likely reflects passive release from necrotic tissue, while the early peak in steatotic transplants—followed by a decline—suggests an active inflammatory response in the absence of extensive necrosis. This initial HMGB1 surge may drive a heightened inflammatory cascade, contributing to graft

failure despite mild necrosis and ALT levels. Interestingly, a recent clinical study indicated intensified hyperacute phase inflammation, including post-reperfusion syndrome, in moderately steatotic grafts⁸. However, the precise mechanism by which steatotic grafts are more prone to deterioration despite relatively mild ALT elevation remains uncertain, and further research is needed to clarify this unique vulnerability in steatotic grafts.

The present study revealed that knocking out TLR4 in the recipient significantly reduced serum HMGB1 levels, thus mitigating IRI. Interestingly, even though the BMDMs infiltrating the transplanted liver lacked TLR4 receptors under this condition, the transplanted liver itself retained its TLR4 receptors. This indicates that the TLR4 on infiltrating macrophages plays a more critical role than that on resident macrophages and hepatocytes within the grafts. Furthermore, TLR4 regulates the transcription of pro-caspase-11, pro-interleukin-1 beta, tumor necrosis factor- α , IL-6, CXCL1, and CXCL2, and indirectly controls the activation of caspase-1 through pro-caspase-11 activation^{33,34}. Lipopolysaccharide (LPS) is not only a separate ligand for TLR4, but it can also bind with HMGB1 to activate pro-caspase-11 through a TLR4-independent pathway^{35,36}. In this experiment, gene expression of caspase-11 was amplified in steatotic liver transplantation (Figure 4B), and it was shown that this gene expression was attenuated in TLR4 knockout recipients (Figure 6D). In addition, the dramatic improvement in survival rates shown in Figure 6A may be difficult to explain by the interaction of HMGB1 and TLR4 alone. This suggests that, in addition to the interaction between HMGB1 and TLR4, the transcription of pro-caspase-11 and its activation

into cleaved caspase-11 via the LPS/TLR4 pathway could play a role in the excessive immune response in steatotic liver transplantation. LPS is one of key mediators aggravating MASLD³⁷. The involvement of intestinal bacteria in steatotic LT is also expected to be studied in more detail in the future.

This study identified a significant upregulation in the transcription of caspase-1 and caspase-11, which partially correlated with their activated forms and the activation of gasdermin D, as observed in WB analysis. Caspase-1, caspase-11, and gasdermin D are key molecules involved in pyroptosis³⁸. Additionally, a prior study on warm IRI demonstrated an interaction between TLR4 and caspase-1³⁹. Pyroptosis has been implicated in the inflammatory deterioration seen in the progression of metabolic-associated steatohepatitis⁴⁰. In the present study, since the ablation of recipient TLR4 did not ameliorate pyroptosis signaling pathways in WB analysis using whole liver tissue, the role of pyroptosis in the context of steatotic LT remains unclear. To further elucidate this role, it will be essential to perform analyses using specific cell populations or alternative knockout mouse models. These future investigations will be critical for understanding the contribution of pyroptosis to graft injury and its broader implications for steatotic LT.

The limitations of this study are as follows. First, only CDAHFD was used to induce steatosis in the mouse model. While CDAHFD was widely used in rodent studies for steatotic liver⁴¹⁻⁴³, further investigation is required to determine if the fragility and post-reperfusion injury of CDAHFD-induced steatotic grafts parallels that of human clinical steatotic grafts. Second,

the mechanisms of worsened IRI in steatotic liver transplantation are diverse. This study focused on TLR4, but it did not address the metabolic pathways specific to steatosis. Since other significant pathways associated with metabolism ³¹ and immune checkpoints ¹⁷ may be involved, future studies should adopt a more comprehensive approach. Nevertheless, one striking advantage of this study is that it highlights the significant role of the recipient TLR4 in steatotic LT.

The study provides novel insights into the role of recipient TLR4 in the context of IRI in steatotic liver transplantation. These findings may inform the development of targeted therapies to mitigate graft injury and improve transplantation outcomes.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Data availability

All raw data that support the findings of this study are available from the corresponding author, Yoichiro Uchida, upon reasonable request.

Figure legend

Figure 1

Induction of Steatosis in Donor Mice. (A) Mice in the normal liver donor group received a regular diet ad libitum, while those in the steatotic liver donor group were fed a CDAHFD for 2, 3, or 4 days. Following each day of feeding, liver and serum samples were collected for analysis. **(B-D)** Comparison of serum ALT (B), triglycerides (C), and total cholesterol (D) between normal and steatotic liver donors. **(E)** Evaluation of Oil-Red O-positive staining area in high power field. Mice on a 3-day CDAHFD exhibited approximately 35% Oil-Red O-positive area in liver samples. **(F)** Representative images of H&E staining (upper panel) and Oil-Red O staining (lower panel) of liver samples, showing macrosteatosis (visible in red on Oil-Red O staining) in the steatotic donor group (x400, scale bar, 100 μ m). Data are depicted as the mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with a Turkey test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. ALT, alanine aminotransferase; ANOVA, analysis of variance; CDAHFD, choline-deficient L-amino acid-defined high-fat diet; H&E, hematoxylin and eosin; SEM, standard error of the mean.

Figure 2

Establishment of the steatotic liver transplantation model. (A) Donor mice were fed CDAHFD for 3, 4, or 5 days. Liver grafts were extracted from these donor mice and stored for either 1 or 5 hours before transplantation into syngeneic recipient mice. Serum ALT and recipient survival were subsequently assessed. **(B)** Macro image of steatotic liver grafts, showing a slightly paler appearance compared to normal liver grafts after reperfusion. **(C)** Serum ALT levels measured at 6 hours after reperfusion (n = 6-10/group). Data are depicted as the mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with a Turkey test; ns, not significant; **p < 0.01. **(D)** Recipient survival in the setting of 1-hour CIT. Recipient survival decreased progressively with an increased duration of CDAHFD administration. (p < 0.0001). **(E)** Recipient survival in the setting of 5-hour CIT. Recipient survival of steatotic LT (3-day CDAHFD) was significantly lower than that of normal LT (p = 0.003). Statistical significance of recipient survival was determined by Kaplan-Meier method with a log-rank test. ALT, alanine aminotransferase; ANOVA, analysis of variance; CDAHFD, choline-deficient L-amino acid-defined high-fat diet; CIT, cold ischemic time; LT, liver transplantation; SEM, standard error of the mean.

Figure 3

Analysis of post-reperfusion injury in steatotic LT. (A) Steatotic LT was performed in the setting of 3-day CDAHFD feeding and 5-hour cold storage. **(B)** Histological comparison between two models at each timepoint (before procurement, after cold storage, and after

reperfusion) (H&E, $\times 400$; scale bar, 100 μm). **(C, D)** Surface marker-positive cells per high-power field were analyzed across four groups (C, CD11b as black arrowheads; D, Ly6g as yellow arrowheads; $\times 400$ magnification, scale bar, 100 μm). LT samples were collected at 6 hours after reperfusion. Data are depicted as the mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA with a Turkey test (C, D); ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. ANOVA, analysis of variance; CDAHFD, choline-deficient L-amino acid-defined high-fat diet; H&E, hematoxylin and eosin; LT, liver transplantation; SEM, standard error of the mean.

Figure 4

The time-elapsed analysis of post-reperfusion injury in steatotic LT. (A) Serum ALT, serum HMGB1, and cleaved caspase-3 levels in liver tissues were measured at three timepoints: sham, 1 hour, and 6 hours after reperfusion. Cleaved caspase-3 bands were analyzed by western blot, with band intensity normalized to beta-actin. **(B)** Relative mRNA expressions of cytokines, chemokines, and caspases were measured ($n = 5/\text{group}$). **(C)** Representative immunofluorescence staining of HMGB1 (green), stabilin-2 (red), and merged images of normal LT (upper panel) and steatotic LT (lower panel) ($\times 1200$; scale bar, 20 μm) at 6 hours post-reperfusion. Data are depicted as the mean $\pm$ SEM. Statistical significance was determined by unpaired t-test (A, B); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ALT, alanine aminotransferase; CXCL1, C-X-C motif chemokine ligand 1; CXCL2, C-X-C motif chemokine ligand 2; DAPI, 4',6-diamidino-2-phenylindole; HMGB1, high mobility group box 1; IL-1 β ,

interleukin 1 beta; IL-6, interleukin 6; IL-10, interleukin 10; LT, liver transplantation; SEM, standard error of the mean; TNF- α , Tumor necrosis factor α .

Figure 5

Analysis of vulnerability in cold-preserved steatotic graft. (A) A schematic of the experimental procedure. Following a 5-hour cold storage period, a liver graft was flushed with 2 ml of normal saline via the portal vein. The resulting liver flush, or effluent, along with liver tissue were analyzed in pairs. **(B)** Western blot-assisted detection of HMGB1 in the liver flush. **(C)** Western blot-assisted analysis of HMGB1 in liver tissue, with beta-actin serving as the loading control. Data are depicted as the mean $\pm$ SEM. Statistical significance was determined by unpaired t-test; * $p < 0.05$; ** $p < 0.01$. ALT, alanine aminotransferase; HMGB1, high mobility group box 1; SEM, standard error of the mean.

Figure 6

Outcomes of steatotic LT in TLR4 knockout recipient mice. (A) Recipient survival comparison among three groups ($n = 4-6$ /group). Statistical significance was determined by the log-rank test. **(B)** Time-course analysis of serum ALT and HMGB1 levels. **(C)** Histological evaluation at 6 hours post-reperfusion in TLR4 knockout recipients (H&E, left panel $\times 100$; right panel $\times 400$; scale bar, 100 μm). **(D)** Relative mRNA expression levels of cytokines, chemokines, and caspases measured in liver samples collected at each timepoint ($n = 5$ /group). **(E)** Quantification of CD11b- and Ly6g-positive cells per high-power field in liver samples at 6 hours post-reperfusion. **(F)** Western blot analysis of cleaved caspase-3 at 1 and

6 hours after reperfusion across three groups. Data are depicted as the mean $\pm$ SEM. Statistical significance was determined by unpaired t-test (B, D, F) and one-way ANOVA with a Turkey test (E); ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. The asterisk indicates the p-value comparing outcomes between steatotic LT into wild-type recipients and TLR4 KO recipients (B, D, F). ANOVA, analysis of variance; CXCL1, C-X-C motif chemokine ligand 1; CXCL2, C-X-C motif chemokine ligand 2; H&E, hematoxylin and eosin; HMGB1, high mobility group box 1; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; IL-10, interleukin 10; KO, knockout; LT, liver transplantation; SEM, standard error of the mean; TNF- α , Tumor necrosis factor α ; TLR4, toll-like receptor 4.

Supporting information statement

Additional supporting information may be found online in the Supporting Information section.

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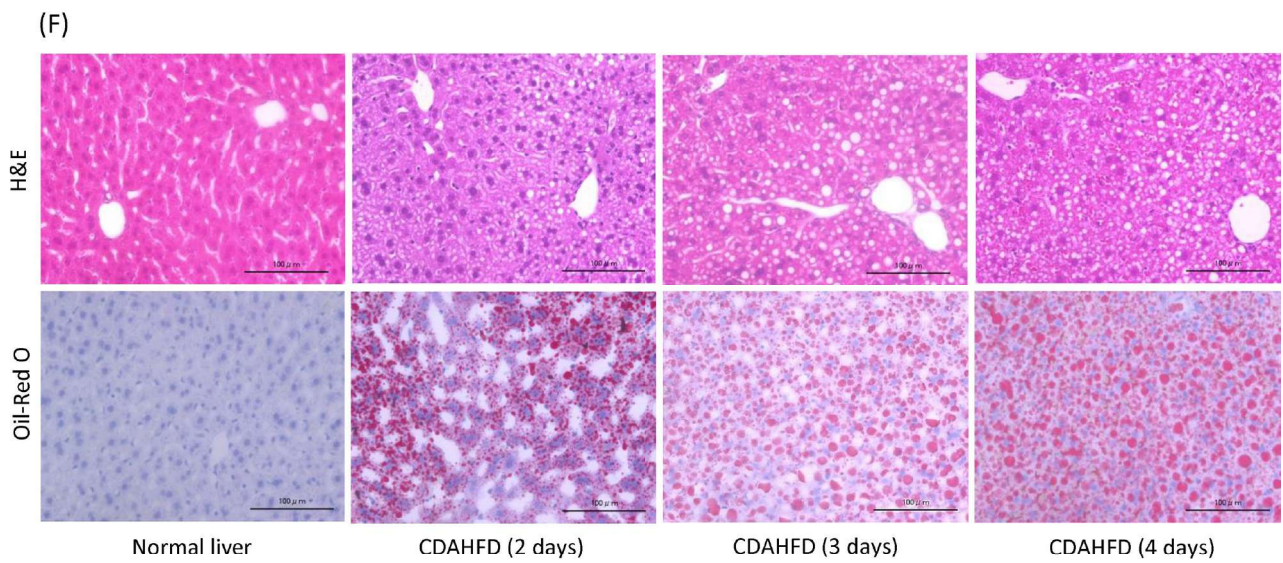
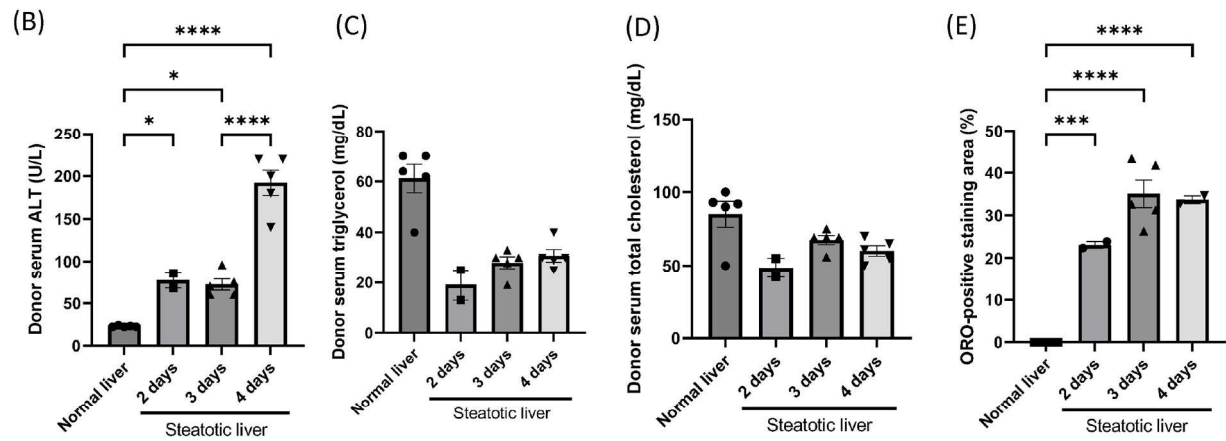
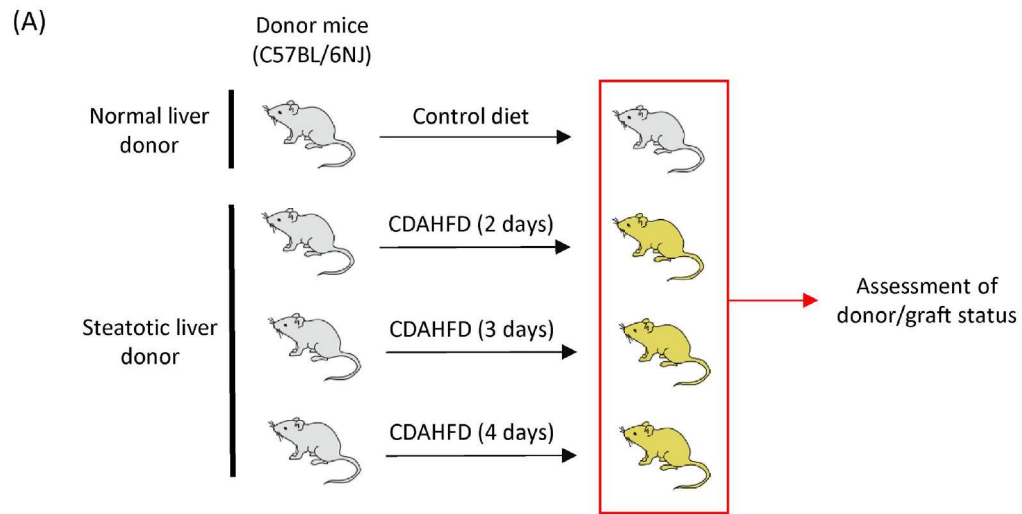
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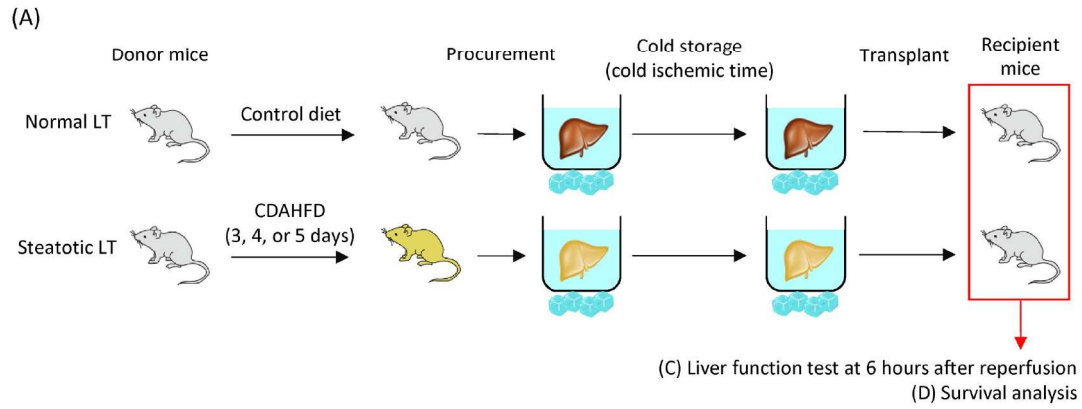
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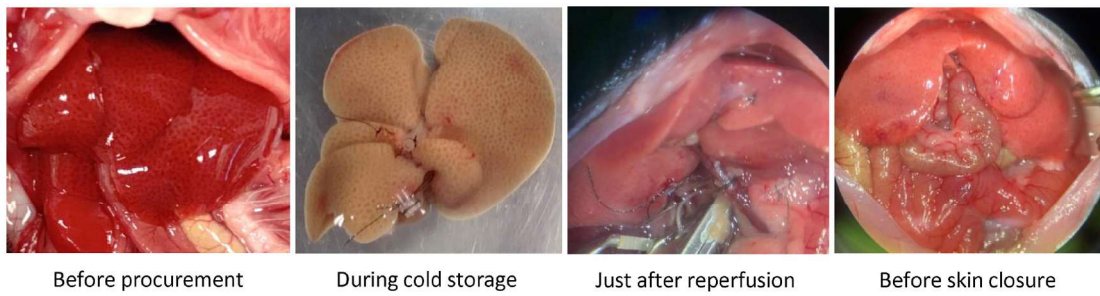
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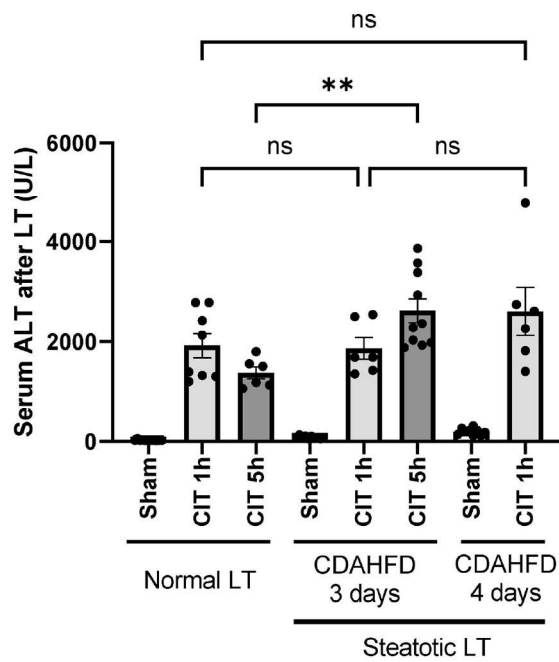




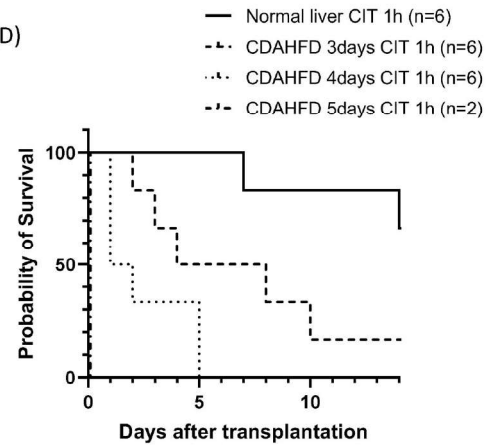
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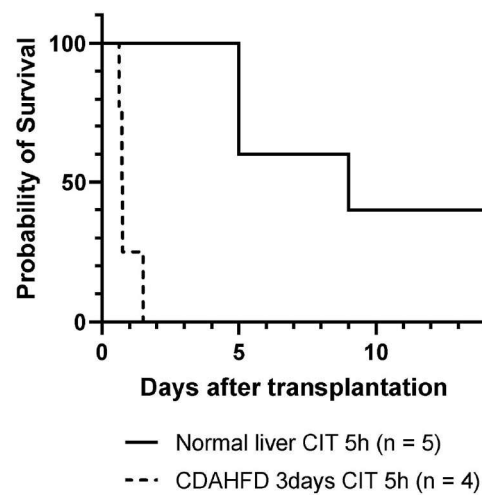
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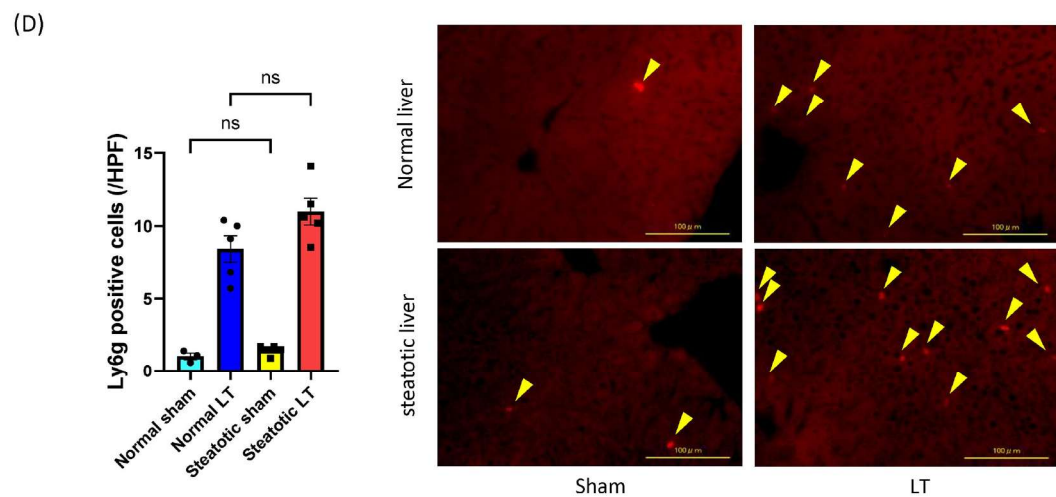
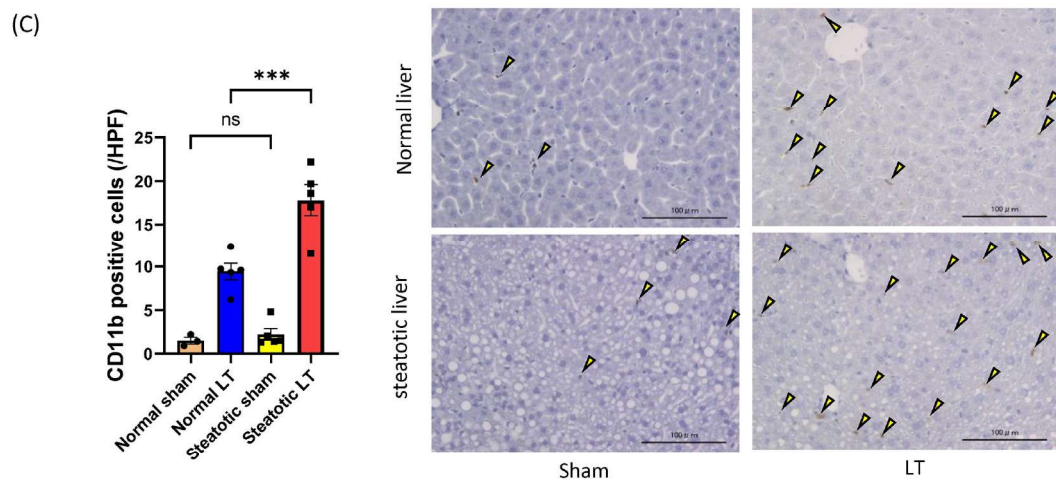
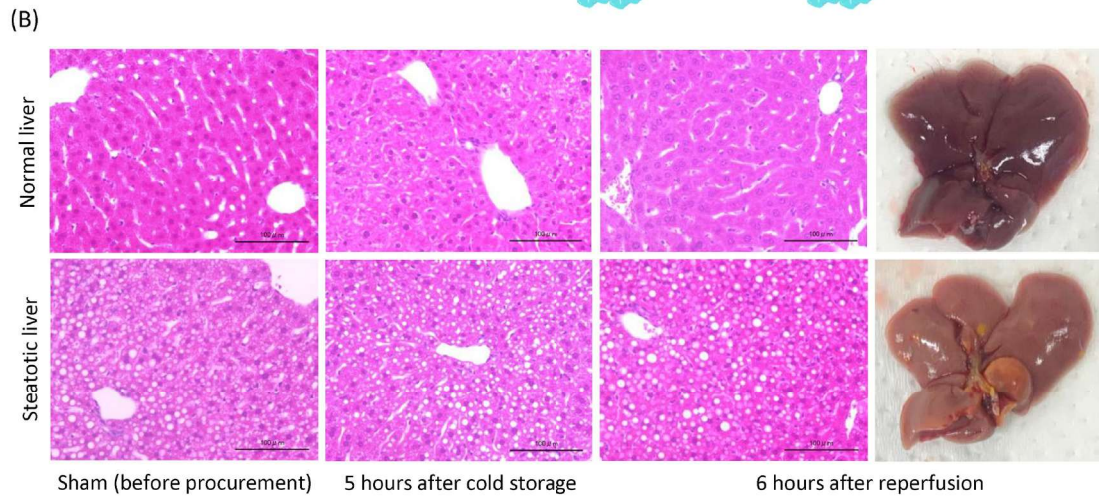
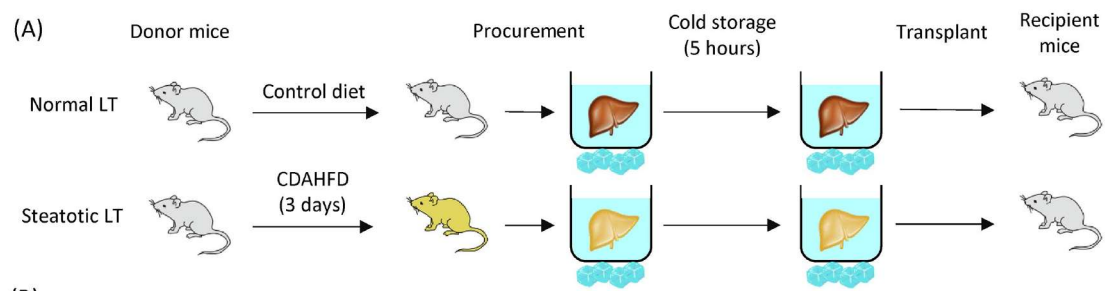


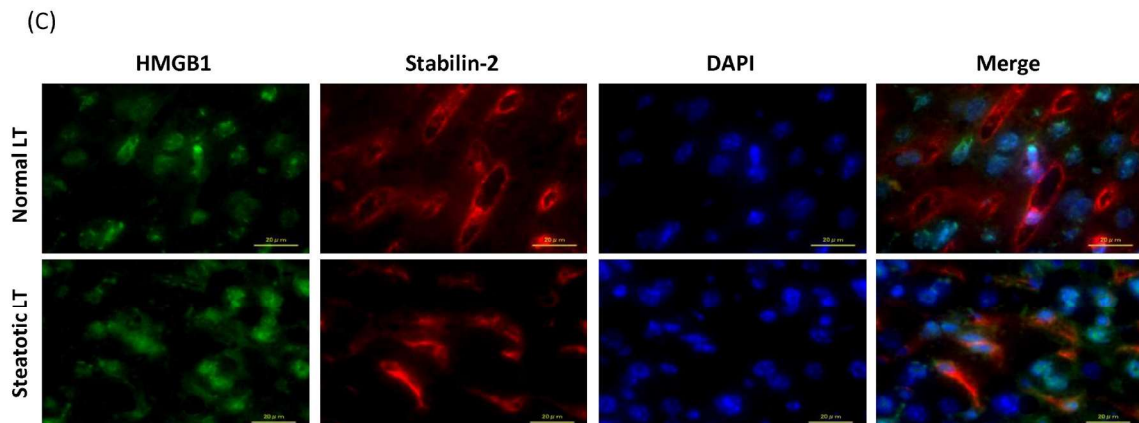
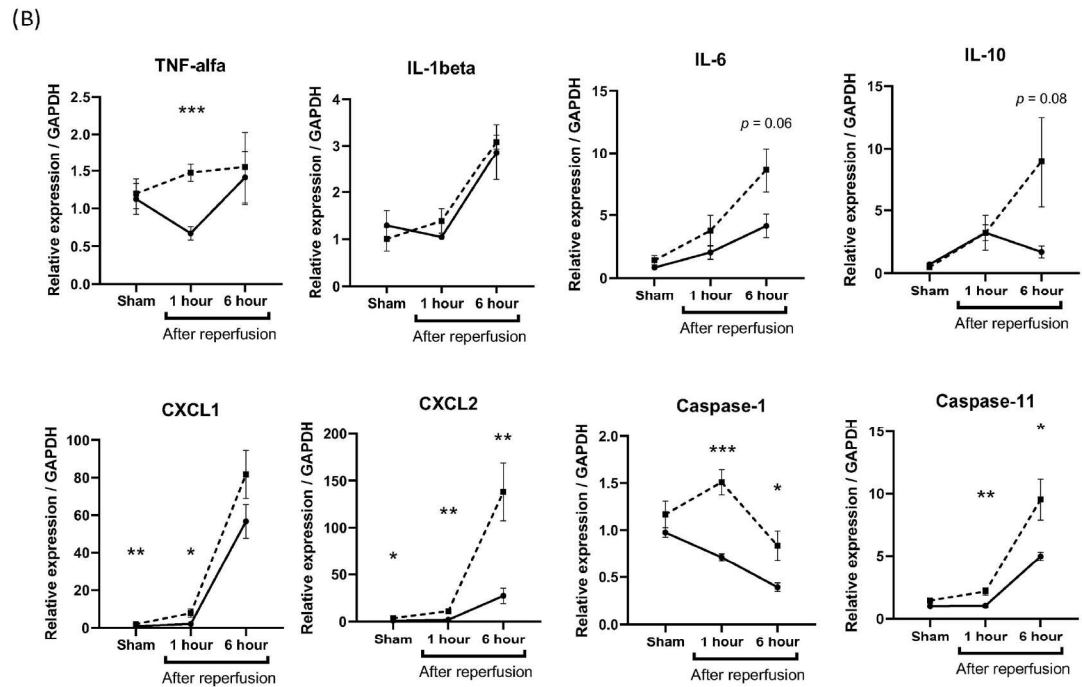
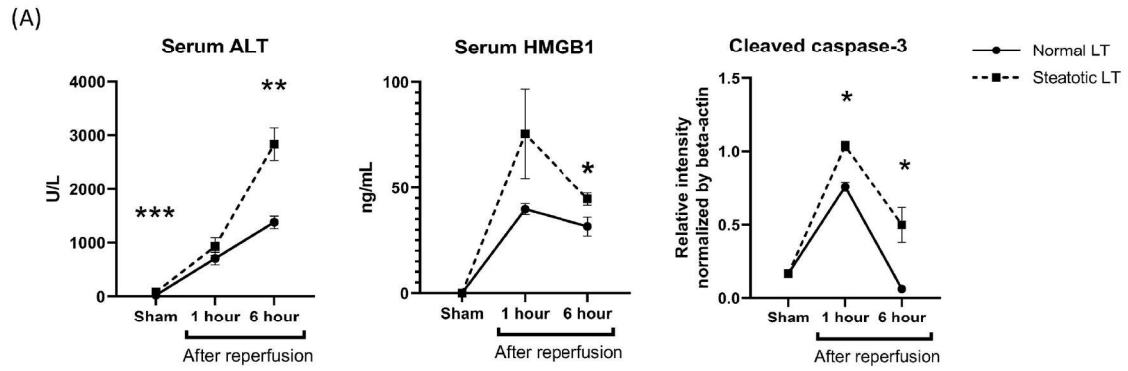
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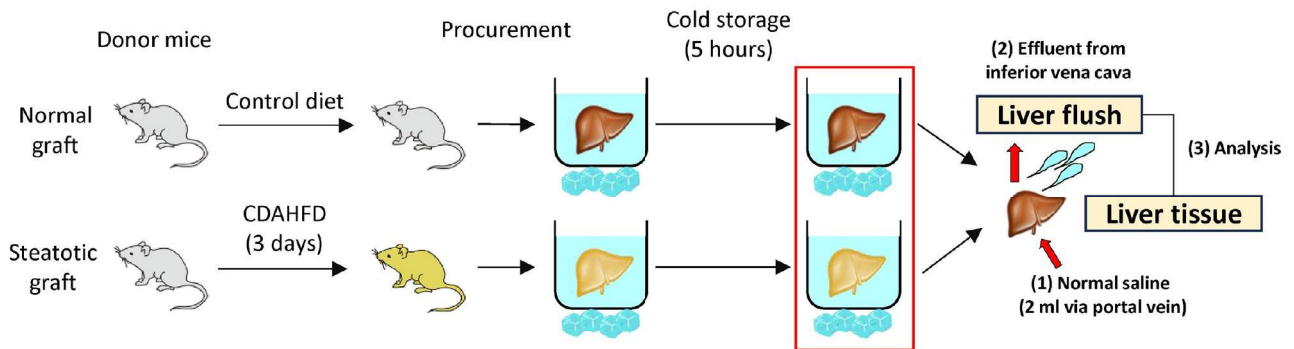
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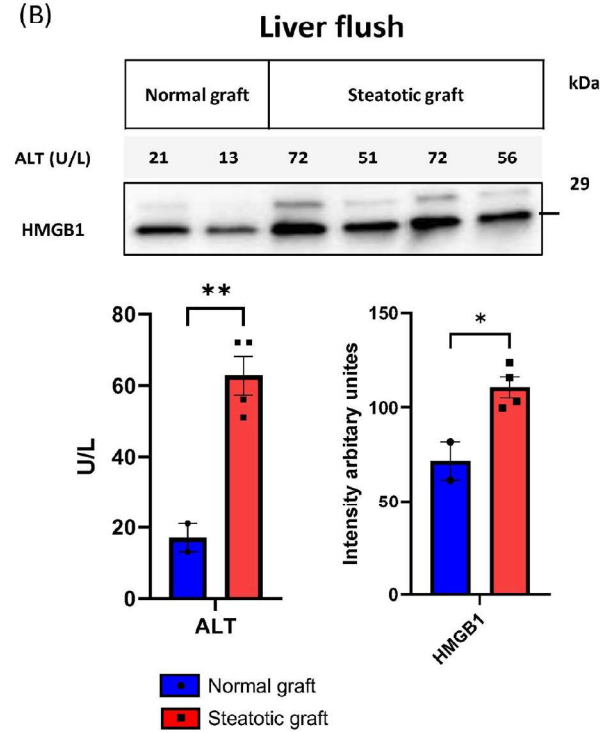




(A)



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