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BRIEF COMMUNICATION

AJT

Serelaxin induces Notch1 signaling and alleviates hepatocellular damage in orthotopic liver transplantation

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Liver ischemia-reperfusion injury (IRI) represents a risk factor for early graft dysfunction and an obstacle to expanding donor pool in orthotopic liver transplantation (OLT). We have reported on the crucial role of macrophage Notch1 signaling in mouse warm hepatic IRI model. However, its clinical relevance or therapeutic potential remain unknown. Here, we used Serelaxin (SER), to verify Notch1 induction and putative hepatoprotective function in ischemia-reperfusion–stressed OLT. C57BL/6 mouse livers subjected to extended (18-hour) cold storage were transplanted to syngeneic recipients. SER treatment at reperfusion ameliorated IRI, improved post-OLT survival, decreased neutrophil/macrophage infiltration, and suppressed proinflammatory cytokine programs, while simultaneously increasing Notch intracellular domain (NICD) and hairy and enhancer of split 1 (Hes1) target genes. In bone marrow–derived macrophage cultures, SER suppressed proinflammatory while enhancing antiinflammatory gene expression concomitantly with increased NICD and Hes1. Hepatic biopsies from 21 adult primary liver transplant patients (2 hours postreperfusion) were divided into low-NICD (n = 11) and high-NICD (n = 10) expression groups (western blots). Consistent with our murine findings, human livers characterized by high NICD were relatively IRI resistant, as shown by serum alanine aminotransferase (ALT) levels at day 1 post-OLT. Our study documents the efficacy of SER–Notch1 signaling in mouse OLT and highlights the protective function of Notch1 in liver transplant patients.

KEYWORDS
animal models: murine, basic (laboratory) research/science, immunobiology, Ischemia-reperfusion injury (IRI), liver disease: immune/inflammatory, liver transplantation/hepatology, macrophage/monocyte biology, tissue injury and repair, translational research/science

1 | INTRODUCTION

Orthotopic liver transplantation (OLT) has become the standard care for patients with end-stage liver disease and those with hepatic malignancies. Liver ischemia-reperfusion injury (IRI), an innate immune-driven inflammation response leading to hepatocellular death, is an inevitable consequence of multiple clinical conditions, including trauma, sepsis, hepatectomy, and liver transplantation. Indeed, hepatic IRI has been recognized as a major risk factor for delayed early graft function and acute and chronic rejection, as well

Abbreviations: BMDM, bone marrow-derived macrophage; Bx, biopsy; cDNA, complementary DNA; eNOS, endothelial nitric oxide synthase; GR, glucocorticoid receptor; H&E, hematoxylin and eosin; Hes1, hairy and enhancer of split 1; HPF, high power fields; HUVEC, Human Umbilical Vein Endothelial Cells; INR, international normalized ratio; IR, ischemia-reperfusion; IRI, ischemia-reperfusion injury; LPS, lipopolysaccharide; MELD, model for end-stage liver disease; NICD, Notch intracellular domain; NO, nitric oxide; OLT, orthotopic liver transplantation; POD1, postoperative day 1; RLX-2, relaxin-2; RT-PCR, reverse transcription-polymerase chain reaction; RXFP1, relaxin family peptide receptor 1; sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; SD, standard deviation; SEM, standard error of the mean; SER, Serelaxin; siRNA, small interfering RNA; T-bil, total bilirubin; TLR4, Toll-like receptor 4; TUNEL, TdT-mediated dUTP nick end labeling; UW, University of Wisconsin; WT, wild-type.

Shoichi Kageyama and Kojiro Nakamura contributed equally.

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as a key obstacle to expanding the donor pool. However, despite clinical importance, mechanisms that account for liver IRI are partially understood, and there are no effective strategies to combat IRI in humans.[1]

Relaxin, a group of low-molecular-weight peptide hormones of the insulin-growth factor family, consists of 7 members, with relaxin-2 (RLX-2) accounting for most of the biological effects in humans and animals.[2] Originally isolated from ovary and named for its ability to "relax" pubic symphysis during pregnancy,[3] relaxin plays a role in hemodynamic change, such as decreasing systemic vascular resistance, increasing cardiac output, and improving global arterial compliance.[4] Experiments demonstrating cytoprotective,[5] antiinflammatory[6] and antifibrotic[7] functions, possibly due to antioxidant effects, underscore the increasing interest in RLX-2 as a pharmacotherapeutic agent. Indeed, a recent phase III randomized clinical trial showed the efficacy, safety, and tolerability of Serelaxin (SER, recombinant human relaxin-2) in patients with acute heart failure.[8]

By binding with high affinity to cognate receptor relaxin family peptide receptor 1 (RXFP1), the majority of RLX-2–related signal transduction involves the RXFP1 molecule.[9] RXPF1 is expressed in reproductive tissues, heart, kidney, lung, and brain,[10] while protective effects of RLX-2 induction were described in heart,[2] kidney,[11] and lung[12] IRI models. Although barely detectable in normal rat liver, RXFP1 is expressed predominantly in fibrotic lesion after chronic carbon-tetrachloride treatment.[13] The mechanisms and putative clinical relevance of apparent RLX-2 protection seen in ex vivo isolated rat livers[14] remain to be elucidated.

Notch1 is a highly conserved transmembrane receptor involved in cell fate decision, proliferation, differentiation and regeneration.[15] Upon ligand binding, the Notch intracellular domain (NICD) is released by proteolytic cleavage operated by the γ-secretase complex, and translocates into the nucleus followed by the activation of Notch1 target genes, including hairy and enhancer of split 1 (Hes1).[16] Disruption of Notch1 transcription factor RBP-J increased cell apoptosis/necrosis and inflammatory response leading to aggravated liver IRI.[17] We have shown that myeloid-specific Notch1 deficiency augmented macrophage activation and hepatocellular damage in a mouse warm IRI model.[18] The clinical relevance of Notch1 signaling in OLT is unknown, and therapeutic potential of Notch1 induction has not been tested to date. Although SER may stimulate Notch1 in cardiac fibroblast,[19] cardiomyocyte,[20] and Human Umbilical Vein Endothelial Cells (HUVEC)[21] systems, whether SER can modulate Notch1 signaling in liver transplantation remains to be defined.

The present translational study was designed to investigate the therapeutic potential of SER and determine the role of Notch1 in SER-mediated hepatoprotection in a murine IRI-OLT model. In a clinical arm, we assessed the relevance of Notch1 in liver transplant patients. Our findings document the significance of Notch1 in hepatoprotection/innate immune regulation, and highlight SER-mediated therapeutic potential of Notch1 activation in liver transplantation.

2 | MATERIALS AND METHODS

2.1 | Animals

C57BL/6 mice at 6-8 weeks of age were used (Jackson Laboratory, Bar Harbor, ME). Animals were housed in a UCLA animal facility under specific pathogen-free conditions and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23 revised 1985). All studies were reviewed and approved by the UCLA Animal Research Committee.

2.2 | Reagents

Serelaxin (SER; recombinant form of human relaxin-2, RLX030) was provided by Novartis International AG (East Hanover, NJ). Lipopolysaccharides (LPS) and IL4 were obtained from Sigma Aldrich (St. Louis, MO).

2.3 | Mouse liver transplantation

We used an established mouse model of ex vivo hepatic cold storage and OLT.[22] To mimic the "marginal" human OLT setting, donor livers were stored in University of Wisconsin (UW) solution at 4°C for 18 hours prior to transplantation into syngeneic mice. Animals were treated intravenously with SER (5 μg/kg) or Ringer's lactate solution (control) at the time of reperfusion. Optimal dose of SER was determined in a preliminary experiment comparing the effects of 0/1/3/5/10 μg/kg given at the time of reperfusion (n = 2/group, data not shown). Liver and serum samples were collected at 6 hours, the peak of hepatocellular damage in this model. Separate groups of OLT recipients were monitored for 14-day survival. The sham group underwent the same procedures except for OLT.

2.4 | Hepatocellular function assay

Serum alanine aminotransferase (sALT) and aspartate aminotransferase (sAST) levels were measured with Infinity ALT and AST Liquid Stable Reagent (Thermo Scientific, Rockford, IL) and validated with ValidateGC3 (Maine Standards Company, LLC, ME).

2.5 | Liver histology and IRI grading

Formalin-fixed paraffin-embedded liver sections (5 μm) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria.[22]

2.6 | TdT-mediated dUTP nick end labeling (TUNEL) assay

Cell death in formalin-fixed paraffin-embedded liver sections (5 μm) was detected by Apop Tag Plus Peroxidase in Situ Apoptosis Kit (Millipore, Temecula, CA). Results were scored semi-quantitatively by blindly counting the number of positive cells in 10 high power fields (HPF)/section.

2.7 | Immunohistochemistry

The expression of RXFP1 (liver and heart) was examined using rabbit anti-RXFP1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Immunostaining signals were visualized with a labeled polymer in the EnVision+ system horseradish-peroxidase kit (Dako, Carpinteria, CA). Liver-infiltrating neutrophils and macrophages were detected with rat anti-Ly6G Ab (BD Biosciences, San Jose, CA) and rabbit anti-CD11b Ab (Abcam, Cambridge, MA), respectively. Signals were visualized with secondary Alexa Fluor 488 IgG. Results were scored semi-quantitatively by blindly counting the number of positive cells in 10 HPF/section (×400).

2.8 | Cell isolations and in vitro cultures

Femurs and tibias were removed from wild-type (WT) mice, and bone marrow–derived macrophages (BMDMs) were generated, as described.[22] In some experiments, cells pretreated with SER (5 μg/mL, 24 hours) were stimulated with LPS (100 ng/mL, 6 hours) or IL4 (10 ng/mL, 24 hours).

2.9 | Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

RNA was extracted from livers or cell samples using RNAse Mini Kit (Qiagen, Germantown, MD). A total of 5.0 μg of RNA was reverse-transcribed into complementary DNA (cDNA). Quantitative PCR was performed using Quant studio 3 (Applied Biosystems, Foster city, CA). The primers sequences are listed (Table S1). The expression of the target gene was normalized to the housekeeping HPRT or β2M.

2.10 | Western blot assay

Proteins were extracted from liver or cell samples, followed by concentration measurement (BCA Protein Assay Kit, Thermo Scientific). Equal amount of protein was electrophoresed, blotted, and incubated with primary Ab and secondary HRP-conjugated Ab, and developed. Primary Ab detecting NICD, pIκBα (Ser32), cleaved caspase-3, glucocorticoid receptor (GR), β-actin (Cell Signaling Technology, Danvers, MA), Hes1, RXFP1 (Santa Cruz Biotechnology), and CD206 (R&D Systems, Minneapolis, MN) were used.

2.11 | Clinical liver transplantation study

Twenty-one adult OLT recipients recruited between May 2013 and August 2015 received routine standard of care and immunosuppressive therapy, as specified by UCLA liver transplant protocols.[23] Study data were managed using REDCap electronic data capture tools. Recipient blood was collected prior to the transplant and at postoperative day 1 (POD1). Liver function was evaluated by sALT/sAST. Posttransplantation biopsy (Bx) samples were collected from the left liver lobe 2 hours after portal reperfusion (prior to the abdominal closure) followed by western blot analyses. To compare target protein expression in multiple human OLT samples, densitometry quantification was conducted using a reference sample and normalization with β-actin, as reported.[23]

2.12 | Statistical analysis

In mouse experiments, group comparisons were performed using a Student *t*-test. For human data, continuous values were analyzed by Mann-Whitney *U* test and categorical variables by Fisher's exact test. The cumulative survival rate was analyzed by the Kaplan-Meier method, and differences between groups were compared using a log-rank test. JMP for Windows 8.0 (SAS Institute, Cary, NC) was used for statistical analyses. A *P*-value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | SER ameliorates hepatic IRI and improves OLT survival

We first aimed to determine the efficacy of SER treatment in mouse OLT with extended cold storage (18 hours), which mimics marginal human liver transplantation. Recipient mice (n = 5/group) were infused intravenously with SER (5 μg/kg) or Ringer's lactate (control) at the completion of surgery (immediately prior to reperfusion). At 6 hours posttransplantation, SER-treated OLT displayed attenuated sinusoidal congestion, edema/vacuolization, and hepatocellular necrosis as compared with controls (Figure 1A). These correlated with decreased Suzuki's histological grading of liver IRI (control-OLT = 5.0 ± 1.9 vs SER-OLT = 3.2 ± 0.6; *P* = .030, Figure 1B); attenuated hepatocellular damage (sALT: control-OLT = 8791 ± 1032 vs SER-OLT = 6762 ± 551 IU/L; *P* = .005; and sAST: control-OLT = 2754 ± 992 vs. SER-OLT = 1676 ± 486 IU/L; *P* = .042; Figure 1C); and decreased number of TUNEL+ cells (control-OLT = 47.6 ± 20.8 vs SER-OLT = 9.6 ± 4.2; *P* = .008, Figure 1A,B). These effects of SER at 6 hours postreperfusion correlated with improved (*P* = .030) overall OLT survival in SER-OLT (69.2%; n = 13) as compared to control-OLT (35.7%; n = 14) recipient groups at 14 days posttransplantation (Figure 1D). Thus SER treatment ameliorated ischemia-reperfusion (IR)–induced hepatocellular injury and improved OLT outcomes.

3.2 | Marginal hepatic RXFP1 expression in OLT with/without SER treatment

Having shown the hepatoprotective effect of SER treatment in IR-stressed OLT, we next assessed RXFP1 (cognate receptor of SER) expression, aiming to gain insight into underlying SER cytoprotection. Consistent with previous reports,[13] unlike in normal murine heart (positive control), RXFP1 was undetectable by immunohistology in sham-operated livers (Figure S1A). Neither OLT nor SER treatment affected hepatic RXFP1 expression—data confirmed by western blots (Figure S1B). Moreover, SER treatment failed to enhance RXFP1 levels in IR-stressed OLT. Hence, it is unlikely that RXFP1 signaling is essential in SER hepatoprotection.

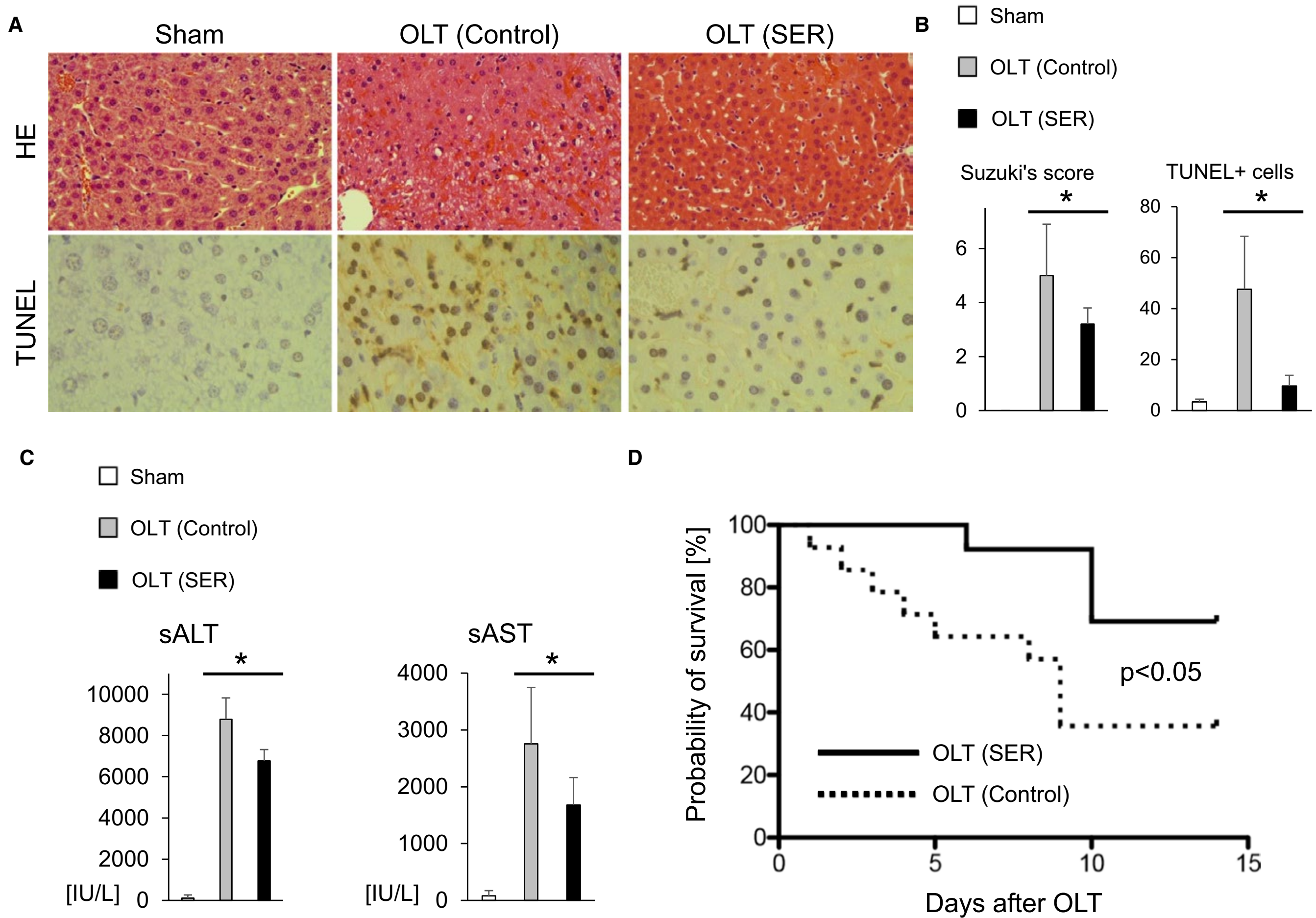

FIGURE 1 SER attenuates hepatic IRI and improves mouse OLT survival. C57/Bl6 donor livers subjected to extended cold storage (18 hours) were transplanted to syngeneic mice. OLT recipients were treated intravenously with SER (5 μg/kg) or an equivalent amount of Ringer's lactate solution (control) at reperfusion. Liver and serum samples were collected at 6 hours postsurgery. (A) Representative H&E (upper panels, original magnification, ×100) and TUNEL (lower panels, original magnification, ×400) staining. (B) Suzuki's histological grading of liver IRI and quantification of TUNEL+ cells/HPF (n = 5/group). (C) Serum ALT and AST levels (IU/L, n = 5/group). Data shown as mean ± standard deviation [SD] (*P < .05, Student t-test). (D) Animals were monitored for 14 days and cumulative survival was analyzed by the Kaplan-Meier method. The solid line indicates SER-treated (n = 13) while the dotted line indicates control (n = 14) mice (*P < .05, log-rank test)

3.3 | SER mitigates leukocyte infiltration in IR-stressed OLT

Because cytokine/chemokine-producing leukocytes in OLT accelerate IR-damage, we next evaluated the SER effect on neutrophil (Ly6G) and macrophage (CD11b) infiltration (n = 5/group). As shown in Figure 2A,B, decreased numbers of Ly6G+ and CD11b+ cells were observed in SER treated as compared with control OLT (Ly6G: control-OLT = 44.4 ± 3.5 vs SER-OLT = 27.2 ± 4.8/HPF; P = .001; and CD11b: control-OLT = 39.6 ± 2.2 vs SER-OLT = 26.6 ± 5.7/HPF; P = .002).

3.4 | SER suppresses proinflammatory IR-signature and induces Notch1 signaling in OLT

Because our recent study has identified the regulatory function of Notch1 for innate immune activation in liver IRI,[18] we next asked whether SER may promote Notch1 signaling in OLT. Indeed, as shown in Figure 2C, SER treatment was accompanied by upregulation of NICD and Hes1 (one of downstream Notch1 target genes). In addition, SER decreased pIκBα expression (Figure 2C), depressed mRNA levels of pro-inflammatory MCP1, IL1β, CXCL1, CXCL2, and CXCL10, and increased mRNA levels of antiinflammatory Arg1 and IL10 (Figure 2D). Consistent with Figure 1A,B findings, SER treatment decreased cleaved caspase-3 expression (terminal apoptosis executer) in OLT. Thus, hepatoprotective (Figure 1) and antiinflammatory (Figure 2A,B,D) function of SER was accompanied by Notch1 induction in IR-stressed OLT.

3.5 | SER induces Notch1 signaling and inhibits LPS-stimulated macrophage activation in vitro

Because macrophages play a key role in innate immune activation in IR-stressed OLT, we next evaluated the impact of SER on BMDM cultures under LPS stimulation. Consistent with Figure 2C,D data,

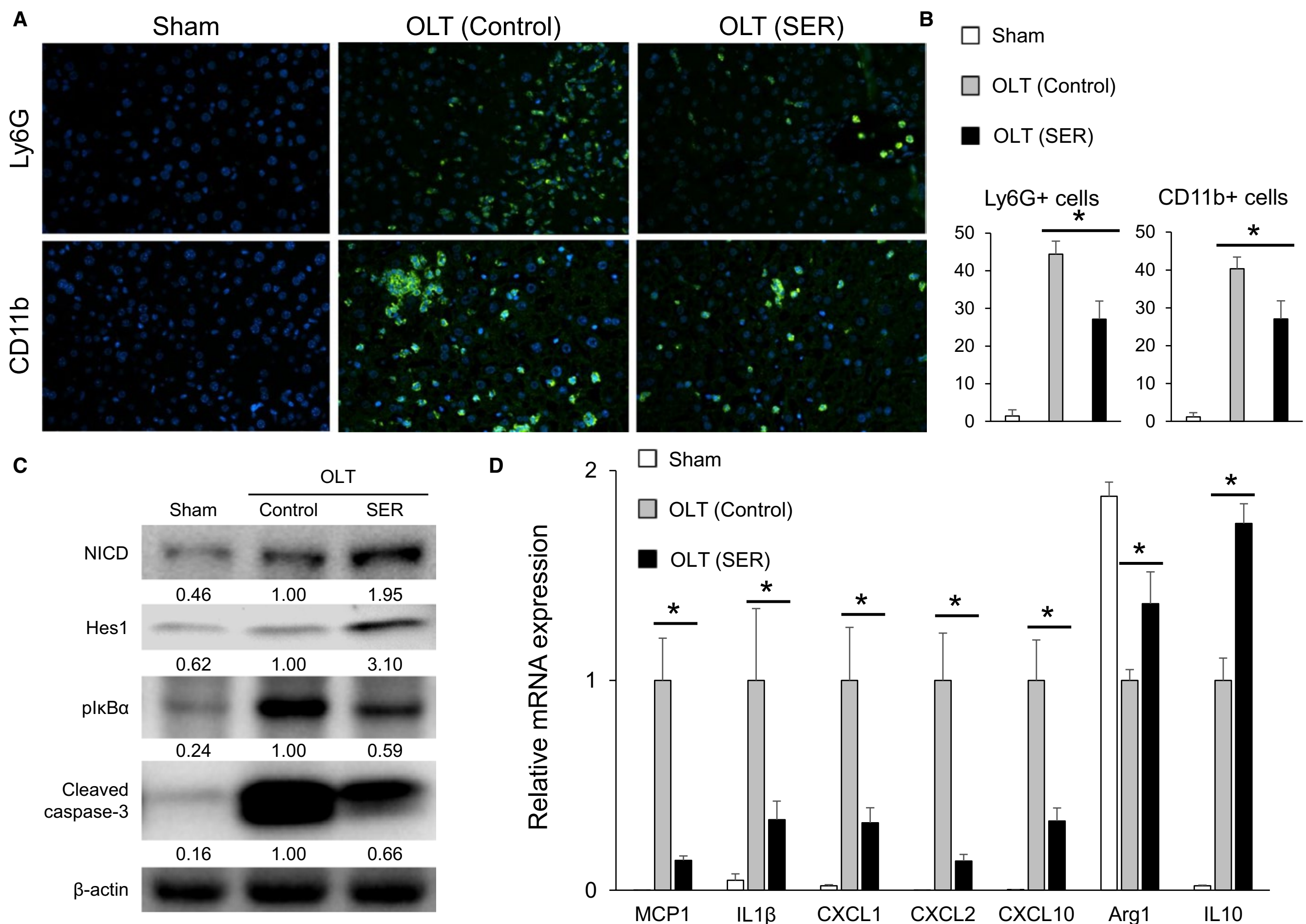

FIGURE 2 SER alleviates IR inflammation and induces Notch1 signaling in mouse OLT. C57/Bl6 donor livers subjected to extended cold storage (18 hours) were transplanted to syngeneic mice. OLT recipients were treated intravenously with SER or an equivalent amount of Ringer's lactate solution (control) at reperfusion, followed by sampling (6 hours postreperfusion). (A) Representative immunohistochemical staining of OLT-infiltrating Ly6G+ (neutrophil) and CD11b+ (macrophage) cells. (B) Quantification of Ly6G+ and CD11b+ cells/HPF (n = 5/group). (C) Western blot–assisted detection of NICD, Hes1, pIκBα (Ser32), and cleaved caspase-3. β-actin expression served as an internal control for normalization. The values under the bands represent the relative ratio of normalized intensity compared to OLT (Control). Representative of 3 experiments is shown. (D) Quantitative RT-PCR–assisted detection of mRNA coding for MCP1, IL1β, CXCL1, CXCL2, CXCL10, Arg1, and IL10. Data were normalized to HPRT gene expression (n = 4/group). Data are shown as mean ± SD (*$P < .05$, Student *t*-test)

SER enhanced Notch1 signaling (NICD and Hes1, Figure 3A); decreased pIκBα protein expression (Figure 3A); suppressed proinflammatory gene program (MCP1, IL1β, CXCL1, CXCL2, and CXCL10); and increased immunoregulatory Arg1 signaling in LPS-stimulated BMDM (Figure 3B). As shown in Figure S2, and increasing dose of SER treatment upregulated NICD and CD206 while decreasing pIκBα levels in cultured BMDM. Of note, the expression of RXFP1 and glucocorticoid receptor (or GR, another SER receptor) remained unchanged, implying the importance of Notch1 signaling in macrophage regulation.

3.6 | SER enhances antiinflammatory gene expression in IL4-stimulated macrophage cultures

We next examined whether SER may affect antiinflammatory gene programs in vitro. Consistent with Figures 2C, 3A data, SER increased NICD and Hes1 expression in IL4-stimulated BMDMs (Figure 3C). In parallel, SER adjunct enhanced mRNA levels coding for antiinflammatory Arg1, CD163, and CCL24 (Figure 3D). These findings, consistent with our studies on the critical role of Notch1 in macrophage immunoregulation,[18] imply Notch1 in SER-mediated macrophage regulation.

3.7 | Increased NICD expression correlates with attenuated liver damage in human OLT

Having shown the regulatory function of Notch1 in SER-mediated hepatoprotection in mouse OLT, we next aimed to determine the clinical relevance of Notch1 signaling in human OLT. Postreperfusion liver Bx from 21 human OLTs screened by western blot, were classified into low-NICD (n = 11) and high-NICD (n = 10) expression groups (Figure 4A left column; representative western

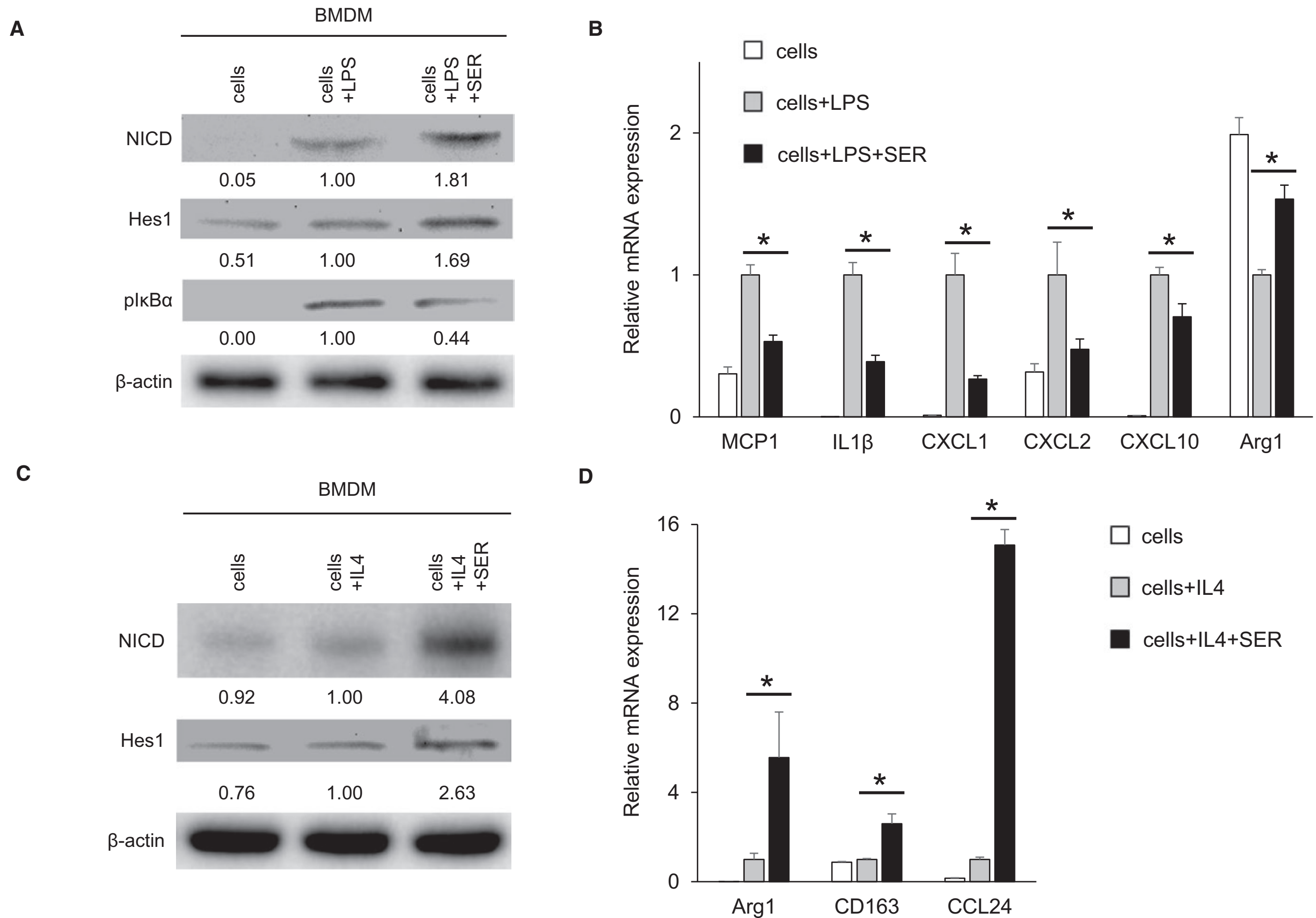

FIGURE 3 SER inhibits proinflammatory but enhances antiinflammatory signature and upregulates Notch1 signaling in BMDM cultures. (A/B) BMDM stimulated with LPS (100 ng/mL, 6 hours) were pretreated with or without SER (5 µg/mL, 24 hours). (A) Western blot–assisted detection of NICD, Hes1, and pIκBα (Ser32). β-Actin expression serves as an internal control for normalization. The values under the bands represent the relative ratio of normalized intensity compared to cells + LPS. Representative of 3 experiments is shown. (B) Quantitative RT-PCR–assisted detection of mRNA coding for MCP1, IL1β, CXCL1, CXCL2, CXCL10, and Arg1. Data were normalized to β2M gene expression (n = 4/group). (C/D) BMDM stimulated with IL4 (10 ng/mL, 24 hours) were pretreated with or without SER (5 µg/mL, 24 hours). (C) Western blot–assisted detection of NICD and Hes1. β-Actin expression served as an internal control for normalization. The values under the bands represent the relative ratio of normalized intensity compared to cells + IL4. Representative of 3 experiments is shown. (D) Quantitative RT-PCR–assisted detection of mRNA coding for Arg1, CD163, and CCL24. Data were normalized to β2M gene expression (n = 4/group). Data are shown as mean ± SD (*$P < .05$, Student *t*-test)

blots are shown in right column). The patients' demographic data and clinical parameters are shown (Table S2). There was no correlation between NICD expression and donor/graft background, including age, gender, weight, BMI, cold ischemia time, or preprocurement blood tests (ALT, AST, T-bil [total bilirubin], and INR [international normalized ratio]). We found no correlation between NICD expression and recipient factors, including age, gender, weight, BMI, race, disease etiology, presence of hepatocelluular carcinoma, ABO-compatibility, MELD score, or pretransplantation blood tests. Notably, the high-NICD expression group had significantly lower sALT (216 ± 46 vs 517 ± 127 IU/L, mean ± standard error of the mean [SEM], *P* = .0486) and lower sAST (196 ± 45 vs 619 ± 208 IU/L, mean ± SEM; *P* = .0725) levels at POD1 (Figure 4B), indicating that OLTs with increased NICD expression were relatively more resistant to IR damage. To examine the relationship between NICD graft levels and clinical outcomes, we analyzed postoperative hospital stay and cumulative posttransplantation survival with median follow-up of 712 days (range, 27-1009 days). None of the patients underwent secondary liver transplantation. Consistently, high-NICD expression group exhibited shorter post-OLT hospital stay (32.4 ± 4.3 vs 40.5 ± 10.4 days, *P* = .5253) and better post-OLT survival (500 days: 93.3% vs 80.0%, *P* = .3673). However, these differences did not reach statistical significance.

4 | DISCUSSION

A recent phase III randomized clinical trial in a cohort of 1161 patients with acute heart failure demonstrated that SER treatment was associated with an improvement of patients' symptoms and significant

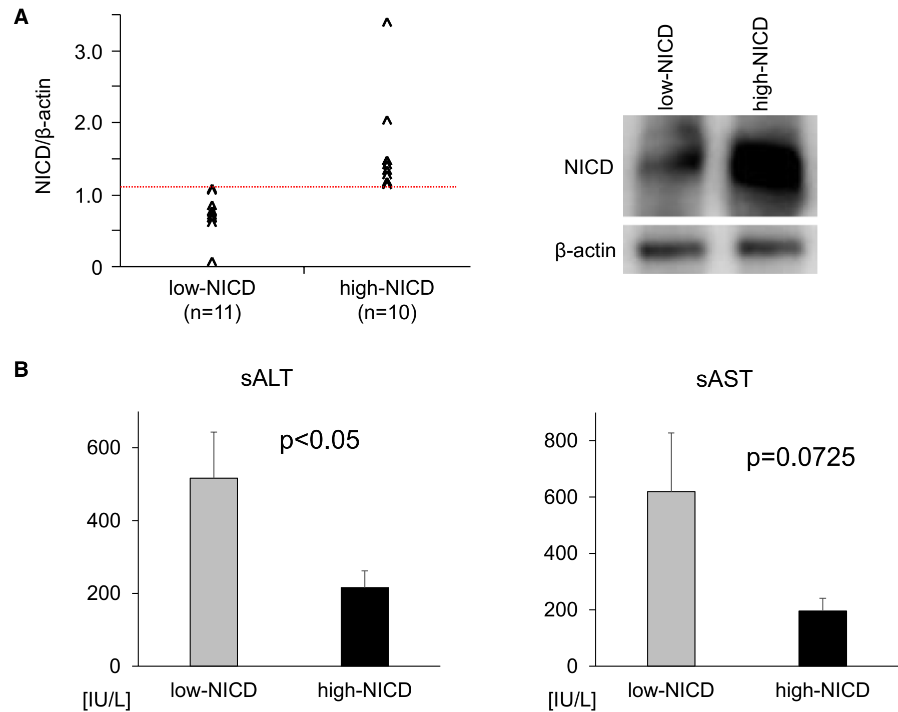

FIGURE 4 NICD levels correlate with the hepatocellular function in human OLT. (A) Based on Western blot–assisted relative protein expression, 21 human OLT biopsies were classified into low-NICD (n = 11) and high-NICD (n = 10) groups (left panel). Representative NICD expression profile is shown in the right panel. (B) Hepatocellular function, assessed by serum ALT and AST levels at POD1. Data are shown as mean ± SEM (Mann-Whitney U test)

reduction of cardiovascular mortality through day 180.[8] By documenting the hepatoprotective effects of SER in a clinically relevant mouse IRI-OLT model, our findings support a new application of SER in organ transplantation. Indeed, SER given at the time of reperfusion in mice mitigated liver damage, as evidenced by preserved hepatic architecture and decreased hepatocellular death (Figure 1A-C); improved post-OLT survival (Figure 1D); inhibited local neutrophil/macrophage infiltration (Figure 2A,B); and depressed proinflammatory signature (Figure 2C,D). The aforementioned clinical trial has also demonstrated that except for a controllable hypotension, SER treatment was well tolerated and safe, with equivalent adverse effects compared with placebo. With no adverse effects during SER treatment and improved OLT survival, our murine findings fortify its tolerable therapeutic use.

Liver IRI represents an innate immune-driven local inflammation followed by hepatocellular death. By recognizing initial danger-associated molecular patterns and triggering Toll-like receptor 4 (TLR4)-mediated inflammation, macrophages are key components of this immune response.[1] Macrophage-derived MCP1 promotes monocyte/macrophage recruitment in IR-stressed OLT, which in turn accelerates local inflammatory cell death.[24] Macrophages also produce neutrophil chemoattractant (CXCL1, CXCL2), which trigger neutrophil infiltration, reactive oxidative species diffusion, homeostasis disturbances, and cell death.[25] Consistent with these findings, in our current study, SER treatment suppressed MCP1/CXCL1/CXCL2 expression in macrophage cultures (Figure 3B) and IR-stressed OLTs (Figure 2D); attenuated macrophage/neutrophil infiltration into OLTs (Figure 2A,B); and alleviated hepatocellular death (Figure 1A,B), indicating that immunoregulatory role on macrophage is one of the central features of SER-mediated hepatoprotection. On the other hand, we recently reported that myeloid Notch1-Hes1 signaling axis suppressed proinflammatory (MCP1/I1β) but enhanced antiinflammatory (Arg1) gene programs, and as a consequence myeloid Notch1 knockout mice had aggravated liver damage in the warm IRI model.[18] In our current study, SER-mediated in vivo hepatoprotection and in vitro macrophage regulation were accompanied by induction of Notch1-Hes1 and Arg1 (Figures 2 and 3), whereas SER failed to alter RXFP1- or GR-receptor expression in BMDM cultures (Figure S2). These findings identify Notch1-Hes1 axis as the key pathway underlying macrophage regulation by SER. With previously reported contradictory RXFP1/GR function in macrophage regulation and divergent responses to relaxin between different macrophage origins,[26,27] their regulatory mechanisms await future in-depth studies.

To the best of our knowledge, our study is the first to document the beneficial impact of Notch1 induction in liver transplant patients. Indeed, high-NICD levels in hepatic Bx at the time of reperfusion correlated with lower sALT levels at POD1 (Figure 4B, left panel), with no correlation between NICD expression and donor/recipient baseline parameters (Table S2). As our failure to detect significant differences in sAST levels (Figure 4B right panel), post-OLT hospital stay, and post-OLT patient survival might be due to a limited patient cohort (n = 21), future studies with a larger number of OLT recipients are warranted.

RXFP1 was barely detectable in mouse livers, and neither OLT nor SER treatment altered its marginal hepatic expression (Figure S1A,B). Indeed, Fallowfield reported that no RXPF1 protein was detected in naive human or rat livers, whereas RXFP1 became detectable on stellate cells/myofibroblasts and sinusoidal endothelial cells in fibrotic livers.[13] However, despite minimal RXFP1 expression in our study, we cannot exclude a possibility that RXFP1 signaling may function as a component of the beneficial molecular crosstalk of SER in OLT. For example, one of the distinct actions of relaxin is mediated

by RXFP1-PI3K-Akt-endothelial nitric oxide synthase (eNOS) axis on artery and endothelial cells, whereas relaxin dilated sinusoid in unstressed rat liver via an nitric oxide (NO)-dependent manner.[28] Therefore, endothelial RXFP1-signal induction followed by sinusoid dilatation might be a part of the hepatoprotective SER mechanism in OLT. Studies using RXFP1 knockout mice or chemically modified RXFP1-inactive relaxin[29] are needed to substantiate the involvement of RXFP1 in SER-mediated hepatoprotection.

Notch1 signal deficiency by genetic RBP-J disruption decreased manganese superoxide dismutase while increasing ROS and hepatocyte apoptosis.[17] In line with this finding, SER-mediated Notch1 upregulation may exert direct cytoprotection in hepatocytes. On the other hand, SER-primed hepatocyte GR signaling may also promote an anti-apoptotic pathway.[30] Indeed, SER cytoprotection was abolished after glucocorticoid receptor (GR) silencing using small interfering RNA (siRNA) in mouse primary hepatocyte cultures, indicating dominant contribution of GR in hepatocyte protection by SER.

In conclusion, our preclinical study documents the efficacy of SER in graft protection and survival via Notch1 signaling in mouse OLT. Parallel clinical screening highlights the protective function of Notch1 in liver transplant patients. In the context of a recent phase III clinical trial demonstrating promising outcomes of SER in patients with acute heart failure,[8] our translational study supports the rationale for using SER in clinical liver transplantation.

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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*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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