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New therapeutic concepts against ischemia-reperfusion injury in organ transplantation

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Abstract

Introduction: Ischemia-reperfusion injury (IRI) involves a positive amplification feedback loop that stimulates innate immune-driven tissue damage associated with organ procurement from deceased donors and during transplantation surgery. As our appreciation of its basic immune mechanisms has improved in recent years, translating putative biomarkers into therapeutic interventions in clinical transplantation remains challenging.

Areas covered: This review presents advances in translational/clinical studies targeting immune responses to reactive oxygen species in IRI-stressed solid organ transplants, especially livers. Here we focus on novel concepts to rejuvenate suboptimal donor organs and improve transplant function using pharmacologic and machine perfusion (MP) strategies. Cellular damage induced by cold ischemia/warm reperfusion and the latest mechanistic insights into the microenvironment's role that leads to reperfusion-induced sterile inflammation is critically discussed.

Expert opinion: Efforts to improve clinical outcomes and increase the donor organ pool will depend on improving donor management and our better appreciation of the complex mechanisms encompassing organ IRI that govern the innate-adaptive immune interface triggered in the peritransplant period and subsequent allo-Ag challenge. Computational techniques and deep machine learning incorporating the vast cellular and molecular mechanisms will predict which peri-transplant signals and immune interactions are essential for improving access to the long-term function of life-saving transplants.

Keywords

CEACAM1; HO-1; inflammation; ischemia-reperfusion injury; machine perfusion; macrophages; NETs; organ transplantation; oxygen stress; TLR

1. Introduction

Among the perch of great miracles of twentieth-century medicine is the widespread use of antibiotics, mass vaccinations to limit the spread of infectious diseases, and the

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development of solid organ transplantation (SOT) to treat end-stage organ failure [1–3]. Introduced in the 1950s as a last-place therapy for patients with terminal and irreversible kidney failure, SOT emerged from an era where renal replacement and dialysis were standardized medical treatments [3]. Even after conventional immunosuppression therapies were introduced using azathioprine and steroids, graft rejection rates remained high. The introduction of cyclosporine some 30 years ago significantly reduced incidences of graft failure, especially for nonrenal SOT programs that lacked associated supportive therapies [4]. By all measures, SOT success has led to lower incidences of comorbidity, better patient survival, improvement of labor life, and global quality of life in the transplant population [5,6].

After kidney transplantation, orthotopic liver transplantations (OLT) remain the second most frequently performed SOT option for various end-stage liver diseases and certain malignancies [7]. The most common indications for OLT include alcoholic liver disease (40%), nonalcoholic steatohepatitis (NASH; 30%), hepatitis C virus (HCV; 20%), and an assortment of metabolic disorders (acute fulminant hepatic failure, hepatocellular carcinoma, and hilar cholangiocarcinoma) [8]. In the United States, the frequency of liver organs donated from living donors (LD-LT), donation after circulatory death (DCD-LT), or donation after brain death (DBD-LT) differ widely [9]. Data from the Scientific Registry of Transplant Recipients (SRTR) database from January 1, 2012, to December 31, 2017, recently showed that DBD-LT (n=22,497) is by far more common than DCD-LT (n=1,431) or LD-LT (1,223). Despite this significant difference, the common comorbidity of liver transplant candidates is diabetes mellitus (26.2%, 28.0%, 22.2%) vs. previous cancers (9.36%, 10.3%, 12.8%), respectively. The highest survival rates are seen in young and pediatric recipients [4], with the overall patient survivability ranging from 90-80% at one and three year post-OLT. A recent meta-analysis of patients undergoing liver transplantation supports the correlation between age and post-transplant survival rates [10]. Data for patients ≥70 years demonstrate significantly lower 1-year and 5-year survival rates compared to patients <70 years, with comparable hospital length of stay. Thus, despite substantial progress in regenerative medicines and scientific research affecting clinical outcomes, the ultimate dysfunction of the implanted organ remains a significant problem.

Some patients encounter acute peri-operative liver failure immediately following organ reperfusion, defined as primary nonfunction (PNF), or develop early allograft dysfunction (EAD) after impaired graft function occurs after some time has passed [11,12]. Both entities involve the multifactorial interplay of donor (graft) and recipient procedure-related factors. An unavoidable complication leading to donor organ PNF and EAD that may result in graft insufficiency is called ischemia-reperfusion injury (IRI) [13]. This peritransplant clinical condition is characterized by distinctive yet interrelated phases involving donor-tissue ischemic necrosis followed by recipient reperfusion, which activates the innate-adaptive immune interface and triggers pro- and anti-inflammatory immune cascades [14,15]. The consequences of IRI in SOT and potentially in other disease states involving ischemic injury/sterile inflammation, such as stroke and myocardial infarction, are vast, so understanding the contributing mechanistic factors leading to IRI remains a top priority.

IRI complications resulting from donor hepatectomy make OLT a race against the clock. The impact of hepatectomy time on liver graft post-transplant outcomes varies from around 35–40 min and serves as an independent predictor of overall graft failure [16]. The unavoidable IRI that follows involves the combinatorial effects of ‘warm’ and ‘cold’ liver insult in the peri-transplant period [17]. Warm organ damage occurs *in situ* during low flow states and leads to profound microcirculatory dysfunction, impaired vasodilatory capability, endothelial activation, increased hepatic vascular resistance, liver inflammation, leukocyte infiltration, oxidative stress, and cell death [18]. Notably, donor warm ischemia times, with a median implantation time of 41 min, in DCD-LT negatively correlate with organ viability and increases the risk of ischemic biliary type strictures [19], [20]. By contrast, organs experience cold insult when the liver is transported to the transplant center during *ex vivo* preservation. Cold ischemia times (CIT) reviewed between March 2002 and September 2016 in the United Network for Organ Sharing database (n= 67,426 recipients) shows that CIT between 1-6 h was protective against posttransplant prolonged length of stay (PLOS), a sensitive marker of morbidity and cost [21]. Cold ischemia causes cellular damage primarily to the liver sinusoidal endothelial cells (LSEC), disrupts the microcirculation, and activates local inflammatory innate immune cell networks [22]. Both types of IRI overlap by triggering Kupffer cell (KC)-derived cytotoxic molecule-mediated hepatocellular injury, neutrophils activation, cytokines/chemokines production, ROS generation, adhesion molecules induction and immune cell infiltration [23]5.

So far, no comprehensive strategy(s) has emerged from Phase II clinical trials to prevent this condition in transplant recipients. The development of novel therapeutics will continue to depend on the elucidation of physiological and molecular pathways activated during IRI. Many scientific reports are published each year that highlight various pathways in IRI. Table 1 shows a representative sampling of genes and targets of study. Recent reviews have highlighted the role of HIF-1α and ferroptosis in myocardial IRI [24,25], mitophagy in cerebral IRI [26], and macrophage polarization and Nrf2/oxidative stress (OS) in liver IRI [27,28]. However, a deeper three-dimensional understanding of how molecular interactions synergize will be needed for novel therapeutics to proceed beyond Phase II trials. A first step may be to take a clue from the dozen clinical trials focusing on hepatic IRI mitigation strategies in OLT recipients. This review aims to summarize the representative molecular pathways targeted for clinical intervention, understand the strengths and limitations of the different treatment approaches, and elucidate the parameters of what modulate IRI severity. A multifactorial intervention approach that manages ischemic stress and reperfusion-related sterile inflammatory processes may be our best hope for establishing a much-needed therapeutic armamentarium that prevents or treats this condition in transplant recipients.

2. Basic molecular mechanisms in organ IRI

IRI is a dynamic process involving necrotic tissue damage and microenvironment circulation (Figure 1) [29]. The preliminary steps of IRI involve activating a classic positive amplification feedback loop where ischemia damages parenchymal cells at the graft site and promotes the generation of ROS in Kupffer cells, i.e., liver-resident macrophages. Cells that produce ROS increase DNA and organelle damage at the interface of hepatocytes and LSECs, leading to the production and local secretion of redox-sensitive damage-

associated molecular patterns (DAMPs), like high mobility group box 1 (HMGB1), into the extracellular space (Figure 1, Phase 1) [30]. The reduction in tissue oxygenation also causes the depletion of adenosine triphosphate (ATP), promoting a transition to anaerobic metabolism in hepatic parenchymal cells [31]. Activation of multiple pathophysiological processes and cells follow that include changes in hypoxia-inducible factors, Toll-like receptor (TLR) signaling, extracellular signaling molecules, inflammatory factors, and mediators of ROS signaling and cell death [15]. The type of cells responsive to IRI-dependent changes in intracellular calcium overload and mitochondrial permeability transition pore opening include organ-specific cells like cardiomyocytes and hepatocytes, fibroblasts, mesenchymal stromal cells, and vascular endothelial cells [32]. Inevitably, tissues secrete DAMPs, which cause the selective recruitment of polymorphonuclear leukocytes (PMNs) to sites of tissue damage [33]. This increases proinflammatory signaling cascades and the complement system in surrounding cells (Figure 1, Phase 2). With the restoration of blood flow, local sequestration of recipient-derived monocytes and neutrophils causes changes in TNF-alpha, IL-6, IFNs, iNOS, TLRs/NFkB signaling, promoting local sterile inflammation and further destruction of the foreign tissue (Figure 1, Phase 3). Studies show the degradation extracellular matrix (ECM) also amplifies the IRI cytotoxic cascade by releasing collagen heparan sulfate, hyaluronan, fibrinogen, fibronectin A domain or tenascin C [34,35]. These events lead to severe clinical consequences in the OLT process as well. Consider a recent study that showed how environmentally triggered chronic liver inflammation can lead to collagen deposits in donor liver tissue that translated to increased susceptibility to hepatocellular damage post-OLT [36].

Despite the well-established molecular mechanisms of organ IRI, some contributing factors need more clarification. For example, the overwhelming evidence show neutrophils, among the largest innate immune cell population in humans, act as the primary proinflammatory sentinel by forming neutrophil extracellular traps (NETs) following the restoration of blood flow during OLT [15]. However, hepatic neutrophils may also mediate anti-inflammatory functions, reverse migration, and tissue-repairing functions to accelerate the resolution of local inflammation [37].

A recent study by Hirao et al. adds to our understanding of how the role played by neutrophils as principal villains in peri-transplant tissue injury may need to be reconsidered [38]. Molecular and functional studies were used to decorticate the controversial role of neutrophils in OLT by functionally dissecting the alternative splice isoforms of biliary CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1; CC1; CD66a). Previously, it has been shown that CC1 acts as a checkpoint regulator of sterile IRI response by suppressing the ASK1/p-p38 cell death pathway [39]. This transmembrane glycoprotein undergoes extensive alternative splicing to generate either the so-called short (CC1-S) or long (CC1-L) cytoplasmic tail isoform, depending on whether the variable exon 7 is present or not [40]. The consequences of these RNA splicing events dictate the cellular expression of CC1-S, associated with epithelial/endothelial tissue, and CC1-L, generally associated with dampening the sustained proinflammatory activity of immune cells. Hirao et al. investigated neutrophil activation, recruitment, and migration into the liver’s atypical sinusoidal vasculature and showed the regulatory role of CC1-L in forming NETs. This newly identified lytic cell death pathway evolved to trap and kill extracellular

microbes as part of a primitive immune defense against pathogens in a cellular process known as NETosis [41]. This process has been shown to be regulated by crosslinking S1P, a bioactive signaling lipid, to its cognate ligands S1PR1-5, during neutrophil activation [42,43]. In the experimental arm, a mouse OLT model was used to document that ablation of recipient-derived neutrophil CC1-L aggravated hepatic IRI by promoting NETs. Notably, in the clinical arm, fifty-five study subjects with donor livers expressing “high” levels of neutrophil CC1-L/cathepsin G (cathG) showed improved OLT function, depressed innate/adaptive immune activation, and lower incidence of EAD compared with “low” CC1-L/cathG individuals. While the double-edged properties of neutrophil target specificity in OLT remain to be fully elucidated, it is worth noting that the regulation of NETosis has already been tested in a clinical trial of the effects of high-dose intravenous vitamin C and syndecan-1, a negative regulator of leukocyte adhesion and migration, in sepsis-induced acute respiratory distress syndrome [44].

Many clinical strategies have exploited the fundamental molecular mechanisms to mitigate liver IRI, including testing agents that enhanced immunosuppression or immunomodulation, reduced oxidative stress and sterile inflammation, and stimulated the production of protective enzymes, such as superoxide dismutase, to promote tissue repair (Table 2). Knowledge of targeted pathways, the treatments that worked, the reported side effects, and the use of appropriate endpoints, combined with the most recent studies, will be crucial for successfully developing any future treatment against organ IRI.

2.1. Targeting oxidative stress and inflammation in IRI therapy

The main functional cells of the liver are the parenchymal cells, the hepatocytes and biliary epithelial cells, whose functions include the synthesis of proteins, the metabolism of carbohydrates and fats, the detoxification of toxins and drugs, and the production of bile [45]. Other functional liver cells include Kupffer cells (KC), specialized macrophages which remove waste products and damaged cells from the liver, found in the sinusoids [46]. Many studies initially identified KCs as the primary producer of ROS, initiating and propagating the cellular damage in the early phase of hepatic IRI [47]. Depletion studies of resident KCs showed no amelioration of liver damage after IRI [48], implicating a ROS role for other cells of the liver. Later it was established that hepatocytes, making up to 78–80% of the total liver tissue, were also an important source of ROS during hepatic IRI [49]. The generation of ROS is a complex process influenced by various factors. It has been established that this highly reactive molecule can be generated in the liver by multiple pathways, including electron leakage during oxidative phosphorylation in the mitochondria, inflammation-dependent recruitment of neutrophils/macrophages at sites of injury, and through the enzymatic activity of xanthine oxidase that produces it as a byproduct of purine metabolism [50]. Chouchani et al., showed that accumulation of the citric acid cycle intermediate succinate occurs through a reversal of succinate dehydrogenase, which is responsible for the observed ROS generation by reverse electron transport at mitochondrial complex I [51]. Pharmacological inhibition studies of ischemic succinate accumulation and its oxidation after reperfusion showed protection against tissue IRI, suggesting a novel therapeutic targeting pathway for IRI injury in pathologies such as cardiac infarction and stroke.

Some mediators that are activated by inflammation and oxidative stress influence transcription factors and enzymes that indirectly alter the generation of ROS [52]. One example is the production of alpha-ketoglutarate (α-KG), a key intermediate of the tricarboxylic acid (TCA) cycle [53]. The role of alpha-ketoglutarate in cellular metabolism and signaling includes acting as an intermediate of the TCA cycle, being produced by the oxidative decarboxylation of isocitrate and serving as a precursor for the production of energy-rich molecules such as NADH and FADH2 [54]. It also has a role to play in the metabolism of several amino acids, cellular signaling, and antioxidant activity. In this last role, α-KG can scavenge ROS and reduce oxidative stress. A recent study investigated the role of α-KG at the level of the oxoglutarate dehydrogenase (OGDH) complex in the Krebs cycle [55]. The substrate for the E1 component of the OGDH complex is α-KG. In this clinical study investigating kidney IRI, metabolomic data was obtained from sequential arteriovenous blood sampling and tissue biopsies collected at various timepoints after reperfusion. Their data showed that failing high-energy phosphate recovery, which is a condition of the inability to efficiently replenish or restore high-energy phosphate compounds, such as ATP and/or creatine phosphate in cells or tissues, occurs post-reperfusion. This metabolic collapse contributed to clinical delayed graft function and was related to a defect at the level of the OGDH complex in the Krebs cycle. This finding contrasts with rodent IR injury which shows increased succinate levels attributed to rapid re-oxidation during reperfusion, paralleled by an oxidative burst [56,57]. More studies are needed to understand why rapid metabolic recovery contrasts with rodent and the clinical context. It will especially be important to dissect whether warm vs. cold storage IR stress contributes to the prolonged, but reversible metabolic defects seen in clinical IRI studies.

Studies show that failure of endogenous antioxidants, such as heme oxygenase 1 (HO-1) and superoxide dismutase (SOD), to scavenge excessive ROS leads to cell damage by organelle, cell membrane, and nuclei DNA destruction [58]. In one study designed to increase ectopic expression of HO-1 by adenoviral delivery, significant improvement of portal venous blood flow, increased bile production, and decreased hepatocyte injury was demonstrated in genetically obese Zucker rats [59]. A similar study showed that gene delivery of Cu/Zn-superoxide dismutase improved graft function after the transplantation of fatty livers in the rat model [60]. Notably, graft steatosis poses its own challenges in adult-living donor liver transplantation [61,62]. This was revealed in a recent study that showed steatotic grafts displayed distinct mitochondrial dysfunction, including membrane, calcium overload, and energy homeostasis dysregulation in rat OLT [63]. These insights and many others prompted the experimental employment of antioxidant reagents that function by scavenging ROS [64]. In one recent study, edaravone (5-methyl-2-phenyl-2,4-dihydro-3H-pyrazol3-one) was tested in a renal warm ischemia-reperfusion injury (wIRI) in a rat model [65]. Endpoint measures such as restoration of spontaneous circulation, serum creatinine, blood urea nitrogen levels, and cystatin-C all showed marked increases compared to the Sham group. However, these findings should be tempered by the known side effects of edaravone, ranging from bruising to skin inflammation and rashes [66].

Moreover, the recognition of the importance of the antioxidant Nrf2-HO-1 pathway in mitigating ROS has recently been exploited in Phase II trials in a renal transplantation model [67]. In this study, the authors investigated the role of ANG-3777, a hepatocyte growth

factor mimetic that binds to the c-Met tyrosine kinase receptor. C-Met plays a crucial role in growth and survival in liver tissue models, protecting cells from ROS, oxidative stress, and cytotoxicity [68]. Notably, the mechanism of cytoprotection is attributed to the increased expression of the anti-apoptotic proteins (Bcl-2 and Bcl-xL) and inhibition of apoptotic cleaved caspase-3. Additionally, c-Met promotes nuclear NRF2 (Nuclear factor erythroid 2-related factor 2) while hindering its binding to the inhibitory protein Keap1. In a clinical kidney transplant trial investigating C-Met, patients treated with ANG-3777 experienced fewer and shorter duration of dialysis sessions, fewer hospital days, and better graft function, all while demonstrating good safety profiles [67]. Future studies may investigate whether patients treated with C-Met led to boosted antioxidant Nrf2-HO-1 properties and whether these data are translatable across other organs that benefit from the “healing” properties of HO-1, such as in the liver [69]. Taken together, these studies show the promise of how antioxidants may serve as a clinical strategy to protect against graft failure during or after organ transplantation.

2.2. Targeting ROS in IRI therapy using machine perfusion strategies

Several new and promising data have emerged over the past decade using ex vivo machine perfusion (MP) as a clinical strategy to reduce peri-transplant ROS and improve outcomes [70]. In general, MP is a technique that perfuses the organ, e.g., the liver, with oxygenated blood, mimicking conditions that the liver would experience in situ during transplantation [71]. Since organs donated after cardiac death experience prolonged times of warm ischemia followed by cold perfusion flush to slow down the metabolism to 4 °C, many studies have investigated the benefits and drawbacks of hypothermic, normothermic, and sub-normothermic MP (reviewed in [72]). Although the number of randomized controlled trials (RCTs) involving liver MP has steadily increased in recent years, recommendations for normothermic vs. hypothermic machine MP from small cohort studies have led to little clinical relevance [73]. One study, however, made significant progress in establishing the conditions for using normothermic MP of discarded human livers for OLT [74]. Mergental et al. conducted a prospective non-randomized, adaptive Phase II trial that showed 71% of perfused discarded livers had 100% 90-day patient and graft survival. Another compared the benefits of normothermic MP to conventional static cold storage in a randomized trial of 220 OLTs [75]. Primary endpoints were defined as the difference between the two treatment arms in the peak level of serum AST within seven days after transplant. Nasralla et al. showed normothermic preservation associated with a 50% lower level of graft injury and rate of organ discard and 54% longer mean preservation time [75]. These studies, as well as others, show that MP strategies may have a complementary role to play in reducing the number of discarded organs, thereby reducing the clinical and economic burden for wait-listed patients and safe lives.

Another new RCT took an alternative approach to reduce ROS in peri-OLT by investigating the uptake, distribution, and efficacy of antioxidant cerium oxide nanoparticles during normothermic perfusion of discarded human livers [76]. Studies show that cerium oxide nanoparticles have various beneficial properties, including antioxidant and anti-inflammatory effects [77]. One recent study was conducted using nine discarded human liver grafts that were randomized into groups that underwent four hours of normothermic MP

with/without cerium oxide nanoparticles conjugated with albumin (Alb-NC). Measurement of glutathione, superoxide dismutase, and catalase activity levels was notably consistent with antioxidant activity in Alb-NC treatment groups. This translated to restoring the effects of a 4,977-bp common mitochondrial DNA deletion, decreased lipid droplet peroxidation, and lipofuscin granules in the treated grafts [76]. An extension of this study would be to initiate molecular studies to determine whether engineered nanoparticles targeting compensatory tissue, myeloid, and lymphocyte cell fates *in vivo* provide a boost of IRI protection over normothermic MP treatment alone. This question was addressed recently by Markmann et al. in a multicenter RCT conducted at twenty US liver transplant programs [78]. Deceased donor livers were perfused in a solution rich in nutrients and oxygen, while maintaining the organ at an average body temperature during normothermic MP. The study trial compared outcomes for 300 recipients of livers preserved using normothermic MP (n = 153) or conventional static cold storage (n = 147). The analytical measures for improved graft function included recording a decrease in EAD, histopathologic evidence of IRI after reperfusion (e.g., less moderate to severe lobular inflammation), higher use of livers from donors after cardiac death, and a significant reduction in the incidence of ischemic biliary complications at 6 and 12 months after transplant. The results showed that liver preservation using ex vivo normothermic MP was associated with superior posttransplant outcomes and increased donor liver use.

Another recent multicenter, controlled trial of 156 patients aimed to assess whether hypothermic oxygenated MP (HOMP) of livers reduced the incidence of biliary complications associated with IRI [79]. The main advantages of using HOMP rather than other dynamic preservation methods, such as normothermic MP, include the low temperatures the organ is maintained at, so technical malfunctions of insufficient hepatic perfusion would limit organ loss. In this study, one-half of the patients (e.g., 78) received a machine-perfused liver, and the other group only received a liver after static cold storage. The study's primary endpoint was the incidence of symptomatic nonanastomotic biliary strictures, a complication in fibrotic narrowing of the bile-duct lumen, and obstruction of bile flow at six months post-OLT. The data showed that when patients obtained a liver subjected to HOMP after circulatory death, symptomatic nonanastomotic biliary strictures were significantly lower than after static cold preservation.

Outstanding questions raised by MP studies that remain to be answered include whether and how standard clinical parameters of serum lactate and liver transaminases (measured every 30 min), glucose metabolism, pH normalization, and bile production provide the best objective assessment of liver viability pre- vs. post-OLT. Second, will MP of any specific type (e.g., hypo, normo, or sub-normo), eventually be equally suitable for all age and history-matched donors? Another line of inquiry involves demarcating how MP alters donor resident immune cells independent from the alloimmune effector response that occurs locally at the graft following transplantation. Finally, how will new technologies such as normothermic regional perfusion (NRP), a technique that involves the restoration of blood supply to the abdominal organs after death using extracorporeal circulation for a limited period before organ recovery, serve in a complementary role (alone or in combination to MP) to reduce the organ discard rates in the coming years. Collectively, these studies are promising but more bench-to-bedside translational studies are needed to determine how

machine preservation corrects impaired redox equilibrium, suppresses sterile inflammation, and prevents biliary complications in SOT patients.

2.3. Targeting CI/WR injury in IRI therapy

Cold ischemia/warm reperfusion (CI/WR) injury is another area of active research into parenchyma cells' role in liver IRI [80]. This can occur during the transplantation process when the typically temperature-sensitive liver is stored in a solution that keeps it cold and oxygenated before it is transplanted into the recipient (cold ischemia) or when the liver is reperfused with oxygenated blood after transplantation (warm reperfusion). Some CI/WR injury consequences are increased risk of bleeding, infection, and rejection of the transplanted liver. Several early studies showed that apoptosis is a critical mechanism during the initial phase of hepatic CI/WR injury in both animals [81] and human liver allografts [82]. Apoptosis, a form of cell death that removes damaged cellular components, is mediated by the activation of cysteine proteases, referred to as caspases. The findings that caspases mediate liver CI/WR led to an early clinical study that aimed to assess the utility of the pan-caspase inhibitor IDN-6556 during human OLT [83]. Organ storage with IDN-6556 was compared to 99 adult liver transplant recipients that received or did not have various IDN-6556 concentrations. Application of the caspase-inhibitor reduced the initial phase of reperfusion-mediated apoptosis, which reduced liver injury during postoperative days 1–7, as evidenced by serum AST/ALT levels. This data was also supported by lower immunohistochemical myeloperoxidase staining for infiltrating neutrophils in day seven post-OLT specimens. Intravenous IDN-6556 was well-tolerated, and while it may represent an effective tool for affecting clinical outcomes, well-controlled and larger cohort studies are needed. A central question that requires further investigation is the significance of altering the molecular expression and cellular milieu using agents that inhibit pathways (e.g., apoptosis) that generally play a role in regaining homeostasis and maintaining well-being in humans.

At the molecular level, profound changes in gene expression occur under warm vs. cold ischemic stress, presumably because of several factors involving the activity of temperature-sensitive enzymes, like ribosomes [84], heat shock molecular chaperone proteins [85], and the overall physiological and cellular environment. This was the key finding in a recent study that investigated the control of HO-1, the rate-limiting enzyme for microsomal heme degradation and catalysis of heme breakdown in the formation of biliverdin, iron, and carbon monoxide [59], by human antigen R (HuR), the stabilizer of adenylate-uridylate (AU)-rich mRNAs [86,87]. In this study, murine hepatocytes were induced with hypoxia and cytokine mixes (CM) composed of TNF-α, IFN-γ, IL-1β, and LPS as coculturing stimulants to mimic acute liver ischemia [87]. Experimental data showed that CM treatment potentiated the strong cytoplasmic translocation of HuR and HO-1, suggesting a positive correlation induced by the proinflammatory stress response (Figure 2A). Importantly, when the authors tested HuR and HO-1, HIF-1, and bone morphogenetic protein 4 (BMP4), markers of ischemic stress, the effects of warm vs. cold ischemic stress became evident (Figure 2B). Surprisingly, regulated expression of HO-1 by HuR only occurred under warm but not cold ischemic stress (left panel vs. right panel). This data brings to light the need for clarity among future studies on the changes in gene expression that may occur during CI/WR

injury. Knowing the peak induction potential of a protein may offer insight into antioxidant, detoxification, and homeostatic functions that may mitigate IRI while offering a clue as to why specific therapeutic strategies fail to advance through clinical trials.

2.3. Targeting cholesterol biosynthesis pathways in IRI therapy

A feature that makes hepatocytes a good target for clinical intervention of IRI is their ability to synthesize large amounts of cholesterol, which is secreted into circulating blood via apolipoprotein particles [88]. Cholesterol, produced from acetyl-CoA during the catabolism of carbohydrates and fats, produces hormones, bile acids, and vitamin D in the liver [89]. Importantly, cholesterol-secreting hepatocytes are the clinically relevant cells targeted by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, which remain among the most prescribed medications in the United States [90]. Statins effectively treat dyslipidemia and act by converting HMG-CoA to mevalonate during cholesterol biosynthesis. Of the limited animal studies available, one showed that using atorvastatin before partial hepatic ischemia/24-h reperfusion in mice led to hepatoprotection against IRI in lean, fatty, and NASH livers by a mechanism involving downregulation of TLR4, and NF-κB activation, suppression of adhesion molecules, chemokines/cytokines, and thromboxane B2 production [91].

A recent study extended these findings by linking Western steatotic diets with hepatic IRI outcomes. Liss et al. showed that hepatic steatosis, whether predominantly macrovesicular or microvesicular, increased the incidence of liver injury following liver transplantation, which led to higher inflammatory cytokine profile, and histological necrosis scoring [92]. Interestingly, this study reported that steatosis in a hepatic IRI model correlated with the induction of necroptosis factors, like receptor-interacting protein kinase (RIPK) 3, RIPK1, and mixed-lineage kinase domain-like (MLKL). These proteins are part of a family of enzymes that regulate apoptosis and inflammation by interacting with various receptors on the surface of cells leading to signal transduction cascades activating caspases that degrade specific proteins during cell death [93–95]. Whether targeting necroptotic signaling, in the context of RIPK3, RIPK1, and MLKL, will be a viable strategy for clinically reducing IRI remains to be determined, but it is notable that long-term worldwide trends show that the incidence of nonalcoholic fatty liver disease (NAFLD) and NASH are rising [96]. Since these animal studies establish the curative approach of using statins to combat IRI in OLT, it follows that a recent clinical study reported the use of Simvastatin (Zocor/FloLipid) on liver transplant recipient outcomes in a double-blind, randomized, prospective trial of 58 patients [97]. Previous studies showed that Simvastatin has antithrombotic properties [98] and vasoprotective capacities via the activation of Krüppel-like Factor 2 (KLF2), an effector identified with a protective role in rat hepatic/renal IRI [99,100]. Measures of statistical evaluation included the incidence of patient/graft survival at 90/180 days, the severity of secondary complications, and gene expression differences in post- vs. pre-OLT biopsies for *KLF2*, endothelial nitric oxide synthase (*NOS3)*, intercellular adhesion molecule 1 (*ICAM1*), and hepatocyte growth factor (*HGF*) in grafts pretreated with Simvastatin in comparison with grafts under placebo conditions. Survival probability curves and other metrics showed higher graft survival after pretreatment with Simvastatin than controls,

leading to the conclusion that a single dose of donor administrated statins may be a simple and inexpensive clinical option, as compared with sophisticated device-based therapies.

2.4. Targeting liver sinusoidal endothelial cells (LSECs) in IRI therapy

Other cells of the liver that play a role in facilitating IRI include LSECs (liver sinusoidal endothelial cells) and hepatic stellate cells [101]. LSECs line the endothelial sinusoids and function by exchanging nutrients, waste products, and gases between the liver and the circulation [102]. They selectively allow substances to pass through their cell walls and into the liver while preventing the passage of others [103]. The influence of oxygen stress on the structure and function of in vitro isolated rat LSEC has been investigated [104]. Indeed, hyperoxia reduced scavenger receptor-mediated endocytic activity while also increasing the production of the proinflammatory IL-6 and decreasing the production of the anti-inflammatory IL-10. This study also documents the changes in LSEC morphology, specifically in the transcytoplasmic holes called fenestrae that filter plasma components toward the liver parenchyma, resulting from the endogenous production of hydrogen peroxide. Notable gaps of knowledge that need more study include understanding how oxygen tension differences between portal and arterial blood flow entering the liver alters the distribution of essential factors produced locally at the liver sinusoids or other cells that interact with native ECM components [105].

A consensus number of studies show that an initial causative factor of IRI is damage to LSECs caused by cold stress (CS) [106]. The changes that occur to LSECs include platelet activation, persistent vasoconstriction, poor graft microcirculation, upregulation of adhesion molecules, Kupffer cell activation, and neutrophil infiltration [107]. However, few recent studies have addressed how oxidative stress affects the zonal heterogeneity of LSECs resulting from CS-induced IR-insult during OLT while looking at the downstream effects of changes in fenestration that occur on OLT recipients. Recently, Kojima et al. investigated the role of CS-induced ferroptosis in LSECs in an IR-damage model of mouse OLT [108]. This type of cell death is characterized by the accumulation of toxic levels of iron and the breakdown of lipid fat droplets triggered by the accumulation of ROS caused by the depletion of glutathione, which is usually responsible for protecting cells from oxidative stress [109]. The study showed that when wild-type mouse liver grafts were incubated with Fer-1, a ferroptosis inhibitor, during *ex vivo* cold preservation storage (4°C/18 h) in University of Wisconsin (UW) solution, the incidence of mouse IRI-OLT damage significantly decreased compared to untreated controls, as evidenced by serum AST/ALT levels and Suzuki's histological scores. Molecular analyses of NRF2KO mice showed that LSEC susceptibility to CS was enhanced after ex vivo treatment (cell death: WT vs. NRF2KO, $p<0.001$). Importantly, in the clinical arm of the study, NRF2 expression in human donor liver biopsies correlated well with GPX4, a key regulator of ferroptosis. This relationship was negatively related to mRNA levels coding for *TLR2*, *TLR9*, *IL-17*, *CXCL10*, and *cathepsin G*. Collectively, these studies provide another clue that preclinical IRI strategies should consider the role of LSECs as a new avenue for therapeutic intervention in oxidative stress-triggered sterile inflammation.

3. The role of the microenvironment in IRI pathophysiology

A great deal of clinical interest has also aimed to manipulate the effector phase of the host's alloimmune repertoire in the graft by targeting micro-circulating cells during IRI (Table 2). Platelets, macrophages, and polymorphonuclear cells are critically involved in the inflammation process by producing toxic proteolytic enzymes, ROS, altering the adhesion of leukocytes and platelets, activation of the complement pathway, enhancing thrombosis and vascular permeability, and altering the rate of proliferation of blood and lymphatic vessels. In the liver, an intricate system of sinusoids also circulates resident T-cells, gamma delta (γδ) T cells [110], and adaptive lymphocytes, such as αβ T cells and B cells. These, as well as natural killer (NK) cells [111], are estimated to account for up to 50% of total hepatic lymphocytes [112], and serve the role of keeping an optimal degree of immune-tolerance vs. foreign antigens while ensuring a correct immune-surveillance against potential threats (i.e., infections, tumors, aberrant inflammation, and autoimmunity). In the case of hepatic IRI, a well-orchestrated activated innate immune response occurs involving both residential and circulating immune cells that leads to a remodeled intrahepatic immune microenvironment that renders the graft susceptible to primary disease recurrences, such as steatosis, fibrosis, viral hepatitis, and malignancies (Figure 1). The collective insight into how the microenvironment influences the alloimmune response has led in recent years to studies inhibiting signaling pathways targeting different transplant organs (Figure 3).

3.1. Targeting innate immune cells in IRI therapy

During the early phase of hepatic IRI, as DAMPs are secreted from damaged or dead cells, their stimulatory program causes local sentinel Kupffer cells to increase the expression of IL-1β, which activates ICAM1 on LSECs. Activated Kupffer cells, and other parenchymal cells, release a volley of chemokines (e.g., CXCL1 and CXCL2) that are recognized by neutrophil G protein-coupled receptors (e.g., CXCR2), leading to their recruitment to the inflammatory site. Early IRI-triggered innate immune responses include activation of neutrophils and the mononuclear-phagocyte system, including professional antigen-presenting cells such as macrophages and dendritic cells (DCs), which are aimed at sensing danger and maintaining tissue homeostasis. Studies have investigated whether inactivating neutrophils is an effective strategy to decrease organ IRI. First, mice treated with monoclonal antibodies (mAB) directed to HMGB1 alleviated IRI-induced renal dysfunction by suppressing the activation of the HMGB1-TLR4-IL-23-IL-17A signaling axis [113]. Second, a single-center Phase II study investigated whether recombinant P-selectin glycoprotein ligand IgG (rPSGL-Ig) administration improved early OLT function in deceased-donor OLT recipients [114]. Animal studies showed that blocking the interaction between the adhesion molecule P-selectin and its endogenous ligand, P-selectin glycoprotein ligand 1 (PSGL-1), leads to decreased neutrophil infiltration, ameliorated hepatocyte injury, and improved survival in non steatotic rat livers [115]. Though rPSGL-Ig treatment in patients showed improved early hepatic performance, measured by the incidence of EAD, it was not clinically feasible to deplete neutrophils as a strategy, given their role in pathogen clearance and immune surveillance. A refined approach should selectively block circulating leukocytes from entering the IRI-stressed inflammatory site.

In animal models, IRI also causes massive recruitment of monocytes into the liver, followed by the maturation of monocyte-derived DCs and a rapid increase in the frequency of intragraft macrophages. These effects are further potentiated by allogeneic antigen pressure from the graft itself, with increasing numbers of infiltrating DCs and induction of macrophage-mediated inflammation and cytotoxicity, which concur in causing graft damage and transplant rejection both directly and through effector T cell activation [116]. Guo et al. demonstrated that pre-ischemic renal lavage in rat models reduced the numbers of infiltrating $CD68^+$ macrophages and myeloperoxidase $(MPO)^+$ neutrophil populations, which led to the reduction in the gene expression of specific proinflammatory mediators [117]. When transplant-induced IRI models were investigated in mice, Aiello et al. detected IL-1R8 as a regulator of donor-derived renal macrophages [118]. While an IL-$1R8^{-/-}$ graft transplanted to an IL-$1R8^{+/+}$ mouse acquired an M1 phenotype inducing IFNγ and IL-17 responses, renal macrophages from an IL-$1R8^{+/+}$ graft caused an M2 polarization phenotype that increased surface expression of IL-1R8 while causing diminished TLR4 activation. The importance of further delineating the mechanisms of myeloid cell activation was recently elaborated in an ischemia-stressed mouse and human OLT settings [119]. Thus, Kadono et al. showed that Ikaros (IKZF1), a well-established transcriptional regulator in leukocyte lymphopoiesis and differentiation, coordinated with Sirtuin 1 (SIRT1), a histone/protein deacetylase [120] involved in cellular senescence, inflammation, and stress resistance in IRI-stressed OLT recipients. Other recent studies of interest include Kurihara et al., which focused on donor non-classical monocyte (NCM) and classical recipient monocyte (CM) populations in lung transplantation [121]. They reported that IRI activated donor NCM and alveolar macrophages, inducing recipient neutrophil and CM trafficking to the allograft through CXCL2 and IL-1β. Importantly, Li et al. showed that the blockage of thrombospondin-1 (TSP1)-CD47 signaling could alleviate the severity of IRI and acute rejection in the mouse OLT model [122]. The use of CD47 mAb alleviated the recruitment of neutrophils and lymphocytes to the allografts and protected them from IRI and acute rejection, with decreased levels of TNF-α, IL-2, and IFN-γ. These preclinical findings, while promising, will require further experimental, translational investigation before it can be implemented in clinical therapies.

3.2. Targeting secretion of cytokines and DAMPS in IRI injury

The innate immune system is a rapid defense system triggered by molecules released by pathogens (PAMPs) and host tissue (DAMPs) [123]. Ischemia-induced metabolic stress causes the initial tissue damage, which generates DAMPs, which then activate pattern recognition receptors, such as TLR4, to initiate the inflammatory response [124]. DAMPs include HMGB1, Hsp60 and Hsp70, ROS, fibrinogen, fibronectin, hyaluronic acid, and heme [125]. Among them, HMGB1 has been characterized as a significant factor underlying liver IRI via its capacity to activate macrophages, which produce an array if proinflammatory cytokine programs. However, the precise mechanism of how HMGB1 activates TLR4 signaling remains to be clarified. With the progression of the ischemic cascade, damaged/dead cells release HMGB1 by acetylation, which can activate IL-23 secretion by macrophages by binding to TLR4. Zhang et al. demonstrated that the HMGB1/TLR4/IL-23/IL-17A signaling axis was pivotal in mediating renal dysfunction in mice IRI models [113]. Following their previous research confirming that recombinant human

soluble thrombomodulin (rTM) ameliorated IRI in a TLR4 pathway-dependent manner [126], Kawasoe et al. revealed the protective effect of the isolated lectin-like domain of rTM (rTMD1) in mouse IRI models [127]. Pretreatment with rTMD1 suppressed serum HMGB1, the expression of TLR4 in liver tissue, and the production of proinflammatory TNF-α, IL-6, IL-1β, and CXCL-2. The fact that TLR4 drives liver IRI has also been confirmed in human studies [128]. Sosa et al. demonstrated that HMGB1 was released from donor hepatic tissue into patient portal blood following reperfusion and that HMGB1 translocated into the cytoplasm of macrophages in IRI-positive OLT patients [124]. Lee et al. used murine renal transplant models to discover that treatment with rAIM (recombinant apoptosis inhibitor of macrophages) enhanced phagocytosis of necrotic cell debris and limited DAMPs release [129]. Li et al. described that liraglutide treatment, a glucagon-like peptide-1 receptor agonist, inhibited IRI-induced nucleocytoplasmic translocation, and release of HMGB1 in mice [130]. These agents can be therapeutically valuable for the clinical prevention and treatment of organ IRI. As oncostatin M (OSM), a member of IL-6 family cytokines with pleiotropic functions increased proinflammatory cytokine programs in a murine intestinal IRI model [131] and induced liver fibrosis by suppressing fibrolysis [132], its causality with IRI stress and tissue damage needs further elucidation.

3.3. Targeting Activation of TLR in IRI therapy

Toll-like receptors (TLRs) are type I transmembrane glycoproteins that activate downstream signaling pathways [133]. Among different types of TLRs, TLR4 is the best-characterized in the mechanism of tissue IRI [134,135]. While TLR4 in the liver is expressed by both the hepatocytes and non-parenchymal cells, including LSECs and Kupffer cells, it is widely expressed in parenchymal cells and resident immune cells in the liver. Functionally, TLR4 is involved in multiple signaling pathways, influencing NF-κB or interferon regulatory factors (IRFs) and enhancing distinct cytokine/chemokine programs. Since numerous exogenous and endogenous TLR4 ligands have been identified, including both pathogen-associated molecular patterns (PAMPs, molecules released by pathogens) and DAMPs (molecules released by host tissue) [136], the regulation of TLR4 signaling is an important key for ameliorating IRI tissue damage in both the liver and liver. Although TLR-4 has been recognized as a key mediator in IR-triggered sterile inflammation, the latest studies have added some updates to the existing evidence.

Hassan et al. demonstrated that pre-administration of carvedilol, a well-known drug that acts as a competitive non-selective adrenergic antagonist, could mitigate liver damage in rat IRI models through upregulating and downregulating different types of isoforms of nitric oxide synthase (NOS); endothelial NOS (eNOS) and inducible NOS (iNOS) [137]. Increased eNOS-derived nitric oxide has a protective effect against hepatocyte damage, and downregulated iNOS could be attributed to its inhibitory effect on TLR4/IL-6 pathway. Liu et al. used rat LT models and suggested that salidroside, one of the most active chemicals from Rhodiola plants, alleviated hepatic IRI by inhibiting the activation of the TLR4/NF-κB/NLRP3 pathway [138]. Kawasoe et al. reported that the supplementation of propionic acid, one of the short-chain fatty acids, exhibited a suppressive effect on the TLR4 signaling pathway in mouse liver IRI by the reduction of TLR4 ligand, HMGB-1, and direct suppression of the inflammation in macrophages through downregulation of TLR4/

NF-kB pathway [139]. Jung et al. presented the reno-protective effects of pretreatment in the kidney using a multiple-TLR-blocking peptide, named TLR-inhibitory peptide 1 (TIP1), which exerts the strongest action on TLR4, against tubular injury, apoptosis, inflammatory cytokines, and oxidative stress caused by IRI in mice [140]. TIP1-pretreated and reperfused kidneys showed a tendency for lower macrophage infiltration and a decrease in the proportion of Th17 cells in CD4+ T cells, which reconfirmed that TLR plays a crucial role in the pathogenesis of renal IRI. A recent in vitro study investigated the affinity between human peroxiredoxin-5 (PRDX5), a molecule acting as a DAMP, and TLR4 [141]. Since human PRDX5 has been shown to enhance brain IRI by activating TLR4-mediated inflammation, further research is warranted to reveal its role in hepatic and renal IRI.

3.4. Targeting NK cells in IRI therapy

In warm IRI, NK cells mainly aggravate liver injury and promote inflammatory cell infiltration depending on IL-15 signaling [142]. In cold IRI, like liver transplantation, recipient NK cells will gradually replace donor NK cells after transplantation, producing IFN-γ and mediating rejection. Therefore, using NK cell depletion, inhibiting NK cell activating receptors, or blocking the signaling pathway of NK cell maturation will become an effective approach for the intervention of hepatic IRI [111]. Recently, research on invariant natural killer T (iNKT) cells is ongoing [143]. Goto et al. reported that activated iNKT cells facilitate liver repair after hepatic IRI in mice by accelerating macrophage polarization to M2 in both the early and late phases [144,145]. However, iNKT cells are rare in the human blood pool, comprising just 0.01-1% of peripheral blood mononuclear cells; thus, its function in human IRI remains unknown.

3.5. Targeting adaptive immune cells in IRI therapy

Antigen-specific T cells are involved in IR stress-triggered innate inflammatory tissue damage. Kageyama et al. suggested that CD4 T cells, especially recipient-derived CD4 T cells, play a key pathogenic role in hepatic IRI of allogeneic OLT. They found that CD4 depletion in mice inhibited intragraft neutrophil/macrophage infiltration and proinflammatory gene expression, including TNF-α, IL-1β, CCL2, CXCL1, and CXCL10 [146]. Research on T-regulatory cells (Tregs), which are involved in the convergence and suppression of immune responses, is ongoing, too [147]. In obese recipients, Th17 cells are increased, while Tregs are decreased. Yang et al. reported that an increased level of IL-17A, mainly produced by CD4 Th17 cells, could upregulate hepatic IRI in fatty liver models through the recruitment of the neutrophils and increases in mitochondria-driven apoptosis [148]. IL-17A deficiency in mice attenuated mitochondria-driven cell apoptosis and inflammation by inhibiting NF-κB signaling. Akimova et al. focused on IL-18, which increased more in obese lung transplant recipients, accompanied by decreased levels of FOXP3, the key Treg transcription factor, and hence promoting lung IRI [149]. It was also described by Liu et al. that IRI promoted the elaboration of IL-18 from endothelial cells to selectively expand alloreactive IL-18R1+ peripheral T helper cells in allograft tissues to promote donor-specific antibody formation [150]. With regard to FOXP3 expression and Treg function, both were reported to be inhibited under an acidic microenvironment in liver IRI [151]. These animal studies suggest that targeting T-lymphocytes for removal from the microcirculatory environment might be an advantageous strategy to pursue in future

human clinical trials. Early studies using thymoglobulin (TG) were reported to protect liver allografts from IRI [152]. TG is involved in the blockade of adhesion molecules, decreased cell surface expression of b2 integrin, and dose-dependent T-lymphocyte depletion [153,154]. Though TG has been successfully used as an induction agent in liver, kidney, and pancreas transplantation, it will be important to determine whether it can salvage more compromised grafts with less clinical evidence of IRI and to what extent any increases in the rate of infectious complications result from T cell depletion, as compared to controls.

4. Mitigating IRI by optimizing the quality of the donor liver

Decades of progress in the liver transplantation field have optimized organ retrieval techniques and implantation procedures to minimize ischemia and minimize the risk of unintended organ injury. Despite this, the independent effects of these phases have not been fully unified by an underlying biological mechanism that drives IRI. With organ shortages remaining a top priority for clinicians deciding how to allocate limited resources, it is worth considering five strategies that may improve graft outcomes [155]. First, the duration of each ischemic phase, from the start of organ retrieval to implantation procedures, should be reduced to as short as possible. This may not be straightforward as surgical experience (fellows vs. attending surgeon) appears to be an independent covariable associated with longer donor hepatectomy times [156]. For example, appropriate training may be required in the case of DCD-LT since DCD-LT organs are more sensitive to surgical injury during procurement than DBD-LT organs and may need more time to recover from ischemic stress [157,158]. Second, better cooling technologies are required to reduce IRI. Some large organs such as the liver may benefit from more than surface cooling storage strategies. New studies may require investigating cold preservation fluids that reduce organ rewarming during donor and recipient back-table work, though at the expense of recipient hypothermia. Novel MP strategies may help regulate vascular/biliary flush and organ rewarming in this regard. Third is the development of clinical approaches to reduce the incidence of IRI using novel therapeutics (Table 2). Our understanding of the disturbances to cellular metabolic processes profoundly affecting the inflammatory immune response with direct and indirect cytotoxic mechanisms is only at the beginning stages. Our knowledge of how post-transcriptional mechanisms (e.g., RNA splicing) control gene expression of local ischemia-related genes that lead to parenchymal cell death is missing. Another area ripe for further research is how (epi-)genome modifications induce long-term changes. The fourth strategy involves refining surgical and organ perfusion techniques that altogether avoid ischemia [159]. This innovative strategy, called ischemia-free transplantation, is complex and would require costly medical center adaptations that make it impractical. However, combining this technique with normothermic regional perfusion might be helpful for DCD-LT or DBD-LT donation procedures, eliminating many cold/warm temperature storage steps that cause cellular damage [155].

The last strategy to reduce IRI involves optimizing the quality of the donor's liver before implantation. This is not a trivial undertaking as it consists of a host of complex factors, where at least in the United States, an aging population is increasingly at risk for obesity and other liver-related diseases (e.g., NASH, NAFLD). Individual behaviors, societal norms, and environmental influences have altered the donor pool creating organs,

and expanded criteria donors (ECDs), that are at higher risk of complications or failure after transplantation compared to a standard liver. Other ECDs include elderly or older donors, donors with hypernatremia, donors with hypotension, donors with viral infections, donors with malignancies, and donors with infections. Importantly, ECDs all share a common poor prognosis after liver transplantation [160,161]. Competing against this decrease in liver quality is the increasing demand for donor organs that have outpaced the supply of available organs. Whereas transplantation centers factor the age and health of the donor, the degree of steatosis (fat accumulation), the length of time the liver has been preserved, and the overall quality of the liver tissue in the past when determining the suitability for transplantation, now centers are turning to four rejuvenation strategies that offer the promise of improving the quality of organs and increasing their likelihood of successful transplantation. The first two strategies involve using MP strategies to improve liver function and integrity, which will increase the success rates of transplantation. In one recent study, hypothermic oxygenated MP had a profound effect on lowering the risk of nonanastomotic biliary strictures following OLT from livers DCD, as compared to conventional static cold storage livers [79]. Another study used normothermic machine preservation (NMP) to resuscitate marginal allografts using pulsatile flow through the hepatic artery and non-pulsatile low-pressure flow through the portal vein [162,163]. A third strategy involves ex vivo liver repair outside the body. In this technique, damaged cells are removed, or new cells are introduced to replace damaged tissue. A proof of principle study recently showed that using a combination of gene therapy and NMP improved liver quality and function, translating to better OLT success rates [164]. Finally, Table 2 shows several studies that have employed pharmacological agents to improve the quality and “rejuvenate” marginal livers. These include anti-inflammatory agents, antioxidants, and drugs with pathway-specific targeting of genes involved in liver injury and repair.

5. Conclusion

The challenge of rationally designing a successful clinical strategy to treat tissue IRI will involve a comprehensive understanding of the complex molecular crosstalk between signaling and metabolic networks. Determining the peak induction potential of the signaling networks that attenuate antioxidant, detoxification, and homeostatic functions may be the key to understanding why certain therapeutic strategies fail to advance through clinical trials. Potential therapies in development include pharmacological treatment, ischemic preconditioning, blood flow maintenance, hypothermia management, and even therapeutic medical gases like hydrogen, hydrogen sulfide, NO, and carbon monoxide. The importance of distinguishing therapeutic strategies for both ischemia and reperfusion, developing a combination of agents may be required to elicit maximum clinical benefit.

6. Expert opinion

The major problem facing transplantation worldwide is the shortage of donor organs because of the widening disparity between the increasing numbers of potential recipients who vie for a constant donor supply [6]. According to the United Network for Organ Sharing (UNOS), on January 23, 2023, there were 114,751 individuals on transplant waiting lists in the U.S., while the total number of transplants performed in 2022 was 42,888 (https://

unos.org/transplant/). During that time, 5,716 patients died while waiting for the life-saving organ, and another 6,150 individuals were removed from the waiting lists because they became too sick. Of note, more than 28,000 donor organs are discarded yearly in the U.S. due to poor quality and function. The major contributing factors of such a status quo are the decreasing donor organ quality in the aging population and associated pathological conditions. As these “suboptimal” organs are particularly susceptible to the harmful effects of peri-transplant tissue damage, there is a consensus that IRI contributes to the acute organ shortage. Hence, improving donor organ management is paramount to saving lives, to benefit patient outcomes, and enhance the overall success of organ transplantation.

Although the current prevailing dogma is to treat transplant recipients with cocktails of immunosuppressive agents to prevent immune rejection response, adjunctive use of modalities promoting tissue rejuvenation/regeneration in the peri-transplant period (e.g., during MP) should be considered. Each year sees the expansion of our understanding of known genes or the discovery of new ones associated with innate immune activation, inflammatory responses, and cell death pathways, among many others (Table 1). Genome-wide gene profiling investigating local and systemic inflammatory machinery that follow oxidative stress and reoxygenation-enhanced tissue-damage estimates more than 1000 genes are involved, of which 70% are up-regulated [165]. However, as IRI in organ transplantation is a multifactorial condition, one of the great challenges will be to design strategies that target pathways that complement each other. For example, the B-cell lymphoma-2 (BCL-2) family proteins have opposing activities that regulate cell death [166]. Some BH3 proteins interact with specific anti-apoptotic BCL-2 subsets, yielding combinatorial signaling pathways toward cell death.

New technologies like single-cell sequencing (sc-seq) should also help to design and re-evaluate hypotheses about similarities and differences between morphologically distinct cell types. Another challenge for the field is to stratify the sometimes-conflicting details seen for complex molecular pathways at work in the mechanism of IRI that result from divergent experimental conditions and the vast array of organs subjected to often cell-specific IR-triggered tissue damage. Future well-designed translational studies should focus on computational techniques that synergize with the growing informational networks, such as Gene Expression Omnibus (GEO), to better understand the underlying biological mechanisms involved in the inflammatory and reparative IRI phases. Even though recent studies have focused on the transcriptomic profiles of human livers undergoing rewarming MP before transplantation [167] and proteomic profiles in early renal dysfunction after OLT [168], largely missing are studies that focus on other levels of gene expression (e.g., epigenetic, post-transcriptional, and post-translational) that could fill-in existing knowledge gaps.

Another persistent challenge concerns how to accurately translate mechanistic in vitro and in vivo studies to clinical developmental activities when small human subjects potentially compromise the ability to draw statistical conclusions that may not have generalizable interest to the broader transplant population. The design of clinical studies for effective IRI treatment will require sample sizes that are large enough to detect meaningful differences, patient populations that account for comorbidities that may limit the outcome

of the study, and appropriate endpoints that are accurate and reproducible. It may be that the use of bioinformatics tools (e.g., eLSA [local similarity analysis], LEfSe [linear discriminant analysis effect size]), and logistic/random-forest classifiers, combined with novel machine learning and deep learning techniques will help to identify regions and features that are similar or different between groups of sequences or samples that lead to the elusive comprehensive therapeutic. The data generated from these techniques may create the classifiers to prospectively predict which molecular peri-transplant signals are indeed essential and which are irrelevant or redundant for long-term clinical outcomes.

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Article highlights:

- Organ IRI in solid organ transplantation may lead to delayed graft function, non-function, increased incidence of rejection crises, and hence compromised graft/patient survival, and the shortage of organs available for life-saving surgery.
- Efforts to improve clinical outcomes and increase the transplant donor pool will depend on better appreciating the complex cellular and molecular mechanisms triggered by peri-transplant IR stress at the innate-adaptive immune interface.
- The complete mechanisms encompassing IRI in organ transplantation remain undetermined, explaining why no effective and specifically tailored clinical interventions have emerged.
- Research to improve donor organ quality is imperative, while elucidating IRI-specific molecular signaling pathways in clinically-relevant translational models should benefit from computational techniques to better predict which peri-transplant signals are essential and which are redundant for clinical outcomes.

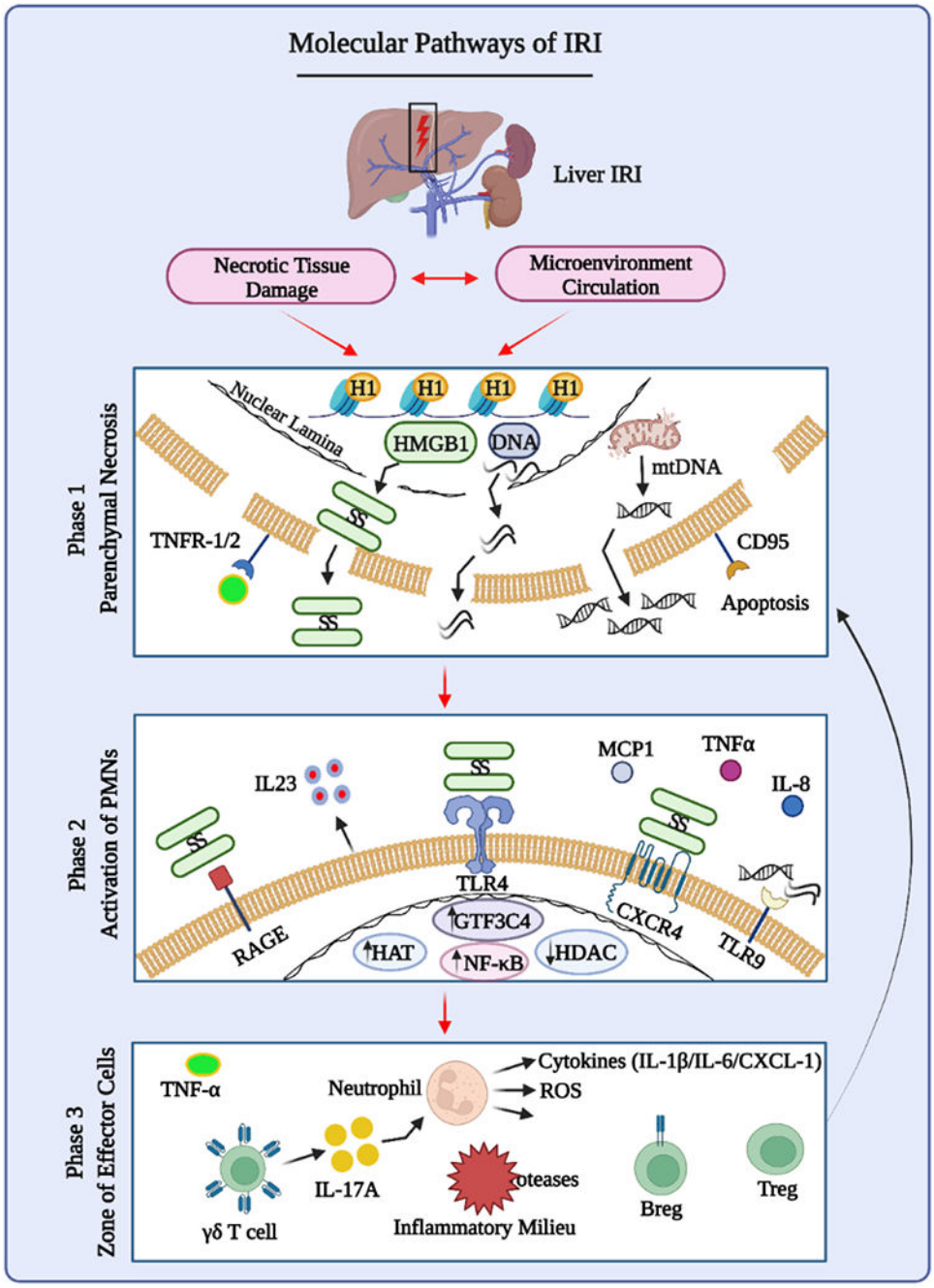


Figure 1:

Ischemia-reperfusion injury (IRI) is a classic positive feedback loop that leads to an amplified cascade response in liver transplant patients. It is characterized by three phases that involve the interplay between necrotic tissue damage and alternations in the microcirculatory environment. *Phase 1* involves parenchymal cell death resulting from ischemic stress that stimulates danger molecules, such as HMGB1 and DNA fragments. *Phase 2* consists of the activation of PMNs by cytokine/chemokines such as IL-23, MCP1, TNFα, and IL-8. *Phase 3* is characterized by the activation of host innate and adaptive

immune cells, like inflammatory cytokine IL-23, which causes gd T cells to secrete IL-17A, recruiting the circulating monocytes and neutrophils to the expanding inflammatory milieu that causes more parenchymal damage, *Phase 1* (large black arrow, right-side panel). Extrapolated from studies in liver and renal injury models [113,200]. Adapted from [13] with copyright permission to reuse and created using Biorender.com.

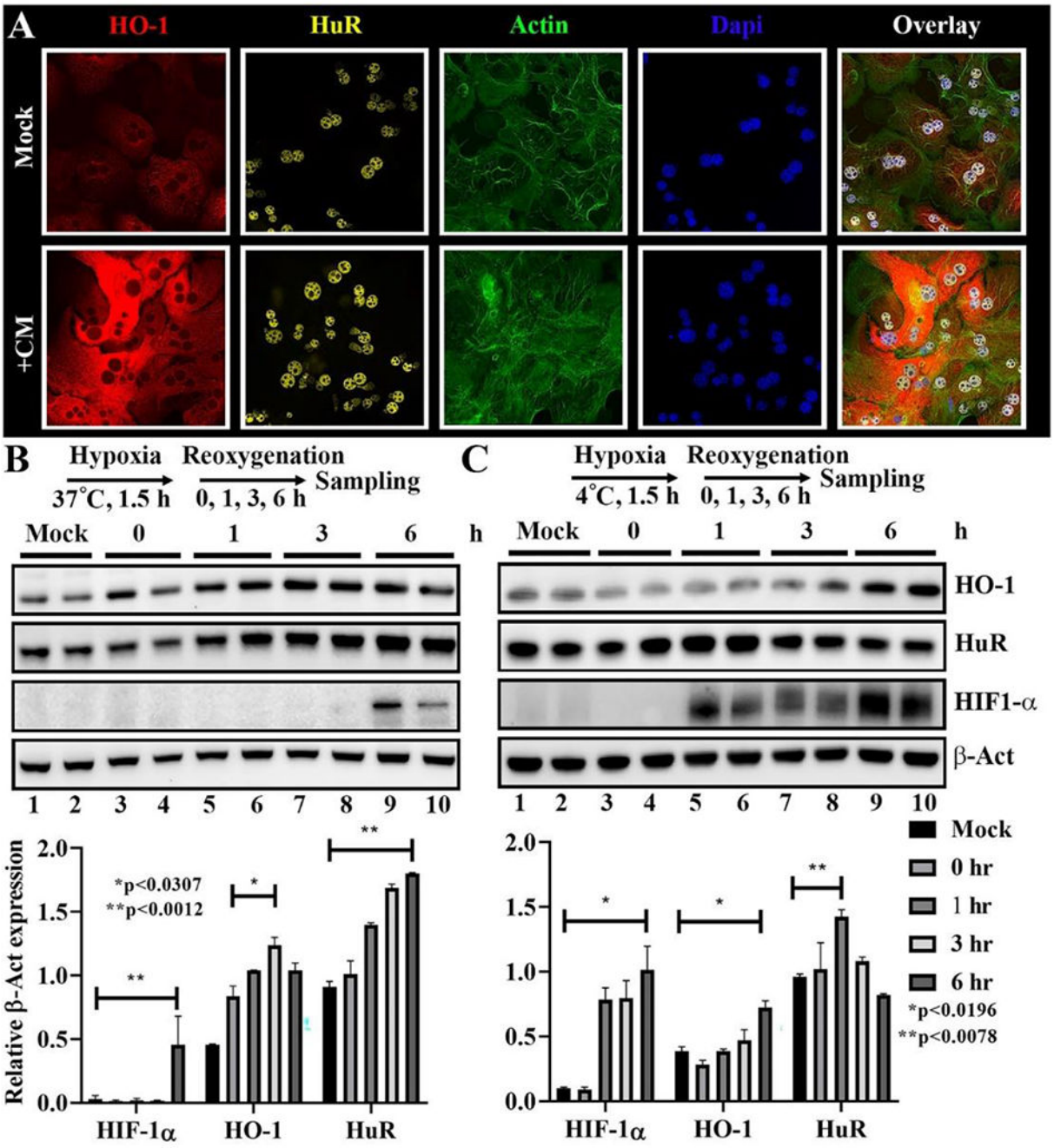
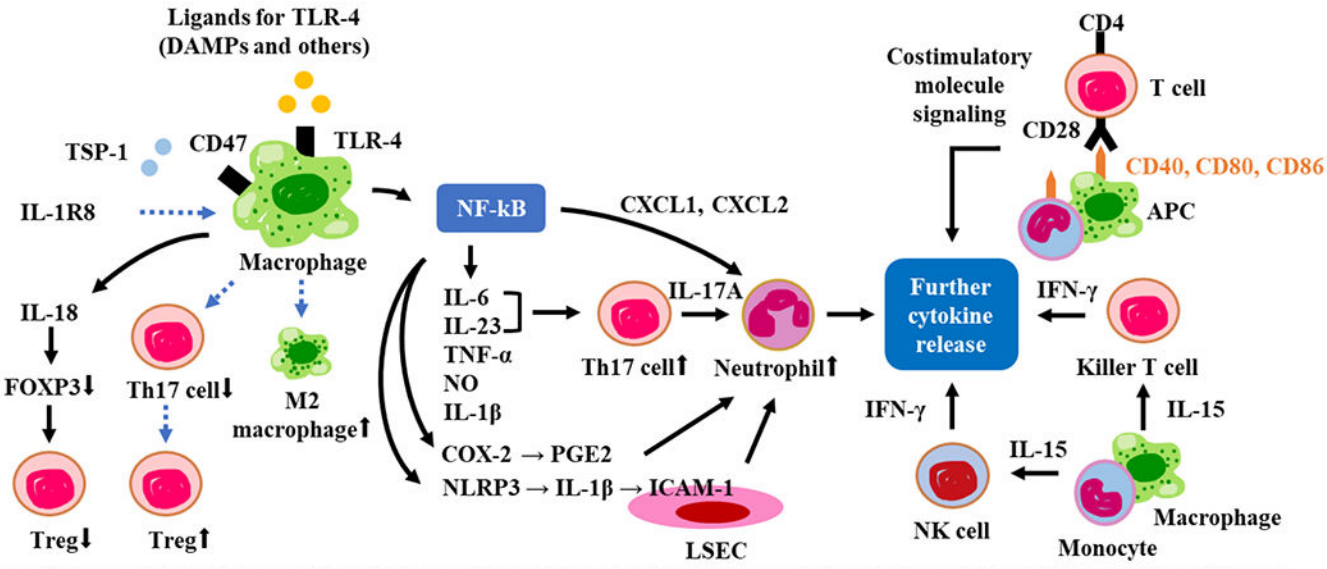


Figure 2.

Warm but not cold ischemic stress of cytokine-stimulated primary-derived hepatocytes causes the up-regulation of HO-1 by HuR protein. (A) Representative immunohistochemical detection of hepatocyte HuR and HO-1 after 12 hours of CM conditioning (n = 3/group; original magnification, ×40). Act, actin; Dapi, 4′,6-diamidino-2-phenylindole. Primary-derived hepatocytes were cultured in serum-free medium incubated under acute (1.5 hours) (B) warm or (C) cold hypoxia conditions followed by standard normoxia incubation for the indicated times. Total lysates were probed by western blot for differences between HO-1,

HuR, HIF-1α, BMP4, Bcl-x_L, and β-Act, as a loading control. Bottom: data below figures show unpaired two-tailed Student *t*-test of representative samples showing HIF-1α, HO-1, and HuR calculated relative to β-Act expression. Data shown are mean ± SEM, n = 2 (repeated at least three times). Adapted from [87], with permission for copyright from John Wiley & Sons Ltd.



Possible inhibitor of signaling pathways in different transplant organs

- Carvedilol (liver)[137]
- Lavage (Kidney)[201]
- Proton pump inhibitor (liver)[151]
- Thrombomodulin (liver)[126]
- CD47mAb (liver)[122]
- Liraglutide (Kidney)[130]
- rAIM (Kidney)[129]
- TIP1 (Kidney)[140]
- anti-CD4 mAb (liver)[146]
- Propionic acid (liver)[139]
- Salidroside (liver)[138]

Figure 3.

Newly discovered signaling pathway modifiers associated with tissue IRI in organ transplantation. TLR4/NF-kB signaling pathway in macrophages can be activated by various ligands, including DAMPs and others, resulting in the activation of neutrophils, leading to inflammatory cytokine release and tissue damage. Recent studies revealed that many modalities, such as carvedilol, salidroside, propionic acid, thrombomodulin, CD47mAb, TIP1, rAIM, and liraglutide, could potentially block this pathway. TIP1 may also decrease Th17 cells and increase Tregs. IL-1R8 may drive macrophage polarization toward the "cytoprotective" M2 phenotype. As for T-cells, anti-CD4 mAb could mitigate neutrophil/macrophage infiltration and proinflammatory gene expression programs. While NK cell involvement in organ IRI is known to be IL-15 signaling-dependent, no specific inhibitor has been reported.

Table 1.

Representative experimental studies targeting tissue IRI stress and organ damage (2021-2022).

Area of Interest	Year	Novelty	Source
Hypoxia Inducible Factor-1 alpha (HIF-1α)	2022	Primary rat hepatocytes were tested with pan-PHD small-molecule inhibitor ethyl-3,4-dihydroxybenzoate (EDHB) on the activity of HIF-1 and its downstream target gene expression assessed. PHD inhibition mitigated allograft injury during liver transplantation.	[169]
	2022	Proinflammatory cytokines during cerebral IRI correlated with HIF-1α. Reducing nitric oxide (NO) species using the L-NAME inhibitor reduced HIF-1α and established an anti-inflammatory phenotype.	[170]
	2021	Diazepam, a well-known anesthetic, was shown to reduce myocardial IRI and improve HIF-1α mRNA expression. It also reduced C-C chemokine receptor type 2 (CCR2) expression alongside other anti-inflammatory cytokines.	[171]
	2021	Hypobaric hypoxia methods were shown to be more beneficial than normobaric hypoxia methods in mitigating myocardial IRI, which correlated with a reduction of HIF-1α expression in hypobaric hypoxia-treated rats.	[172]
Immune cell targets	2023	A bioinformatics approach was used to identify macrophages, DCs, neutrophils, CD4 T cells, and ADM, KLF6, SERPINE1, and SLC20A1 as potential biological biomarkers underlying IRI post-transplant infiltrated the IRI that occurred after LT	[173]
	2023	Profiling datasets of liver tissues and hospital samples were collected to reveal changes in the expression of ARNTL, BTG2, CXCL10, CHI3L1, IER3, FOS, and PPARGC1A and the proportion of dendritic cells may be associated with aging livers being more prone to IRI.	[174]
	2022	This study explored the roles of key pyroptosis-related genes in liver ischemia-reperfusion injury. Eighteen pyroptosis-related genes were significantly and differentially expressed between disease and normal samples.	[175]
	2021	CD4 T cells were identified to be critical markers for IRI in liver allografts. Disrupting CD4 signaling reduced autoimmune responses contributing to IRI by reducing costimulatory immune factors in mice. Correlations were found between CD4 levels and immune-driven liver inflammation early after human OLT.	[146]
	2021	T-cell immunoglobulin and mucin domain-containing protein-4 (TIM-4) deficiency in resident Kupffer cells of mice livers during ischemia and reperfusion worsened IRI and reduced inflammatory recovery 7 d after reperfusion.	[176]
Macrophage polarization	2023	A study investigating the role of Nicotinamide phosphoribosyl transferase (NAMPT) in liver IRI. NAMPT plays an important role in both hepatocytes and liver macrophages.	[177]
	2022	SS-31, an antioxidant peptide, was shown to reduce liver damage at the cellular level, which was correlated with reduced ROS. SS-31 inhibited macrophage M1 polarization by regulating the STAT1/STAT3 signaling pathway.	[178]
	2021	Liraglutide, an activator of glucagon-like peptide 1 receptor (GLP-1R), had anti-inflammatory and hepatoprotective properties. It also inhibited macrophage M1 polarization during liver IRI.	[179]
Mitogen-activated Protein Kinases	2022	c-Jun N-terminal Kinase (JNK) and p38 MAPK (p38), members of MAPK families, activate during liver I/R in rats. Treatment with silibinin reduced liver injury by targeting JNK and p38.	[180]
Microbiota	2022	5% Inulin rich diets led to increased *Bacteroides* species in mouse gut microbiota and feces and various metabolic changes in short-chain fatty acids, with propionic acid to be most significantly increased. When injected peritoneally, propionic acid reduced proinflammatory cytokine expression, suggesting the benefits of inulin diets.	[139]
MicroRNAs and associated targets	2022	Rat bone marrow mesenchymal stem cells (BMMSCs) expressing HO-1 downregulated IREB2 protein through exosomal involvement of microRNA miR-29a-3p, reducing ferroptosis and IRI in rat steatotic livers.	[181]
	2022	Gene markers relating to pyroptosis and liver IRI were identified: *ADORA3, BIRC3, CAMP, CEBPB, CXCL8, GBP1, GJA1, GSDMB, GZMB, IL1β, IL1RN, IRF1, JUN, NFκB, NLRP3, SERPINB1, TNF*, and *TXNIP*. MicroRNA and TF RELA play a role in gene regulation.	[175]
	2022	miR-181a-5p upregulation correlated with downregulation of YYI (Yin-Yang 1 transcription factor), and upregulation of YYI inhibited miR-181a-5p expression. Inhibiting miR-181a-5p elevated ESR1 expression showing ESR1 as a target gene of miR-181a-5p.	[182]
	2021	MicroRNA miR-135b-5p regulates JAK2 as an inhibitor, which worsens liver IRI by upregulating apoptosis. Desflurane administration regulated miR-135b-5p, which led to more JAK2 expression and decreased apoptosis.	[183]

Area of Interest	Year	Novelty	Source
Mitophagic targets	2022	Tongxinluo, a traditional Chinese herbal medication, downregulated ubiquitin response in myocardial IRI, and reduced injury through mitophagy involved with PINK1/Parkin pathway.	[184]
Nuclear Factor Erythroid 2-related Factor 2 (Nrf2)	2022	p21-activated kinase 4 (PAK4) phosphorylates NRF2 suppressing its transcriptional activity. Inhibiting PAK4 protected mice from liver injury, whereas silencing NRF2 led to more liver injury.	[185]
	2022	Sevoflurane mitigated liver IRI in rats by regulating microRNA-122, which promoted the activation of the Nrf2/HO-1 pathway, historically shown to have a hepatoprotective effect.	[186]
	2022	NRF2 bound to tissue inhibitor of metalloproteinase 3 (Timp3) in macrophages inhibited RhoA/ROCK, which led to reduced macrophage activation and proinflammatory immune responses.	[187]
	2022	Treatment of basic fibroblast growth factor (BFGF) *in vivo* reduced liver IRI through the Nrf2/Hippo signaling pathway. Yes-associated protein (YAP) was promoted upon NRF2 activation.	[188]
	2021	Itaconate (anti-inflammatory agent and antioxidant) was found to activate Nrf2, which suppressed Ccl3 and Ccl3 (proinflammatory cytokine) pathways. A cluster of mRNAs Il6, Il1b, Ptgs2, Mmp13, Ccl3, Ccl4, Osm, and Il1f9 were identified to be involved in IRI.	[189]
Transcription Factors	2022	AGBL4, CILP2, and IL4I1 genes are associated with liver IRI.	[190]
	2021	Sufentanil was identified as a possible therapeutic for liver IRI as it suppresses ATF4 and TP53BP2, reducing hepatic inflammation and apoptosis.	[191]

Table 2.

Clinical studies targeting hepatic IRI in OLT patients (1993 to present).

Therapy	Study Design N, Year	Mechanism	Novelty	Outcome	Source
Alprostadil	RCT 20, 1993	Smooth muscle relaxant and vasodilator; acts at prostaglandin receptors increasing cAMP	1st clinical use of prostaglandins	PGE1 therapy during OLT preserved platelet function	[192]
Epoprostenol	RCT 106, 1999	Acts at prostacyclin IP receptors to increase platelet cAMP	Preconditioned donor livers with	Rapidity and homogeneity of graft reperfusion improved	[193]
Tacrolimus	RCT 20, 2003	A macrolide antibiotic that impairs gene expression by inhibiting calcineurin phosphatase	Supplemented to the flush solution to evacuate the preservation solution	Decreased hepatocellular injury after reperfusion	[194]
Aprotinin	RCT 24, 2004	Serine protease inhibitor	Identified relationship MMP-9 and tissue-type plasminogen activator (t-Pa)	MMPs was not altered using aprotinin	[195]
Thymoglobulin	RCT 22, 2005	Rapid T-cell-depleting agent in both the blood and peripheral lymphoid tissues	Compromised liver grafts had less IRI	Reduced alanine aminotransferase and bilirubin	[152]
IDN-6556	RCT 99, 2007	Pancaspase inhibitor	Phase II trial, focused on cold ischemia, warm reperfusion injury (CI/WR)	CI/WR-mediated apoptosis and injury only during cold storage and flush	[83]
rPSGL-Ig	RCT 47, 2011	P-selectin antagonist reduces adherence of leukocytes to activated platelets and endothelium	Phase II trial, 1st clinical trial of an adhesion molecule antagonist	Reduced total hospitalization, liver enzymes and bilirubin, IP-10 IL-10 levels	[114]
Normothermic preservation (NMP)	RCT 220, 2018	Liver was perfused with oxygenated blood, medications, and nutrients at normal body temperature	1st to compare NMP with conventional static cold storage in OLT	Significant reductions in peak AST and EAD rates	[75]
Remote Ischemic Preconditioning	RCT 208, 2021	Transient brief episodes of ischemia at the remote site before prolonged IRI of the target organ	None	Not significant	[196]
Treprostinil	Cohort 23, 2021	A prostacyclin I2 (PGI2) analog that acts as a vasodilator of pulmonary and systemic arterial vascular beds, improving systemic oxygen transport	Safety trial showed intravenous infusion led to no occurrence of primary graft non-function	Improved hepatobiliary excretory function	[197]
ANG-3777	RCT 28, 2021	Used a hepatocyte growth factor mimetic that binds to the c-MET receptor	Phase II trial of patients undergoing renal transplantation	Improved hepatobiliary excretory function, better graft function with a good safety profile	[67]
NMP	RCT 300, 2022	Provides oxygen and nutrition during preservation and allows aerobic metabolism	1st evidence NMP reduces post-OLT EAD and IBC*.	Significant decrease in EAD, histopathologic evidence	[198]
Simvastatin	RCT 58, 2022	Serves as β-Hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase inhibitor to lower the production of cholesterol	Preliminary evidence on the effectivity and safety of oral administration	Improved graft and recipient survival at 6-mo after LT	[199]
Cerium oxide nanoparticles (nanoceria)	RCT 9, 2022	Act as effective scavengers of reactive oxygen species (ROS)	A combined strategy of NMP and nanotechnology	Significant antioxidant activity reported	[76]