Ischaemia–reperfusion injury in liver transplantation—from bench to bedside

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Abstract

Ischaemia–reperfusion injury (IRI) in the liver, a major complication of haemorrhagic shock, resection and transplantation, is a dynamic process that involves the two interrelated phases of local ischaemic insult and inflammation-mediated reperfusion injury. This Review highlights the latest mechanistic insights into innate–adaptive immune crosstalk and cell activation cascades that lead to inflammation-mediated injury in livers stressed by ischaemia–reperfusion, discusses progress in large animal experiments and examines efforts to minimize liver IRI in patients who have received a liver transplant. The interlinked signalling pathways in multiple hepatic cell types, the IRI kinetics and positive versus negative regulatory loops at the innate–adaptive immune interface are discussed. The current gaps in our knowledge and the pathophysiology aspects of IRI in which basic and translational research is still required are stressed. An improved appreciation of cellular immune events that trigger and sustain local inflammatory responses, which are ultimately responsible for organ injury, is fundamental to developing innovative strategies for treating patients who have received a liver transplant and developed ischaemia–reperfusion inflammation and organ dysfunction.

Introduction

Liver transplantation is the standard of care in patients with end-stage liver disease and those with tumours of hepatic origin.[1] In 2011, 16,107 patients in the USA were waiting for a liver transplant, but only 6,341 procedures were performed, indicating a shortage of about 10,000 donor livers per year.[2] During the same time, 1,589 patients died whilst waiting for a liver transplant and an additional 1,349 patients were removed from the waiting list because they became too sick to undergo surgery.[2] Organ shortage has prompted the use of extended criteria donor organs from older, steatotic, or non-heart-beating donors, as well as organs that have been subjected to prolonged periods of warm and cold storage. However, these 'marginal' organs are particularly susceptible to ischaemia–reperfusion injury (IRI) as a result of damage during procurement, preservation and surgery. Indeed, IRI not only contributes to the donor organ shortage (as organs might be harvested and then deemed too damaged for transplant) but might also lead to poor early graft function and primary

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nonfunction. Moreover, the cellular damage surrounding organ removal and storage affects transplantation outcomes as it is a major risk factor for both acute and chronic rejection. Despite its obvious clinical importance, the mechanisms that account for organ IRI are only partially understood and remain one of the most understudied areas in clinical and experimental transplantation.[3,4]

In this Review, we first focus on our current understanding of cellular and molecular mechanisms that trigger local immune activation and inflammatory cascades in procured livers subjected to revascularization. We then summarize research progress in large animal models, and finally address the current status of clinical trials focusing on liver IRI in patients who receive a liver transplant. By presenting new insights into tissue inflammation responses driven by complex innate–adaptive immunity, we had to limit our discussion of other essential IRI pathogenic mechanisms, such as parenchymal cell death programmes, the complement system and the role of mitochondria in generating reactive oxygen species (ROS) and nitrogen species. Progress pertaining to the aforementioned factors might lead to innovative therapies in liver IRI (including preconditioning and postconditioning approaches). These issues have been discussed elsewhere.[5–11]

Types and stages of liver IRI

Two major types of liver injury that are attributable to ischaemia–reperfusion can be distinguished.[4,12] The 'warm' IRI, which is initiated by hepatocellular damage, develops *in situ* during liver transplantation surgery or during various forms of shock or trauma, and might lead to liver or even multiorgan failure. The 'cold' IRI, which is initiated by damage to hepatic sinusoidal endothelial cells and disruption of the microcirculation, occurs during *ex vivo* preservation and is usually coupled with warm IRI during liver transplantation surgery. Although initial cellular targets of the two IRI types might be different, they do share a common mechanism in the disease aetiology; that is, local inflammatory innate immune activation. Mechanistic appreciation of different IRI types is of current interest because, as discussed later, cold static hepatic preservation and warm *ex vivo* liver perfusion are being compared in large animal models of IRI for future clinical use.

The activation of liver Kupffer cells and neutrophils, the production of cytokines and chemokines, the generation of ROS, increased expression of adhesion molecules and infiltration by circulating lymphocytes and/or monocytes are immunological cascades present in both types of IRI.[3,48] Distinct from alloreactive responses against liver grafts, tissue inflammation triggered by ischaemia–reperfusion occurs immediately after reperfusion. This inflammation is predominantly an innate-immune-dominated response that is mediated by the sentinel pattern recognition receptor (PRR) system. Endogenous ligands generated as a result of hepatocellular damage—danger-associated molecular patterns, rather than exogenous pathogen-associated molecular patterns—have a key role in tissue inflammation. However, ischaemia–reperfusion elicits an equally robust adaptive immune response in the liver that is dependent on CD4^{+} T cells. Our own studies suggest that although innate activation might induce ischaemia–reperfusion damage both *in situ* and in liver transplants, the 'cold' preservation injury favours an early and massive T-cell influx into ischaemic liver grafts.[13]

Two distinct stages of liver IRI, with unique mechanisms of hepatic damage, have been identified (Figure 1). Ischaemic injury, a localized process of cellular metabolic disturbances, results from glycogen consumption, lack of oxygen supply and ATP depletion, which lead to the initial parenchymal cell death. Reperfusion injury, which follows the ischaemic injury, results not only from metabolic disturbances but also from a profound inflammatory immune response that involves both direct and indirect cytotoxic mechanisms.

Indeed, the liver's response to inflammation is critical, as prevention of local immune activation uniformly ameliorates IRI. Therefore, dissection of innate immune activation is important for identifying novel therapeutic targets to alleviate proinflammatory mechanisms whilst sparing or augmenting anti-inflammatory mechanisms. Furthermore, crosstalk between the innate and adaptive immune responses triggered by ischaemia–reperfusion readily converts an immunologically quiescent liver into an inflammatory organ, even in a sterile environment.[4] Most of the discussed studies were performed in an *in situ* model of segmental hepatic warm ischaemia–reperfusion in mice. Although only partially reflecting the real-life transplant setting, the model takes advantage of the genetically targeted mouse strains. A clinically more relevant mouse model, combining cold and warm IRI components followed by orthotopic liver transplantation, has only been established in the past decade.[14]

Ischaemia–reperfusion activates TLRs

On the basis of seminal observations in the mid 1990s from patients who received a renal transplant,[15] Professor Walter Land's 'injury hypothesis' states that IRI activates a cascade of innate-dominated proinflammatory immune responses, which then trigger the adaptive immune response that culminates in allograft rejection.[16] Indeed, ample evidence indicates that all vertebrates use the same sentinel innate immune receptor systems—PRRs—in response to tissue damage in the absence of infection.[17–22] PRRs are divided into four classes: Toll-like receptors (TLRs); C-type lectin receptors; retinoic acid-inducible gene I-like receptors and NOD-like receptors (NLRs). The first two are transmembrane proteins and the last two are cytoplasmic proteins. These PRRs are expressed predominantly in macrophages and dendritic cells, and their activation triggers upregulation of the transcription of genes involved in the inflammatory response.[23] TLRs are the most thoroughly studied PRRs in the liver IRI immune cascade (Figure 2). The TLR system consists of at least 13 members, which function as homodimers or heterodimers of type I transmembrane glycoproteins. Most TLRs are expressed on the cell surface, except TLR3, TLR7, TLR8 and TLR9, which are located in the endoplasmic reticulum membrane. Co-receptors are required for some TLRs to recognize their ligands, for example, lymphocyte antigen 96 and CD14 are required for TLR4 to bind lipopolysaccharides. TLR ligation triggers multiple intracellular signalling pathways and ultimately results in activation of transcriptional factors, such as nuclear factor κB (NF-κB), activator protein 1 and interferonregulatory factors (IRFs), which act in concert to initiate expression of genes encoding cytokines, chemokines and co-stimulatory molecules.[24–26]

The liver receives blood that drains from the gastrointestinal system (where commensal bacteria reside) via the hepatic portal vein; therefore, gut-derived endotoxin might be translocated directly into the liver circulation. This phenomenon certainly occurs during liver ischaemia–reperfusion when hepatic portal vein occlusion results in blockage of the intestinal wall, leading to its increased permeability during reperfusion.[4] TLR4 was the first innate immune receptor tested in liver IRI. Indeed, using mouse models of partial warm ischaemia, data from three separate laboratories demonstrated that livers in TLR4-deficient mice were protected against IRI and that local hepatic inflammation against ischaemia–reperfusion insult was suppressed in the absence of TLR4.[27–29] Of note, TLR2 was not necessary for the development of liver IRI, which is unlike its essential role in other organs, such as the kidney or heart.[30,31] The critical role of TLR4-specific activation in triggering liver IRI pathology was also confirmed in orthotopic liver transplantation, which consists of both warm and cold IRI components[32] and in a steatotic liver IRI mouse model.[33] Interestingly, the donor being deficient in TLR4 was sufficient to protect the liver from IRI in the transplant model, and TLR4 signalling on liver nonparenchymal cells seems to be more relevant than TLR4 signalling on parenchymal cells for liver IRI;[18] however, a study has demonstrated that TLR4 has a unique role in liver parenchymal cells at the late stage of

the disease process.[34] These data indicate that TLR4 on both parenchymal and nonparenchymal cells in livers are involved in the process of hepatic IRI.[34]

The activation of TLR4 in liver IRI is dependent on a downstream signalling pathway mediated by IRF3 but independent of myeloid differentiation primary response gene MyD88.[29] MyD88-deficient mice not only developed hepatocellular damage, but their liver IRI 'signature' proinflammatory cytokine (TNF and IL-1β) and chemokine (CXCL10) programmes were largely unaffected. As the MyD88-independent, IRF3-dependent signalling pathway of TLR4 just delays NF-κB activation, it seems that the MyD88-mediated activation of NF-κB in the early phase of IRI is not necessary for the proinflammatory immune response against ischaemia–reperfusion in the liver. This situation is very different from renal and heart IRI models in which either MyD88 and IRF3 or only MyD88 are required.[35–38] The fact that the severity of liver IRI peaks at 6 h of reperfusion and that of kidney and heart injury last for days after reperfusion might partially explain this discrepancy. The other potential reason is that TLR4 activation in the liver is unique, as gut-derived endotoxin might have already tolerized the innate immune system in the liver, which has been shown to target the MyD88-dependent pathway.[39,40] Both proinflammatory and anti-inflammatory gene programmes are induced by TLR4 activation in macrophages *in vitro* and livers *in vivo*.[4] Glycogen synthase kinase 3β, a serine/threonine kinase, was found to differentially regulate these two programmes.[41] We have shown that glycogen synthase kinase 3β inhibitor can be used as an effective therapeutic agent in liver IRI by selectively inhibiting the proinflammatory programme while sparing the immune-regulatory IL-10 gene programme.[42]

Although endotoxin has been implicated in IRI, the question of whether it provides the triggering signal for the liver inflammatory immune response against ischaemia–reperfusion remains controversial. Increased levels of lipopolysaccharide were detected in portal and systemic circulation after ischaemia–reperfusion in both animal models and patients who received a liver transplant.[43] A protective effect of antiendotoxin monoclonal antibodies was detected in a steatotic hepatic IRI model.[44] However, our own study using endotoxin-neutralizing peptides failed to show any organ protection in the early phase of liver ischaemia–reperfusion.[45] One key point that might differentiate the latter result from the former is the time after reperfusion at which the measurements were taken (6 h versus 24 h). Perhaps endotoxin is not responsible for triggering the activation of the innate immune response of the liver during ischaemia–reperfusion, but instead it might participate in sustaining the inflammation response.

More than 20 distinct endogenous TLR2 and/or TLR4 ligands, representing intracellular proteins, extracellular matrix proteins, oxidatively modified lipids and other soluble mediators, have been identified.[46] High mobility group protein 1 (HMGB1), originally discovered as a nuclear protein, has been identified as the key endogenous TLR4 ligand responsible for immune activation in the liver during ischaemia–reperfusion.[47] HMGB1 can be released from damaged hepatocytes to subsequently stimulate liver nonparenchymal cells, including Kupffer cells, via TLR4 signalling (Figure 2). Hypoxic hepatocytes release HMGB1 through an active process facilitated by TLR4-dependent production of ROS. In turn, ROS induces the release of HMGB1 through a CaMK-dependent mechanism, and such a positive loop of HMGB1–TLR4 signalling might encourage a sustained inflammatory response in the liver during ischaemia–reperfusion.[48]

Of note, HMGB1 biology has become quite complex, and how this ubiquitously expressed protein might trigger the inflammation response is a matter of controversy. First, the relative importance of hypoxia and cellular necrosis in HMGB1-mediated stimulation of target cells remains to be determined. Second, questions concerning the molecular nature of TLR4

binding partners and putative roles of other receptors in immune activation, such as receptor for advanced glycation end products (RAGE) need to be addressed.[49,50] Third, additional functions of HMGB1 have been discovered via its binding to nonorthotropic receptors.[51,52] Indeed, RAGE has been shown to be essential in liver IRI by regulating expression of CXCL2 (also known as macrophage inflammatory protein 2) via an epidermal growth factor receptor (EGFR)-dependent mechanism, as well as influencing cell death and production of TNF in an EGFR-independent manner.[51,52] TLR4-mediated upregulation of IRF1 in hepatocytes has also been identified as a necessary step for release of HMGB1 in response to hypoxia,[53] consistent with the essential function of parenchymal rather than nonparenchymal IRF1 in the mechanism of IRI as a result of liver transplant.[54] HMGB1 also promotes recruitment of inflammatory cells to damaged tissue by forming a complex with the chemokine CXCL12 and signalling via CXCR4, independent of RAGE and TLR4.[55] Although mechanisms that govern inflammatory cell sequestration in the damaged organ versus cytokine release might be different, both biological processes do involve HMGB1.

In addition to HMGB1, other DAMPs released from damaged and/or necrotic cells might stimulate innate immune cells via various receptors, such as, heat-shock proteins, S100 proteins via TLR4, RNA via TLR3 and DNA via TLR9. TLR9 was found to function in cells derived from bone marrow, particularly neutrophils, during liver ischaemia–reperfusion, which boosted production of proinflammatory cytokines and chemokines.[56] Inhibition of TLR9 had additive protective effects when HMGB1 was neutralized in livers subjected to ischaemia–reperfusion. [56] Nuclear histone proteins were identified as the potential endogenous ligand of TLR9 in the liver.[57] Indeed, liver IRI resulted in increased levels of circulating histones and neutralization of histones was cytoprotective. Extracellular histones enhanced TLR9 activation mediated by DNA, whereas histone infusion exacerbated liver IRI pathology via TLR9 signalling. TLR3, which recognizes necrotic cell-derived RNA products, has also been shown to sustain inflammation in a mouse gastrointestinal ischaemia model.[58]

The role of other PRRs in liver IRI has only become unravelled in the past 5 years. Necrotic cells can be 'sensed' by inflammasomes as they release panels of proinflammatory mediators. In a model of sterile inflammation, one member of the NLR family, NLRP3 protein, was found to be involved in the mechanism of polymorphonuclear cell recruitment to sites of focal hepatic necrosis. [59] Gene silencing of NLRP3 attenuated liver damage, in association with reduced levels of IL-1β, IL-18, TNF and IL-6, diminished levels of HMGB1 and decreased local inflammatory cell infiltration.[60] Interestingly, haemorrhagic shock-induced liver damage might develop independently of NLRP3, and caspase 1 was found to be cytoprotective during trauma, as it mitigated liver injury and inflammation.[61]

Although an array of PRR-targeting studies have shown promise in different animal models, the caveat is that most of these studies focus on the 'correlation' between genetic deletion and cytoprotection rather than establishing the actual cause of the reduced tissue damage. With limited mechanistic understanding of a successful anti-IRI therapy, exploring multiple PRR pathways with small molecules acting preferably in a synergistic manner might be required.

T cells in liver innate immune response

Despite the fact that IRI can develop in syngeneic grafts, *ex vivo*, or under sterile conditions, T cells, particularly of the CD4+ phenotype, are indispensable for the activation and regulation of the tissue proinflammatory immune response to ischaemia–reperfusion (Figure 2). The observation that systemic immunosuppression (with either ciclosporin or tacrolimus)

attenuated hepatocellular damage provided initial indirect evidence for involvement of T cells in the development of IRI.[62] Studies in T-cell deficient and CD4-deficient mouse systems have proven the pivotal function of CD4^{+} T cells in the mechanism of liver IRI.[63–66] The obvious question that arises is how do T cells function in this predominantly innate response and in the absence of exogenous antigen stimulation?

The role of T cell co-stimulatory pathways in promoting IRI in the absence of alloantigen was initially shown in a study in which CD28 blockade with cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA4Ig) protected rat kidneys from IRI by reducing infiltration of T cells and/or macrophages.[67] Both CD28 and CD154 are important for the activation of liver inflammation pathways that lead to hepatocellular damage. Indeed, livers in CD154-knockout or CD28-knockout mice or in wild-type mice treated with anti-CD154 or CTLA4Ig were all protected from IRI.[65] Furthermore, T-helper (T_H)1-type cells have a major role in the pathogenesis of liver IRI, as *Stat4*-knockout mice (deficient in T_H1 development), but not *Stat6*-knockout mice, were protected from the injury, whereas reconstitution of 'nude' mice with T cells from *Stat6*-knockout, but not *Stat4*-knockout, mice restored cardinal features of liver IRI.[68] T_H17 cells have also been implicated in autoimmune inflammatory diseases, and their putative role in IRI has started to be revealed. Although cellular sources of IL-17 in the liver remain to be determined, Il-17a knockout mice suffer less severe liver IRI concomitant with reduced neutrophil infiltration than wild-type mice. The effect of Il-17a deficiency was associated with fairly late stages of the disease and did not affect acute ischaemia–reperfusion liver damage.[69] In agreement with the Lentsch group,[70] our research group has detected CD4^{+} T-cell sequestration into postischaemic livers before any appreciable local neutrophil accumulation (H. Ji, personal communication). This sequestration occurs via released Il-17, which might then act upon hepatocytes and macrophages to produce CXCL2, a known neutrophil chemotactic factor.

By contrast, IL-22, which is produced by the same subset of CD4^{+} T cells that express IL-17, exerts protective functions in mouse models of acute liver failure.[71–73] Unlike other cytokines, IL-22 is not involved in communication between immune cells, but instead it signals directly to hepatocytes via IL-22R1.[74] Having documented that administration of recombinant IL-22 had a hepatoprotective effect via STAT3 activation,[75] we favour the concept that IL-22 is well-positioned to orchestrate innate–adaptive networks by activating cell survival genes, preventing apoptosis and promoting hepatic regeneration in livers stressed by ischaemia–reperfusion.

Although the role of CD154 in liver IRI has been attributed to its co-stimulatory T-cell function, CD40 ligation on dendritic cells or macrophages by T-cell-derived CD154 is the critical activating signal to innate immune cells.[4] Endogenous ligands that trigger liver IRI might be insufficient to fully activate and sustain a proinflammatory phenotype in an ischaemia–reperfusion-stressed liver. Thus, hepatic Kupffer cells might be less sensitive to TLR4 stimulation than peripheral macrophages because of their exposure to endotoxin from the gut that drained through the hepatic portal vein. Indeed, Kupffer cells might synthesize immune regulatory IL-10 when exposed to lipopolysaccharide *in vitro*.[76] Liver dendritic cells have lower expression levels of TLR4 and are less susceptible to stimulation with lipopolysaccharide than dendritic cells in the spleen.[77] In fact, conventional liver dendritic cells have been shown to exert immuneregulatory functions during ischaemia–reperfusion by producing IL-10 via a TLR9-mediated mechanism.[78]

The T cell, immunoglobulin, mucin-containing molecules (TIM) family of cell surface proteins has attracted much attention as novel regulators of host immunity.[79] Stimulation of T cells amplifies expression of TIM-1, a phosphatidylserine receptor, that is expressed primarily on CD4^{+} T_H2 and T_H1 cells, whereas TIM-4, one of the major TIM-1 ligands, is

expressed mostly by macrophages and antigen-presenting cells. Therefore, interactions between TIM-1 and TIM-4 constitute a novel molecular mechanism of T cell–macrophage regulation at the innate–adaptive immune interface, and might be a therapeutic target. Indeed, treatment with anti-TIM-1 monoclonal antibodies ameliorated the hepatocellular damage, accompanied by decreased local polymorphonuclear cell infiltration and/or activation, inhibition of T cell and/or macrophage sequestration and diminished homing of TIM-4^{+} cells expressing the TIM-1 ligand in ischaemic livers.[80] The induction of proinflammatory cytokine and/or chemokine programmes was also blunted,[80] this data is supported by findings from a renal IRI model.[81] By contrast, the TIM-3–galectin pathway constitutes a 'negative' co-stimulation signalling pathway between T_H1 cells and macrophages, and has been shown to promote immunotolerance in transplant recipients.[79] Interestingly, TIM-3 blockade worsened the hepatocellular damage, and caused increased IFN-γ but decreased IL-10 expression in ischaemia–reperfusion-stressed livers.[82] The programmed death 1 programmed death ligand 1 (also known as B7–H1) 'negative' T cell pathway has also been shown to promote hepatoprotection in warm and cold liver IRI models.[83,84] Thus, multiple co-stimulatory signalling pathways, both positive and negative, might function in a two-way traffic approach to promote versus inhibit liver inflammatory immune responses against ischaemia–reperfusion insult (Figure 2).

In addition to 'traditional' T cells, natural killer (NK) and natural killer T (NKT) cells might also have distinct roles in the mechanism of IRI. Although general depletion of cells expressing the antigen NK1.1 (both NK and NKT cells) fails to affect the severity of IRI at its early stages,[85] this depletion does reduce the hepatocellular damage in the later phases.[86] Activation of NKT cells (which make up almost 50% of liver T cells) that is triggered by ischaemia–reperfusion is mediated by CD1d, which is expressed by most liver cells. This activation also presents glycolipid antigens (that are possibly released by necrotic cells) to NKT cell invariant T cell receptors. Furthermore, subsets of NKT cells have distinct roles *in vivo*. Indeed, type II NKT cells were shown to prevent liver IRI when activated by a specific glycolipid ligand sulphatide (self-glycolipid 3-sulphated β-galactosyl ceramide.[87] NK cell activation triggered by ischaemia–reperfusion is dependent on CD39 to hydrolyse ADP to AMP. Indeed, CD39-deficient livers were resistant to IRI, and NK-derived IFN-γ production was diminished, possibly because of activation of the type 2 purinergic receptor.[88] Thus, T cells, NKT cells and NK cells are all involved, although possibly at different stages, in the activation of the innate immune system by ischaemia–reperfusion, by providing co-stimulatory signalling via direct cell–cell interactions or cytokine stimulation to Kupffer cells and/or dendritic cells. This, in turn, promotes the proinflammatory phenotype of innate immune activation.

Liver IRI in large animal models

Although indispensable for dissecting basic molecular mechanisms of liver IRI, the rodent studies discussed earlier fall short of addressing critical clinical problems, such as using grafts procured from extended criteria donors and donation after cardiac death (DCD) as no models exist that would reproducibly mimic these situations. Approximately 60% of the potential DCD livers are discarded because of irreversible ischaemia–reperfusion damage.[1] In those DCD livers that are used, long-term survival outcomes are inferior compared with those from donation after brain death. With no established therapies for IRI available, limiting the duration of cold preservation and re-warming during surgery remain primary strategies to reduce the detrimental effects of liver IRI. Major efforts are being directed towards developing innovative techniques to circumvent warm IRI in clinically relevant pig liver DCD models. Emerging therapeutic protocols can be categorized on the basis of the timing of organ resuscitative therapy during the different phases of liver preservation. Organ preservation and resuscitation can be instituted immediately after cardiac death, when the

organ is still in the donor, using extracorporeal membrane oxygenation.[89,90] They can also be used after removal of the organ from the donor until its implantation via an *ex vivo* machine perfusion, or during revascularization of the liver after ischaemia, which is termed regulated hepatic reperfusion.[91]

Initiating preservation and resuscitation immediately after cardiac death and before organ removal provides a theoretical advantage of shortening the ischaemic period of the liver. Normothermic recirculation with extracorporeal membrane oxygenation restores warm oxygenated blood flow to the intra-abdominal organs and provides a beneficial preconditioning effect that reverses warm ischaemia-mediated cellular injury.[92,93] Accruing clinical data suggest that normothermic recirculation might improve graft function, and ultimately salvage DCD livers that would have been otherwise discarded.[89,90]

Current research in organ preservation is shifting from a static hypothermic preservation to dynamic *ex vivo* machine perfusion of the liver. Unlike static hypothermic preservation, machine perfusion provides continuous circulation and therefore improved preservation of the microcirculation, effective delivery of nutrients and oxygen for cellular metabolism, removal of metabolic waste products and administration of cytoprotective and/or immunomodulating agents. Machine perfusion also enables the surgical team to assess organ viability and potentially extend the period of organ storage.[94] Liver perfusion protocols, which are based on the temperature of the perfusate, can be categorized as normothermic (37 °C), subnormothermic (20 °C) or hypothermic (0–4 °C).

The rationale for normothermic machine perfusion is to maintain physiological temperature and avoid hypoxia to enable maintenance of cellular metabolic function during organ storage, avoiding depletion of cellular energy stores, accumulation of metabolic waste products and the direct adverse events of hypothermia. By using this approach, stable metabolic and haemodynamic function in a pig liver was reported for up to 72 h in a nontransplant setting.[95] Normothermic extracorporeal perfusion for 4 h at 37 °C in a pig DCD liver model enabled successful transplantation of livers exposed to up to 60 min of warm ischaemia.[96] Superior outcomes of DCD liver transplantation were reported after prolonged periods of normothermic perfusion compared with cold static storage in a pig model.[97]

Hypothermic organ perfusion is based on reducing cellular metabolism by lowering the temperature of the organ. Indeed, hypothermic oxygenated perfusion of pig DCD liver grafts can confer a survival benefit that is associated with a reduction of necrosis and platelet adhesion, improvement of bile flow and the return to usual levels of ATP and glutathione.[98] The first prospective clinical study has confirmed machine perfusion as a safe and reliable preservation strategy.[99] However, cold perfusion limits the duration of organ storage because it might increase vascular resistance and damage to sinusoidal endothelium and endoplasmic reticulum.[100]

A critical factor of reperfusion injury is microcirculatory failure, which is associated with insufficient energy supply, impaired ATP regeneration and incomplete recovery of hepatocellular excretory function.[101,102] Resuscitation of mitochondria (in which the mitochondria are protected from damage and orderly energy production is sustained) offers a potential avenue to mitigate IRI because of its central role in energy production, the generation of ROS and the initiation of apoptosis and necrosis. A group at UCLA, USA, has proposed a novel concept of regulated hepatic reperfusion to modulate IRI during the critical period of organ revascularization.[91] The strategy aims to nurture ischaemic hepatocytes by providing a substrate-enriched, leukocyte-depleted, oxygen-saturated perfusate in a pressure, temperature and flow-controlled milieu to enable compromised cells to withstand

reperfusion insult of warm blood during allograft revascularization (Figure 3). In a pig model, regulated hepatic reperfusion mitigated IRI, provided intraoperative postreperfusion haemodynamic stability, preserved hepatocellular function and improved survival after prolonged periods of warm ischaemia.[91] This novel strategy might have direct applicability to clinical hepatic surgery and transplantation of marginal grafts. In human liver surgery, regulated hepatic reperfusion can be administered after a prolonged hepatic warm ischaemia from portal trial occlusion (the Pringle manoeuvre). In liver transplantation, regulated hepatic reperfusion can be applied during organ procurement after cardiac death and/or before organ revascularization during liver implantation.

Liver IRI transplant clinical trials

Little progress has been made since the seminal study by a group of researchers from Munich, Germany, on the beneficial effects of human recombinant superoxide dismutase in mitigating endothelial cell damage mediated by free radicals and induced by ischaemia–reperfusion or to ameliorate acute and chronic rejection episodes in recipients of deceased donor renal transplants.[15] Targeting hepatic IRI at the bedside can occur in preconditioning or postconditioning transplant settings. Preconditioning applies to pharmacological interventions in the donor or in the graft before implantation or reperfusion, whereas postconditioning refers to the time of reperfusion in transplant recipients. Although an array of agents have shown protective effects against liver IRI in animal models, only a few have been tested in clinical randomized controlled trials (RCTs) in liver transplantation (Table 1).

In agreement with the ability of prostaglandins to exert cytoprotection against liver IRI in rodents,[103,104] systemic bolus of prostacyclin (also known as the drug epoprostenol) in the donor before the graft retrieval improved early sinusoidal perfusion and diminished the sharp increase in levels of hepatocellular enzymes as a result of local IRI after liver transplantation.[105] Confirming experimental findings on the key pathogenic function of adaptive–innate immunity crossregulation,[4] administration of immunosuppressive drugs that target T cells seems to be an attractive approach to suppress the inflammation response that results from brain death and ischaemia–reperfusion.[106,107] For instance, continuous treatment of brain dead donors with methylprednisolone resulted in a considerably reduced incidence of hepatic IRI and lowered the rate of acute rejection.[106] The rationale behind this study relates to the enhanced immune activation in brain dead donors, which in turn might trigger inflammation and damage mediated by ischaemia–reperfusion in liver transplants. Donor pretreatment with methylprednisolone not only reduced serum levels of IL-2, IL-6, CCL2, TNF and CXCL10 but also downregulated macrophage (CD68) local sequestration along with expression of major histocompatibility complex class II, tumour necrosis factor ligand superfamily member 6, TNF, CXCL10 and intercellular adhesion molecule 1 at the graft site.[107]

Following encouraging data from patients who received a renal transplant,[108,109] the effects of thymoglobulin (an anti-thymocyte globulin) on IRI were studied in deceased donor liver transplantation.[110] Indeed, treatment with thymoglobulin during the anhepatic phase (when the patient's liver has been removed and the donor liver has not been transplanted) and shortly after surgery enabled compromised livers to be transplanted, as this strategy improved the initial graft function and ameliorated the hepatocellular damage. This effect might have resulted from blockade of the adhesion cascade, decreased β2 integrin expression and depletion of activated CD4+ T cells, which are all known to be instrumental in the liver IRI immune cascade.[4] These findings imply that combined rather than individual donor and recipient immunosuppressive treatment is more efficacious against IRI in patients who receive a liver transplant.

Conclusion

One of the major problems facing organ transplantation is the widening disparity between the numbers of potential recipients who vie for the donor supply.[1] Although the number of liver transplants in the USA has increased 3.7-fold between 1988 and 2011, the number of patients on waiting lists has increased 15-fold, and many of them die while waiting for the life-saving organ.[2] The leading factor in the decreasing number of donor livers available for transplantation is IRI, that is, local tissue damage that occurs during organ harvesting and the subsequent peritransplant period. Cellular immune activation, cytoprotective functions and immune regulation are all critically involved in the pathogenesis of hepatic IRI. Despite their distinctive roles in different liver cell types, these pathways might function in series and in a synergistic or counteractive fashion. The intricate network of functional interactions among molecular targets can be amplified, highly regulated and in many cases flexible to be either cell or tissue-type specific. Therefore, dissecting innate–adaptive immune cross-regulation in clinically relevant animal models of liver IRI is warranted to provide the rationale for much needed novel agents to fill the current therapeutic gaps. However, a great deal of work is yet to be done to translate our current experience from bench to bedside in the continuing effort to improve donor organ quality, save lives, benefit patient outcomes and ultimately enhance the overall success of clinical liver transplantation.

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Key points

- The cellular damage incurred by organ procurement and preservation affects transplantation outcomes, contributes to donor organ shortage and represents a major risk factor for acute and chronic liver graft rejection

- Liver ischaemia–reperfusion injury (IRI) is a local proinflammatory response that is mediated by the innate immune system

- Several pattern recognition receptors, including Toll-like receptor (TLR)4, TLR9 and the inflammasome, are involved in liver immune activation against ischaemia–reperfusion in distinct cell types at different stages of IRI development

- Different subsets of T cells participate in innate immune responses triggered by ischaemia–reperfusion via positive and negative co-stimulatory pathways

- Studies of regulated hepatic reperfusion in large animals might lead to successful clinical use of liver grafts procured from extended criteria donors and donation after cardiac death

Review criteria

Relevant literature on the pathophysiology of liver ischaemia–reperfusion injury (IRI) in experimental settings and in liver transplant patients were identified by searching the PubMed database for articles published up to June 2012. The search terms used in combination with "liver IRI" were "transplantation", "inflammation", "T cells", "innate immunity", "Toll-like receptors" and "macrophages". All papers identified were English-language, full-text original research articles published in peer-reviewed journals.
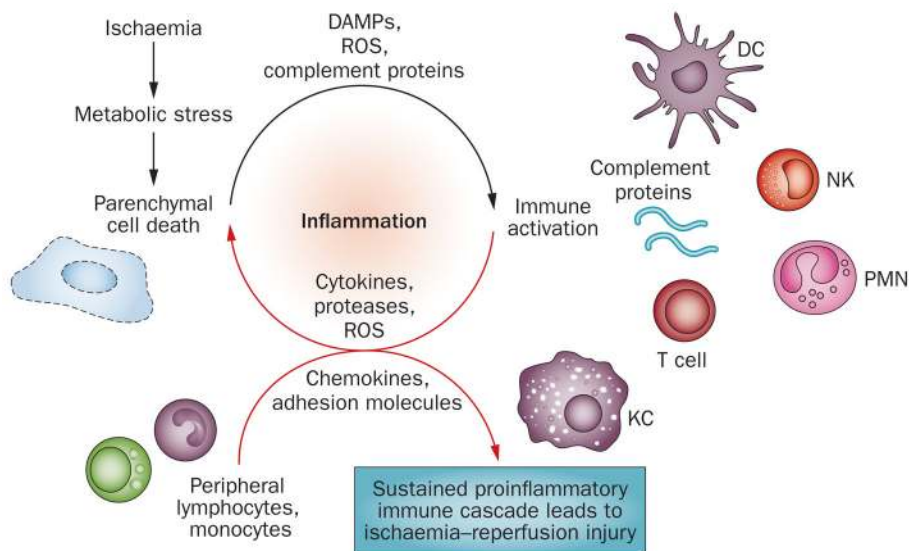
Figure 1.
The distinct stages of liver ischaemia–reperfusion injury. Ischaemic injury, a localized process of hepatic metabolic disturbances, results from glycogen consumption, lack of oxygen supply and ATP depletion. The cell-death-released DAMPs, activation of complement (a group of proteins that are involved in tissue injury and/or repair) induced by tissue injury and mitochondrial ROS production triggered by oxygenation all contribute to liver immune activation after reperfusion, which involves multiple liver nonparenchymal cell types, including Kupffer cells, dendritic cells, T cells, NK cells and neutrophils (PMNs). The ischaemia–reperfusion-activated proinflammatory immune cascade sustains itself by recruiting peripheral immune cells from the circulation, and is responsible for the ultimate liver reperfusion injury. Abbreviations: DAMPs, danger-associated molecular patterns; DC, dendritic cells; KC, Kupffer cells; NK, natural killer cell; PMN, polymorphonuclear cells; ROS, reactive oxygen species.
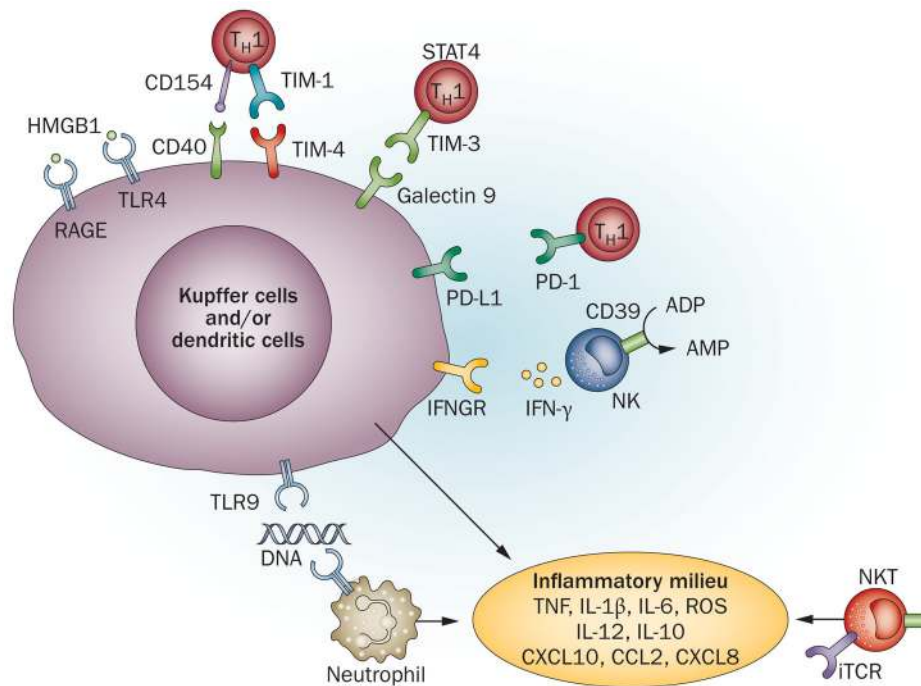
Figure 2.
A scheme of liver immune activation against ischaemia–reperfusion injury. The ischaemic insult induces initial cell death, which results in diverse 'danger' molecules, such as HMGB1, DNA fragments and histones activating TLR4, RAGE and TLR9 signalling on Kupffer cells and/or dendritic cells and neutrophils. T cells, particularly CD4$^+$ T_H1 effectors, might also facilitate and regulate local innate immune activation via CD154–CD40, TIM-1–TIM-3, TIM-4–galectin 9 and PD-L1 pathways. In addition, CD1d-mediated NKT and CD39-mediated NK cell activation contribute to hepatic immune activation against ischaemia–reperfusion. IFN-γ produced by activated NK cells promotes Kupffer cell and/or dendritic cell activation. The proinflammatory milieu, composed of TNF, IL-1β, IL-6, IL-12, CXCL10, CCL2, CXCL8 and ROS, further activates local immune cells and recruits circulating immune cells, culminating in inflammatory reperfusion injury. Abbreviations: HMGB1, high-mobility group protein B1; IFNGR, IFN-γ receptor; iTCR, invariant T cell receptor; NK, natural killer cell; NKT, natural killer T cell; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; PMN, polymorphonuclear cell; RAGE, receptor for advanced glycation end products; STAT4, signal of transducer and activator of transcription 4; T_H1, T-helper type 1 cell; TIM, T cell, immunoglobulin, mucin-containing molecules; TLR, Toll-like receptor.
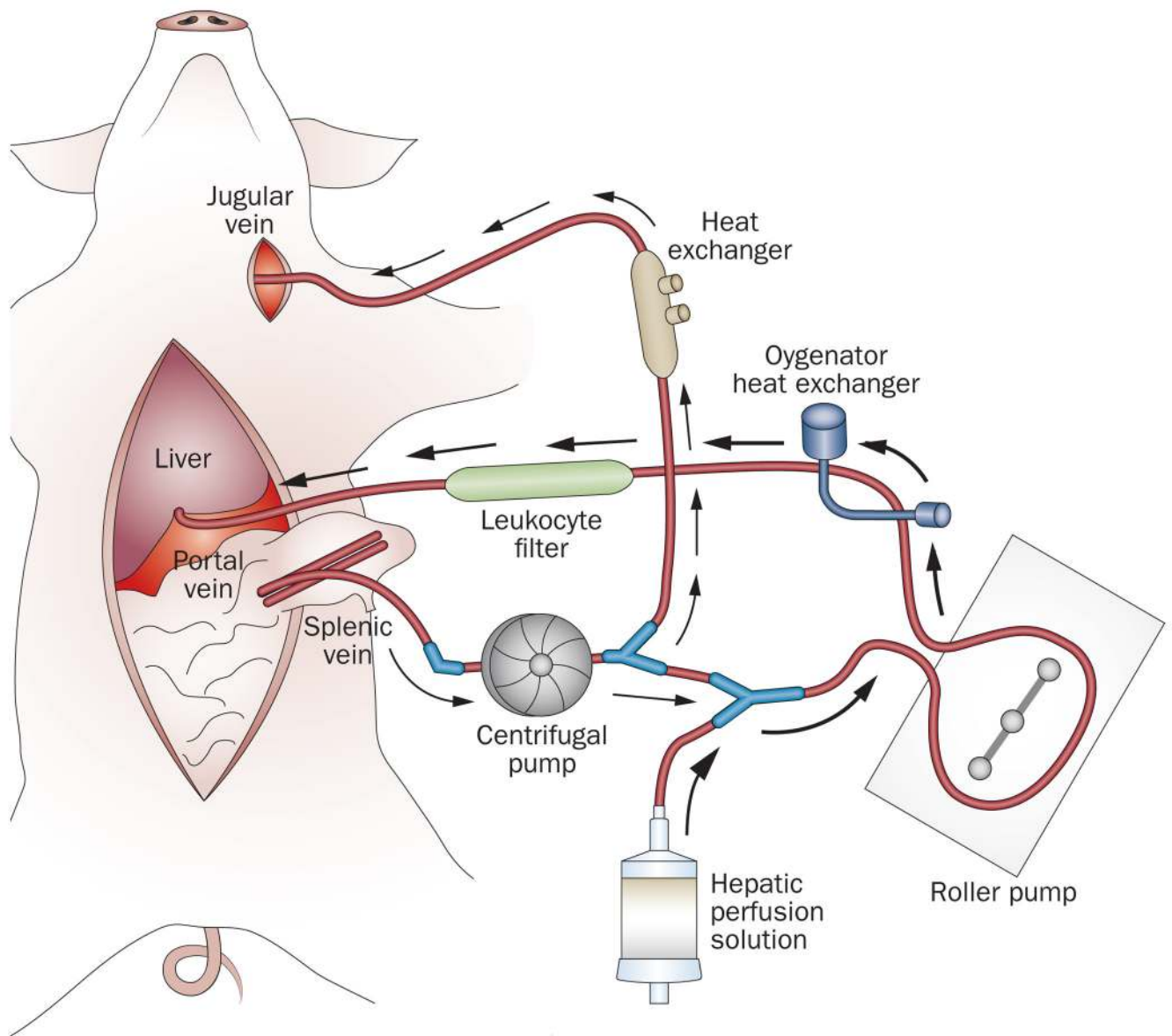
Figure 3.
Regulated hepatic reperfusion and splenojugular venovenous bypass circuits. An extracorporeal centrifugal pump recirculates the animal's splanchnic venous blood to the heart through a splenojugular venovenous bypass (the direction of the blood flow is indicated by white arrows) to avoid congestion of the splanchnic circulation during total portal occlusion. During regulated hepatic reperfusion, an amount of the animal's splanchnic venous blood is diverted through a Y-connector from the centrifugal pump and mixed with hepatic perfusion solution in a 4:1 dilution ratio (perfusate). Another extracorporeal roller pump recirculates the perfusate through a paediatric oxygenator heat exchanger and leukoreduction filter before perfusion of the liver through the portal vein (the direction of the perfusate flow is indicated by black arrows). The roller pump regulates the reperfusion pressure (8–12 mmHg) and the oxygenator heat exchanger maintains the perfusate oxygen saturation (to 100%) and temperature (30–32 °C). Permission obtained from Elsevier Ltd © Hong, J. C. *et al. Am. J. Coll. Surg.* **214**, 505–515 (2012).

Table 1

Randomized clinical trials on pharmacological strategies to minimize hepatic IRI in deceased donor liver transplantation

Study	Pharmacological intervention	Mechanism	Patients (placebo/study drug)	Findings for intervention group
Klein *et al.* (1999)[105]	Epoprostenol (iv bolus of 500 µg) before cross clamp	Improvement of sinusoidal perfusion	53/53	Decreased levels of AST and ALT after surgery
Bogetti *et al.* (2005)[110]	Thymoglobulin (1.5 mg/kg) during anhepatic phase and 2 doses after surgery	Suppression of inflammatory immune response	11/11	Decreased levels of AST and bilirubin after surgery, improved initial allograft function
Khan *et al.* (2005)[124]	NAC iv and portal flush of donor liver	Antioxidant hepatoprotection	9/9	No protective effects on liver IRI or on acute cellular rejection
HEGPOL trial[126]	HEGPOL (glycin) in multiple iv doses in liver transplant recipients	Decreased Kupffer cell activation	65/65	Trial completed but not yet published
Baskin-Bey *et al.* (2007)[111]	IDN-6556 in organ storage solution and recipient	Inhibition of pan-caspase (apoptosis)	23 (placebo)/23/27/26 *	Decreased apoptosis and decreased liver injury for the group with study drug in preservation and flush solution
Lang *et al.* (2007)[118]	Inhaled NO (80 ppm) during liver transplantation	Downregulation of endogenous NO production	10/10	Decreased levels of AST after surgery, decreased hepatocyte apoptosis, improved rate of liver function
Kotsch *et al.* (2008)[106]	Donor treatment with iv methylprednisolone before recovery	Suppression of inflammatory immune response	50/50	Decreased levels of AST, decreased serum levels of cytokine, improved levels of biomarkers after surgery and decreased incidence of

Study	Pharmacological intervention	Mechanism	Patients (placebo/study drug)	Findings for intervention group
				acute rejection
Hilmi *et al.* (2010)[125]	NAC (12 doses) in liver transplant patients	Antioxidant/GSH-mediated hepatoprotection	50/50	No effects on liver/renal injury, no increase in GSH levels in some patients (possibly because of inadequate dose/ duration of NAC)
Kristo *et al.* (2011)[123]	Intraoperative intraportal organ perfusion with tacrolimus	Suppression of inflammatory immune response	13/13	No effects on early graft function despite decreased immune response and inflammation on a genome-wide basis
Busuttil *et al.* (2011)[113]	Preimplantation allograft and recipient treatment with rPSGL-Ig	Blockade of leukocyte adhesion cascade	24/23	Decreased levels of AST after surgery in recipients with high DRI, and improved biomarkers (IL-10, CXCL10)

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 The first group received placebo through the process, the second group received IDN-6556 during cold storage and flush and placebo was given to the recipient, the third group received a solution of 5 μg/ml IDN-6556 during cold storage and flush and then 0.5 mg/kg of IDN-6556 every 6 h for 24 h after the surgery, the fourth group received a solution of 15 μg/ml IDN-6556 during cold storage and flush and then 0.5 mg/kg of IDN-6556 every 6 h for 48 h after the surgery.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DRI, donor risk index; GSH, glutathione; IP-10, inducible protein 10; IRI, ischaemia–reperfusion injury; iv, intravenous; NAC, *N*-acetyl cysteine; NO, nitric oxide; rPSGL-Ig, recombinant P-selectin glycoprotein ligand IgG.