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Cell Therapy for Advanced Liver Diseases: Repair or Rebuild

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Relevant disclosures: S.J.F. has patents pending, entitled 'Macrophage-based therapy' in national territories of USA, Europe, Japan, China and Australia. These patents have been derived from PCT/GB2017/052769 filed 18/09/2017 and claim priority from UK application 1615923.8 filed 19/09/2016. Both of the original patents have now been abandoned because the original UK patent and PCT patent are no longer live and have now been replaced by the national patents. B.J.D. and S.J.F have patents pending regarding macrophage cell therapy, UK applications 2007903.4, 2007906.7 and 2007905.9, filed on 27/05/2020. S.J.F. is supported by funds from Wellcome Trust, Medical Research Council, UKRMP and Syncona Ltd.

Keywords: Cell therapy, Stem cells, Liver Regeneration, Acute Liver Failure, Liver Cirrhosis, Metabolic Liver Disease

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Key points:

1. Cell therapy can be used to add cells to the liver, or to remodel and repair the damaged liver.
2. Hepatocyte therapy is a paradigm for adding cells to the liver that has been translated to the clinic.
3. Cell therapies that attempt to remodel and repair the liver and tested in humans include mesenchymal stromal cell and macrophage cell therapy.
4. Where the liver is damaged, then techniques to protect the donor cells will be required, such as cell encapsulation.
5. The gap from animal models to clinical testing of cell therapies need to be lessened. Ways to do this may include the use of clinically realistic animal models and the development of predictive mathematical models.

Abstract (121 words):

Advanced liver disease presents a significant worldwide health and economic burden and accounts for 3.5% of global mortality. When liver disease progresses to organ failure the only effective treatment is liver transplantation which requires lifelong immune suppression and brings associated risks. Furthermore, the shortage in suitable donor organs means patients may die waiting for a suitable transplant organ. Cell therapies have made their way from animal studies to a small number of early clinical trials. Here we discuss the current state of cell therapies for liver disease and the mechanism underpinning their actions to repair liver tissue or rebuild functional parenchyma, cellular therapies that are on the clinical horizon and challenges to be overcome before routine clinical use is a possibility.

Main text:

Introduction

Liver disease represents a significant health and economic burden worldwide with liver cirrhosis and cancer the 11th and 16th leading causes of death, respectively [1]. The only curative therapy for end stage liver disease is orthotopic liver transplantation (OLT). Suitable available donor organs fall short of clinical need. In the United States, 11,844 adult and 700 paediatric patients were added to the liver transplant list, versus 8250 adult and 563 paediatric transplants performed in 2018 [2]. In Europe, approximately 7300 liver transplants are performed annually and almost half of patients wait more than three months for transplant [3]. Efforts to overcome this donor shortage have led to surgical grafting techniques such as heterotopic or partial orthotopic auxiliary transplantation to provide functional support enabling host liver regeneration [4]. Split liver transplantation, where two grafts are generated from a single donor, can service both an adult and paediatric recipient [5]. This is advantageous in paediatric cases, where the grafted liver can grow with the recipient. In 2018, 19.2% of paediatric liver transplants in the US were split liver grafts [2]. Although current clinical therapies use whole organs or segments to achieve this benefit, therapy at the level of cell transplantation is emerging and may further overcome shortages of organs and reduce the need for invasive surgical procedures.

There are several theoretical advantages to cell therapy compared to traditional OLT or adjunct grafting. Efficacious hepatocyte transplantation involves reconstitution of as little as 1% of functional tissue for metabolic diseases such as glycogen storage disease 1a [6], 2.5% for acute-on-chronic liver failure [7], and potentially greater requirements for bridging time to OLT/regeneration in acute liver failure, raising the possibility of using one donor organ for multiple recipients, particularly where cells can be cryopreserved. Cells may be delivered endovascularly which is less invasive than OLT and also preserves the native liver, which, in the context of metabolic disorders will continue to perform the majority of hepatic functions even if graft failure occurs. Additionally, cell therapy approaches to repair the injury niche may allow host parenchymal regeneration without the need for organ transplant. In this review, we summarise the history and state-of-the-art liver cell therapies.

Hepatocyte transplant (HT) for liver diseases

Inborn errors of metabolism (IEM)

HT is theoretically promising for treating IEMs, representing a form of 'cellular gene-therapy', whereby transplanted hepatocytes contain functional versions of dysfunctional disease-causing mutations present in host hepatocytes. The expansion capacity of serially transplanted hepatocytes in fumarylacetoacetate hydrolase-deficient ($FAH^{-/-}$) mice, which models hereditary tyrosinemia, type-1, demonstrated that adult hepatocytes possess an extraordinary in vivo expansion capacity rivalling that of hematopoietic stem cells [8]. HT for IEM has demonstrated encouraging short-term clinical results, partially correcting an array of disorders and delaying OLT [9]. The first sustained effect of HT for an IEM was the partial correction of hyperbilirubinemia, up to 11 months post-HT, in a 10 year-old Crigler-Najjar (CN) patient who was infused with $7.5x10^9$ hepatocytes via the portal vein [10]. There are examples which demonstrate the utility of HT in overcoming donor shortage by treating numerous patients with the same donor, or transplantation of marginal tissue. High quality hepatocytes can be isolated and cryopreserved from cadaveric neonatal livers, with greater post-thawing recovery than adult hepatocytes [11]. Cryopreserved hepatocytes from a 9-day old neonate used to treat children with carbamoyl phosphate synthase 1 deficiency (CPSD; n=1), citrullinemia (n=1) or OTC deficiency (n=2), demonstrated clinical and metabolic stabilisation during initial follow-up for all patients, with one patient with severe OTC subsequently dying from a fatal metabolic decompensation [12]. Similarly, OTC deficiency was corrected using cryopreserved hepatocytes from remnant liver tissue from a hyper-reduced left lateral segment from a living donor transplantation. The patient was transplanted at 11 days ($7.4x10^7$ cells) and 14 days of age ($6.6x10^7$ cells) via the umbilical vein, was discharged 56 days post-HT and healthy at 3 month follow-up, albeit with continued protein restriction, medication for OTC deficiency and immunosuppression [13]. Domino transplant allows the use of tissue from a patient with a metabolic disease that would otherwise be discarded to treat a patient with a different metabolic disease, since only a fraction of functional tissue is required to fulfil function. This approach has demonstrated clinical improvement for a number of disorders including Phenylketonuria [14, 15], CN syndrome, propionic acidaemia and CPSD [16].

In its current form, cell therapy for IEMs is a useful bridge to more permanent therapy. Further work is required to bridge the gap between efficacious pre-clinical HT and sustained clinical success. Theoretical considerations concerning graft function and expansion are outlined herein.

Transplanted hepatocytes require a competitive proliferative advantage over host cells and appropriate niche signals to expand and contribute significantly to liver repopulation [17] (Figure 1). For IEMs, this can be observed in animal models of Tyrosinemia type 1 [8] , Wilson's disease (Long-Evans cinnamon rat; [18, 19]) and Alpha 1 Antitrypsin deficiency, (PiZ mice; [20]). These disorders affect liver function and architecture and large-scale repopulation by transplanted hepatocytes is observed. For disorders such as Crigler-Najjar syndrome (Gunn rat; [21]), hypercholesterolemia (Apolipoprotein E knockout mouse; [22]) and urea cycle disorders such as OTC deficiency (Spf-ash mouse [23]), negative effects of these diseases are largely observed extrahepatically, and large-scale repopulation and complete functional correction is more difficult into intact parenchyma. Another theoretical consideration is in order for functional repopulation to take place, withdrawal from effective therapy (e.g. 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione for Tyrosinemia type 1) may be required to give transplanted cells a competitive advantage over host hepatocytes, risking further disease complications.

Acute Liver failure

Acute liver failure (ALF) is a rare condition affecting patients without pre-existing liver disease, with significant morbidity and mortality [24]. ALF aetiology demonstrates geographical variation, with acetaminophen (APAP) overdose the most common cause of ALF in the UK, USA and parts of Europe [24], and viral hepatitis most common within Eastern Asia. A significant proportion of ALF cases are idiopathic; so called non A-E, seronegative or indeterminate hepatitis [24]. HT for ALF has met some clinical success, bridging patients to OLT and, rarely, complete recovery. Some barriers exist for HT to be reliably curative. Transplanted cells encounter a hostile niche for engraftment and expansion with abundant cellular necrosis and apoptosis. Activated macrophages phagocytose cellular debris and initiate compensatory regeneration, but also secrete TGFβ, which induces hepatocyte replicative senescence, in turn, transmitting senescence to transplanted cells, presenting a barrier to host/donor-mediated regeneration [25] (Figure 1). Pre-clinically, strategies to inhibit paracrine senescence are being developed to overcome this [25, 26]. Another strategy to circumvent the microenvironment is extrahepatic HT. Sites examined include the peritoneum [27] and lymph nodes [28] of mice which enable graft vascularisation and functional support of injured liver.

Alginate microencapsulation of hepatocytes in microbeads protects cells from immune destruction and permits molecular exchange (Figure 1). The safety of this approach was tested in 8 paediatric ALF patients. Microbeads were infused intraperitoneally, avoiding the hepatic microenvironment, without immunosuppression. 4 patients recovered, avoiding OLT, 3 were bridged to OLT and 1 patient died [29]. There are a number of clinical examples of HT for ALF. Relevant cases of acute variants of liver failure treated with HT are shown by indication (Table 1).

HT to treat various drug-induced ALFs have been reported including halothane [30], dilantin [31] and multiple polysubstance misuses [31, 32] with improvements in encephalopathy and ammonia concentration. Splenic vein HT successfully bridged two adults to OLT at 2 and 10 days post-procedure, respectively [31], and a 35 year old adult completely recovered with an isolated intra-portal HT of $3.7x10^9$ cells. In a single 23-year old patient with cryptogenic acute liver failure, two splenic infusions of donor hepatocytes (2.86 and 1.52 x 10^9 cells) bridged the patient to OLT (day 5). However, the patient succumbed to overwhelming sepsis and multi-organ failure at day 13 [33]. Regarding studies of individuals with acute viral pathology and multi-organ dysfunction treated with HT, 2 patients have been successfully bridged to OLT [30, 34], while a single individual achieved spontaneous recovery following infusion. The majority of these cases were related to acute hepatitis B virus (HBV), with 2 incident cases of acute Herpes Simplex II Virus [32, 35]. A variety of delivery routes were used, including intra-portal, intrasplenic or intraperitoneal administration. Within this group there was an episode of non-lethal splenic vein thrombosis [33] and lethal mesenteric vein thrombosis [35] highlighting the risks of such approaches.

Acute on Chronic Liver Failure (ACLF)

Acute-on-chronic liver failure (ACLF) is considered as an acute deterioration of liver function in patients with cirrhosis resulting in failure of one or more organs and high short-term mortality [36], posing unique clinical challenges [37]. Wang and colleagues trialled intrasplenic HT for ACLF, with a 5 year follow-up interval [7]. Following transplantation of $4.2–6.0x10^{10}$ hepatocytes, 3 patients demonstrated recovery from liver failure, 1 survived however, subsequently required OLT, while the remaining 3 patients died 2.5 to 12 months post-HT.

Conclusion

Overall, the literature predominantly comprises individual or small case series with a heterogeneous populous and intrinsic variation in both transplanted cell number and delivery method, making efficacy hard to determine. Future controlled trials with standardised methodology and predetermined and validated endpoints are required to support widespread use of HT. Reassuringly, there appears to be demonstrable longevity in this approach given that functional hepatocytes were identified at the 48-month interval scan and complete recovery at 5 years for almost half of patients [7]. Such therapies hold promise as an adjunct to standard of care or bridging to OLT. Efforts to improve engraftment and expansion of hepatocytes into intact parenchyma are vital in achieving significant therapeutic efficiency (Figure 1).

Overcoming the challenges of cell therapy for liver disease

Optimising routes of delivery

Whilst intra-splenic injection is commonly used to access the portal circulation in animal models, the portal vein is typically accessed via three routes in humans; ultrasound guided transcutaneous puncture of an intrahepatic portal vein tributary, intrahepatic transcutaneous splenic vein tributary puncture or via the hepatic venous system with a transjugular intrahepatic portosystemic shunt. These procedures incur bleeding risk [38-40], compounded by impaired primary haemostasis which can accompany liver failure. Portal vein hypertension frequently accompanies chronic liver disease (CLD), which may progress to the development of portosystemic shunting through varices, reversal of portal vein flow or portal vein thrombosis, impeding delivery of cells to the liver via this route. The hepatic artery offers an alternate route for cell delivery and is accessed endovascularly, from either common femoral arteries, via the coeliac trunk. Unlike the portal system, retrograde flow and thrombosis do not occur in CLD and the hepatic artery therefore offers an alternate route for cell delivery in cases of portal hypertension. There remains an associated risk of vessel injury which, for the hepatic artery, is dangerous in the context of concurrent portal vein thrombosis. Despite this, hepatic artery access is commonly performed during trans-arterial chemoembolization and selective internal radiation therapy and has been used as a route for cell therapy administration. There is evidence that it may be a more efficient way of delivering cells to the hepatic sinusoid in humans [41]. Furthermore, the biliary tree is supplied exclusively by the hepatic artery. Therefore, in biliary

injury conditions, the hepatic artery offers a more direct delivery route to the portal venous system [42].

Improving engraftment and homing

A significant hurdle to successful cell therapy is the ability of donor cells to access the liver, and survive long enough to exert a therapeutic effect. Freshly isolated cells are not readily available, particularly for acute injury where a more “off the shelf” approach is required. Hepatocytes are susceptible to cryopreservation and thawing, with negative effects on survival, engraftment and function, downregulating important adhesion proteins including integrin-β1 and E-cadherin [43]. Improvements in parameters such as in vitro apoptosis, in vivo survival, and engraftment by four-fold in APAP-injured mice, was achieved by optimising cryopreservation medium [44]. Thawed hepatocytes display features consistent with early apoptosis. Caspase inhibitors mitigate stress response pathway activation, restoring cell attachment ability and metabolic function [45]. The use of apoptosis inhibitors is also effective in improving thawed hepatocyte viability and function of alginate-encapsulated and cryopreserved hepatocyte microbeads [46].

The microenvironment in which cells are transplanted is a critical consideration in the success of cell therapy. Cells enter the liver via sinusoids where they integrate with the parenchyma [47-49]. Specific liver insults, such as portal vein embolization and partial hepatectomy can pre-condition remaining host hepatocytes to proliferate, positively influencing donor hepatocyte engraftment and proliferation [50, 51]. Irradiation-induced apoptosis of sinusoidal endothelial cells allows greater engraftment and integration of donor hepatocytes into hepatic cords [52]. Irradiating individual liver lobes to give donor hepatocytes a competitive advantage has been performed in primates and a patient with phenylketonuria (PKU). Biopsy of the PKU patient 6 months post-transplant confirmed homing of donor hepatocytes to irradiated areas and Ki67-positive proliferating donor hepatocytes [15]. Other methods to disrupt the sinusoidal endothelial barrier to cellular engraftment include using vasodilating chemicals or agents inducing endothelial injury such as cyclophosphamide, which increases hepatocyte engraftment in rodents [53, 54].

Influencing the host environment with co-transplantation of cells or factors may also be an effective strategy. HT induces pro-inflammatory cytokine production by neutrophils and macrophages, reducing hepatocyte engraftment, which can be improved by depleting innate immune cells [55]. An approach to modulate this response that could benefit HT is to co-perfuse mesenchymal stromal cells (MSCs) to utilise their paracrine immunomodulatory potential, as has been demonstrated with islet transplantation. Long-term improvements were made in the engraftment of allogenic pancreatic islet cells into the liver when co-transplanted with GMP-compatible umbilical cord perivascular MSCs, enabling delayed rejection of islet grafts [56]. The immunomodulatory function of MSCs alters macrophage phenotype and enhances the pro-regenerative effect of macrophage therapy (discussed later) in pre-clinical murine fibrosis [57], and co-transplantation of MSCs improves foetal hepatocyte engraftment in retrorsine-injured mouse livers [58]. Hydrogels have been used to coat cells in substances containing growth factors and matrix proteins to maintain the cells in a pro-regenerative state. A number of materials have been developed including synthetic polyvinyl alcohol [59], natural decellularised ECM [60], and coating human EpCAM+ HPCs with hyaluronan increases liver engraftment efficiency over uncoated cells by 3.75-fold when delivered via intrasplenic injection [61](Figure 1).

Alternative sources of hepatocytes

Foetal liver progenitor cells

The foetal liver contains EpCAM+/NCAM+ progenitor cells capable of *in vitro* expansion and *in vivo* differentiation into hepatocytes [62, 63] (Figure 2). Epithelial cells isolated from foetal human livers have been transplanted into injured murine livers, promoting fibrosis resolution and functionally regenerating parenchyma [64, 65]. Comparisons between the matched repopulation capacity of foetal and adult hepatocytes in immune deficient liver repopulation mouse models have demonstrated superior repopulation capacity of adult over foetal hepatocytes [66, 67], which may be a result of the adult liver providing inadequate signals to retain or promote proliferation of immature cell types. Liver engraftment is significantly improved in mice by combining direct intrahepatic injection of cells within a hyaluronan matrix compared to injecting cells in suspension which lowers engraftment efficiency, with cells migrating to ectopic sites [68]. Small case series exist of human foetal liver cell transplants into patients with CLD [41, 69], with reported improvements in biochemistry and clinical findings.

In a retrospectively case-controlled study of intrasplenic administration of foetal hepatocytes to nine patients with CLD, model for end-stage liver disease (MELD) score was stabilised [70]. Further, adequately controlled, and powered studies are required to establish efficacy, and problems of inefficient engraftment and expansion must be overcome. Furthermore, given ethical controversies of sourcing foetal tissues have limited clinical adoption of foetal liver tissue transplantation.

Harnessing innate liver plasticity for cell therapy

Lineage tracing models of mouse liver injury have established the bi-directional plasticity of liver epithelial cells in rodents [71, 72]. When hepatocyte-mediated regeneration is impaired, ductular reactions containing hepatic progenitor cells (HPCs) develop, reconstituting a proportion of hepatocytes (10-15%) [72, 73]. Mouse HPCs can be isolated, expanded and differentiate into hepatocytes when transplanted into pre-clinical models of liver disease. Lgr5+ HPCs are stimulated to proliferate following liver injury, can be isolated and grown as organoids with high clonogenicity. Intrasplenic administration of 0.5-0.8 $x10^6$ HPCs cultured in hepatocyte differentiation medium gave rise to functional parenchymal nodules, up to 1% of the liver, when transplanted into $FAH^{-/-}$ recipients but were unable to provide functional rescue [74]. Comparable biliary-derived HPCs can be harvested and expanded from human liver, and when transplanted intrasplenically as undifferentiated HPCs in immune-deficient CCl_4/retrorsine-injured mice (1-2 x 10^6 per mouse), generate small numbers of albumin-producing cells in liver [75]. HPCs can be purified with antibodies against suppression of tumorigenicity 14 (ST14) [76], or antibody-based enrichment of non-hematopoietic $EpCAM^+/CD24^+/CD133^+$ clonally-derived progenitors [77]. Clonally-derived progenitors can be expanded in vitro, and transplanted intrasplenically into MCD-injured mice with induced hepatocyte senescence (imparting selective advantage for transplanted progenitors; AhCre-$MDM2^{flox/flox}$), reconstituting ~15% of liver parenchyma from $5x10^6$ starting cells [77]. Studies re-capitulating in vivo hepatocytes de-differentiation to HPC-like cells during chronic liver injury could generate expandable hepatocytes. Using chemical inhibitors of Rho-kinase, TGFβ receptor and glycogen synthase kinase 3, rat hepatocytes are converted into HPC-like cells (chemically induced liver progenitors) that can expand and re-differentiate into functional hepatocyte-like cells, contributing to 72-89% of parenchyma regeneration in urokinase-type plasminogen activator-severe combined immunodeficient mice [78].

Despite encouraging pre-clinical data, several milestones must be addressed to meet functional and regulatory requirements for clinical translation including development of good manufacturing practice (GMP)-compliant conditions for culture and cryopreservation, and proving safety of using stem/progenitor cells for cell therapy. Current methods for organoid growth use clinically incompatible animal tumour-derived matrices [75]. Efforts to enable clinical translation of liver and other epithelial organoids have explored replacement of undefined culture components with GMP-compatible techniques such as using defined ECM proteins [79], development of synthetic hydrogels to replace tumour-derived matrices [80], and bioengineering scaffolds to recreate organotypic niche interactions [81]. De-cellularised liver ECM provides support for complex in vitro liver cell development of foetal liver cells [82], and proteomic assessment of intestinal ECM gels display an overlapping profile with other endodermal organs, including liver, and support human adult and foetal organoid expansion, comparable to non-defined matrices [83].

Long-term pre-clinical safety studies are required to ascertain a clinically safe differentiation state of HPCs with no significant tumorigenicity risk. Of particular concern is that biliary cells with constitutively active Wnt and in vivo xenograft tumour-forming ability can be enriched from cirrhotic patients using the biliary lineage marker EpCAM [84]. EpCAM-enriched cells from donors with uninjured livers do not appear to contain a tumour-forming component [84], and organoid lines from normal human livers are genetically stable over long-term culture [75]. These studies provide key proof-of-principle that in vitro expansion and transplantation of HPCs, rather than in vitro matured progeny, may be a viable strategy for clinical translation with limited tumorigenic risk in the future (Figure 2). However, the poor engraftment and expansion of immature cells needs to be overcome progenitor approaches to be clinically viable.

Pluripotent stem cells

Protocols to differentiate hepatocyte-like cells from pluripotent sources use factors to recapitulate differentiation via relevant in vivo cell types observed embryonic development, including Activin A and Wnt3a to induce hepatic endoderm formation [85, 86], bone morphogenetic protein and fibroblast growth factor to promote hepatocyte differentiation, and maturation factors hepatocyte growth factor and oncostatin-M [87]. Protocols have been adapted to produce clinical-grade hepatocyte-like cells from ES and iPSC cells that are capable of liver engraftment and function in pre-clinical mouse models [88, 89]. However, a shortfall exists in current protocols in the ability to produce fully differentiated hepatocytes from pluripotent cells *in vitro* and established methods produce cells that are akin to foetal hepatocytes [90], and remains a barrier to clinical utility. This has lead to further investigation of different ECM substrates, small molecules, 3D culture systems and assessment of cell of origin for derivation of iPSCs to bridge this gap.

Culture of hESC-derived hepatocyte-like cells on laminins -521 and -111 suppresses gene expression associated with inhibition of terminal differentiation, enhances CYP1A2 and 3A function and suppressed unwanted fibroblastic and colonic differentiation compared to Matrigel [91]. Culture of human pluripotent cell-derived hepatocyte-like cells in spheroids under defined culture conditions enables a stable hepatocyte-like phenotype, albeit immature compared to primary hepatocytes, for up to one year in vitro, and cells and mitigate the progression of liver injury, but do not rescue function, in FAH-deficient mice [92]. Comparison between isogenic hepatocyte and fibroblast-derived iPSCs determined that the tissue of origin in deriving iPSCs for hepatocyte derivation does not affect functional capacity of hepatocyte progeny [93], and it is donor-dependent genetic variation that affects hepatocyte-like differentiation capacity of iPSCs [94]. Development of hypoimmunogenic pluripotent cells may overcome immune rejection issues with allogenic transplants [95]. Editing HLA class I and class II molecules [96-99], combined with overexpression of immunoregulatory factors to evade immune recognition facilitates development of ‘universal donor’ stem cells [100]. The establishment of hypoimmunogenic universal donor iPSCs, that have been developed on a donor genetic background for optimal differentiation to hepatocytes, or non-epithelial cells for niche modulation, would greatly accelerate clinical translation. A limitation to translation is the immaturity of progenitor/stem cell derived hepatocyte-like cells which preclude significant engraftment and functional rescue. Further studies recapitulating organotypic conditions will aid the development of fully functional cells for transplant, and may also benefit protocols for

hepatocyte culture and HPC differentiation, to bridge the functional gap between transplanting progenitors versus mature hepatocytes, enabling clinical translation (Figure 2).

Remodelling the injury niche with cell therapy

An alternative approach to replacing hepatocytes is promoting liver repair by modulating the liver microenvironment and/or systemic immune responses. The hepatic mononuclear phagocytic system and gut co-operate to prevent major immune activation from immunogenic molecules (e.g. bacterial endotoxin), via pattern recognition receptors and prevent intestinal bacterial translocation via the portal vein to the liver [101]. These cells also mediate the immune response to liver cell death via damage-associated molecular pattern-mediated cytokine/chemokine production and phagocytosis of cellular debris [102]. Macrophages play a critical role in this gut-liver axis, representing a second-line defence in sensing invading bacteria or their pathogenic toxins and antigens [103], forming a barrier to peritoneal bacterial infection and sensing bacterial load [104]. Macrophage phagocytic function is diminished in severe injury [105], compromising their barrier function and leading to infection and innate immune dysfunction, further heightening infection risk [106]. With compromised immunity, bacterial and/or fungal infection is a common occurrence in patients with ALF [107-110], cirrhosis [111] and ACLF [112, 113], driving dysregulated proinflammatory innate immune cell activation, cytokine overactivation and eventually multiorgan failure [37, 114-116].

Cell therapies may have a number of positive functions within the injured liver promoting: 1) debris removal, 2) scar resolution, 3) epithelial regeneration and 4) host immune cell recruitment. Wider functions may be: 5) restoring innate immunity and 6) limiting systemic inflammation. Although not applicable to IEMs requiring hepatocyte replacement, ALF, ACLF and cirrhosis are particularly amenable to remodelling strategies to reverse the effects of injury where uncontrolled injury and inflammation contribute significantly to morbidity and mortality. The bone marrow, and its progeny, contains several candidate cell-types for this application, including hematopoietic progenitor cells, mesenchymal stromal cells (MSCs) and macrophages. The uses of these cell types for treating acute (Figure 3) and chronic liver injures (Figure 4) are discussed herein.

Hematopoietic progenitor cells

Hematopoietic progenitor cells can differentiate into all blood lineages and can be induced to proliferate with granulocyte colony stimulating factor (G-CSF) and enriched using CD34 or CD133 antibodies [117, 118]. Bone marrow cell (BMC) contribution to parenchymal replenishment in liver injury is minor, representing mostly cell fusion events [119-121]. Injections of whole BM worsens fibrosis in experimental mouse models [122] which may reflect component pro-fibrotic elements such as fibrocytes and MSCs [123, 124]. BM also contains matrix metalloprotease (MMPs)-producing macrophages that can be anti-fibrotic [125]. This complexity is reflected in conflicting pre-clinical and clinical observations of manipulating hematopoietic progenitors in liver disease. G-CSF improves regeneration in murine acute and chronic liver injury [126], and repeat peripheral administration of c-kit+/sca1+/lin- purified BM cells in liver fibrosis reduces collagen deposition in mice [127]. Interestingly, hematopoietic progenitor cell administration resulted in recruitment of host neutrophils and monocyte-derived macrophages to fibrotic areas. Committed lymphoid progenitors exerted similar anti-fibrotic effects to hematopoietic progenitors via recruitment of host innate immune cells, suggesting that, rather than exerting a direct anti-fibrotic effect via differentiation to macrophages, hematopoietic progenitors could modulate host innate immunity to promote injury resolution [127] (Figure 4).

Uncontrolled and small cohort clinical studies indicate potential benefit in improving liver function [128]. Larger, randomised controlled studies show mixed benefits of HSC therapy. A randomised controlled trial of G-CSF injection, followed by purification and infusion autologous hematopoietic stem cells via the portal vein (0.5×10^8 HSCs; n=90) in HCV-associated end-stage liver disease patients showed stabilisation of liver biochemistry, improved Child-Pugh score in 48 cell-treated patients and increased survival at 6 month follow-up (81/90 alive) compared to placebo water-injected controls (24/50 alive) [129]. However, multicentre, open label phase two trial assessed subcutaneous G-CSF (n=26), or G-CSF with three doses of peripherally infused autologous CD133+ cells (0.2×10^6/kg; n=28), compared to standard care (n=27) in compensated cirrhosis observed no clinical benefit, with worsening of serious adverse events in treatment arm patients [130]. These studies differed in aetiology, with the latter representing a mixed cirrhotic cohort compared to only HCV-associated disease, peripheral versus portal vein infusion, and differing G-CSF/cell dosing regimens.

Mesenchymal stromal cells

MSCs are a multipotent fibroblast-like cell that are characterised by their expression of cell surface antigens CD73, CD90 and CD105, with a lack of expression of CD45, CD34, CD14,Cd11b, CD19, CD79α and HLA-DR, are adherent to plastic and are capable of differentiating into osteoclasts, chondrocytes and adipocytes [131]. MSCs were originally identified in bone marrow [132], but can be sourced from umbilical and adipose tissue and represent an ideal cell for clinical development as they are easy to isolate, expand and cryopreserve [133]. MSCs exert immunomodulatory effects on T cells, B cells and macrophages, and anti-fibrotic effects both via immunomodulation and directly inhibiting hepatic stellate cell proliferation and ECM synthesis [134, 135]. Although some peripherally-injected MSCs do reach the liver, cell tracking experiments in humans and rodents have shown a significant proportion of cells accumulate in lung and spleen [57, 136, 137], and are detectable at lower levels 10 days post-infusion in human cirrhotic patients [136]. Even so, transient effects on the immune environment and fibrotic niche may be beneficial in liver disease. The use of MSCs in ALF models attenuates injury via anti-inflammatory IL-10 production, inhibiting hepatocyte death [138] (Figure 3), and in chronic liver injury modulates macrophage phenotype, increasing the pool of alternatively activated macrophages expressing MMPs to remodel collagen deposits [139] (Figure 4).

Clinical trials utilising MSCs have been completed for a variety of liver indications with mixed results. A randomised placebo-controlled trial of peripherally infused autologous MSCs with decompensated cirrhosis showed no beneficial effect on biochemical parameters of liver cirrhosis, with 3 out of 15 patients in the cell treatment group dying in the first 5 months of the study compared to none of the control patients (n=12) [140]. Where some efficacy is observed, MSCs effects are generally transient. In post-HCV end stage liver disease patients, MSC-treated patients showed stabilisation of biochemical measures of liver function compared to worsening in control patients, but no significant difference in markers of collagen remodelling in a randomised, controlled trial of peripherally infused BM-MSCs (n=20 per group) [141]. A randomised, controlled study of cirrhotic patients assessed one or two cycles of portal vein infusion of HSCs followed by peripheral infusion of $1x10^6$/kg differentiated BM-MSCs 8 days-post HSC infusion (n=30 per group), showing an average MELD reduction of 3.27 in doubly

infused patients compared with 0.88 reduction in controls. Improvements in Child-Pugh grade and liver biochemistry were also observed with MSC therapy. Importantly, HCC incidence was not altered with MSC therapy [142]. Likewise, a study assessing autologous BM-MSC therapy via the hepatic artery in 53 chronic HBV liver failure patients versus 105 injury-matched controls showed improvements in MELD, bilirubin, albumin and prothrombin time in both treated and control groups, with significant improvements in MELD in treatment versus control groups and no change in HCC incidence or survival at 192 weeks [143]. A phase 2 randomized open-label study in 72 patients with alcoholic cirrhosis assessed one or two infusions of 50 million BM-MSCs via the hepatic artery and found 25% and 37% reduction in collagen area in liver biopsies from pre- and 6 months post-single or double BM-MSC-infused patients, respectively, with improvements in Child-Pugh score, but not MELD, with cell treatment, and no difference in adverse events between groups [144]. An open label, non-blinded randomised control trial of 56 patients with repeat infusions of allogenic BM-MSCs, improved survival (73.2% versus 55.6%, p=0.03) and reduced the incidence of severe infections in hepatitis B-associated ACLF. Cell infusion was associated with fever in these patients [145], but despite this side effect this data suggests the immune modulatory function of MSCs could potentially be beneficial in ACLF.

MSCs can be partially differentiated into hepatocyte-like cells with some in vitro function, including CYP3A4 activity and Urea production similar to fresh hepatocytes. But, these MSC-derived cells retain CD90 expression, express less albumin and do not upregulate HNF4α or CYP2B6 [146], providing evidence of a lack of complete transdifferentiation (Figure 2). Pre-clinically, they can support some metabolic functions and liver repopulation, but still retain mesenchymal marker expression [147] and have been assessed in a phase I safety trial in paediatric metabolic liver disorders, although without comparison to undifferentiated MSCs [148]. Conversely, a phase 2 non-randomised study comparing undifferentiated MSCs (n=9) to hepatocyte-like MSCs (n=6), or standard care control (n=10) showed short-term improvements in MELD, bilirubin, albumin and clinical signs of liver disease, in patients receiving MSC therapy for HCV. No difference in clinical effect was observed between patients receiving undifferentiated MSCs or hepatocyte-differentiated MSCs, [149], suggesting that contribution to hepatocyte functions from MSCs is negligible. Reprogramming somatic cells, such as fibroblasts or MSCs, with hepatic transcription factors to produce induced hepatocytes (iHeps) is also being explored [150, 151]. iHeps display functionality and

transcriptomic profile approaching human hepatocytes and significantly repopulate livers of $FAH^{-/-}$ livers, improving survival to 40% of mice compared to 0% survival after 27 days in control mice [152]. Long-term expanded MSC-derived iHeps undergo uncontrolled proliferation and a reversion to a mixed epithelial/adipocyte phenotype, with adverse outcomes when transplanted into a model of hepatocellular injury and senescence –the AhCre-$MDM2^{flox/flox}$ mouse [153], raising concerns regarding phenotype stability of iHeps when transplanted into an adverse hepatic niche.

Macrophages

Recruited monocyte-derived macrophages play a dual role in orchestrating the pro-inflammatory response to liver injury, which mediates the recruitment of immune cells to the injury niche, activation of hepatic stellate cells to promote liver fibrosis [154, 155] and initiation of progenitor-mediated liver regeneration and hepatocytic differentiation [156, 157]. In ALF, administration of CSF-1 in mouse APAP-induced injury promotes macrophage differentiation of infiltrating monocytes, restoration of liver innate immunity and accelerated recovery [158]. Phagocytosis of debris by macrophages and interaction with neutrophils mediates a phenotypic shift from pro-inflammatory ('M1-like') to pro-resolution ('M2-like') macrophages via downregulation of the pro-inflammatory sensor, NLRP3, upregulation of anti-inflammatory IL-10 which reinforces a pro-phagocytic phenotype by downstream STAT3 signalling and autocrine IL-6 stimulation [159-161]. Pro-resolution macrophages upregulate pro-phagocytic genes and collagen-degrading MMPs [161]. Mer tyrosine kinase (MerTK) is a phagocytic receptor that recognises apoptotic cells, facilitating the functional switch from pro-inflammatory to pro-resolution macrophages following efferocytosis of cargo [162], and plays a key role in facilitating macrophage clearance of neutrophils in ALF allowing injury resolution [163]. These data provide a fundamental link between liver inflammation, regeneration, wound healing and resolution processes orchestrated by macrophages, with a critical role for interactions between neutrophils and macrophages in the switch from a pro-inflammatory to pro-resolution phenotype with immunomodulatory and injury resolution properties (Figure 3).

Given that recruited macrophages play a crucial role in orchestrating liver repair and regeneration this raises the possibility that exogenous, ex-vivo differentiated macrophages with the appropriate phenotype could be administered to accelerate liver disease regression. In acute liver injury, intravenous administration of either mouse or clinical grade human IL4/IL13-

treated alternatively activated macrophages, with enhanced phagocytic capacity over naïve macrophages, reduced liver necrotic area, reduced liver infiltration of Ly6C^{hi} inflammatory monocytes and reduced pro-inflammatory cytokines in serum and liver [164] (Figure 3). In chronic disease, M-CSF differentiated bone marrow derived macrophages administered via venous or intrasplenic injection reduced liver scar formation via the production of MMPs, increased the regenerative ductular reaction and recruited host immune cells such as neutrophils and monocytes to the injury niche to reinforce injury repair mechanisms [122, 165] (Figure 4). Macrophages administered via the circulation home to both the liver and spleen in uninjured and APAP-injured mice [164, 166]. In chronic liver injury, donor macrophages engraft transiently into the liver and can be observed one week, but not one month post-transplant in chronic liver injury [122], suggesting little Kupffer cells reconstitution by donor cells in pre-clinical models. However, recruitment of host innate immune cells is a critical mechanism for macrophage therapy and can enhanced by polarising macrophages to a classically-activated macrophage (CAM) phenotype with LPS and IFNγ [167]. Although this phenotype is associated with increased inflammation, hepatic stellate cell activation, and fibrogenesis [102], CAMs upregulate the monocyte chemoattractant CCL2, which increases recruitment of host monocytes to the injury niche that become Ly6C^{low} restorative macrophages, expressing key phagocytosis genes such as *MerTK*, *MARCO* and *TREM2* [167], analogous to post phagocytic pro-resolution macrophages [161], with an overall effect of inducing hepatic stellate cell apoptosis and reducing liver collagen [167] (Figure 4). Clinically, a dose escalation trial of autologous macrophage therapy for liver cirrhosis demonstrated safety in nine patients with compensated cirrhosis, up to a maximum assessed dose of up to 10^9 cells per patient of M-CSF differentiated monocyte-derived macrophages delivered via peripheral vein injection [168], with efficacy currently being assessed in an ongoing phase 2 randomised controlled trial (ISRCTN 10368050).

Conclusions

There has been encouraging progress in the development of cell replacement methods to reconstitute liver parenchyma including hepatocyte cryopreservation and thawing, cell encapsulation to prevent immune rejection, and host pre-conditioning studies to improve engraftment and survival. Much progress has been made in developing stem/progenitor sources of hepatocytes. However, the development of GMP-compatible cell production methods are

required for clinical safety and efficacy testing. Alternative remodelling strategies act directly to reverse liver injury dynamics, and harness host innate immunity to reignite stalled resolution processes and are being assessed in clinical trials with encouraging results, but much more work is required to rigorously establish efficacy. For cell types which are not as far advanced along the clinical pipeline, particularly stem cell-derived therapies, barriers to be overcome include proving long-term safety and improving engraftment and maturity of cells to enhance efficacy. Nonetheless, with approaches to repair or rebuild the injured liver being assessed in patients, cell therapy for liver disease is on the cusp of meaningful clinical utility.

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Figure legends:

Figure 1. Optimisation of hepatocyte transplantation.

The first barrier to successful hepatocyte transplant is cell quality, which has been improved by optimising cryopreservation and thawing techniques. Cells are administered via the venous or arterial circulation and enter the liver via the hepatic sinusoids. Engraftment into unperturbed liver is low. Liver preconditioning can increase engraftment by inducing apoptosis in sinusoidal endothelial cells to allow donor cell access and integration into hepatic cords, and induces damage in host hepatocytes to provide a selective growth advantage to allow donor hepatocyte growth. The distorted microenvironment of severe injury is challenging for engraftment and proliferation of transplanted cells. Macrophage TGFβ spreads p21-induced senescence in host and donor hepatocytes. Strategies to mitigate the severity of the niche including co-transplantation of immunomodulatory cells, inhibiting senescence, or inducing proliferation with growth factors. Alternatively, hepatocytes can be encapsulated and transplanted into extrahepatic sites to circumvent the liver injury niche and avoid immune destruction. Successful cell transplant currently provides a functional bridge to host regeneration or orthotopic liver transplantation (OLT).

Figure 2. Hepatocyte donor cell lineages for cell replacement therapy.

A number of different cell types are currently being explored to replace functional parenchymal tissue. Hepatocytes can be isolated and transplanted and are a paradigm for clinical liver cell replacement therapy. Strategies have been developed to enable in vitro hepatocyte expansion to overcome donor shortages including 3D organoid expansion, and inducing de-differentiation using chemical inhibitors to revert hepatocytes to chemically induced liver progenitor cells (CLiPS). Foetal liver progenitors, which can be purified based on EpCAM expression, express markers such as LGR5, NCAM, CDCP1 and CD90, are capable of expansion and transplantation have also been used clinically. Hepatic progenitor cells (HPCs) can be isolated from subsets of the EpCAM+ ductal epithelium using antibodies recognising CD24, CD133

and ST14, and are capable of expansion and in vivo differentiation to hepatocytes in pre-clinical models of liver disease. Pluripotent cells, including ES and iPS cells can generate hepatocyte-like cells capable of fulfilling hepatocyte functions in vivo, and somatic cells including fibroblasts and mesenchymal stromal cells (MSCs) can be reprogrammed to form induced hepatocytes (iHeps).

Figure 3. Cell therapy to modulate the injury niche in acute liver injury.

Following hepatocyte injury, damage-associated molecular pattern (DAMPs) molecules activate liver resident Kupffer cells (KCs) to initiate the inflammatory response to injury. Secretion of chemokine (C-C) motif ligand 2 (CCL2) recruits $Ly6C^{hi}$ inflammatory monocytes from the circulation via C-C chemokine receptor 2 (CCR2) which contribute to inflammatory macrophages. Inflammatory macrophages activate γδT-cells via IL-23, which secrete IL-17A to recruit neutrophils to the injury niche. Macrophage function is impaired in severe injury, allowing gut bacteria translocation, leading to further pathogen associated molecular pattern (PAMP)-induced inflammation , systemic inflammation, and eventually multi-organ failure. Resolution of acute injury occurs via the lineage switch of pro-inflammatory to pro-resolution macrophages through interaction with neutrophils and increased phagocytosis via MerTK, leading to anti-inflammatory IL-10 secretion and initiation of hepatocyte-mediated regeneration. For cell therapy, administration of alternatively activated macrophages (AAMs) increases the pool of pro-resolution macrophages, downregulates the recruitment of inflammatory monocytes and restores the innate immune barrier to bacterial translocation. Administration of mesenchymal stromal cells (MSCs) facilitates pro-resolution phenotypic switching of macrophages and inhibition of inflammation via IL-10.

Figure 4. Cell therapy to modulate the injury niche in chronic liver injury.

Inflammatory monocytes are recruited via chemokine (C-C) motif ligand 2 (CCL2), contributing to the pro-inflammatory macrophage pool. Inflammatory macrophages activate hepatic stellate cells to become fibrogenic extracellular matrix (ECM) producing myofibroblasts via TGFβ and PDGF. Severe injury drives innate immune dysfunction, increasing infection risk, innate immune activation, and systemic inflammation. Macrophage phenotypic switching following phagocytosis drives fibrosis resolution via myofibroblast apoptosis and matrix metalloprotease (MMP) breakdown of ECM. Resolution is further reinforced by the recruitment of pro-resolution innate immune cells to injury areas.

Hepatocytes regenerate via proliferation, or TWEAK-induced ductular reaction (DR), containing regenerative hepatic progenitors. For cell therapy, human monocyte-derived macrophages (HMDMs) increase the pool of pro-resolution macrophages, and recruit host immune cells to reinforce injury resolution mechanisms. Classically activated macrophages (CAMs) support recruitment of host pro-restorative macrophages to the injury niche via CCL2 secretion. MSCs influence macrophage phenotype via IL-10 induced alternative activation to amplify pro-resolution pathways, and limit systemic inflammation via their immunomodulatory properties. Hematopoietic stem cells (HSCs) may contribute to macrophages and can recruit host immune cells to support fibrosis resolution.

Tables:

Table 1. Hepatocyte transplantation for acute liver failure (ALF).

	No of Patients	***Age Range (Yrs)***	***Survival (Death* or time to OLT**)***	***Reference***
Drug Induced	3	32-55	6h* – 20* days	[32]
	4	21-51	1 day*->1year (disease Free)	[35]
	2	26,27	2 days**-10 days**	[31]
	1	43	35 days*	[30]
Cryptogenic ALF	1	23	5 days**-13 days*	[33]
Virus-associated ALF	3	28-43	1 day** – 5 days*	[34]
	1	28	3 days**	[30]
	2	29, 65	18hrs*, 52 days*	[32]
	1	37	Fully Recovered	[169]
	1	54	Day 7*	[35]
	1	40	13hrs*	[170]
Acute-on Chronic (ACLF)	7	19-48	3 patients fully recovered 1 survived and subsequently underwent OLT	[7]

	3 patients* (2.5-12 months)