Instant-Blood Mediated Inflammatory Reaction in Hepatocyte Transplantation: current status and future perspectives

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Running Header: IBMIR in Hepatocyte Transplantation
Abstract

Hepatocyte transplantation (HT) is emerging as a promising alternative to orthotopic liver transplantation (OLT) in patients with certain liver-based metabolic disease and acute liver failure. Hepatocytes are generally infused into the portal venous system, from which they migrate into the liver cell plates of the native organ. One of the major hurdles to the sustained success of this therapy is early cell loss, with up to 70% of hepatocytes lost immediately following infusion. This is largely thought to be due to the instant blood-mediated inflammatory reaction (IBMIR), resulting in activation of complement and coagulation pathways. Transplanted hepatocytes produce and release tissue factor (TF), which activates the coagulation pathway leading to the formation of thrombin and fibrin clots. Thrombin can further activate a number of complement proteins leading to activation of the membrane attack complex and subsequent hepatocyte cell death. Inflammatory cells including granulocytes, monocytes, Kupffer cells and natural killer (NK) cells have been shown to cluster around transplanted hepatocytes leading to their rapid clearance shortly after transplantation. Current research aims to improve cell engraftment and prevent early cell loss. This has proved successful in vitro using pharmacological interventions such as melagatran, low-molecular weight dextran sulphate and N-acetylcysteine (NAC). Effective inhibition of the IBMIR would significantly improve hepatocyte engraftment, proliferation and function, providing successful treatment for patients with liver-based metabolic diseases.
Key words: Hepatocyte transplantation, complement, coagulation, innate immunity, thromboinflammation

Introduction

Hepatocyte transplantation (HT) offers a promising alternative to liver transplantation for patients with certain liver-based metabolic diseases and acute liver failure. Human hepatocytes are isolated from whole donor livers or tissue obtained from split liver procedures using a collagenase perfusion technique and purified using centrifugation (Fig. 1.) 1-4. The number of cells transplanted aims at approximating 5% of the normal liver mass and are usually administered under radiological guidance via the portal vein or spleen.

HT has potential advantages over orthotopic liver transplantation (OLT) in certain cases because the procedure is less invasive with significantly less complications than organ transplantation. The technique can also provide temporary lifesaving treatment until a patient can undergo whole organ transplantation or potentially future gene therapy 2,5. HT can improve the phenotype of a number of metabolic liver diseases. To date, HT in patients with Crigler Najjar syndrome has been shown to decrease serum bilirubin levels by 30-60% and maintain the expression of UDP glucuronosyltransferase activity for up to 9 months 5-10. Furthermore, partial hepatectomy followed by hepatocyte transplantation increased concentrations of hepatocyte growth factor (HGF), leading to increased hepatocyte function 11. Patients with factor VII deficiency have shown up to a 70% decrease in the requirement for recombinant factor VII treatment 12 and in familial hypercholesterolemia there was up to a 70% decrease in LDL production 13. Phenotypic improvement has also been seen in glycogen storage disease (GSD) 1a and 1b with normal glucose-6-phosphatase activity and increased triglyceride levels observed 14,15. Hepatocyte transplantation has also been shown to improve a number of urea cycle
defects including: ornithine transcarbamylase (OTC) deficiency where patients showed a decrease in ammonia production and an increase in urea production\textsuperscript{16,17} argininosuccinate lysase (ASL) deficiency in which there was sustained hepatocyte engraftment with increased ASL activity\textsuperscript{18} and tyrosinemia type 1 which improved clotting factor levels and decreased bilirubin concentrations\textsuperscript{19}. Such promising results suggest HT has clinical potential in alleviating the severity of metabolic liver diseases, making day to day management easier and improving quality of life for patients. Although hepatocyte transplantation has great potential, there are still many bottlenecks that limit the efficacy of the technique. This includes the scarcity of donor organs, the decreased viability of cells due to cryopreservation, early cell loss and the lack of engraftment into the liver. So far, only rarely have patients with metabolic disease demonstrated a sustained long-term improvement in their condition following HT\textsuperscript{18}.

**Pathophysiology of the IBMIR**

Though immunosuppression is generally used in hepatocyte transplantation for liver-based metabolic conditions, early cell loss due to the innate immune response has not been successfully targeted. Transplantation of hepatocytes, islets of Langerhans and therapies involving mesenchymal stem cells have all been associated with cell damage and significant cell loss very soon after administration, leading to poor engraftment and cell function\textsuperscript{20}. Early hepatocyte cell loss has been demonstrated in dipeptidyl peptidase IV-deficient F344 rats, which showed hepatocytes present in large numbers in portal areas shortly after transplantation\textsuperscript{21}. There was significant clearance of hepatocytes from these portal areas and low engraftment into the liver sinusoids with an average 3.3 fold decline of the total number of transplanted hepatocytes between 2 and 24 hours\textsuperscript{21}. This significant cell clearance was attributed to the presence of granulocytes, phagocytes and activated macrophages surrounding the hepatocytes in portal areas within 6 hours post transplantation\textsuperscript{21}. This has been confirmed clinically with explanted livers from metabolic liver disease patients who had undergone hepatocyte transplantation followed by OLT. Patients were shown to have inflammatory thrombi containing lymphocytes, histocytes and multinucleated macrophages post HT\textsuperscript{22}. 
This inflammatory reaction is now commonly referred to as the instant blood-mediated inflammatory reaction (IBMIR), in which cells are recognised by the innate immune system, leading to rapid activation of both complement and coagulation pathways. Additionally, inflammatory cells including granulocytes, monocytes, kupffer cells and natural killer (NK) cells are activated leading to rapid clearance and death of transplanted hepatocytes. IBMIR induced cell loss has been well described in islet transplantation in both in vitro blood perfusion systems, rodent and primate studies and in patients undergoing clinical islet transplantation.

The coagulation cascade is usually activated following endothelial cell surface damage, leading to activation of fVIIa. Hepatocytes have been shown to trigger activation of the extrinsic coagulation cascade by producing tissue factor which binds to factor VII (fVII) and fVIIa, leading to the activation of fVIIa (Fig 2). The TF-VIIa complex initiates the activation and cleavage of factor X (fX), producing fXa which associates with co-factor Va to form the prothrombinase complex which converts prothrombin to thrombin. Thrombin initiates the conversion of fibrinogen to fibrin and activates factor XIII to promote fibrin cross linking and the formation of fibrin clots. Thrombin also amplifies its own expression through activation of cofactor VIII to VIIIa which binds factor IXa to further activate fX. Thrombin activates platelets by binding to receptors on their cell surface and is required for their aggregation and formation of clot structure.

The complement and coagulation cascade share similar characteristics with both consisting of circulating zymogens, that rely upon a series of enzymatic reactions, producing an active protease and a non-enzymatic ligand. There are over 30 complement proteins, however the most important is complement component 3 (C3). The complement cascade consists of 3 distinct pathways including the alternative, classical, and lectin pathway, however all lead to the cleavage of C3 into C3a and C3b by C3 convertase (Fig 2). The alterative pathway is the first to act and is directly activated by binding of the C3b fragment to the foreign cell surface, targeting it for destruction by phagocytes, while the smaller C3a fragment acts as a chemoattractant for phagocytes and other inflammatory cells. The lectin pathway is activated by mannose binding lectin (MBL) which circulates in plasma in its inactive form.
in complex with MBL-associated protease (MASP) 1 and 2. The MBL complex binds to mannose containing carbohydrates on the foreign surface. Lastly, the classical pathway is activated when C-reactive protein or immunoglobulins such as IgM, IgG bind to specific antigens on a target surface. All 3 pathways lead to the covalent binding of C3b to the foreign surface. C3b binds to C3 convertase producing C5 convertase which results in the cleavage of C5 into C5a and C5b. C5b triggers the terminal pathway and initiates the formation of the membrane attack complex, which creates pores in the target cell membrane. The liver is a major source of complement proteins, primarily produced by inflammatory cells and hepatocytes. Interestingly it has been shown that hepatocytes synthesise up to 15 times more C3 than macrophages.

Interactions between the complement and coagulation pathways are poorly understood (Fig. 2.). Complement proteins may directly enhance coagulation through platelet activation and modification of the lipid membrane, resulting in activation of TF. Furthermore, thrombin may also have a role in amplifying the complement cascade. Krisinger et al. showed thrombin efficiently cleaved C5 at a newly identified site, R947 generating C5\textsubscript{T} and C5b\textsubscript{T}. The crosstalk between such related biological cascades has clinical importance with evidence suggesting an inhibitor of the coagulation cascade may also prevent activation of the complement pathway, reducing the effects of the IBMIR with a single inhibitor.

Activation of both complement and coagulation pathways leads to the stimulation of a number of inflammatory cells including neutrophils, monocytes, macrophages and natural killer (NK) cells (Fig 3). Macrophages or kupffer cells in the liver are responsible for phagocytosis of foreign cells, and translocation of NF-\kappa B to the macrophage nucleus initiates translocation of a number of inflammatory cytokines including IL-1, IL-2, IL-6, IL-12 and CXCL-8. These act as chemokines for a number of inflammatory cells such as neutrophils and NK cells. Neutrophils function in a similar way to macrophages, producing a number of degradative enzymes and granules to lyse the target cell. NK cells are lymphocytes that circulate in an active state and act through IFN-\gamma production and through receptor mediated binding that allows distinction between foreign and innate cells.
Thus, activation of processes such as coagulation, complement and inflammatory cell recruitment have the potential to cause significant harm to transplanted cells, preventing engraftment and the success of the transplant. Current research has aimed to discover the possibility and mechanisms of IBMIR induced hepatocyte cell loss and investigate reliable and effective methods of preventing this occurring.

**Activation of coagulation in Hepatocyte Transplantation**

A single stage clotting assay showed hepatocytes in contact with human plasma resulted in pro-coagulant activity (PCA) that increased significantly with increasing concentrations of hepatocytes. PCA was absent in factor VII-deficient plasma, further suggesting the role of TF in coagulation activation. A chandler loop model was designed in which polyvinyl chloride (PVC) tubing was coated with heparin, incubated at 37 degrees and placed on a rocker to generate a flow of 45ml/minute to mimic portal vein blood flow. Using this model, it was shown $5 \times 10^5$ hepatocytes induced clot formation in ABO-matched blood circulating for 30 minutes. This correlated with a significant drop in platelet count and increased D-dimer levels. The same authors have shown pro-coagulant activity in human adult liver-derived mesenchymal progenitor cells. Thromboelastography revealed shorter clotting times in plasma and whole blood treated with hepatocytes, while the tubing loop model revealed a significant drop in platelet count and increased D-dimer levels.

The source of TF in HT was directly tested in mouse models of acetaminophen (APAP) overdose. Following HT, thrombin anti-thrombin (TAT) expression in plasma was significantly higher in wild type mice transplanted with control hepatocytes compared to wild type mice receiving hepatocytes from genetically modified mice with selectively deleted TF expression. This proves donor hepatocytes are the source of pro-coagulant TF, triggering the IBMIR. Activation of coagulation following HT has been demonstrated clinically in a child with ornithine transcarbamylase deficiency. An hour following intraportal infusion of $8.3 \times 10^7$ cryopreserved hepatocytes there was a significant increase in TAT, FXI-anti-thrombin, FXII-anti-thrombin and
platelet depletion. This suggests activation of the IBMIR within 60 minutes of HT. Similarly, a 9 month-old girl with Crigler-Najjar syndrome received 2.6 billion cryopreserved hepatocytes over 2 weeks in 14 infusions. The patient showed increased D-dimer levels but normal fibrinogen, prothrombin time and activated partial thromboplastin time. The authors suggest cell dependent coagulation may occur in the small liver sinusoids and therefore may not be detected in high peripheral and portal flow. Evidence of thrombosis has also been demonstrated in explant livers of children with metabolic livers that received hepatocyte transplantation. Hepatocytes, identified by the bile salt export protein (BSEP) were found in the portal vein thrombi.

Activation of complement in Hepatocyte Transplantation

Complement activation of C3a and C5b-9 has been demonstrated in a tubing loop model containing ABO matched human blood and 1x10^5 fresh hepatocytes. The addition of melagatran, a direct thrombin inhibitor and low molecular-weight dextran sulfate (LMW-DS), an anti-thrombotic agent, reduced both TAT expression and C3a expression, further suggesting the association between the complement and coagulation cascade. Melagatran, a thrombin inhibitor reduces the generation of thrombin through the production of chondroitin sulphate and indirectly inhibits the complement system. Complement activation has been implicated in the acute death of allogenic rat models following intraportal infusion of rat hepatocytes. Transplantation of WAG-Rij hepatocytes into Lewis rat participants resulted in high morbidity rates and large thrombi containing complement components including C3 and IgM. No deaths occurred when WAG-Rij hepatocytes were transplanted into WAG-Rij rats. Lewis rat recipients pre-treated with the complement inhibitor, cobra venom factor had high survival, suggesting complement activation was a vital contributor to the higher morbidity rates. The role of complement activation in HT has further been investigated using transfected hepatocytes. Hammel et al. infected hepatocytes with an adenovirus containing the genes encoding the hCMV promoter and the human CR1 receptor, which is a potent inhibitor of the C3 and C5 convertases, essential in the classical and alternative complement pathways. Transplantation of hepatocytes
expressing the CR1 adenovirus prevented immediate rejection in immunocompetent Nagase analbuminemic rats and increased albumin production. Such genetic modification of hepatocytes may be useful in reducing complement activation following transplantation into sensitized recipients 43.

Infiltration of inflammatory cells

Once administered via the portal vein, transplanted hepatocytes become entrapped into the liver sinusoids, migrate across the endothelial barrier and integrate into the parenchyma 23,44. However, the presence of transplanted hepatocytes within the sinusoids temporarily occludes blood flow and activates ischaemia-reperfusion events 45. Ischaemia-reperfusion leads to the activation of Kupffer cells which are specialised macrophages residing in liver sinusoids, responsible for the clearance of transplanted hepatocytes and the 70% cell loss observed following transplantation 45. Gadolinium chloride (GdCl3) has been used to deplete Kupffer cells and increase hepatocyte cell survival 45. Control rats showed transplanted cells in 34% of liver radicles compared to 66% in GdCl3 treated rats 72 hours following transplantation. Furthermore, approximately 2-fold more hepatocytes were observed in the periportal sinusoids and the liver parenchyma 1, 2 and 7 days following transplantation. The authors suggest inhibition of Kupffer cell activation offers a way to prevent early cell loss following transplantation and improve engraftment of transplanted hepatocytes.

Inflammatory cells from the recipient liver have also been demonstrated to stimulate activation of a number of cytokine-chemokine genes within 3 weeks post hepatocyte transplantation 46. This included chemokine ligands, chemokine receptors, and regulatory cytokines. In the DPPIV rat model, gene expression levels of cytokine-chemokine genes increased from 2- to over 120-fold. Depletion of Kupffer cells and neutrophils resulted in the normalisation of 19 of 25 genes. This was associated with increased cell engraftment for up to 2 weeks 46.
NK cells have also been shown to accumulate around transplanted hepatocytes. Wesolowska et al. developed a combined protocol of non-lethal whole body irradiation, administration of anti-asialoGM1 anti-serum (to eliminate NK cells) and reconstitution with bone marrow cells, to eliminate inflammatory cell activation following transplantation. Lewis rats received this combined treatment over 3 days before transplantation of syngenic hepatocytes into the spleen. Such treatment led to the formation of hepatocyte islands 14 days following transplantation which increased in number at 30 days. Immunohistochemical staining revealed the absence of cytotoxic granulocytes, macrophages and NK cells. Combined treatment may be a potential way to eliminate inflammatory cells involved in the innate immune response and prevent early hepatocyte cell loss. However, clinically the risks of such treatment may need to be considered.

It is also important to consider the potential benefit of the inflammatory response. To a certain extent, kupffer cell production of cytokines such as interleukin, TNF-α and free radicals are important for endothelial disruption which is necessary for transplanted hepatocytes to cross the endothelial barrier and enter the sinusoidal plates. It may be important to consider the effects complete inhibition of the inflammatory response has upon the hepatocyte translocation and engraftment. It may be more effective to dampen the response but not cause complete inhibition.

Current therapeutic strategies to inhibit the IBMIR

Inhibition of the IBMIR has been well explored in the setting of islet transplantation using a variety of different methods. This includes systemic anti-coagulant drugs such melagatran, low-molecular weight dextran sulphate and NAC. These have been shown to inhibit platelet consumption, decrease the expression of complement and coagulation proteins and reduce the number of inflammatory cell subtypes in blood perfusion models. Several anti-inflammatory proteins have also been investigated in vivo. This includes alpha-1 antitrypsin (AAT) and activated protein C, which have been shown to down-regulate the expression of a number of inflammatory cytokines and improve islet survival and engraftment in mouse models of islet transplantation. An anti-TF antibody...
decreased post-transplant markers of coagulation following islet transplantation in cynomolgus monkeys, resulting in higher C-peptide levels and prolonged graft function. A novel approach has been to coat cell surfaces with macromolecular heparin complexes. Tubing loops with heparin coated islets resulted in no macroscopic clotting and the generation of TAT and drop in platelet count were significantly attenuated compared to untreated loops. Alternatively, it is possible to coat the cell surface of islets with poly-ethylene glycol (PEG), which allows binding of a number of different anti-inflammatory proteins such as sCR1.

Mesenchymal stem cells have also been shown to trigger the IBMIR after exposure to human blood, with activation dependent on the cell passage number. The authors showed visible clot formation in the Chandler loop model which was prevented by addition of the anti-TF blocking agent FVIIai and by monoclonal anti-TF antibodies. Currently only a limited number of anti-inflammatory drugs have been tested to inhibit the IBMIR in the context of hepatocyte transplantation.

**Therapeutic strategies to inhibit the IBMIR in hepatocyte transplantation**

**Systemic Administration**

Gustafson et al. studied a number of systematic coagulation inhibitors to minimise the IBMIR in HT and investigated the effect of thrombin inhibition, a factor VIIa inhibitor (iFVIIa) and an anti-TF monoclonal antibody. In a tubing loop model, both inhibitors prevented platelet consumption after 15 minutes but this was not maintained after 30 minutes. iFVIIa did not significantly decrease the concentration of coagulation factors TAT, FXIIa-AT, FXIa-AT, and complement factors C3a and C5b-9G. In contrast, the direct thrombin inhibitor Melagatran (10µM) and low molecular weight dextran sulphate prevented platelet depletion and significantly decreased concentrations of TAT, FXIIa-AT, FXIa-AT, C3a and C5b-9G after 60 minutes.

The anti-oxidant NAC, has been shown to inhibit the activity of thrombin and coagulation factors VII and X. NAC has been shown to inhibit PCA in whole blood containing hepatocytes in a dose
dependent manner. Pro-coagulant activity was significantly lower in plasma containing hepatocytes and 10-25mM/L NAC. Furthermore, platelet counts and D-dimer concentrations were maintained at a similar level to the control.

In human adult liver-derived mesenchymal progenitor cells (hALPCs), the combination of bivalirudin and heparin treatment inhibited reduced clotting time and improved both engraftment and cell survival.

**Encapsulation**

An alternative approach to prevent activation of the IBMIR response following HT, is to encapsulate cells in microbeads made from purified alginate. The semi-permeable membrane within the microbeads allows glucose and oxygen to pass through, maintaining necessary metabolic function, whilst also protecting against immunocompetent cells by preventing the entry of antibodies.

Encapsulating hepatocytes in alginate microbeads improved the survival of mice with acute liver failure by providing metabolic support, decreasing the number of cytokines and reducing the inflammatory stress on the liver. However, the encapsulation of cells using alginate or agarose can produce large capsules that make transplantation into the liver through the portal vein difficult, as major blood vessels would be occluded. Teramura et al. carried out microencapsulation of cells in an ultra-thin polymer membrane, which showed reduced platelet aggregation, decreased TAT levels and lower levels of C3a and C5b compared to alginate coated cells when exposed to human whole blood and serum.

**Cytotopic Therapeutic Agents**

Currently, novel cytotoxic therapeutic agents are being investigated as a novel way to target the IBMIR in both whole organ transplantation and cell transplantation. Such agents are designed to bind to the cell membrane of transplanted cells and provide local inhibition of complement and coagulation pathways without the need for systemic treatment which may result in bleeding risk. Currently cytotoxic agents such as Mirococept are being developed and investigated for kidney, islet and hepatocyte transplantation as well as the prevention of myocardial and intestinal ischaemia reperfusion injury.
Mirococept is derived from the human complement regulatory protein CR1, a transmembrane protein found on the cell surface of inflammatory cells. CR1 is a potent inhibitor of the C3 and C5 convertases. In both rat kidney transplantation and human tumour nephrectomy specimens, immunohistochemical analysis showed Mirococept localized to the glomerular and tubular structures of the kidney. After 24 hours, neutrophil activity and deposition of C3a and C5-9 was significantly lower in Mirococept treated grafts compared to controls. In 2012, 76 patients had received Mirococept including one liver patient. These initial results showed Mirococept had no adverse side effects and the next step is a multicentre phase 2b clinical trial.

Conclusions

Evidence of the instant blood-mediated inflammatory reaction has been demonstrated following HT in both blood perfusion models, animal experiments and patients. Transplanted hepatocytes produce and release TF, leading to activation of coagulation and complement pathways and eventually cell lysis. Inflammatory cells are further activated leading to the rapid clearance of hepatocytes shortly after transplantation. Such activation of the innate immune response following HT is a limiting factor to the success of this technique. Current research in HT aims to reduce this early cell loss and improve cell engraftment. This includes pharmacological interventions, cell encapsulation and novel cytotoxic therapeutic agents. Improved cell engraftment and survival will significantly increase the application of HT and provide treatment for patients who are unsuitable or unable to receive orthotopic liver transplantation.

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References


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### Table 1: Inhibitors of the IBMIR and their mechanisms of action in islet transplantation

<table>
<thead>
<tr>
<th>IBMIR inhibitor</th>
<th>Mechanism</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-molecular weight dextran sulphate</td>
<td>Anti-thrombin inhibitor</td>
<td>Inhibited macroscopic clotting, reduced platelet consumption and inhibited the production of C3a, FXIa-AT and TAT</td>
<td>49</td>
</tr>
<tr>
<td>N-acetyl cysteine</td>
<td>Inhibitor of coagulation factors II, VII and X</td>
<td>Inhibited TF expression and decreased pro-coagulant activity</td>
<td>66</td>
</tr>
<tr>
<td>Melagatran</td>
<td>Anti-thrombin inhibitor</td>
<td>Decreased TAT, FXIa-AT and C3a expression</td>
<td>39</td>
</tr>
<tr>
<td>Alpha-1 antitrypsin</td>
<td>Serine protease inhibitor that can cleave pro inflammatory and coagulation proteins.</td>
<td>Down-regulated inflammatory cytokines and increased islet survival in mouse models.</td>
<td>51</td>
</tr>
<tr>
<td>Activated protein C</td>
<td>Anti-inflammatory, anti-thrombotic properties</td>
<td>Improvements in factor VII levels, reduction in clot weight, improved platelet, neutrophil and monocyte counts and improved fibrinogen and D-dimer levels.</td>
<td>52</td>
</tr>
<tr>
<td>Monoclonal anti-TF</td>
<td>Anti-tissue factor</td>
<td>Decreased coagulation factors, higher CP levels and increased graft survival.</td>
<td>29</td>
</tr>
<tr>
<td>Heparin</td>
<td>Inhibitor of anti-thrombin</td>
<td>Heparin coated islets resulted in no macroscopic clotting and the generation of TAT and drop in platelet count were significantly attenuated.</td>
<td>28</td>
</tr>
<tr>
<td>Surface Modification With Poly(ethylene glycol)-Lipid and Urokinase</td>
<td>Coating of islets to prevent direct exposure to blood</td>
<td>Insulin levels increased after transplantation in recipient mice.</td>
<td>67</td>
</tr>
</tbody>
</table>
Figure 1. Isolation of human hepatocytes. Hepatocytes are isolated from donor liver tissue using a collagenase perfusion technique and purified using centrifugation. Cells are either transplanted directly into the portal vein or cryopreserved for emergency cases. Viability and metabolic activity is checked before administration via the portal vein.

Figure 2. Activation of the instant blood-mediated inflammatory reaction following hepatocyte transplantation. Transplanted hepatocytes produce and release TF which binds to the coagulation factor VIIa and initiates the activation and cleavage of factor Xa, which is responsible for the conversion of prothrombin to thrombin. This is followed by platelet binding to the cell surface and the conversion of fibrinogen to fibrin forming fibrin clots. Thrombin has also been shown to activate complement proteins. C3 activates C3 convertase resulting in the cleavage of C3 to C3a and C3b, which activates C5 convertase, resulting in the cleavage of C5 to C5a and C5b-9. C5b is responsible for the production of the membrane attack complex (MAC) which binds to the membrane of target cells resulting in cell lysis.

Figure 3. Intravenously transplanted hepatocytes cause activation of inflammatory cells including: neutrophils, monocytes, Kupffer cells and NK cells. Such inflammatory cells recognise transplanted hepatocytes and activate mechanisms including; receptor mediated phagocytosis, cytokine and chemokine production, and the production of cytotoxic granules such as fas and perforin. Activation of such inflammatory responses contribute to hepatocyte cell lysis and death.
Figure 1
Figure 2

Cell Transplantation: The Regenerative Medicine Journal
Figure 3