Induced pluripotent stem cells for the treatment of liver diseases: challenges and perspectives from a clinical viewpoint

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Contributions: (I) Conception and design: L Tolosa; (II) Administrative support: None; (III) Provision of study materials or patients: E Pareja, L Tolosa; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: L Tolosa; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Abstract: The only curative treatment for severe end-stage liver disease (ESLD) is liver transplantation (LT) but it is limited by the shortage of organ donors. The increase of the incidence of liver disease has led to develop new therapeutic approaches such as liver cell transplantation. Current challenges that limit a wider application of this therapy include a limited cell source and the poor engraftment in the host liver of cryopreserved hepatocytes after thawing. Induced pluripotent stem cells (iPSCs) that can be differentiated into hepatocyte-like cells (HLCs) are being widely explored as an alternative to human hepatocytes because of their unlimited proliferation capacity and their potential ability to avoid the immune system. Their large-scale production could provide a new tool to produce enough HLCs for treating patients with metabolic diseases, acute liver failure (ALF), those with ESLD or patients not considered for organ transplantation. In this review we discuss current challenges for generating differentiated cells compatible with human application as well as in-depth safety evaluation. This analysis highlights the uncertainties and deficiencies that should be addressed before their clinical use but also points out the potential benefits that will produce a great impact in the field of hepatology.

Keywords: Cell transplantation; stem cells; hepatocyte; liver therapy; safety assessment

Submitted Nov 14, 2019. Accepted for publication Feb 14, 2020.
doi: 10.21037/atm.2020.02.164
View this article at: http://dx.doi.org/10.21037/atm.2020.02.164

Introduction

Despite the fact that current medical and surgical therapies are available for early stages of liver diseases, a significant clinical need exists for alternative treatments of intractable liver diseases. Cell-based therapies are being developed as promising tools, alternative to liver transplantation (LT), to treat degenerative disorders, inborn hepatic metabolic diseases and organ failure (1-5). The earliest attempts made in this field involved the transplantation of allogeneic hepatocytes which is hindered by the increasing shortage of suitable donor livers for hepatocyte isolation as well as by the insufficient functional quality and great susceptibility to cryopreservation and thawing of hepatocytes (2,3,6). Therefore, the major challenge for hepatic cell therapy is to identify alternative reliable cell sources, equivalent to hepatocytes, expandable, bankable and engraftable, which can be derived from reproducible methods, thus making them available for transplantation to large numbers of patients. Current research on induced pluripotent stem cells (iPSCs), which are adult cells that have been genetically reprogrammed to an embryonic stem cell (ESC)-like state, point to these cells as an appealing option to face these challenges. iPSCs possess the unique properties of hepatic differentiation, self-renewal and in vitro expansion which make them a very promising cell source for generating
large-scale production of suitable functional hepatocyte-like cells (HLCs) (1,4,7,8). Large numbers of HLCs can be made readily available to any patient on an as-needed basis for hepatic cell-based therapies, both for programmed treatment for liver-based metabolic disorders and emergency use in patients with acute liver failure (ALF), acute-on-chronic liver failure (ACLF) or end-stage liver disease (ESLD) (4,7-10).

On the other hand, using patient-specific iPSCs has been made available likely solving the problem of allogeneic immune rejection. In this case, ex vivo gene-corrected patient-specific iPSCs lines raise the possibility of autologous transplantation for the treatment of hereditary liver metabolic diseases (10,11). Ideally, these cell lines should be highly viable preparations with robust hepatic function and engraftment capacity. Recent preclinical studies have shown that transplantation with HLCs differentiated from human iPSCs ameliorated inherited liver diseases (12,13) and ALF (14). Nonetheless, critical aspects to be addressed in clinical trials are long-term safety, tolerability, efficacy as well as the tumorigenic potential of the iPSC-derived cell based treatments to define the target patient and standardize the protocols.

This review focuses on the different strategies recently described to reprogram somatic cells to the pluripotency, their differentiation to HLCs and their potential use to provide a real prospect of bringing cell-based therapy for liver diseases in two main areas: to make unlimited numbers of HLCs available to extend treatments to many patients and to treat hereditary liver diseases using autologous genetically corrected HLCs (Figure 1). The review also reflects about challenges and uncertainties of their clinical application and the needs of clinicians (Figure 2).

### iPSC derivation

Based on the findings showing that Oct3/4, Sox-2 and Nanog play essential roles in the maintenance of early embryos and embryonic stem cells (ESCs) (15-17), Yamanaka’s group selected a pool of 24 genes to identify the reprogramming factors that could induce pluripotency in somatic cells. Finally, they selected the now known as Yamanaka factors (Oct3/4, Sox2, c-Myc and Klf-4) as they were able to successfully reprogram mouse fibroblasts into iPSCs (18). A year later they reprogrammed human fibroblasts into iPSCs by using the same combination of factors (19).

Since Yamanaka reported the first generation of iPSCs from somatic cells, two major aspects have been continuously under investigation: methods to induce somatic cell reprogramming and which somatic cells to reprogram. Currently, iPSCs can be obtained from different cell sources and through distinct strategies that have been reviewed in detail (8).

### Cell sources for iPSCs derivation

Although initially iPSCs were obtained from fibroblasts, distinct cell sources have been used to derive them [for a review (20,21)]. Skin fibroblasts are simple to culture and easily accessible with a skin biopsy, although, since it is an invasive process, alternative cell sources have been explored. These alternative cells such as blood cells, urine cells, hair-follicle derived keratinocytes, menstrual blood cells or amnion cells have been derived into iPSCs.

### Reprogramming methods

Important efforts have been made to understand the reprogramming process involved in the generation of iPSCs [reviewed by (20)]. The first method, used for the introduction of reprogramming transcription factors to human differentiated cells, was based on retroviral vectors (19), although, upon transduction, retroviral vectors are randomly integrated into the host genome, increasing the risk of tumor formation, which would be unsuitable for the generation of clinical-grade iPSCs.

For the future clinical applicability of iPSCs-derived cells, the development of non-integrative iPSCs derivation methods that do not introduce genetic changes is required. Among these the use of adenoviral vectors (22) or TransGen-free induction of human pluripotent stem cells (PSCs) by the vectors derived from Sendai virus (23) have been explored. The use of Sendai virus involves viral particles raising questions about the safety of the generated iPSCs. Due to this, others have focused their research on DNA-free and viral-free protocols based on the introduction of reprogramming-inducing molecules into cells such as recombinant proteins (24), microRNA (25), mRNA (26) or small molecule-mediated reprogramming (27). It has recently compared the efficiency of different RNA-based footprint methods through the use of self-replicating RNA (srRNA) and the use of synthetic mRNA, showing that srRNA had a better efficiency indicating that could be an appropriate approach for clinical applications (28). It should be also considered that direct reprogramming of
Figure 1 Summary of the key aspects to consider about the use of iPSCs-derived HLCs in the treatment of liver disease. For the treatment of liver diseases with HLCs derived from iPSCs there are several steps to consider from the iPSCs obtaining to the final used in selected patients with metabolic diseases or other liver diseases. HLC, hepatocyte-like cell; iPSCs, induced pluripotent stem cells.
somatic cells into HLCs using lentiviral vectors (29,30) has been proposed.

On the other hand, recently, human primary hepatocytes have been reprogrammed into a population of proliferating bipotent cells with regenerative potential by adding two small molecules and HGF, providing a new tool for personalised cell-based therapy (31). Finally, it should be considered that the iPSCs derivation efficiency of

Figure 2 Summary of the needs of clinicians for the application of iPSCs for the treatment of liver disease. After considering the critical steps for the obtaining of hepatocyte-like cells from pluripotent stem cells, safety issues should be addressed before its translation to the clinics. iPSCs, induced pluripotent stem cells.
all the proposed methods is low. Improving both the reprogramming efficiency and safety using integration-free and virus-free methods under feeder-free conditions is the most promising step in the safe translation of iPSCs to their clinical application in personalized regenerative medicine.

**iPSCs correction**

The gene correction of patient-specific iPSCs should be applied for patients with monogenic inherited metabolic diseases such as Crigler-Najjar disease or alpha-1 antitrypsin (A1AT) deficiency. The most widely used tools for genome editing are zinc-finger endonucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated system (Cas9) [reviewed by (32)]. After gene editing, iPSCs could be differentiated into HLCs and then transplanted. In this sense, it has been described the gene correction of A1AT deficiency in iPSCs by combining two technologies: ZFNs and PiggyBac technology which resulted in the in vitro restoration of the structure and function (33).

In another study, researchers generated iPSCs from a patient with Wilson’s disease (with a mutation in the ATPase Cu^{2+} transporting beta polypeptide gene) and corrected them using a lentiviral vector. These corrected iPSCs differentiated into HLCs showed copper metabolism capacity (34). In a study of Omer et al. [2017] the CRISPR/Cas9 system was used to correct a LDLR mutation of iPSCs derived from a patient with hypercholesterolemia. In this case, the genetic correction restored LDLR-mediated endocytosis (35).

The efficacy of gene editing has shown the potential for the application of patient-derived iPSCs for the correction of underlying genetic defects that could allow the autologous transplantation and, thus, reduce problems of immune rejection.

**iPSC differentiation, characterization and safety**

PSCs, including both ESCs and iPSCs can differentiate into all cell types of the body and are a promising tool for regenerative medicine, drug discovery and development studies. Great advances have been made to differentiate initially ESCs and now also iPSCs toward the hepatocyte lineage although the maturation levels and the characterization of HLCs vary in different studies.

**Differentiation of iPSCs into HLCs**

The generation of HLCs from iPSCs is a complex process. Although several protocols have been defined for the generation of HLCs from PSCs, this process includes 3 basic stages by administering different soluble factors (i.e., growth factors) in a time-dependent manner to mimic ontogenetic liver development (9,36). The first step includes endoderm induction by Activin A, BMP4, LY294002 and Wnt3a. The second step uses as inducers FGF2, FGF4 and BMP4 to produce hepatoblast cells (hepatic specification), whereas the final step is hepatic differentiation and maturation (using HGF and oncostatin M). For clinical applications HLCs need to be produced in a large scale and different bioreactors have been proposed (1,37).

In addition to the use of growth factors, some groups have proposed the use of microRNAs (38) or small molecules (39) to differentiate PSCs into HLCs as a simple, highly efficient, and cost-effective alternative for generation. On the other hand, different improvements of the standard protocols such as co-culture with other cell types (40), genetic manipulation (41) and/or culture in 3D configuration (42) trying to simulate what happens in vivo have been also proposed. Although even a 2-fold increase in some of the functions has been demonstrated with some of these new methods (42), HLCs-derived iPSCs are closer to a fetal than adult hepatocyte phenotype and in other cases there is a lack of appropriate controls that allow a clear conclusion about the results. Moreover, it has been described that the origin of the donor cells and not the derivation method can determine the variation in hepatic differentiation (43), which really complicates the therapeutic use of iPSCs–HLCs because the quality of HLC could be different depending on the donor.

**Characterization of HLCs**

Hepatocytes are the most predominant cell type of the adult liver mass and perform essential functions, including plasma protein secretion, ureogenesis, metabolic homeostasis or detoxification (37). Figure 3 summarizes important hepatic specific functions that HLCs should exhibit although, depending on the disease to be treated with these cells, the studies may focus on the lack of a specific liver function.
For example, in the case of the treatment of patients receiving extensive medication, clinicians may focus on CYPs activities and drug-metabolism enzymes, whereas for the treatment of inborn metabolic errors, characterization should focus on the specific lacking function. Although numerous studies have demonstrated that iPSCs can be differentiated into HLCs, the characterization of the cells sometimes is only based on the analysis of the expression of few hepatic markers by means of immunofluorescence or RT-PCR and do not include functional analysis. Before their clinical use, specific functional assays should be also routinely included and standardized. Moreover, it should be also considered that cells should be phenotypically stable over a long period and safe before being applied clinically (1) and that in vivo maturation is expected (44), which may compensate the lack of a fully mature phenotype.

Safety assessment of HLCs

Complete characterization of the inherent immunogenicity profiles of iPSCs is also essential to define the best immunosuppressive strategy to favour their homing and engraftment (45). On the other hand, genetic modifications have profound functional implications and promote tumorigenic qualities, such as increased proliferation or higher frequencies of tumor-initiating cells (10). In this sense, the development of well-defined methods to reduce the expression of oncogenic genes in iPSCs is necessary to reduce the tumorigenicity of transplanted cells (46). The prospective removal (e.g., removal before transplantation) of
tumorigenic cells using surface antigens has been proposed and would provide the highest level of safety (47). Finally, the safe distribution of the cells should be also assessed and it has been proved in different animal models.

**Challenges of HLCs derived from iPSCs**

Long-term safety, tolerability and efficacy of iPSCs-derived hepatic cell-based treatments are key issues to be addressed prior to the translation of cell therapy to the clinical practice. Human iPSCs technology is still in its infancy and a number of hurdles need to be overcome before these cell therapies become a reality. Reprogramming itself can induce both genetic and epigenetic defects in iPSCs (48), and it is possible that those defects can directly or indirectly promote immunogenicity and tumorigenicity in vivo, raising safety concerns. In fact, it has been recently reported that the genomic translocation detected in the iPSCs will create fusion proteins and new immunogenic determinants (49).

**Immunogenicity of HLCs derived from iPSCs**

Theoretically, the autologous HLCs derived from a patient should be immune tolerant without any concern of immune rejections after transplantation into the same patient (50). However, it has been reported that even syngeneic iPSC-derived cells can be immunogenic in syngeneic hosts by using a teratoma transplantation model (51).

On the other hand, it has been shown a differential immune recognition between differentiated and undifferentiated pluripotent cells (11,46,52). Undifferentiated, but not differentiated, PSCs have been reported to possess immune privilege properties, and would thus be less susceptible to immune recognition than their derived differentiated cell types (46,52). iPSCs are epigenetically abnormal and inherited epigenetic signature of parental cells could explain abnormal expression of immunogenic proteins expressed during the differentiation of iPSCs (53). In fact, undifferentiated iPSCs show a lower expression of MHC class I, and the complete absence of MHC class II antigens compared to their differentiated progeny (54). Generation of normal HLA-typed iPSCs banks homozygous for HLA-A, -B, and -DR, the most important loci to match, could be a solution to use compatible donors and reduce allograft rejection (55). Nevertheless, further in vivo studies will be needed to determine to what extent appropriately and terminally differentiated pluripotent cell lineages will induce the immunoresponse after transplantation (10).

**Tumorigenicity and safety of gene editing**

Genome editing has evolved to address the need for improving the efficiency and specificity of traditional genome modification achieved by homologous recombination. However, due to the possibility of off-target effects (edits in the wrong place) and mosaicism (when some cells carry the edit but others do not), safety is of primary concern. An important safety issue for genome editing is the accurate assessment of off-target cleavage by endonucleases and the effects of non-specific activity (56). The enhancement to efficiency and safety of genome editing will bring cell-based therapies closer to the clinic for patients with inborn metabolic diseases (56).

In this context, tumorigenicity is a serious bottleneck for developing individualized hepatic cell therapy using patients’ own or compatible banked iPSCs (48,49). The intrinsic qualities of self-renewal and pluripotency that make PSCs so therapeutically promising are also responsible for an equally fundamental tumorigenic potential. The induction of pluripotency by reprogramming somatic cells has been linked to tumorigenic transformation by creating genomic aberrations at chromosomal and sub-chromosomal stochastically generated levels (57). This genomic instability of iPSCs can create new immunogenic determinants like the tumor antigens developed in cancer cells (48,49). The potential risk of tumorigenicity has been evaluated in recent years in small and large animal studies. Ultimately, genetic modifications can promote tumorigenic qualities, such as increased proliferation, growth factor independence and higher frequency of tumor-initiating cells.

Protocols for the elimination of the remaining PSCs in the HLCs cultures have been described but the risk of teratoma formation after transplantation remains and could be an obstacle for clinical grade manufacturing (58).

**Engraftment of transplanted cells**

Engraftment potential of iPSC-HLCs, both short- and long-term, is another relevant key issue associated with the success of hepatic cell therapy. The proliferative advantage of transplanted native hepatocytes over resident hepatocytes to efficiently repopulate the liver has been shown in a number of animal models (59). Concerns associated with safety and engraftment potential of iPSC-HLCs are currently being addressed using these animal models.
models. Among the many strategies aimed at increasing homing, engraftment and proliferation of transplanted cells, partial reversible embolization of the portal vein (60) and irradiation of the native liver could be applicable to human iPSC-HLCs therapy (61).

**Large-scale and GMP production of HLCs**

Although stem cell technology offers multiple treatment options, important technical advances are necessary before the clinical application of HLCs derived from iPSCs. In this sense, the starting material should be obtained and processed under good manufacturing practices (GMP) guidelines (62). Additionally, maintenance, expansion and differentiation of iPSCs will require GMP compatible conditions. Generation of HLA-typed iPS cells banks will lead to minimize the risk of allograft rejection. Finally, challenges remain to generate large quantity of well-differentiated cells to achieve enough material for transplantation and the obvious tremendous cost of getting enough tissue mass to maintain the hepatic functionality (63). On the other hand, a total of $2.0 \times 10^8$ viable cells/kg for each patient has been proposed as an optimal and safe dose in humans (64), but, considering that most of the patients are children and a possible future routine use, the costs would be reduced. Alternatively, it has been proposed the use of rats as bioreactors to get the sufficient amount of cells (65).

**Potential therapeutic use of iPSCs in liver diseases**

The demand for LT outweighs supply which leads to an increased morbidity and mortality among waiting-list patients. Cell-based liver therapies are envisaged as a useful therapeutic option to replace or complement whole organ transplantation by recovering and stabilizing the lost metabolic function for acute and chronic liver diseases (Table 1). However, success is hampered by the scarce availability of liver tissue to isolate good-quality cells, the low engraftment capability of the cells into the host liver mainly due to the rejection of transplanted cells as well as the difficulties to monitor and predict rejection (64). Human iPSCs differentiated towards the hepatic lineage could establish the basis for producing autologous cell therapies that would avoid immune rejection but that would require gene correction and/or help to create biobanks of readily available HLCs for the emergency treatment of ALF. Table 1 summarizes liver diseases susceptible to being treated with hepatic cell transplantation.

**Inborn metabolic diseases**

Liver-based inborn metabolic disorders are rare diseases characterized by defects in hepatic enzymes or proteins with metabolic functions, such as receptors or transporters. The management of patients with metabolic disorders is complex and LT may not always be the first therapeutic option in children due to invasiveness, recipient’s age or the need of lifelong immunosuppressive therapy (93,94). For those patients for whom the risks of LT are not justified, cell transplantation could be an appropriate therapeutic option to provide the missing liver function without replacing the whole organ. In this sense, hepatocyte transplantation has been used in pediatric patients with a number of inborn hepatic metabolic disorders (Crigler-Najjar disease, deficiencies in enzymes of the urea cycle, AAT1 deficiency) (93,94) with encouraging results.

Cell therapy for hereditary liver diseases with patient-specific iPSC-derived HLCs would require gene correction before or after reprogramming. Patient-specific iPSCs are considered a promising alternative for an ex vivo gene therapy approach that could be used for cell therapy applications and curing diseases. Personalized cell therapy using iPSCs would likely avoid rejection, and thus immunosuppression, which would be an important advantage over LT and hepatocyte transplantation. However, the immunogenicity of iPSCs and their derivatives is still controversial (50,51).

**Other liver diseases**

Hepatocyte transplantation has also been foreseen as a useful therapeutic approach for bridging patients to LT and is indicated for providing metabolic support during ALF and ACLF in which the only hope for survival for most patients is either LT, or facilitating liver regeneration of the native organ (93) (Table 1). Additionally, some patients suffering from ESLD, but with preserved liver function and no indication of LT could benefit from HLCs transplantation because cell therapy could delay disease progression and associated complications (95). Recently, the use of this strategy has been proposed to reverse the inflammation and fibrosis in non-alcoholic fatty liver disease, one of the commonest chronic liver diseases (96).
In these cases, time to make, mature and expand patient's cells into iPSCs and then into HLCs may be prohibitive to particularly ALF treatment (8). For this reason, an allogeneic source of HLCs should be prepared for and readily available for their use in the treatment of ALF or ACLF which would imply the use of immunosuppression as when human hepatocytes are transplanted.

**Final remarks**

There is an urgent requirement for an unlimited source of human hepatocytes for transplantation that could be solved by using HLCs derived from iPSCs. The current challenge in this field is to develop reliable processes to differentiate stem cells into functional and engraftable cells that exhibit phenotypic stability and with no risk of tumorigenicity. Key issues should be addressed to improve clinical outcomes of hepatic cell therapy: (I) development of well-defined methods to generate iPSCs without viral integration and reduce the expression of oncogenic genes, (II) evaluation of unpredictable risks of using gene editing, (III) refinement of protocols for their complete and safe hepatic differentiation into HLCs comparable to their in vivo counterparts, (IV) expansion and production of cells under GMP conditions, (V) creation of iPSCs and HLCs biobanks, (VI) prospective removal (e.g., removal before transplantation) of tumorigenic cells through utilizing intrinsic cell properties, such as surface antigens, to minimize the tumorigenicity of transplanted cells, (VII) optimization and refinement of immunological strategies for transplant recipients, (VIII) defining preconditioning treatments of the recipient liver to enhance the engraftment and proliferation of donor cells, and (IX) development of non-invasive and accurate tracking or monitoring methods for cell survival and engraftment.

**Table 1** Indications of cell therapy for liver diseases

<table>
<thead>
<tr>
<th>Indications of cell therapy for liver diseases</th>
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<tr>
<td><strong>Inborn metabolic disorders</strong></td>
</tr>
<tr>
<td>- Criggler-Najjar syndrome type 1 (66,67)</td>
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<tr>
<td>- Urea cycle defects (67-71)</td>
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<tr>
<td>- Ornithine transcarbamylase</td>
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<tr>
<td>- Carbamoyl phosphate synthetase type 1 deficiency</td>
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<td>- Citrullinemia</td>
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<tr>
<td>- Argininosuccinatelyase deficiency</td>
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<tr>
<td><strong>Glycogen storage disease type I (67,72-75)</strong></td>
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<tr>
<td>- Type I a</td>
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<tr>
<td>- Type Ib</td>
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<tr>
<td>- Refsum disease (76)</td>
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<tr>
<td>- Phenylketonuria (64,75)</td>
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<tr>
<td>- Tyrosinemia type 1 (67)</td>
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<tr>
<td>- Factor VII deficiency (77,78)</td>
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<tr>
<td>- Primary hyperoxaluria (79)</td>
</tr>
<tr>
<td>- Familial hypercholesterolaemia (80,81)</td>
</tr>
<tr>
<td>- Progressive familial intrahepatic cholestasis (78,82)</td>
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| **Other liver diseases**                     |
| - Patients with indication of LT (83-89)    |
| - Acute liver failure                       |
| - Acute on chronic liver failure            |
| - Non-alcoholic steatohepatitis             |
| - Patients with no indication of LT (90-92) |
| - Patients with postoperative liver failure after partial hepatic resection |
| - Patients with chronic decompensation of an end-stage liver disease |
| - Other patients with no indication of LT (i.e., advanced age, alcoholism) |

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post-transplantation. Finally, such new strategies should be rigorously tested and validated in preclinical studies before they can be safely transferred to clinical practice with patients.

**Acknowledgments**

The authors acknowledge financial support from the ALIVE Foundation.

**Funding:** This work has been supported by the Institute of Health Carlos III (Plan Estatal de I+D+i 2013-2016) and cofinanced by the European Regional Development Fund “A way to achieve Europe” (FEDER) through grant CP16/00097 and PI18/00993. L Tolosa was supported by ISCIII CP16/00097.

**Footnote**

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Front Immunol 2017;8:645.


