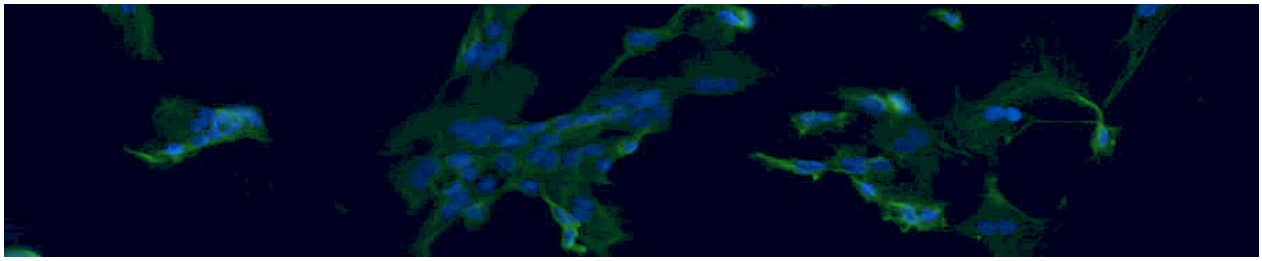
Université de Liège
Faculté de Médecine
Service de Chirurgie Abdominale, des Glandes Endocrines et de Transplantation

Mesenchymal Stromal Cells in Liver Transplantation

Translational Investigations

from a phase I-II Clinical Trial to

a Rodent Model of Acute Graft Rejection



Dr Morgan VANDERMEULEN

Promoteurs: Professeur François JOURET

Professeur Olivier DETRY

Thèse présentée en vue de l'obtention du grade de Doctorat en Sciences Biomédicales et Pharmaceutiques

Année académique 2023-2024

« La science est une histoire sans fin. La question n'est pas de savoir qui a raison, qui a tort. Le but est de progresser.

[…]

Les plus grands défis ne sont pas surmontés quand nos prédictions sont exactes, mais quand elles ne le sont pas ! Nous découvrons alors de nouvelles informations qui nous obligent à modifier tout ce que nous croyions savoir. »

Stephen Hawking

Avant-propos

Je souhaiterais remercier chaleureusement toutes les personnes qui m'ont aidé, de près ou de loin, à réaliser ce travail.

Je tiens tout d'abord à exprimer ma profonde gratitude envers mes promoteurs, les Professeurs F. Jouret, chef de service de néphrologie du CHU de Liège, et O. Detry, chirurgien dans le service de chirurgie digestive et de transplantation du CHU de Liège, pour votre guidance, votre soutien infaillible et votre expertise tout au long de cette aventure scientifique. Vos conseils éclairés, votre disponibilité et votre bienveillance ont été essentiels dans la réalisation de cette thèse, et je suis extrêmement reconnaissant de vous avoir eu comme guides tout au long de ce parcours.

Je souhaite également remercier sincèrement le Pr J.-O. Defraigne qui a accepté de présider le Jury de cette thèse ainsi que les Professeurs Y. Beguin, P.-V. Drion, A. De Roover et J. Delwaide, membres de mon comité de thèse. Vos critiques constructives, vos remarques éclairées et vos encouragements lors des réunions de Comité de thèse ont enrichi mon travail de recherche et ont été une source d'inspiration et de motivation.

Je tiens également à exprimer ma profonde reconnaissance envers les membres du jury extérieurs, les Professeurs J. Pirenne et G. Oldani, qui me font l'honneur de participer au jury de ce thèse. Un remerciement tout particulier au Professeur G. Oldani, dont les nombreux conseils m'ont permis d'apprendre le modèle de transplantation hépatique chez le rat.

Je remercie aussi toutes les personnes qui ont permis la réalisation et l'interprétation de la partie clinique de ce travail, les Professeurs Y. Beguin et F. Baron, les Docteurs M. Hannon, C. Lechanteur, A. Briquet, O. Giet et E. Baudoux du service d'Hématologie, du laboratoire d'hématologie du GIGA et du LTCG, le Professeur F. Jouret et le docteur P. Erpicum, du service de néphrologie, le Professeur O. Detry et le Docteur M. Mohamed-Wais en chirurgie abdominale, les coordinatrices de transplantation, les docteurs N. Bletard et J Somja du

département d'anatomopathologie. Je remercie particulièrement Monsieur G. Maggipinto, du laboratoire d'immuno-hématologie, pour les nombreuses heures passées à mes côtés pour m'aider à analyser et à interpréter les Luminex et anticorps anti-HLA.

Je remercie chaleureusement les membres de la merveilleuse équipe du « LTRN » et du CREDEC (Centre de Recherche et Développement en Chirurgie), les actuels et les anciens, qui ont rendu cette expérience au laboratoire très agréable. Merci tout particulièrement à Pascal et Pauline, qui ont été mes « exemples à suivre », pour les pauses-café et les nombreux conseils, mais également à Badr et Justine, pour l'agréable bout de chemin parcouru ensemble, et les galères partagées. Merci aussi à Jean-Paul, pour son aide précieuse et ses bons conseils. Merci à Laurence. Merci également aux « voisines », Cécile et Christelle pour les temps de midi, l'ambiance chaleureuse, leur écoute et leurs conseils.

Merci à S. Raafat et Céline Vaniwinge, de la plateforme GIGA d'imagerie cellulaire et cytométrie en flux pour leur aide au paramétrage et à l'interprétation des données du FACS. Merci également à tous les membres du GIGA cardiovasculaire avec qui j'ai eu le plaisir de travailler.

J'aimerais également adresser mes sincères remerciements à mes maintenant collègues du service de chirurgie abdominale, des glandes endocrines et de transplantation du CHU de Liège (Pr A. De Roover) ainsi qu'à tous les chirurgiens rencontrés lors de ma formation. Leurs conseils bienveillants à mon égard, leur soutien et leur énorme contribution à ma formation chirurgicale ont été inestimables.

Je ne peux que remercier de tout cœur ma famille, et particulièrement mon papa et ma sœur, pour leur soutien et leur amour indéfectible. Vos encouragements constants, vos conseils avisés et votre bienveillance ont été les fondements sur lesquels j'ai pu construire ma vie et mes réussites. Merci pour tout ce que vous avez fait pour moi.

Je tiens à exprimer ma profonde gratitude envers mes amis, d'ici et d'ailleurs, qui persistent malgré les années. Ils ont été une source inestimable de soutien et ont souvent été la bouffée

d’oxygène nécessaire à la poursuite de cette aventure; particulièrement Nico, Martin, Colt, Dam, Francis, Bo, Alex, Jerem, Clem, Didjé, NicoW, Chris, Mouks, Marty, Philou, OBK, les copains du tctc, de MMO, et tous les autres… Merci !

A Mathilde, merci pour ta présence, ton soutien, ta compréhension, ta confiance et ton amour. Merci pour tous ces beaux moments passés et à venir. Merci d’être là pour moi.

La réalisation de ces travaux a été possible grâce à l’aide financière du Fonds National de la Recherche Scientifique via un mandat de Candidat Spécialiste Doctorant et un Crédit de Recherche, au soutien de la Fondation Léon Frédéricq, de l’Université et du CHU de Liège. Je les en remercie.

SUMMARY

Liver transplantation (LTx) is presently considered as the most effective treatment for the majority of end-stage liver diseases, with highly favorable results in both the short- and long-term. However, the long-term use of immunosuppressive drugs (ISDs) exposes liver transplant recipient (LTR) to numerous side effects that restrict these excellent outcomes. Furthermore, the use of marginal livers to address the shortage of liver grafts exposes the recipient to more complications, primarily due to ischemia-reperfusion injuries (IRI). Many strategies, aiming at preventing and attenuating IRI in the liver and limiting the use of ISD and their linked side-effects, are studied. Mesenchymal *stromal* cells (MSCs), thanks to their immunosuppressive properties including their beneficial effect on regulatory T cells (Treg), could have the potential to modulate immunity to prevent acute rejection (AR) after LTx without (or with lower doses of) ISDs as well as to reduce IRI. In addition to very encouraging preclinical results, the safety and efficacy of MSC therapy in human subjects require additional validation before being expanded for broader use in large-scale LTx programs. Furthermore, many parameters about the use of MSCs including the optimal ISDs to combine with MSCs in order to achieve a synergistic effect, still need to be defined. Here, we report on the first prospective phase I-II controlled clinical trial investigating the safety and tolerability of a single allogeneic bone marrow-derived MSC injection following deceased donor LTx in addition to conventional ISD regimen. No potential adverse effect related to MSC treatment was observed, particularly there was no increase in the rate of opportunistic complications which were comparable in both the MSC and control groups. Nevertheless, our data suggest that MSC may promote the emergence of donor-specific antibodies (DSAs) against liver or MSC donors, encouraging the monitoring of DSA in future studies. Moving from bed to bench-side, we showed, *in vivo,* that MSC and the association of everolimus and MSC could be beneficial for regulatory Treg expansion. However, in our LTx model in rat, 2 MSC injections after LTx were inefficient in preventing severe AR. Additionally, when compared to everolimus alone, there was no difference in the effects of the association of MSCs with everolimus. One of our hypotheses is that MSCs should be injected earlier.

As a whole, our work brings new data supporting the use of MSC for LTx. Further investigations on MSCs in LTx will probably help to design more efficient (pre)clinical studies.

RÉSUMÉ

La transplantation hépatique (TH) est actuellement considérée comme le traitement le plus efficace pour la plupart des maladies du foie en phase terminale, avec des résultats très favorables à la fois à court et à long termes. Cependant, l'utilisation à long terme de médicaments immunosuppresseurs (MIS) expose le receveur de greffe de foie (RGF) à de nombreux effets secondaires qui limitent ces résultats favorables. En outre, l'utilisation nécessaire de greffons marginaux pour pallier le manque de greffons expose les RGFs à plus de complications, principalement en raison de lésions d'ischémie-reperfusion (IRI). De nombreuses stratégies sont étudiées, visant à prévenir et atténuer les IRI dans le foie et à diminuer l'utilisation de MIS ainsi que leurs effets secondaires. Les cellules stromales mésenchymateuses (CSM), grâce à leurs propriétés immunosuppressives y compris leur effet bénéfique sur les cellules T régulatrices (Treg), pourraient avoir le potentiel de moduler l'immunité pour prévenir le rejet aigu (RA) après TH sans ou avec des doses plus faibles de MIS, ainsi que pour réduire l'IRI. Malgré des résultats précliniques encourageants, la sécurité et l'efficacité des traitements par CSM chez l'homme nécessitent une validation supplémentaire avant qu'ils soient étendus en routine aux programmes de TH. En outre, parmi beaucoup d'autres paramètres, le choix des DIS à associer aux CSM pour tenter d'obtenir un effet synergique doit encore être défini. Ici, nous rapportons le premier essai clinique contrôlé prospectif de phase I-II étudiant la sécurité et la tolérance d'une seule injection de CSM après TH (au 3ème jour post-TH) en plus d'un traitement immunosuppresseur conventionnel. Aucun effet indésirable potentiel lié à l'utilisation de CSM n'a été observé, en particulier aucune augmentation du taux de complications opportunistes. Néanmoins, nos données suggèrent que les CSM pourraient favoriser l'apparition d'anticorps spécifiques aux donneurs de foie et de CSM. En parallèle, *in vivo*, nous avons confirmé que les CSM de même que leur association avec de l'everolimus étaient bénéfiques pour les Tregs. Cependant, dans un modèle de TH chez le rat, 2 injections de CSM après TH se sont révélées inefficaces pour prévenir le RA et, par rapport à l'éverolimus seul, il n'y a pas eu d'avantage démontré à associer les CSM et l'everolimus. L'une de nos hypothèses est que les CSMs devraient être injectées plus tôt, au moment de la TH par exemple.

Dans l'ensemble, notre travail apporte de nouvelles données qui soutiennent l'utilisation des CSM en TH. Des recherches supplémentaires sur l'utilisation de CSM dans la TH aideront probablement à concevoir de nouvelles études (pré)cliniques pour démontrer leur efficacité.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AR	acute rejection
APC	antigen presenting cells
ATP	adenosine triphosphate
ABMR	antibody mediated rejection
BM	bone marrow
BPAR	biopsy-proven acute rejection
CD	cluster of differentiation
CM	conditioned medium
CNI	calcineurin-inhibitor
CsA	cyclosporine A
CTLA4	cytotoxic T lymphocyte-associated antigen 4
C4d	complement component 4d
DA	Dark Agouti
DBD	donation after brainstem death
DC	dendritic cell
DCD	donation after circulatory death
DSAs	donor-specific antibodies
EV	extracellular vesicles
EVR	everolimus
FoxP3	forkhead box protein 3
GVHD	graft-versus-host-disease
HO	heme oxygenase
HLA	human leukocyte antigen
HSCs	hepatic stellate cells
IDO	indoleamine dioxygenase
IFN	interferon
IL	interleukine
IRI	ischemia-reperfusion injury
IV	intravenous
ISD	immunosuppressive drug
KC	Kupffer cell
KTR	kidney transplant recipient
KTx	kidney transplantation
LSECs	liver sinusoidal endothelial cells
LTx	liver transplantation
LTR	liver transplant recipient
MAPCs	multipotent adult progenitor cells
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
MSC	mesenchymal stromal cell
mTOR	mammalian target of rapamycin
NK	natural killer

NMP	normothermic machine perfusion
OLT	orthotopic liver transplantation
PDL-1	programmed cell death protein ligand
PGE2	prostaglandin E2
PVG	Piebald Virol Glaxo
rATG	rabbit anti-thymocyte globulins
SOT	solid-organ transplantation
TCMR	T-cell mediated rejection
TCR	T-cell receptor
TGF	tumor growth factor
Th	T helper cell
TNF	tumor necrosis factor
Treg	regulatory T cell
UC	umbilical cord
+	positive
-	negative

CHAPTER I. INTRODUCTION

CHAPTER I. INTRODUCTION

1 Liver Transplantation

1.1 History of liver transplantation

In 1963, Thomas Starzl performed the first human liver transplantations (LTx) at the University of Colorado, Denver, USA. Nevertheless, outcomes from his and other pioneers' experiences were very poor with a survival of less than 1 month, and LTx remained experimental until the 80's [3]. The improvement of organ preservation techniques as well as advancements in the management of the recipient with improvement of anesthesia, perioperative care and surgical techniques led to improved LTx outcomes, but the real revolution in LTx came with the discovery and the development of cyclosporine A (CsA) during the 70's [4]. The works of Roy Calne and his team from the Cambridge-King's group who initiated in 1979 the cyclosporine clinical trials in LTx changed the face of transplantation [5]. From there, the introduction of a combination of CsA (followed some years later by tacrolimus) and prednisone in the early 80's by Starzl, has made it possible to achieve prolonged survival after LTx [6]. Very importantly, in 1983, the U.S. National Institutes of Health Consensus Development Conference concluded that LTx was "clinical service", leading to the adoption of LTx as a validated clinical procedure worldwide [3].

1.2 Outcomes and limitations

Nowadays, LTx is recognized as a life-saving treatment modality for an increasing number of indications with excellent long-term survival. Nevertheless, due to the limited organ supply, approximatively 20% of patients die while on the waiting list. To overcome this organ shortage, the criteria for organ selection, such as livers donated after circulatory death (DCD) or steatotic livers, has been extended. However, these high-risk grafts are more

susceptible to ischemia-reperfusion injury (IRI), with increased graft loss rates due to dysfunction or ischemic cholangiopathy (see infra) [7, 8].

On the other hand, immunosuppressive drugs (ISDs) used to prevent and cure allograft rejection, are also a significant factor limiting long-term outcomes due to their toxicities (malignancies, infections, metabolic syndrome, renal, cardiovascular and neurological toxicity,…) [9].

New strategies aiming at preventing and attenuating IRI in the liver graft and limiting the use of ISD and their associated side-effects are needed to increase access to this life-saving treatment modality and to improve long-term outcomes of LTx.

1.3 Hepatic ischemia reperfusion injury

IRI is defined as tissue damages that occur when an organ blood flow is interrupted (ischemia) and subsequently resumed (reperfusion) [10]. It is an unavoidable consequence of transplantation that the allograft sustains some degree of ischemia. This happens during organ harvesting as well as during organ transportation to the transplant facility (known as the cold ischemia time since the liver is stored in the cold), and during organ implantation (known as the warm ischemia time). Compared to donation after brainstem death (DBD), an additional warm ischemia is unavoidable in the case of DCD due to the delay between circulatory collapse and liver procurement [11]. As a result of ischemia, intracellular adenosine triphosphate (ATP) depletion occurs in liver sinusoidal endothelial cells (LSEC) and hepatocytes, leading to cell injury and death [12, 13]. After reperfusion, the recruitment and activation of neutrophils and Kupffer cells (KC) within the liver graft exacerbate IRI through the release of reactive oxygen species and pro-inflammatory cytokines such as interleukine (IL) -1, tumor necrosis factor (TNF) -α and interferon (IFN) -γ, eliciting further damages with recruitment of leukocytes to the allograft. Ultimately, IRI activates hepatic stellate cells (HSCs), which facilitate long-term recovery from IRI but can result in allograft fibrosis [14].

Hepatic IRI negatively affects allograft outcomes with an increased rate of complications such as graft dysfunction, biliary strictures and acute and chronic rejection. The intensity of IRI is affected by the characteristics of the donors. The shortage of available organs

for transplantation leads to the use of “extended criteria donors” who present a higher risk for IRI, especially for steatotic and DCD livers. Finally, patients experiencing severe IRI show poorer graft function and survival after LTx [2, 15]. Many therapeutics have been studied to alleviate IRI after LTx including anti-inflammatory drug cocktails, gene-based therapy and cell-based therapy, but to date, none has shown convincing clinical results [12, 16].

1.4 Liver transplant rejection

Liver allograft rejection is due to the recognition of the “non-self” alloantigens by host immune cells after LTx. The most common mechanism of rejection is T cell-mediated rejection (TCMR) with an infiltration of the liver by T cells and activated innate immune cells leading to allograft damage. Antibody-mediated rejection (ABMR) was considered as a rare phenomenon after LTx. Nevertheless, recent data suggest that liver damage due to donor-specific antibodies (DSAs) may occur.

1.4.1 T cell-mediated rejection

1.4.1.1 Basis of TCMR

The major histocompatibility complex (MHC) molecules represent the main antigens responsible for rejection. In humans, the MHC complex is represented by the Human Leukocyte Antigen (HLA). MHC class I molecules are expressed at the surface of all nucleated cells and present intracellular epitopes to cluster of differentiation (CD)8 positive (+) cytotoxic T cells. MHC class II molecules are generally expressed only on antigen-presenting cells (APC) such as dendritic cells (DCs) and KCs and present epitopes derived from extracellular material to $CD4^+$ helper T cells (Th) [2]. In case of liver inflammation, expression of both MHC-I and MHC-II is upregulated and rejection is thus promoted **(Figure 1)**.

After LTx, alloantigen presentation to T cells (mainly by DCs) is a major step in the rejection process. The initial actor of T-cell activation is represented by a large number of donor-derived DCs (the so-called “passenger leukocytes”) migrating to the recipient’s

lymphoid tissues. These DCs provide a first stimulus for naive $CD4^+$ T cells by recognition of the presented alloantigen but also of MHC molecules by T-cell receptors (TCR). This constitutes the **direct pathway**. DC expressing MHC class I and II are able to activate both $CD4^+$ helper and $CD8^+$ cytotoxic T cells. The successful activation of T cells by DCs depends on different adhesion molecules and co-stimulatory molecule interactions expressed on their surface [17]. The priming of T cells leads to the activation of the calcineurin enzyme within T cells activating transcription factors that upregulate the expression of IL-2. IL-2 represents the main stimulus for T-cell proliferation through its interaction with the IL-2 receptor on the cell membrane. Besides the direct pathway, alloantigen recognition also depends on **the indirect pathway** in which the presentation of alloantigens is mediated by recipient DCs and other APCs presenting alloantigens through self-MHC. **The semi-direct pathway** activates T cells through presentation of intact donor MHC molecules on the membrane of recipient DCs. *In toto*, TCMR thus requires i) the presentation of alloantigens to $CD4^+$ naive T cells by APCs ii) interaction between the MHC and TCR iii) the presence of costimulatory molecules iv) IL-2 and other stimulatory cytokines, to lead to clonal expansion of alloreactive T cells. This leads to the expansion of effector B and T cells emerging from recipient lymphoid tissues and infiltrating the liver graft. These cells then drive an inflammatory response into the graft (infiltration by effector T cells, activated macrophages, secretory B cells, …) finally leading to allograft damage **(Figure 1)** [18].

Most of the time, TCMR occurs within 6 weeks after transplantation. Although minor increases of liver enzymes are commonly associated with TCMR, the sensitivity and specificity of abnormal tests are generally poor. Consequently, allograft core needle biopsy continues to be the gold standard for TCMR diagnosis [19-21]. The histological grading of rejection is based on the Banff working group classification and is given by a quantitative score based on the intensity of 1) portal inflammation [0-3] 2) bile duct inflammation damage [0-3] and 3) venous endothelial inflammation [0-3] [19]. Interestingly, fluorodeoxyglucose positron emission tomography combined with computed tomography has been shown to be linked with AR in LTR and could represent a potential noninvasive tool for AR detection but this requires further evaluation [22]. As long as the episode of rejection is appropriately managed, TCMR of the liver allograft has not been demonstrated to have a detrimental effect on graft or patient survival [20].

1.4.1.2 Effector cells

$CD8^+$ cytotoxic T cells

The main effector responsible for graft tissue damage is primed $CD8^+$ cytotoxic T effector cells ($CD62L^-$,$CCR7^-$,$CD45RA^-$ $CD45RO^-$). Binding of the TCR to donor-derived MHC class I molecules widely expressed on hepatocytes, endothelium and biliary epithelial cells activates cytolytic activity **(Figure 1)**. In addition to TCR:MHC interaction, activation of $CD8^+$ cytotoxic T cells also depends on interactions with other adhesion molecules such as LFA1-ICAM1 and CD2-LFA3 molecules [2, 23]. After priming, a part of naïve $CD8^+$ T ($CD62L^+$,$CCR7^+$,$CD45RA^+$ $CD45RO^-$) also differentiate into $CD8^+$ memory T cells ($CD62L^{+/-}$,$CCR7^{+/-}$,$CD45RA^-$ $CD45RO^+$)[24].

$CD4^+$ helper T cells

Depending on cytokine environment, different $CD4^+$ subsets are generated from naive $CD4^+$ T cells, each subset producing a different type of IL. The main driver of TCMR is considered to be the pro-inflammatory **Th1 response**. In the presence of IL-12, Th1 cells, continuously produce IL-2 and INF-γ which activate macrophages and $CD8^+$ cytotoxic T cells **(Figure 1)** [25]. On the contrary, in the presence of IL-4, the polarized **Th2 response** is generally considered as tolerogenic through the secretion of IL-4 and IL-10 that inhibit the Th1 response [26]. Nevertheless, Th2 cells have also been shown to be implicated as direct mediators of rejection notably through interaction with B cells leading to the production of DSAs **(Figure 1)** [2, 27].

As IL-17 is a potent neutrophil attractor, **Th17** cells also play an important role in tissue damage. They promote allograft rejection and have been associated with impaired tolerance [2, 28]. The secretion of IL-6 and IL-1ß by biliary epithelial cells seems to play an important role in maintaining Th17 differentiation. There appears to exist a dynamic equilibrium between Th17 and regulatory T-cell (**Treg**) differentiation, and these cell populations direct the immune response towards rejection or tolerance, respectively **(Figure 1)** [29].

Different markers allows to identify naive $CD4^+$ T cells ($CD25^-$, $CD45RA^+$, $CD45RO^-$, $CD127^+$) which can differentiate from naive to effector T cell ($CD25^+$, $CD45RA^{+/-}$, $CD45RO^{+/-}$, $CD127^-$) migrating to the site of rejection and memory T cell ($CD25^{-/+}$, $CD45RA^-$, $CD45RO^+$, $CD127^+$) responding faster in case of re-exposure to antigen [24].

Memory T cells

After exposure to a novel antigen, a small part of naive T cells also differentiates into memory T cells that will reside for a long time in peripheral tissues. In case of repeated exposure to the antigen, these cells will respond faster and stronger than naive T cells. Paradoxically, memory T cells appear to play also an important role in initial acute rejection (AR) despite the fact that the exposure of the allograft to the recipient is “new”. This is explained by the pre-transplant presence of primed alloreactive memory T cells due to previous environmental antigen exposure through blood transfusions, pregnancies, cross-reactivity between allogeneic peptides and previously encountered pathogen-related antigens, etc. [30, 31]. $CD4^+$-derived memory T cells are able to enhance the production of DSAs by B cells and so to induce ABMR, while $CD8^+$-derived memory T cells exert direct cytotoxic effects. Of note, immunosuppressive drugs are in most cases less effective on memory than on naive T cells. This could explain why some patients sometimes do not fully respond to conventional treatment for acute TMCR. Memory T cells are also believed to be one of the barriers to establishing allograft tolerance [2, 31, 32]

B cells

B cells probably play a minor role in the context of TCMR but can still enhance it by promoting the activation of T cells and the generation of memory T cells. B cells play a major role in ABMR through the production of antibodies **(Figure 1)** [2].

Macrophages

In a simplified view, macrophages can be polarized into two phenotypes, depending on numerous cytokines and growth factors. The so-called **M1 macrophages**, stimulated by

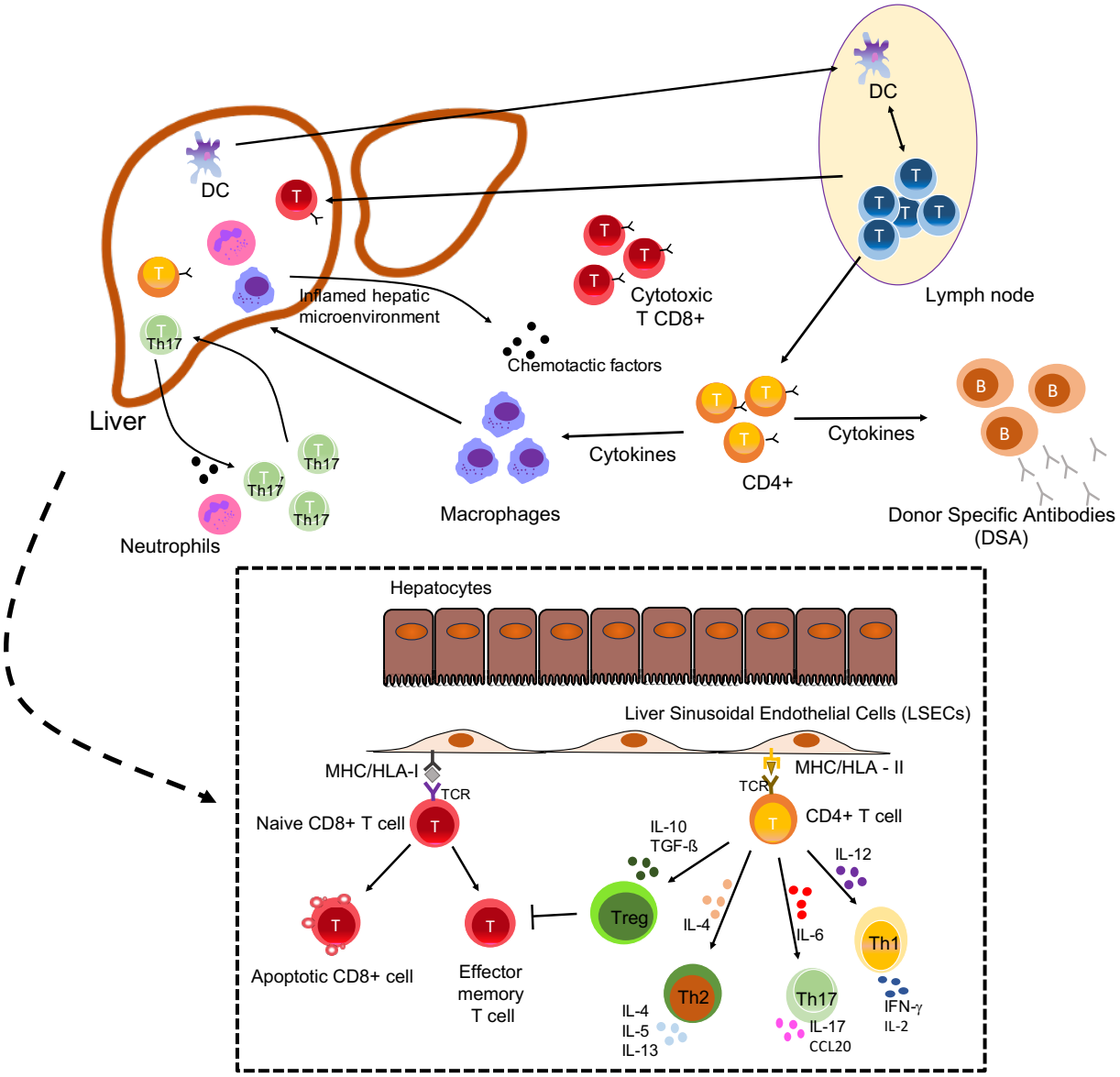


Figure 1. (A) Schematic view of the immunological basis of TCMR. Once activated, DCs migrate to lymphoid tissue and present foreign antigens on both MHC class I and II molecules. When they interact with naive alloreactive T cells, along with the presence of appropriate co-stimulatory molecules and a pro-inflammatory cytokine environment, this leads to the proliferation of alloreactive CD4+ and CD8+ effector T-cells, followed by the proliferation of B-cells. The migration of these immune cells to the liver is controlled by chemokines like CXCL9 and CXCL10, which interact with the CXCR3 receptor on lymphocytes, and involves complex interactions with specialized immunomodulatory liver sinusoidal endothelial cells. Specific mechanisms exist for the recruitment of lymphocyte subsets like Th17 cells. Additionally, cells of the innate immune system such as macrophages, neutrophils, and eosinophils are recruited to the liver. Together with effector T cells, they contribute to tissue damage. **(B)** Antigen presentation within the liver. Antigens are presented within the transplanted liver by various cell types, including endothelial cells, macrophages, hepatic stellate cells, hepatocytes, and biliary epithelium. Increased antigen presentation is observed during inflammatory episodes. Depending on the cytokine environment, interactions between naive lymphocytes and the liver lead to rejection or tolerance. The latter is characterized by the apoptosis of effector cells and a shift in T-cell differentiation toward the regulatory T-cell phenotype, facilitated by an immunosuppressive cytokine profile. CD, cluster of differentiation; DC, dendritic cell; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; TCR, T-cell receptor; TGF, tumor growth factor; Th, T helper cell; Treg, regulatory T cell. Adapted from Ronca *et al., 2020 [2].*

IFN-γ and lipopolysaccharides, are considered as pro-inflammatory. Most of the macrophages are polarized toward a M1 phenotype and produce inflammatory cytokines such as TNF-α, IFN-γ, IL-1, IL-12, IL-18, IL-6 and IL-23. In addition to cytokine-mediated effects, infiltrated macrophages produce reactive oxygen and nitrogen species leading to allograft damage and promoting AR[2, 33]. Intrahepatic macrophages (both donor- and recipient-derived) are also able to promote transplant rejection via activation of the adaptive immune response through the presentation of alloantigens in MHC class II molecules [33]. On the opposite, **M2 macrophages**, which are stimulated by IL-4 and IL-13, are considered as immunosuppressive, facilitating wound healing and tolerance [33].

Neutrophils

In acute TCMR, neutrophils are commonly recruited in large numbers to the liver allograft. They are an important actor of the inflammatory process that regulates the link between innate and adaptive immunity [34]. They can be recruited into the graft after IRI and are early effector cells in response to adaptive immunity (mainly in Th17 responses) **(Figure 1)**. Neutrophils are able to generate reactive oxygen species and a lot of enzymes mediating cell damage. In contrast, neutrophils can in some conditions show induced anti-inflammatory properties inhibiting T-cell activation, which may be important to promote or maintain tolerance [34, 35].

Eosinophils

The maturation and the migration of eosinophils in TCMR is generally stimulated by Th2 cytokines such as IL-4 and IL-5. Eosinophils have long been recognized as a major actor of TCMR in the liver and peripheral eosinophilia has been linked to rejection **(Figure 1)** [2, 36].

Natural killer cells

Natural killer (NK) cells represent up to 15% of blood lymphocytes but do not express T- (CD3) nor B-cell (CD20) antigen receptors, expressing instead CD16 and CD56 [2, 37]. Activation of NK cells can be stimulated by both the presence of activating signals and the loss

of inhibitory signals. In the allograft, activating signals are generally molecules expressed by the allograft tissue in response to a stress in a pro-inflammatory environment. Self MHC class I molecules normally interact with the inhibitory receptor on NK cells to protect “normal” cells against cytotoxicity mediated by NK cells. In the allograft, non-self MHC class I molecules expressed on the cells of the graft are not able to inhibit NK cells, making them susceptible to NK cell-mediated cytotoxicity (the “missing self” theory) [2, 31, 37, 38]. The role of NK cells in solid-organ transplantation (SOT) is still poorly understood, but it has been shown that they are implicated in both TCMR by boosting it but also in tolerance induction by different mechanisms (e.g., by killing donor-derived DCs) under some immunosuppressive conditions [31, 38].

1.4.2 Antibody-mediated rejection

Hyperacute rejection is the most severe kind of ABMR leading to graft failure within hours or days, but is exceedingly rare in LTx (e.g in ABO-incompatible grafts, xenotransplantation, …). While other forms of ABMR frequently occur after kidney, lung or heart transplantation (between 10 and 50%), the liver seems to be at low risk of ABMR even if the true incidence is unknown and under-recognized (estimated at 1% of liver transplant recipients, LTR) [2, 39]. Although long considered of little relevance in the field of LTx, a growing body of evidence suggests a potential deleterious role of both pre-formed and *de novo* DSAs causing DSA-mediated injuries in the liver (and most of the so-called “chronic allograft failures”) and influencing LTx outcomes [40-42].

1.4.2.1 DSA production

DSAs may be present in the recipient before transplantation (pre-formed DSAs) or may arise *de novo* after transplantation (*de novo* DSAs). In the case of pre-formed DSAs, the presence of alloantibodies before transplantation can be explained by mechanisms comparable to those explaining the presence of pre-transplant primed alloreactive memory T cells (see supra). On the other hand, the interaction of MHC molecules with naive B cells leads to the production of *de novo* antibodies (classical adaptive pathway) [2, 43]. The presence of

pro-inflammatory signals such as IL-1 leads to the activation of B cells that are able to internalize antigens and to present them through MHC class II molecules directly interacting with primed Th2 cells. Th2 cytokines (such as IL-4 and IL-5) and Th1 cytokines (such as IL-2) promote this process. Part of the activated B cells differentiate into plasma cells and start the production of DSAs. The other part of activated B cell migrates to lymph nodes to undergo a process of maturation, refinement and amplification of the antibody response [20, 31].

1.4.2.2 DSA target and effector function

A "two-hit" theory has been described to explain the mechanism of ABMR of the liver transplant[44]. An initial injury to the allograft, such as TCMR, viral hepatitis, hepatic ischemia or IRI, leads to the production of pro-inflammatory signals significantly upregulating the expression of non-self class I and II MHC molecules on endothelial cells of the liver allograft. These non-self molecules constitute the main targets of DSAs. Antibodies against MHC class I molecules usually appear earlier than anti-MHC class II antibodies (especially anti-HLA-DQ), which develop later in the post-transplant period. When DSAs and their target antigen interact, the complement component 1q binds to the Fc regions of bound DSAs, activating the classical pathway of the complement system and initiating an enzymatic cascade. This leads to the release of inflammatory mediators and chemotactic signals, which are known to be important for the activation of mast cells, basophils, and macrophages, for the recruitment of these cells and other granulocytes, including eosinophils and for increased vascular permeability [45].

Additionally, the complement also interacts with the adaptive immune system and promotes ABMR. As a sign of complement activation and ABMR, immunohistochemical complement component 4d (C4d) deposition on the allograft vasculature is used as a diagnostic marker. Despite the fact that the complement seems to be the primary cause of tissue injury in ABMR, complement-independent mechanisms also appear to be important. One mechanism involves the binding of DSAs to MHC molecules through Fc receptors on neutrophils, macrophages and NK cells[2, 46]. DSA binding to the allograft endothelium leads to endothelial swelling and injury as well as the development of platelet aggregates, microthrombi and inflammation. These changes typically appear as portal edema and

hemorrhage, bile ductular response and dilatation of portal microvessels, resulting in graft injury [2].

Acute ABMR

Acute ABMR generally occurs within the first weeks after LTx in highly sensitized patients (high preexisting DSA titers with high mean fluorescence intensity (MFI)) and is commonly described as mild allograft dysfunction (hyperbilirubinemia, thrombocytopenia, ...) while hyperacute allograft failure is anecdotal. Less commonly, late-onset acute ABMR (>6 months after LTx) can also develop in the case of *de novo* DSAs [19, 39, 47]. The h-(histopathology)-score (scoring from 0 to 3) and the C4d-score (scoring from 0 to 3) are both used to grade ABMR lesions [19]. The actual Banff criteria for definite diagnosis of acute ABMR is defined by the four items listed below (all four criteria are required) [19, 39]:

1) Histology: portal edema, microvasculitis (with eosinophils, neutrophils and monocytes), ductular reaction and endothelial cell hypertrophy
2) Increased DSAs in the serum
3) Diffuse microvascular C4d deposition
4) Exclusion of other complications or liver conditions causing the same kind of graft damages

Diagnosis can also be suspicious for ABMR in case of DSA positivity with a C4d-score + h-score >3 [19].

Chronic ABMR

Because of the lack of specific biochemical and histologic characteristics, the prevalence of chronic ABMR is probably underestimated and, despite histological evidence of allograft injury, the majority of patients with chronic ABMR have normal liver tests [48]. Chronic ABMR can be caused by different mechanisms including antibody-mediated effector pathways and antibody-mediated complement activation, which result in inflammation and fibrosis of the graft. Along with other factors including transplant vasculopathy with intimal

hyperplasia and endothelial cell death, non-inflammatory fibrosis has also been observed in the presence of DSAs and may be connected to HSC activation [39]. LTR who are inadequately immunosuppressed (low ISD levels or poor adherence) are at higher risk of developing DSAs [49].

The actual consensus criteria for suspected chronic ABMR are [19]:

1) Histological injury compatible with chronic ABMR (mild portal and/or perivenular mononuclear inflammation with interface and/or perivenular necroinflammatory activity + moderate sinusoidal, periportal and/or perivenular fibrosis)
2) Focal C4d deposition in more than 10% of portal tracts
3) DSA detection in serum within 3 months of biopsy
4) Realistic exclusion of other liver insults

Plasma cell-rich rejection

Formerly called *de novo* auto-immune hepatitis, plasma cell hepatitis or plasma cell-rich rejection remains a poorly understood and atypical cause of late allograft dysfunction resembling liver autoimmune hepatitis. The so-called “plasma cell-rich rejection” is due to a mixed TCMR/ ABMR etiology overlapping with autoimmunity in patients without a pre-existing auto-immune hepatitis and is associated with C4d staining of portal capillaries [19].

Effect of DSA on liver graft function and outcomes

The precise role of DSAs in LTx remains uncertain. As opposed to the kidney, the liver has been thought for a long time to be unaffected by humoral responses and a cross-match test is still not considered as useful in LTx, although a series of LTR with a positive cross-match showed decreased graft survival [50]. Even if it has not been confirmed in large RCT, experts think that the presence of DSAs influences liver graft outcomes and may be potentially associated with i) hyperAR ii) acute ABMR iii) TCMR iv) chronic rejection v) steroid-resistant rejection vi) plasma cell-rich rejection vii) idiopathic fibrosis viii) anastomotic biliary stricture viii) nodular regenerative hyperplasia and portal venopathy [43].

DSAs may target class I or class II HLA antigens as well as non-HLA antigens like the angiotensin II type 1 receptor. DSAs that probably have the main impact on clinical outcomes are class II DSAs as class II antigen expression on the liver endothelium, hepatocytes and bile ducts occurs after an initial injury to the liver allograft. While pre-formed class I DSAs have been usually shown to disappear after LTx, pre-formed class II DSAs tend to persist and have been linked to worse results [51].

In a large retrospective study, it has been shown that approximatively 8% of LTR develop *de novo* DSAs 1-year post-transplantation. Nearly all *de novo* DSAs were against class II antigens with the bulk of these being anti-DQ. In multivariate analysis, non-adherence to treatment and low immunosuppression levels were identified as risk factors for *de novo* DSA formation. The appearance of *de novo* DSAs after LTx has been shown to be an independent risk factor of graft loss and death [42]. In a recent study, it was also shown that *de novo* DSAs developed within the first year after LTx are associated with a significantly higher risk of AR and liver graft fibrosis [52]. In another study in liver-transplanted children, a high MFI sum (sum of the MFI values of the detected DSAs) of class II DSA was associated with fibrosis [53]. In a French cohort also evaluating high MFI sums in adult LTR, such an association between pre-formed DSAs and fibrosis could not be confirmed [54]. Of note, the positivity cut-off of MFI for DSA positivity with potential clinical significance is not clearly standardized and varies according to laboratories and manufacturers but a cuff-off of 1500 is commonly used for DSA positivity [19].

Overall, DSAs are observed in a small percentage of adult LTx patients, with a small global impact on graft and patient outcomes. Nevertheless, this topic clearly needs to be more extensively studied.

1.4.3 The liver: an “immune-privileged organ”

The particular anatomy of the liver gives it a privileged immunological status. Due to its particular blood supply, the low flow in fenestrated sinusoids facilitates the interaction of antigens and immune cells without sensitization [39]. Moreover, the large endothelial surface is capable of absorbing circulating antibodies and KCs lining in the sinusoid are able to clear circulating immune complexes of soluble MHC class I molecules and alloantibodies [55].

In simultaneous liver-kidney transplantation, the liver provides protection against hyperacute rejection and acute ABMR of the kidney, regardless of the cross-match, but also reduce the occurrence of acute and chronic cellular rejection [56]. Very interestingly, it has been shown in highly sensitized, cross-match positive recipients that with a combined partial auxiliary liver and kidney transplantation from the same donor, the liver protected the kidney from harmful DSAs [57]. This protective effect of the liver can also be seen in simultaneous liver heart/intestine with lower rejection rates in the associated organ [39, 58, 59].

1.4.4 Prevention and treatment of graft rejection

1.4.4.1 Generalities

As described above, allotransplantation induces a robust humoral and cellular immune response against the liver allograft. In the absence of prophylaxis and/or therapy, acute TCMR would swiftly progress to severe injuries of bile ducts and vessels (both predominantly expressing MHC antigens), resulting in the loss of the graft. Insufficient prevention and/or treatment can also lead to humoral and chronic rejection, also associated with graft loss.

1.4.4.2 Immunosuppressive medications

For SOT to be effective, it is absolutely essential to control immune responses to foreign donor HLA antigens and to prevent both cellular and humoral rejection. Nowadays, very efficient ISDs are available to control rejection after SOT. Steroids are usually used within the first weeks or months after LTx before being discontinued. In addition, to maintain immunosuppression, three categories of medications are routinely used:

1. calcineurin inhibitors (CNIs): cyclosporine and tacrolimus
2. antiproliferative agents: mycophenolate mofetil (MMF) and azathioprine
3. mammalian target of rapamycin (mTOR) inhibitors: everolimus (EVR) and sirolimus

CNIs function by impeding calcineurin, an intracellular phosphatase that dephosphorylates cytosolic nuclear factor of activated T cells to enable nuclear translocation and transcriptional

activation of cytokine genes like IL-2, which regulate the proliferation, survival, and maturation of all subtypes of T cells [60].

In vivo, **MMF** is converted into its active form of mycophenolic acid and inhibits the *de novo* synthesis of purines. B and T cells are dependent on this pathway for their proliferation [61]. mTOR is a serine/threonine kinase that functions through two different complexes (mTORC1 and mTORC2) which mediate distinct cellular activities. mTORC1 is required for the differentiation of $CD4^+$ T cells into Th1 and Th17 cells while mTORC2 is required for Th2 differentiation [62]. Both mTOR complexes enhance effector $CD8^+$ T-cell differentiation and glycolytic metabolism in $CD8^+$ T cells at the expense of memory $CD8^+$ T-cell development. **mTOR inhibitors** are more effective at blocking mTORC1, but can also block mTORC2 following prolonged exposure [60, 63].

These drugs are usually used in combination to improve immunosuppression and to lower adverse effects by limiting exposure to each drug. The choice of this association depends on recipient's characteristics, comorbidities and treatment side effects and is based on transplant center/physician usual practice without established worldwide norms [1]. High levels of ISDs are generally maintained during the first 1 to 3 months after LTx because AR occurs predominantly during this period. The doses are then progressively decreased to reach maintenance immunosuppression levels. Currently, CNIs (most often tacrolimus) remain the pillar of the immunosuppressive treatment in most transplant centers, usually in combination with MMF to keep CNI doses as low as possible to avoid adverse effects, but other combinations exist (e.g.: CNI monotherapy, mTOR-inhibitor + MMF, CNI + mTOR-inhibitor, etc.) [64-66].

Compared to other SOT (kidney, heart, lung), rejection rates are lower and early allograft rejection is less deleterious, with a lower impact on long-term outcomes, and is more easily treated in LTx. The major concern is thus not to try to find the most potent immunosuppression but rather to avoid excessive immunosuppression and its consequent toxicity. Indeed, long-term and excessive exposure to these drugs have heavy consequences on LTx outcomes, such as diabetes, infections, cancers, nephrotoxicity, etc.... It is therefore necessary to strike a balance between insufficient and excessive immunosuppression, yet there is no objective method to exactly tailor immunosuppression to each individual.

In case of liver AR, performing a liver biopsy is highly recommended. In case of low through levels of CNI, an increase of CNI doses is generally efficient to treat rejection. In case of appropriate CNI through levels, intravenous (IV) steroid boluses are required [1, 67]. The mechanisms of action and adverse effects of ISDs commonly used in LTx are described in the Table below **(Table 1)**.

Table 1. Mechanisms of action and adverse effects of ISDs commonly used in LTx.

Types of agents	Drug	Mechanisms of action	Adverse effects	Frequency	Severity
Steroids		⛔ IL-1 dependent lymphocyte activation ⛔ antibody and complement binding ⇓ synthesis of IFN-γ and IL-2	Diabetes	+++	+++
			Osteoporosis	+++	+++
			Dyslipidemia/Obesity	+++	++
			Hypertension	++	++
			Opportunistic infections	+	++
Calcineurin inhibitors	Cyclosporine	⛔ IL-2 transcription ⛔ calcineurin through cyclophilin	Nephrotoxicity	+++	+++
			Diabetes (T.>C.)	++	++
			Neurotoxicity (tremor, paresthesia, headache, convulsion)	++	+
	Tacrolimus	⛔ IL-2 transcription ⛔ calcineurin through FKBP	Hypertrichosis$^{\psi}$	++	±
			Dyslipidemia	+	+
			Gingival hyperplasia$^{\psi}$	+	±
			Microangiopathy and PRESS	±	+++
Antiproliferative agents	Mycophenolate mofetil	⛔ T and B cell proliferation by blocking *de novo* synthesis of nucleotides ⛔ inosine-5'-monophosphate dehydrogenase	Diarrhea	++	+
			Leukopenia	+	+
			Thrombocytopenia	+	+
			Anemia	±	±
mTOR-inhibitors	Sirolimus/Everolimus	⛔ mTOR pathway ⛔ T- and B-cell proliferation	Oral ulcers	+++	++
			Delayed wound healing	+++	+
			Dyslipidemia	++	+
			Hepatic artery thrombosis	+	+++
			Nephrotoxicity / proteinuria	+	++
			Thrombocytopenia	+	+
			Leukopenia	+	±
			Anemia	+	±
			Diarrhea	+	±
			Interstitial pneumonia	±	+++

Legends: C., Cyclosporin; FKBP, human peptidyl-prolyl isomerase FK-binding protein; PRESS, posterior reversible encephalopathy syndrome; T., Tacrolimus; ⛔, inhibition/blockage of; ⇓,: reduction in; ψ, only with cyclosporine; Adapted from [1]

1.5 Tolerance

Acquired immune tolerance was first described in rodent models in the 50's by Medawar and his team and is defined as the specific suppression of the immunological response of an organism towards a given antigen, while this response remains normal to other antigens. In transplantation, tolerance is a very rare event and occurs when there is no observable reaction against the allograft antigen in an immunocompetent host that would reject another allograft from another donor [68]. The so-called "operational tolerance" refers to the long-term preservation of sustained graft function in the absence of a clinically significant, harmful immune response or immunological deficiency [31].

Compared to other solid organs, the liver is considered as a particularly "tolerogenic" organ. Histocompatibility between the recipient and the liver is considered as not important for graft survival. Low-dose regimens of ISDs are usually well tolerated by LTRs and, after a few years, in selected stable patients, about 20 to 40% of LTRs can be withdrawn from ISDs without rejection, whereas comparable "spontaneous tolerance" in recipients of other organs has only been anecdotally recorded [69]. In case of rejection when the recipient is weaned or withdrawn of ISDs, reinstatement of conventional immunosuppression (or for some, a steroid bolus) easily resolves rejection without graft loss in most cases [61]. Nevertheless, achieving such an objective may prove very challenging with any specific protocol because of the significant variability among organ graft donors and recipients, such as tissue compatibility, natural immune responses, and the potential for disruptions to a state of tolerance caused by infections or allergies. Therefore, Calne *et al.* also made the case for the concept of "prope" or almost tolerance in which graft acceptance is sustained through a minimal, non-harmful level of ongoing immunosuppression that may not be necessary indefinitely [70].

Further proof of the liver tolerogenicity can be seen in the reduction of alloimmune damages to other organs (kidney [56] or cardiac grafts [58]) that were simultaneously transplanted during a combined transplantation including the liver.

1.5.1 The liver: a particular immune environment

In an immunocompetent subject, central tolerance to auto-antigens is essentially the result of the apoptotic elimination of autoreactive T cells during intrathymic T-cell development. In SOT settings, despite the absence of deletion of donor-reactive T cells through central tolerance, immunity can nevertheless lean toward tolerance by increasing the ratio of $CD4^+$ forkhead box protein 3-positive (FoxP3+) Tregs to effector T cells, which allows Tregs to persistently inhibit donor-reactive T cells. This corresponds to **peripheral tolerance** [31]. Of importance, intrahepatic APCs also define the balance between immunity and tolerance in the liver [31]. To date, the mechanisms underlying liver graft tolerance are still poorly understood. No validated biomarkers are available to predict whether ISD can be withdrawn or not.

1.5.1.1 Innate immune cells

Depending on their microenvironment and on their subset, innate immune cells of the liver (i.e. DCs and KCs) can tip the balance towards tolerance or immunity. *Regulatory **DCs*** obtained *in vitro* from differentiation of haematopoietic progenitors in a culture mimicking the hepatic microenvironnement, have low MHC II expression and secrete high levels of anti-inflammatory cytokines (IL-10 ± IL-12) inhibiting T-cell proliferation and allograft rejection [8]. Conventional DCs are mostly found in portal areas. These cells sample antigens and migrate to peripheral lymph nodes, where they are important actor orchestrating and regulating T-cell immunity, consequently integrating adaptive and innate immunity. **KCs** represent the resident macrophage population in the liver. KCs are located in the liver sinusoids where they can interact with T cells. Two populations of liver macrophages (proinflammatory versus immunoregulatory) have been identified with single-cell RNA sequencing. KCs, like other macrophages, are important to eliminate microorganisms and apoptotic cells. They promote liver regeneration and regulate liver injury and tolerance [8, 71]. Comparatively to conventional DCs, KCs express low levels of MHC class II and costimulatory molecules. Furthermore, IL-10 production is induced as a result of the interaction between KCs and Tregs, which is essential in order to successfully establish tolerance to hepatocyte-expressed

antigens [72]. KCs also secrete prostaglandins (e.g. PGE2) which have an important role in enabling cells to inhibit T-cell allo-specific responses through the inhibition of IL-2 secretion, inhibition of tyrosine kinases or suppression of intracellular calcium elevation [8, 71]. Additionally, PGE2 can promote FoxP3+ Tregs[73]. In humans, **NKs** represent 30-50% of hepatic lymphocytes. It has been shown that NKs were functionally maintained in a state of hyporesponsiveness by intra-hepatic IL-10 [8]. Overall, within the liver microenvironment, the diverse residents of the innate immune cell population intrinsically tend to promote tolerance.

1.5.1.2 Liver sinusoidal endothelial cells

Representing the most abundant non-parenchymal cell population in the liver, LSECs play a major and complex role in maintaining and regulating immune homeostasis [74]. LSECs act as APCs by presenting hepatocyte-derived antigens on MHC-I or -II molecules with T-cell co-stimulatory molecules[8]. It was shown in mice that LSECs present exogenous antigens to $CD4^+$ and $CD8^+$ lymphocytes without expressing IL-12, eliciting an antigen-specific T-cell tolerance instead of a Th1 response [8]. LSEC lectin, a binding protein, has been shown to be able to specifically downregulate activated T-cell response. Moreover, it has been shown that $CD4^+$ T cells primed by antigens presented by LSECS do not promote Th cell differentiation but induce the development of FoxP3+ Tregs and that stimulation of naive $CD8^+$ T cells leads to a tolerogenic signaling pathway of programmed cell death protein ligand (PDL)-1 inhibiting cytotoxic function [8, 75]. Furthermore, LSECS have shown their ability to downregulate DC function to prime T cells [76].

1.5.1.3 Hepatic stellate cells

HSCs, which are specialized in hepatic tissue repair, might also have a role in liver tolerogenicity [8]. For example, they were shown to be able to inhibit adaptive immunity and to preferentially expand Tregs in an IL-2 dependent manner [8, 77]. The induction of indoleamine dioxygenase (IDO) production in DC by HSC also inhibits the allo-stimulation of T cells **(Figure 2)** [78]. HSC also exhibit inhibitory properties on B-cell activity via PDL-1 and other innate properties thwarting T-cell effector responses [8].

1.5.1.4 Resident T cells

In the normal adult liver, T cells can be found in the parenchyma and in the portal area (predominantly $CD8^+$ over $CD4^+$ T cells and NK cells). The action of these cells needs to be controlled, depending on many factors and interactions, to avoid immune damage of the liver [8]. Resident T cells can play a role of a kind of immunosurveillance thanks to the slow blood flow in liver sinusoids and their fenestrated architecture favoring interactions between resident hepatic cells and circulating T cells. For example, the expression of PDL1 on hepatocytes, KCs, HSCs and LSECs encourages low T-cell activation and/or $CD8^+$ T-cell apoptosis [79]. Moreover, it has also been shown in mice that the liver microenvironment plays an important role in the depletion of activated NK cells, which, unlike T cells, react rapidly to antigens without prior sensitization [80]. Importantly, after LTx, the presence of these donor-derived T-cells in LTR can sometimes result in a graft-versus-host-disease (GVHD) in which donor immunocompetent cells react against tissues of an immunosuppressed host [81].

1.5.2 Liver allograft tolerance in rats and mice

In many species, the rejection of liver allografts is typically less severe than in other transplanted organs. This is particularly true in rats and mice, in which LTx between specific fully MHC-mismatched strain combinations are tolerated permanently in the absence of immunosuppressive therapy, whereas skin, heart, and renal allografts experience AR [82, 83]. For example, livers from Piebald Virol Glaxo (PVG) rats transplanted into Dark Agouti (DA) rats are not rejected. Liver grafts from ACI rats to Lewis rats are permanently accepted after a short course (4 days) of tacrolimus [8, 84]. Very interestingly, it was shown in a rat model of LTx of MHC-mismatched allografts that the transplanted liver confers protection against rejection of other transplanted organs procured from the same donor and can even reverse donor-specific allo-immunity in sensitized recipients after liver grafting [85, 86]. The hematopoietic function of the liver plays a role in the development of tolerance. For example, it was shown in rats that the lymphoid tissue of the liver is able to produce donor-derived DCs

and it was shown that the persistence of such cells *post*transplantation was correlated with tolerance of the liver (but not the heart) [87].

1.5.3 Mechanisms and cells implicated in tolerance

1.5.3.1 Passenger leukocytes

The role previously attributed to the so-called "passenger leukocytes", according to which donor-derived T cells migrating to recipient lymphoid tissues are responsible for tolerance by a mechanism of clonal deletion, is now questioned. Indeed, rapid migration of passenger leukocytes and immunological activation in recipient lymphoid tissue have been linked to tolerance in rats[8]. Nevertheless, later investigations have shown that chimeric liver containing donor-, recipient- or 3rd party-derived leukocytes were tolerated long-term [88]. Interestingly, liver transplants from PVG-to-DA rats are spontaneously tolerated while kidney transplants from the same strains are rapidly rejected. It was shown that the kidney allograft was rapidly invaded by mononuclear cell infiltrates expressing high levels of cytokines with signs of rejection, while the tolerated liver graft from the same strain showed a rapid infiltration by activated T cells, promptly followed by a high level of apoptosis in the leukocyte infiltrates. This abortive character of the effector response found in livers that have been transplanted and tolerated (in which donor liver cells trigger the death of recipient alloreactive T cells by neglect) may play a key role in the induction of spontaneous liver transplant tolerance [89, 90].

1.5.3.2 Molecular pathways

Many molecules and cytokines have been identified to be associated with liver allograft tolerance. For instance, B7-cytotoxic T lymphocyte-associated antigen 4 (CTLA4) activation inhibits T-cell responses and induces the apoptosis of alloreactive T cells. Its inhibition in mouse liver grafts leads to AR, indicating that CTLA4 signaling is necessary for liver transplant tolerance induction [8, 91]. IFN-γ, classically recognized as a pro-inflammatory cytokine, is thought to play a significant role in the spontaneous acceptance of liver allografts

[92]. Indeed, IFN signaling from alloreactive T effector cells induced a high production of PDL1 by LSECs, which causes T effector cell apoptosis via the PDL1-PD1 pathway [93]. An endogenous lectin expressed in lymphoid organs, Galectin 1, seems to play a major role in prolonging liver allograft survival in the mouse by inhibiting Th1 and Th17 responses and promoting T-cell apoptosis [94]. Overall, the available data from animal experiments seem to point to the existence of multiple and parallel molecular mechanisms.

1.5.3.3 Regulatory T cells

General properties

Tregs constitute a T-cell population that inhibits immunological responses and maintains immune homeostasis and self-tolerance. They develop either in the thymus as the pool of "natural Tregs" or in the periphery as "induced Tregs"[95]. The classical phenotype characterizing Tregs in rodents and humans is $CD4^+$ with high expression of CD25 ($CD25^+$, IL-2 receptor) and intracellular expression of $FoxP3^+$ and, in human only, low levels of CD127 has been demonstrated to distinguish Tregs from other T cells [95, 96]. Nevertheless, the relatively recent advances in immunophenotyping technologies showed that Treg populations are in fact heterogeneous and divided in many subsets. For example, their subpopulations include $CD45RA^+FoxP3^{low}$ Tregs, representing resting/naive Tregs, $CD45RA^-FoxP3^{high}$ Tregs, representing activated/effector Tregs, and $CD45RA^-FoxP3^{low}$ Tregs, representing non immunosuppressive, cytokine-producing cells [97]. As mentioned above, depending on the cytokine environment, the activated $CD4^+$ cells differentiate into tissue-destructive IFNγ-producing Th1 cells in presence of IL-12 or, in presence of IL-4, into Th2 cells producing IL-4 and IL-5. In the presence of transforming growth factor (TGF)-ß without proinflammatory cytokines, $FoxP3^+$ expression is stimulated in $CD4^+$ T cells, which subsequently differentiate into Tregs. Conversely, TGF expression in conjunction with IL-6 or IL-21 inhibits the maturation of tolerogenic Tregs and promotes the differentiation of $CD4^+$ T cells into the highly cytotoxic Th17 cells producing IL-17 [31, 98].

In mice, increased levels of Tregs ($CD4^+CD25^+FoxP3^+$) with enhanced CTLA4 expression was linked to spontaneously accepted MHC-mismatched allograft. Clinically, it has been

shown that increased Treg levels and increased FoxP3 transcripts are associated with operational tolerance or low rejection rates [95, 99].

Mechanisms of action

The mechanisms of action of Tregs can be broadly divided into two concepts: Tregs that target T cells and those that target APCs. The main mechanisms by which Tregs **directly inhibit effector T cells (Figure 2)** are: i) the secretion of suppressive cytokines such as TGF-ß, IL-10 and IL-25 directly inhibiting responder T cells ii) the high expression of IL-2 receptor alpha (CD25) resulting in depletion of IL-2 and in effector cell apoptosis iii) their direct action as cytotoxic cells and the induction of apoptosis in effector cells via granzyme-mediated and Fas-Fas ligand pathways iv) the interaction of some Treg surface molecules (e.g. Galectin-1) with effector T-cell receptors inducing T-cell cycle arrest. The main mechanisms by which Tregs are able to **inhibit APCs** and thus indirectly suppress the activation of effector T cells are: i) the presence of CTLA4 on the Treg surface downregulating the major CD80 and CD86 (B7) costimulatory molecules on APCs ii) the LAG (lymphocyte activation gene)-3 on Tregs interacting with MHC class II molecules of immature DCs and keeping them immature iii) the inactivation of extracellular ATP by CD39 preventing the effect of ATP on APCs and thus decreasing their costimulation [100].

Effect of classical ISDs on Tregs

Tregs probably play a central role in allograft tolerance after LTx. Immunosuppressive drugs can inhibit many of the Treg mechanisms of action described above and thus hinder tolerance while preventing allograft rejection by inhibiting effector T cells. The effects of ISDs on Tregs have been evaluated in different studies[60]. It has been shown that some ISDs have a deleterious effect while others showed a beneficial effect on the proliferation and function of Tregs. Both *in vitro* and *in vivo,* Treg function and proliferation seem to be inhibited by **CNIs** [60, 101]. Indeed, tacrolimus and CsA act by inhibiting the transcription of cytokine genes such as IL-2, which allow T cells (including Tregs) to proliferate, survive and maturate. CNIs negatively affect Tregs by directly inhibiting Treg activation and proliferation and, indirectly,

by preventing conventional T cells from producing IL-2 [102]. *In vivo,* reduced FoxP3 mRNA expression in CsA-exposed Tregs is correlated with decreased suppressor activity [101]. It appears that CNIs have dose- and duration-dependent effects on Tregs [60]. Low doses of CNI seem to have a positive impact on Treg proliferation [103]. *In vitro,* **MMF** does not have a negative impact on Treg viability and function. It appears that MMF therapy increases Treg predominance over Th17 cells. Nevertheless, it was shown that high doses of MMF in Treg-treated mice impaired Treg efficacy [60, 104]. In the clinic, conversion from CNI to MMF with a single dose of daclizumab (anti-CD25 antibody) in LTRs resulted in an increase in the proportion of Tregs from baseline [105]. Overall, preclinical and clinical data to date indicate that MMF has a favorable impact on Treg function and homeostasis. **Steroids** appear to have a beneficial effect on Treg proliferation and function both *in vitro* and *in vivo* [60]. Indeed, it appears that corticosteroids can shift the balance of T cell subsets to favor an increase of Th2 cells and Tregs through several mechanisms including by promoting the expression of glucocorticoid-induced leucine zipper (GILZ) which appears to be a strong inducer of $FoxP3^{+}$ expression [106, 107]. In clinical kidney transplantation (KTx), the use of methylprednisolone to treat rejection episodes leads to a shift in T cell composition that promotes highly suppressive $DR^{high}CD45RA^{-}$ Tregs [108]. With regards to **mTOR inhibitors**, Tregs are able to alternatively use the PIM2 kinase for cell proliferation and activation with the consequence that Tregs are able to expand in the presence of mTOR inhibitors [60, 109]. *In vitro*, it has been shown that rapamycin is able to selectively expand Tregs and that, *in vivo,* these cells presented a greater suppressive activity than freshly isolated Tregs [110]. Gedaly *et al*. have recently shown that mTOR inhibitors could be used to expand *ex vivo* functionally competent Tregs for clinical use [111]. Similarly, in KTx, it has been observed that recipients under rapamycin maintenance have increased circulating Treg levels compared to patients receiving CsA [112]. Another team recently confirmed these observations in LTRs, showing increased Treg levels after being switched from tacrolimus to mTOR inhibitors [113].

Clinical trials using exogenous Tregs

The first clinical (phase I-II) trial studying the effects of Treg therapy after LTx (n=10) was published by Todo et al. (Japan) in 2016. The authors could show, in living related LTx,

that injection of donor-derived *ex-vivo* generated Tregs was safe and effective, allowing ISD reduction (from month 6 post-LTx) and inducting operational tolerance in a majority of LTRs [114]. In another recently published phase I-II study (ARTEMIS study), the authors investigated whether donor alloantigen-reactive Tregs could produce graft-specific immunosuppression in the absence of broad-spectrum immunosuppressive medications. Tregs were administered to only 5 patients (2-6 years post-transplantation) among the 10 patients initially planned because of failure to manufacture the required minimal dose. Among treated patients, no treatment-related adverse events were reported. Two patients could be weaned of immunosuppression [115]. A few other clinical trials studying the use of 3rd party or donor-derived Tregs are in progress in LTx (clinicaltrials.gov: NCT02188719 (USA), NCT03654040 (USA), NCT03577431 (USA), NCT02166177 (UK) NCT01624077 (China)).

1.5.3.4 Mesenchymal stromal cells

Mesenchymal stromal cells (MSC), which can be found in the perivascular space of virtually all organs, are particularly prevalent in two highly vascularized organs, the placenta and the liver, in which they seem to play a central role in maintaining tolerance **(Figure 2)** [116]. It seems that APCs can be reprogrammed towards a tolerant phenotype by interaction with MSCs, as evidenced by an increase in IL-10 production. In addition, MSCs are able to modulate co-stimulatory signals on DCs and can inhibit T-cell activity through different mechanisms (e.g. secretion of IDO, see infra) [117].

1.5.3.5 NK cells

In contrast to recipient-derived NK cells, which often contribute to rejection, donor-derived NK cells transplanted as passenger cells within the liver allograft can directly eliminate alloreactive recipient immune cells through different interactions **(Figure 2)** [118]. Consistent with this theory, increased numbers of NK cells have been observed in LTRs who have successfully discontinued immunosuppressive therapy [119].

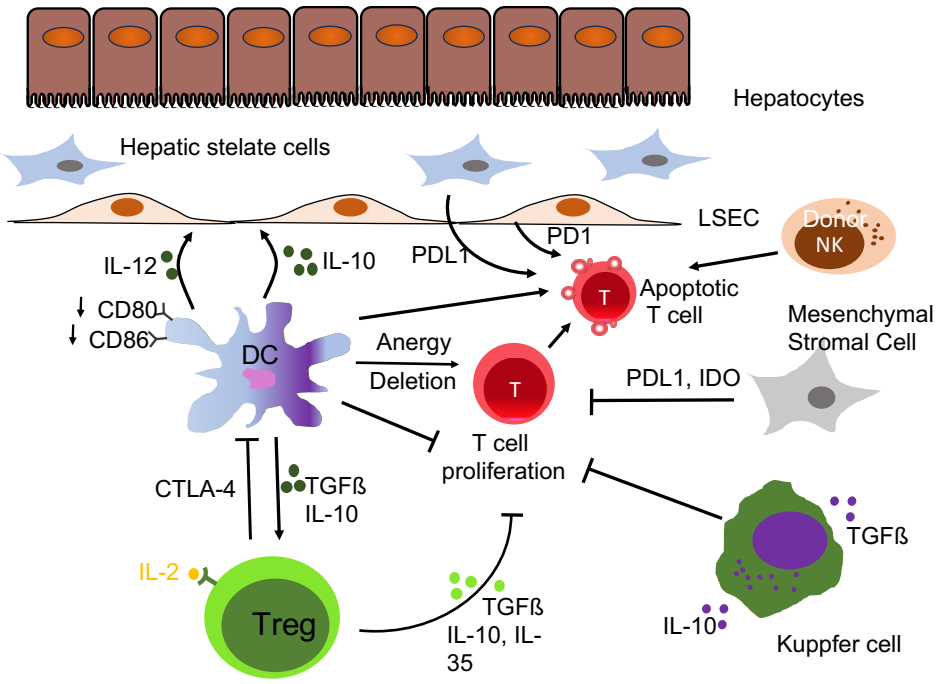


Figure 2. Cellular mechanisms underlying liver allograft tolerance involve various cell types. In a non-inflammatory state, dendritic cells express low levels of co-stimulatory molecules. They also have high levels of PDL1, which induces either T-cell inactivity or the deletion of alloreactive T-cell clones. Dendritic cells contribute to tolerance by releasing IL-10 and TGF-ß, which promote the formation of Tregs. Tregs, in turn, foster a tolerogenic environment by producing TGF-ß, IL-10, and IL-35. They also interact with dendritic cells through CTLA4, which has a stronger binding affinity to B7 on dendritic cells than CD28, hindering DC-T-cell interactions. Additionally, Tregs bind IL-2 on CD25 more effectively than T effector cells and can exert direct cytotoxicity through granzyme, perforin, and the Fas-FasL pathway. In contrast to recipient-derived NK cells, which often contribute to rejection, donor-derived NK cells transplanted as passenger cells within the liver allograft can directly eliminate alloreactive recipient immune cells. MSCs inhibit T-cell proliferation and differentiation via various mechanisms including the IDO pathway and cell-to-cell contact facilitated by PDL1. Kupffer cells can be polarized toward the M2 phenotype, producing IL-10 and TGF-ß, thereby supporting tolerance. They can also release NO if stimulated by IFN-γ to suppress T-cell proliferation. LSECs serve as non-professional antigen-presenting cells with generally low MHC class II expression, promoting antigen-specific tolerance under many conditions. LSECs, along with hepatic stellate cells, induce T-cell apoptosis through interactions involving the PDL1/PD1 pathway.
CD, cluster of differentiation; CTLA4, cytotoxic T lymphocyte-associated antigen 4; DC, dendritic cell; IDO, indoleamine dioxygenase; IFN, interferon; IL, interleukin; LSECs, liver sinusoidal endothelial cells; MHC, major histocompatibility complex; NK, natural killer cell; PDL-1, programmed cell death protein ligand-1; TGF, tumor growth factor; Treg, regulatory T cell; Adapted from Ronca *et al., 2020 [2].*

1.6 Model of liver transplantation

1.6.1 Surgical model of liver transplantation in small animals

In 1973, 10 years after the first human LTx by Starzl and his team, Lee et al. described the first orthotopic liver transplantation (OLT) in rats [120]. In this complex procedure, all vascular reconstructions were performed with microsutures under extracorporeal portal to jugular shunt. It was a very highly complex procedure, in which long-term survival was very low. In 1979, Naoshi Kamada and Roy Calne revolutionized the procedure with the introduction of the cuff technique, shortening the anhepatic phase and simplifying the vascular anastomoses in the recipient animal [121]. There was no rearterialization of the liver graft in this technique that has become the most widely used LTx model for OLT in rats **(Figure 3+4)**.

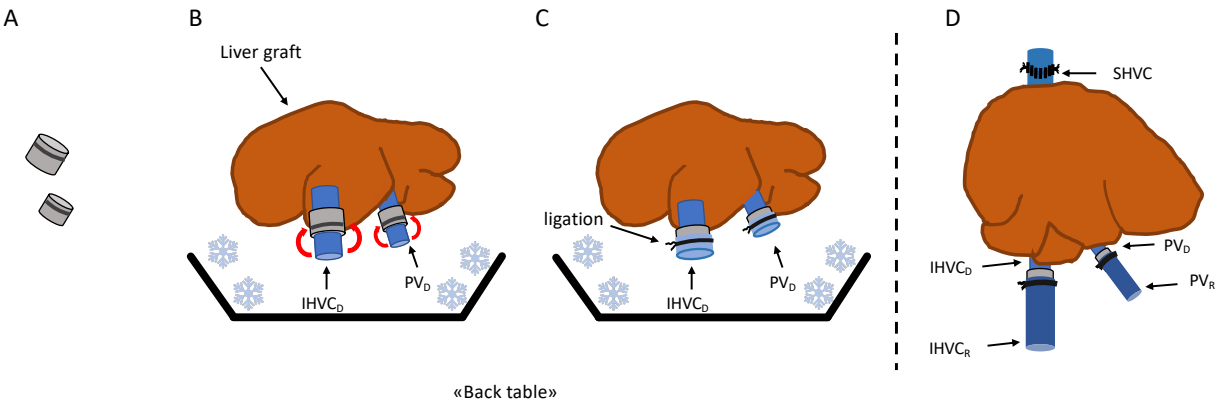


Figure 3. Schematic view of the cuff method. **A. The cuff** is usually "home-made" from a sterilized tube or catheter with a size chosen depending on the vessel (IHVC or PV); **B.** During the **"back table",** the vessel is introduced through and reversed on the cuff using a micro-forceps; **C.** The reversed vessel is attached to the cuff using a small ligature; **D.** Anastomosis of the SHVC with a running suture; PV and IHIVC anastomosis by insertion of the PV cuff and the IHVC into the PV_R and $IHVC_R$, respectively.
$IHVC_{D/R}$, infra-hepatic vena cava of the donor/recipient; $PV_{D/R}$, portal vein of the donor/recipient; SHVC, supra-hepatic vena cava.

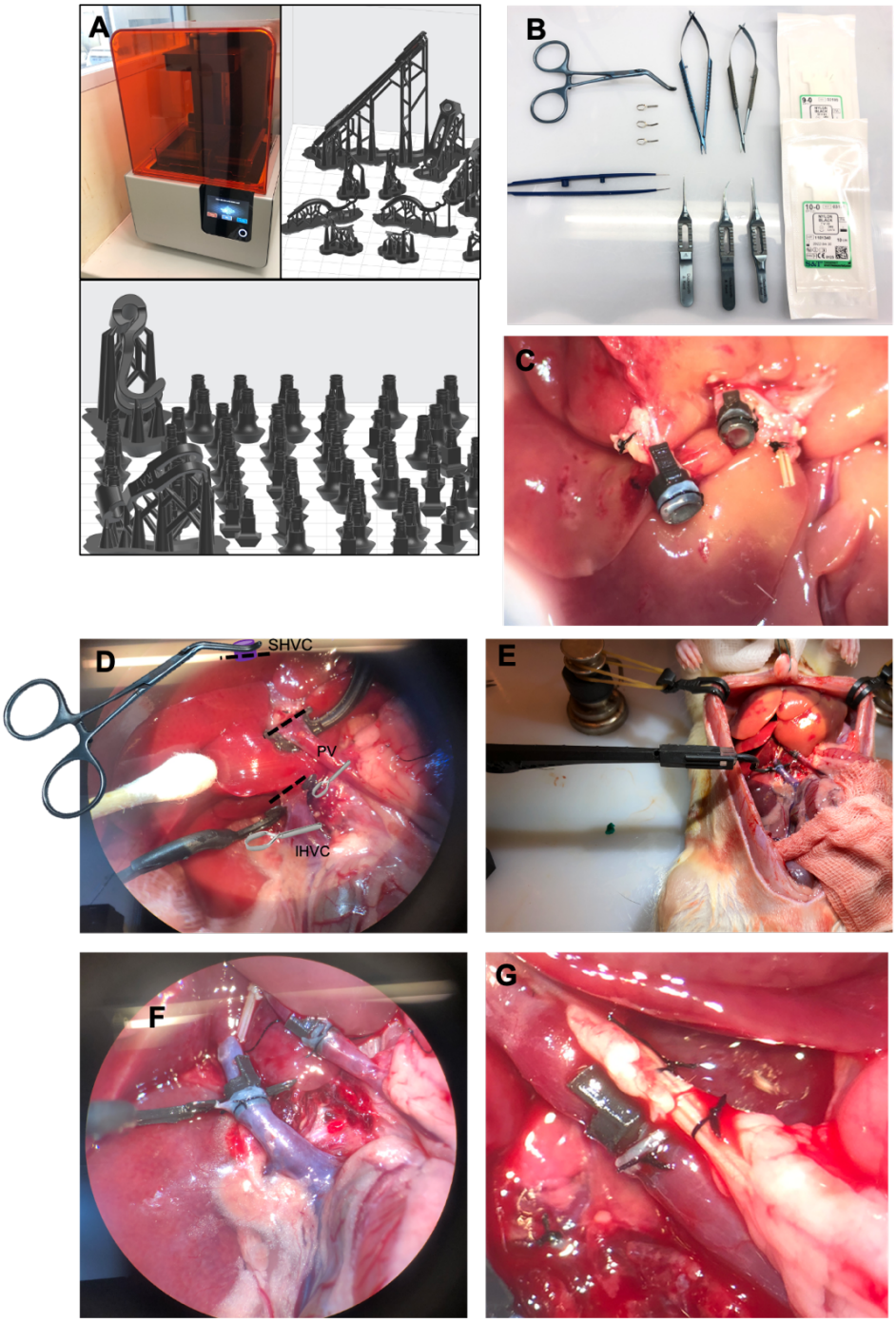


Figure 4. Instruments used during OLT and prodecural steps. **A.** upper-left part: 3D-printer; upper-right and lower part: screenshot of 3D-printer workspace with 3D instruments models to print (cuffs, retractors, etc.); **B.** Microsurgical instruments; **C.** Microscopic view: infrahepatic vena cava (IHVC, on the left) and portal vein (PV, on the right) reversed on cuffs and secured with ligatures; **D.** Microscopic view of the attachment of vessel holders (black plastic) on recipient's IHVC and PV to help maintaining the vessels before cutting ; clamp installation on PV, on IHVC and on the suprahepatic vena cava (SHVC); **E.** External view after vascular anastomosis of the liver graft and unclamping; **F.** Microscopic view of infrahepatic anastomosis of IHVC (left) and PV (right); **G.** Biliary duct reconstruction using a 24-G catheter and secured with ligatures (right) and PV anastomosis (left).

In Kamada's experience, the long-term survival of this non-arterialized model was more than 95% in a series of 530 OLT in rats [122]. Up to now, more then 30 techniques or modification of the existing techniques have been published to help make it easier and more reproducible, some describing "rearterialized" models[123].

Many variables influence the success of this procedure and the survival of the transplanted rat. One of the most important factors is the duration of the anhepatic phase. It has been shown that in case of anhepatic phase superior to 20 minutes, short-term survival is close to 0% [124]. This probably explains in part why OLT is considered as one of the most challenging animal transplantation models. Czigany et al. described it as a "demanding procedure needing a long and often discouraging training. Perseverance, patience […] and frustration tolerance are considered as the cornerstones for achieving success" [123]. Indeed, the learning curve is very demanding and requires beforehand advanced microsurgical skills. At least 30 procedures are generally required before obtaining a first successful OLT and more than 50 cases are needed before obtaining a successful rate of 80%. Below the critical training number of 40 procedures, survival is often less than 30 percent [123, 124].

Amongst the published techniques facilitating the procedure, Oldani et al. recently published the use of 3D-printed devices (cuffs, vessel holders, retractors, etc) [125]. This paper describes a technique of mouse LTx but it can be easily transposed to rats by modifying the dimensions of the 3D printed cuffs allowing to facilitate the procedure, to reduce its duration and to increase its accessibility. This modified "two-cuffs" technique was used in the animal experiments of the present thesis (**Figure 4**). In our experience, 35 procedures were necessary to obtain a first survival and, from the 48th procedure, we reached a successful rate of 80%.
As mentioned above, OLT in mice has also been described. The use of mice in the laboratory assays is particularly attractive due to their large availability with well-defined genome and the possibility to work with knock-out or gene-altered animals. Nevertheless, this procedure is even more difficult than in rats and thus not widely available [124].

1.6.2 Models of allograft rejection in rats

Large animal models are of relatively limited utility in studying allograft rejection due to the difficulty of procuring defined inbred strains. In rats, thanks to the availability of inbred and congenic strains with well-defined MHC phenotypes as well as reliable surgical techniques, it has been possible to study the rejection process in detail [126]. As mentioned above, fully-MHC-mismatched allograft transplantation between well-defined strains results in a very low allograft rejection rate and prolonged survival without ISDs. Nevertheless, in particular inbred rat strain combinations, rejection can occur acutely or with a delay, making this species interesting to study allograft rejection **(Table 2)**. On the contrary, in mice, rejection intensity is always less severe, limiting the use of this model for transplant studies [127]. Otherwise, an example of xenotransplantation of livers from hamsters to Lewis rats resulted in graft survival without treatment of only 3 days (n=6) [127]. Examples of various strains combination of donor and recipient rats and of the associated survival are given in the table 2 below.

Table 2. Liver graft survival, without any immunosuppressive treatment, of various combinations of donor and recipient rat strains.

Donor	Recipient	Graft survival (days)	Ref.
LEW	BN	11 [7 – 14]	Qin *et al.,* 2010
ACI	LEW	11.8± 0.4	Redaelli *et al., 2001*
DA	LEW	11±2	Kunugi *et al., 2011*
PVG	LEW	12 [10-19]	Wang *et al.*, 2010
PVG	DA	>100	Wang *et al., 2010*

Legend: ACI, ACI rat; LEW, Lewis Rat RT1l; DA, Dark Agouti rat; PVG, Piebald Virol Glaxo Rat or Black Hooded rat; BN, Brown-Norway rat). Table adapted from [127]

2 Mesenchymal stromal cells

2.1 Definition and sources

MSCs represent a heterogeneous population of fibroblast-like cells whose definition relies on the combination of the following criteria, according to the International Society for Cellular Therapy (ISCT): (i) adherence to plastic, (ii) specific surface antigen expression of CD105, CD73 and CD90 and lack of expression of CD45, CD34, CD14 and/or CD11b, CD79α and/or CD19 and Human leukocyte antigen (HLA)-DR, and (iii) the potential to differentiate into osteocytes, adipocytes and chondrocytes **(Figure 5) [128]**.

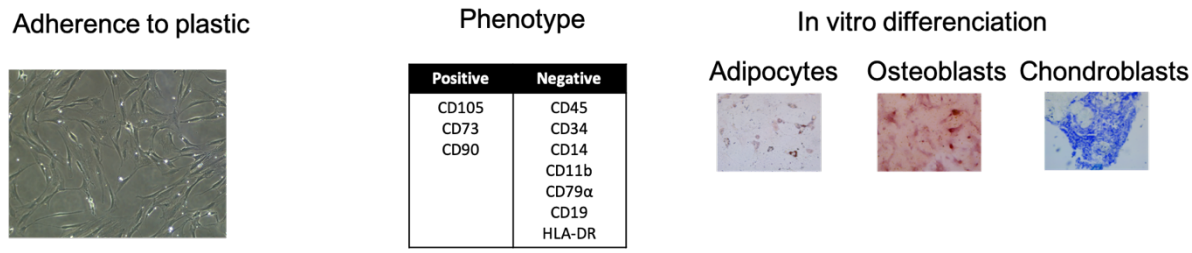


Figure 5. Figure summarizing the minimum criteria for defining MSCs, according to the ISCT [128]

MSCs were initially isolated from the bone marrow (BM) [129]. Since then, cells exhibiting similar *in vitro* characteristics as BM-MSCs have been alternatively obtained from adult tissues (especially adipose tissue [130]), umbilical cord (UC) [131, 132] and other sources. Although sharing analogous features, these cells are not strictly identical, as inter-source heterogeneity has been reported [133, 134]. This results in different functional properties according to the origin of the MSC. [135, 136]. BM-MSCs currently represent the predominant source of MSCs used in SOT [137].

2.2 Mechanisms of action and properties

MSCs exert anti-inflammatory, immunomodulatory and tissue repair/regeneration properties that are mediated by (i) the secretion of soluble factors, (ii) direct cell-cell contacts and (iii) the production of extracellular vesicles (EV) [138, 139].

2.2.1 Implicated factors

A considerable number of chemokines/adhesion molecules, cytokines, pro-angiogenic and/or growth factors constitute the secretome of MSCs [140]. Among immunomodulatory factors, indoleamine 2,3-dioxygenase (**IDO**) is likely to be one of the key molecules involved in the immune-related effects of human MSCs [141]. By catalyzing the rate-limiting step for the conversion of tryptophan - an essential amino acid necessary for T-cell proliferation - to kynurenine, IDO activity leads to a reduction in local tryptophan concentrations, as well as in the production of immunomodulatory tryptophan metabolites that alter the proliferation and function of immune cells [140, 142, 143]. **HLA-G5**, a soluble isoform of the non-classical HLA-G class I molecule, is also critical in mediating the immune response in human MSCs, especially for the expansion of functional Tregs [144, 145]. MSC-secreted prostaglandin E2 (**PGE2**) was also shown to play an important role by inducing an increase of the anti-inflammatory/pro-inflammatory ratio of cytokine secretion in DCs leading to a shift in $CD4^+$ T cells from a Th1 to a Th2 subtype [146-148]. Many other factors have also been shown to play a direct or indirect role in MSC-mediated properties, including **TGF-ß** [147], **heme-oxygenase (HO)-1** [149], **hepatocyte growth factor**, **IL-1 receptor Antagonist**, Peptide LL-37, Matrix Metalloproteinase 3 and 9, angiopoietin-1, TNF-α and insulin-like growth factor-binding proteins [148, 150].

2.2.2 Extracellular vesicles

Additionally, the MSC secretome includes EVs, which are categorized into apoptotic bodies, microvesicles and exosomes according to their size and mechanism of cellular release[151]. MSC-derived EVs may represent a cell-free therapeutic approach[152, 153]. A wide variety of bioactive molecules are included in EVs, such as proteins, lipids, ribonucleic

acid (RNA) subtypes (i.e. at least mRNA and miRNA) and deoxyribonucleic acid (DNA) subtypes[154]. Beside the release of their content into target cells, MSC-derived EVs may help transfer cytoplasmic materials and organelles[154], particularly in a potential bidirectional exchange with T cells[155].

2.2.3 Interaction with immune cells

Regarding the innate immune system, MSCs influence **macrophage** differentiation, with a preferential shift towards an anti-inflammatory immunosuppressive M2 phenotype[138, 156]. M2 macrophages are involved in the repair and maintenance of tissue integrity, and are characterized by efficient phagocytic and immunoregulatory activities **(Figure 6)** [157]. MSCs have also been shown to modulate **neutrophil** activity and to suppress neutrophil activation **(Figure 6)**. The relationship between MSCs and **NK cells** is complex. MSCs have been reported to inhibit NK cell proliferation and cytotoxicity [158]. Conversely, MSCs might be susceptible to lysis mediated by NK cells[158, 159] since they secrete ligand activating NK cell receptors and they express low levels of MHC class I molecules **(Figure 6)** [160]. Interestingly, MSCs can affect major stages of the **DC** cycle [161], i.e. activation, differentiation, maturation and antigen presentation. MSCs may thus favor the reprogramming of mature stimulatory DCs into a more pro-tolerogenic DC phenotype, characterized by (i) lower immunogenicity, (ii) higher secretion of IL-10 but lower production of IL-12, (iii) an ability to inhibit the proliferation and function of alloreactive T cells, and to generate alloantigen-specific Tregs **(Figure 6)** [161-164]. The interaction between MSCs and **T cells** has also been broadly investigated. MSCs suppress T-cell proliferation triggered either by allogeneic [165], mitogenic or antigen-specific stimuli [166]; impair the activation and differentiation of T cells [167]; decrease T-cell cytotoxicity [168, 169]; regulate the Th1/Th2 balance [146, 170]; and favor the differentiation of $CD4^+$ T-cell subsets with a **Treg** phenotype **(Figure 6)** [169]. The enhancement of Treg function is particularly relevant and attractive in the field of SOT [171] since Tregs are crucial mediators of the immune allogeneic response. Moreover, their impact on the T-cell population indirectly results from MSC-mediated modulation of APCs [172]. However, the relative impact of MSCs on memory and pre-activated T-cell effectors compared to naive T cells, in addition to the duration of MSC-induced

immunomodulation, are uncompletely understood[170]. Finally, MSCs have been reported to inhibit **B-cell** proliferation, especially through an arrest in the G0/G1 phases [173].

Of important note, the immunomodulatory properties of MSCs are thought to depend upon the microenvironment to which they are exposed. For example, it has been suggested that MSC activation depends on the levels of inflammatory cytokine such as IFN-γ or TNF-α [174]. In addition, critical MSC- and immune cell-related parameters could impact MSC capacities, including cell culture and expansion conditions, tissue origin, cryopreservation, activation signals and the MSC-to-immune cell ratio [167]. Variability in these parameters may result in heterogeneous outcomes in MSC-based therapy.

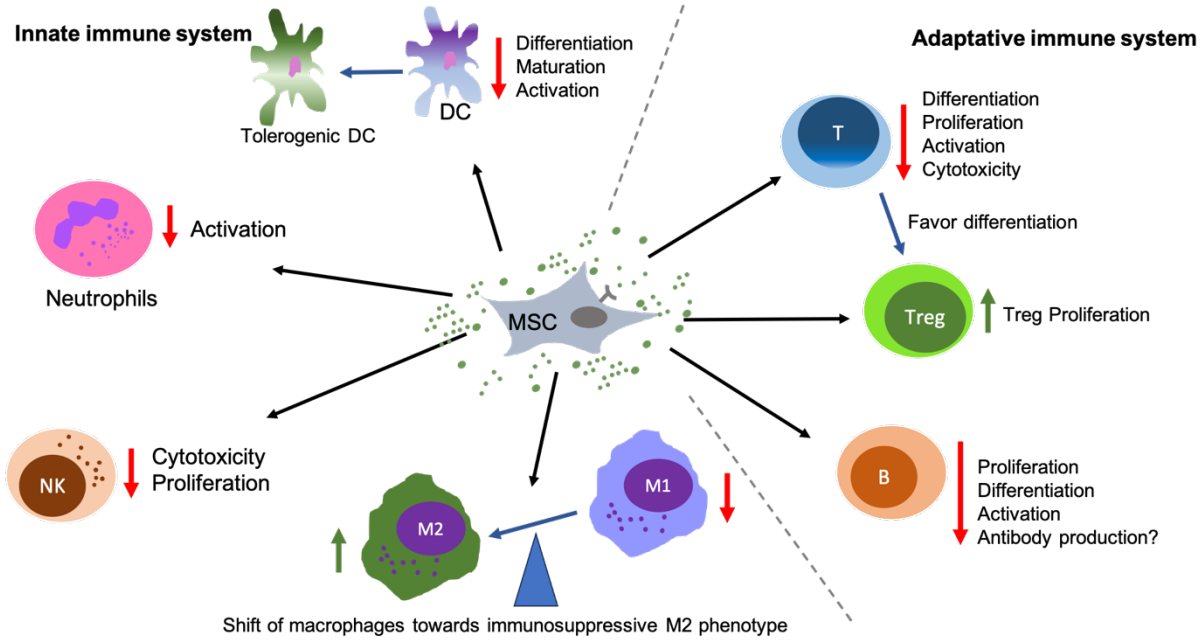


Figure 6. Schematic view of the interactions between MSC and immune cells. NK, Natural killer cells; DC, Dentritic cells; T, T cells; B, B cells; M, macrophages, → : cell-to-cell contact; : MSC secretome; →: differentiation/polarization into…

2.2.4 Tissue repair properties

Besides their immunomodulatory actions, MSCs are likely to contribute to tissue repair through several mechanisms [154, 175, 176], such as (i) promoting angiogenesis, (ii) reducing apoptosis and (iii) enhancing the survival and proliferation of endogenous cells. MSCs were

described to home into primarily damaged tissues via chemotactic factors released by immune cells[177, 178] but this statement remains controversial and several studies suggest that MSCs do not home to sites of inflammation and could even remain trapped in the lung barrier [179-182]. However, accumulating evidence has also highlighted the predominant paracrine role of MSCs in establishing a regenerative microenvironment through their interactions with many cells, including fibroblasts, endothelial cells, epithelial cells and macrophages. The original theory of a trans-differentiation or cell fusion phenomenon has been refuted [183, 184]. Angiogenesis is a crucial step in the process of tissue repair, which can be modulated by MSC paracrine factors, especially vascular endothelial growth factor [185, 186], angopoietin-1, hepatocyte growth factor, TGF-β and stromal cell-derived factor 1-α. MSCs regulate several functions of endothelial cells including their proliferation and migration [185, 187]. An anti-apoptotic effect is also noted in many studies, via the ability of MSCs to prevent oxidative stress and via the activation of the protein kinase B pathway [176]. MSC anti-inflammatory effects result from a reduction of pro-inflammatory molecules, such as IFN-γ and IL-1α, and the production of anti-inflammatory cytokines, such as IL-10 and TNF-α-induced protein-6 [154, 176]. In addition, MSC-derived EVs may help rapidly restoring the ATP supply following IRI by transferring mitochondria into the damaged cells [188]. Finally, the attenuation of acute kidney injury could result from the MSC-mediated modulation of renal metabolism [189] and prevention of lipotoxicity [190].

2.3 Immunogenicity

The broad impact of MSCs on the immune system is widely admitted. By contrast, the potential of MSCs to elicit allogeneic responses remains uncertain. The evidence for an intrinsic immune privileged status of MSCs has been evoked on the basis of (1) their inhibitory functions on various immune cells, (2) the creation of a suppressive micro-environment, and (3) their low immunogenicity [191]. Indeed, culture-expanded MSCs usually express low levels of MHC class I antigens and no MHC class II antigens or costimulatory molecules such as CD40, B7-1, or B7-2[137, 192, 193]. MSCs do not activate allogeneic lymphocytes [192, 194]. However, following exposure to IFN-γ, MSCs act as APC with upregulation of both MHC-I and

MHC-II antigens [195, 196]. After the preclinical documentation of an immune response against MSCs[196], the paradigm of the absolute immune privileged status of MSCs was questioned. MSC immunogenicity needs to be considered as one of their characteristics since these cells are “immune evasive” but not “immune privileged”[193]. These considerations are particularly relevant in SOT. Indeed, in the settings of SOT, MSCs can be isolated from the patient who is the recipient of the graft (autologous MSCs), from the graft donor (allogeneic donor-derived MSCs) or from an unrelated healthy donor not matched with the recipient nor to the graft donor (allogeneic third-party MSCs) **(Figure 5)** [137]. The main concerns with the use of allogeneic MSCs in SOT include (1) the rejection of MSCs by the recipient’s immune system, (2) the similar efficacy between autologous and allogeneic MSCs, and (3) the induction of an immune response that could be deleterious to the host, including the production of additional graft DSAs in cases of shared mismatches between MSCs and graft donors with a potential risk of recipient sensitization[191]. One of the major advantages of 3rd party MSCs is their more rapid availability for a deceased donor transplantation program in which it would be difficult to plan the thawing and expansion of autologous MSCs in the peritransplant period[197].

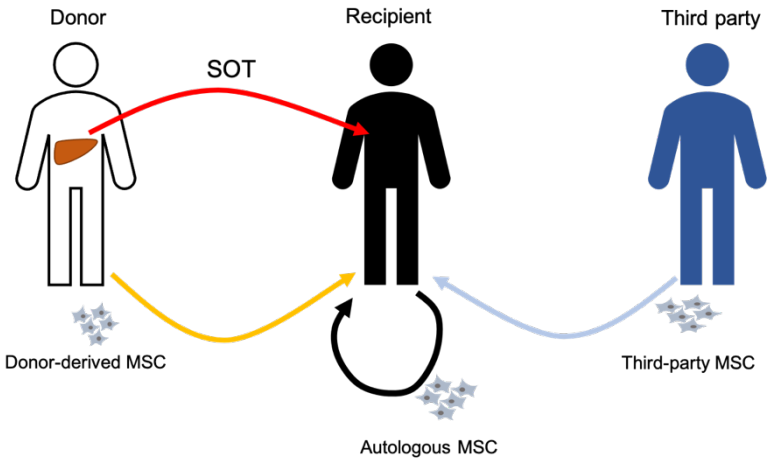


Figure 7. Possible sources of MSC donors.
MSC, mesenchymal stromal cell; SOT, solid organ transplantation;

3 Mesenchymal stromal cells in solid organ transplantation

3.1 Pre-clinical models

3.1.1 Prevention and treatment of liver IRI and enhancement of liver regeneration

MSCs have shown therapeutic effects for the treatment of IRI in the kidney, heart and lung in a significant number of studies[198]. Only a few studies have been published for IRI in the liver, and the exact role of MSCs has not yet been defined **(Table 3)**.

A few years ago, Jin G. et al. [199] evaluated the effect of allogeneic BM-MSCs to attenuate IRI in rats during the first 24h after liver reperfusion. In their model, partial liver ischemia was obtained by vascular clamping during 60 min. BM-MSCs were injected through the portal vein. Injury severity, oxidative stress response and apoptosis of the liver were regularly evaluated during the first 24h and compared to a control group. The conclusion of this study is that allogeneic BM-MSCs partially protect the liver from IRI when injected via the portal vein due to their ability to suppress oxidative stress and to inhibit apoptosis **(Table 3)**. Another related model using repeated adipose-derived MSC injections (within 24 hours after reperfusion) via a peripheral vein in rats also showed a significant protective effect against liver IRI with a suppression of inflammatory response, oxidative stress and apoptosis [200]. It was also found in a model of IRI with major hepatectomy that MSCs protected the liver from IRI and that liver regeneration was enhanced [201] **(Table 3)**.

In addition to liver IRI, research has also focused on the potential beneficial effect of MSCs in partial LTx. A few years ago, 50% reduced-size LTx in rats were used to examine whether MSC-conditioned medium (MSC-CM) could protect hepatocytes and LSEC and enhance their regeneration [202] **(Table 3).** MSC-CM was injected in rats via a peripheral vein directly after orthotopic partial LTx. Compared with the control group, the MSC-CM group showed a significantly lower release of liver injury biomarkers and a clear survival benefit.

Table 3. Preclinical studies using MSC therapy in model of liver IRI and of liver regeneration

Model	Groups	Source Dose	ISD	Timing and route of MSC injection	Time point of analysis	Outcomes	Ref Year
Rat (Wistar) n=24/group IRI model	A) Sham-op B) IRI- MSC C) IRI-Ctl	BM 3p $1x10^6$	/	1 PV injection Immediately after reperfusion	Sacrifice at H2, 6, 12, 24 (n=6/group/timepoint): -blood sample: AST, ALT -liver sample: histological damages, apoptosis, SOD, GSH-PX, MDA, Bcl-2 protein, Bax	In group B (MSC) compared to C: ↓ ALT, AST after 12 and 24h ↑ SOD, GSH-PX activity, Bcl-2 protein, ↓ MDA, Bax ↓ Liver damages, apoptotic hepatocytes → MSC suppress oxidative stress and inhibit apoptosis within 24h of reperfusion	Jin *et al.* [199] 2013
Rat (Fisher) n=30/group IRI model	A) Sham-op B) IRI-CM (ctl) C) IRI-MSC	AT Autologous $1.2x10^6$	/	3 IV injections Immediately after reperfusion, H6, H24	Sacrifice at H72: AST, histologic features, hepatic cytokine profile, oxidative stress, and apoptose	In group C (MSC) compared to B: ↓ AST, TNF, TGFß, IL1ß, IL-6, endothelin-1, MMP-9, Bax, Casp-3, ↓ Apoptotic nuclei ↑ eNOS, Bcl-2, IL-10, HO-1, NQO-1 → MSC suppress inflammatory response, oxidative stress and apoptosis	Sun *et al.* [200] 2012
Rat (Lewis) N=24/group IRI + Hepatect. (70%) model	A) MSC B) Ctl (PBS)	BM Autologous $1x10^6$	/	1 PV injection Immediately after reperfusion	Sacrifice at H6, 24, 72, 168 (n=6/timepoint/group): AST, ALT, histological damages, apoptosis, liver regeneration, detection of MSC (luciferase activity)	Peak of MSC in the liver at H24 rapidly declining ↓ AST/ALT ↓ Histological damages ↓ Apoptosis ↑ Remnant liver regeneration	Kanazawa *et al.* [201] 2011
Rat (SD) n=25/group RSLTx (50%)	1) Sham-op 2) RLSTx + MSC-CM 3) RSLTx + medium	BM MSC-CM Autologous	/	1 IV injection At the day of surgery	Sacrifice at H6, 24, 48, 72, 168 + survival: Graft function, pro-inflammatory cytokines, liver apoptosis, proliferation of hepatocytes, VEGF and MMP9 in the liver	↓ Liver injury biomarkers, proinflammatory cytokines ↑ Survival ↑ Hepatocytes and SEC proliferation and ↓ apoptosis ↑ VEGF and MMP9 in grafts ↓ Proinflammatory cytokines	Du *et al.* [202] 2013
Rat (SD) n=25/group RSLTx (30%)	1) RSLTx + PBS 2) RSLTx + MSC	BM- Autologous $2.4\ x10^6$	/	1 IV injection After reperfusion	Liver biopsy H1, 6, 24, D 3, 7 (n=5/timepoint): Expression of apoptosis-, inflammatory-, anti-inflammatory- and growth factor-related genes Activities of transcription factors AP-1 and NF-kB	↓ IRI and acute inflammation to promote liver regeneration ↑ 1-W survival ↑ mRNA of HGF, Bcl-2, Bcl-XL, IL-6, IL-10, IP-10 and CXCR2 ↓ TNF-α ↑ Activities of AP-1 and NF-kB ↑ expression of p-C-Jun, Cyclin D1 and PCNA Very rare engraftment in the liver of the injected MSCs at D7	Wang *et al.* [203] 2014
Swine n=5/group DCD LTx model	1) HB 2) DCD 3) DCD+ MSC	AT 3p $1x10^7$	/	1 IV injection 2h after reperfusion	Survival + Blood samples at H1, 3, 5, 7, D1, 3, 7: AST, ALT, TNF-α, IL-1ß, IL-6	Better survival in DCD+MSC (>7d: n=3, <4d: n=2) vs DCD (<24h : n=5)	Sasajima *et al.* [204] 2020

Legend: ALT, alanine aminotransferases; AP-1, activator protein 1; AST, aspartate aminotransferase; AT, adipose tissue; Bax :Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2 protein; BM-, bone-marrow; Casp,: caspase-3 protein; CM, culture medium; Ctl, control; CXCR, CXC chemokine receptors; D, day; DCD, donors after cardiac deatheNOS, endothelial nitric oxide synthase; GSH-PX, glutathione peroxidase; H, hour; HB, heart-beating; hepatect., hepatectomy ; HGF, hepatocyte growth factor; HO, heme oxygenase; IL, interleukine; IRI, ischemia reperfusion injury; IV, intravenous; ISD, immunosuppressive drug; MDA, malondialdehyde; MMP, matrix metalloproteinase; MSC, mesenchymal stromal cell; MSC-CM, Mesenchymal stromal cell-conditioned medium; NF, nuclear factor; NQO-1, nicotinamide-adenine dinucleotide phosphate: quinone oxidoreductase 1; op, operated; PBS, phosphate buffered saline; PNCA, proliferating cell nuclear antigen; PV, portal vein; p-C-Jun, phosphorylated C-Jun; RSLTx, reduced size liver transplantation; SD, Sprague-Dawley rats; SECs, sinusoidal endothelial cells SOD: superoxide dismutase; TGF, tumor growth factor; TNF, tumor necrosis factor;VEGF, vascular endothelial growth factor; W, week; 3p, Third-party;

More proliferating hepatocytes and LSECs, and less apoptosis were observed. The levels of many inflammatory cytokines and the infiltration by neutrophils and KC activation were decreased. VEGF and MMP-9 expression was increased in the graft. All these facts suggest that MSC-CM could have potential for the prevention of liver injury and to enhance its regeneration after partial liver transplant. More recently, in another reduced-sized LTx model comparing injection of MSCs versus placebo at the time of transplantation, MSC injections have been shown to be effective in alleviating IRI and acute inflammation of the liver graft and in up-regulating anti-inflammatory cytokines. Histologically, MSCs were effective to protect both hepatocytes and LSECs. One-week survival was also significantly improved[203] **(Table 3)**. Interestingly, very rare engraftment of the injected MSCs could be found in the liver 7 days after transplantation. Recently, a group studied the impact of MSC injection in a swine model of LTx from DCD. The authors concluded that adipose tissue-MSCs could have a protective effect on liver graft function from warm IRI and could improve the viability of DCD liver grafts [204] **(Table 3)**. However, it has been demonstrated in a liver IRI model that intravenously injected MSCs are short-lived, that viable MSCs do not go beyond the lungs, and that they remain in the circulation for a very limited period [182]. It has thus been suggested that other cells should be implicated to mediate the powerful immunomodulatory and regenerative properties of MSCs on target organs.

3.1.2 MSC as alternative to immunosuppressive drugs / tolerance induction in liver allograft rejection models

MSC infusions have shown an ability to prolong graft survival in heart [205-207], skin [165, 208] and kidney [208-210] animal transplantation models. However, one group found no effect of MSCs alone on heart allograft survival in a mouse model [211], and another group found that MSCs infused after KTx could cause premature graft dysfunction [208].

Table 4 (part 1). Preclinical studies using MSC therapy in model of liver allograft rejection and of GVHD.

Model	Groups	Source Dose	IS	Route of MSC injection	Timing of MSC injection	Time point of analysis	Outcomes	Ref Year
IS alternative strategy/ tolerance induction								
Rat n=12/group Allo-LTx SD to Wistar	A) MSC B) Ctl	AT DD (SD) 2 x 10^6	None	IV + PV	3 injections: D-7, D3 and during LTx	Sacrifice at D7: ALT, AST, TB, IL-2, IL-10, histo	↓ ALT, AST, TB in group 1 Histo: no or mild to moderate acute rejection (group A) vs severe (group B)	Wan *et al.* [212] 2008
Rat n=6/group Allo- LTx Lewis to BN	A) MSC-DD B) MSC-RD C) MSC-3p D) Ctl	BM DD /RD / 3p 2 x 10^6	None	IV	7 injections: During LTx and D1, 2, 3, 8, 12, 16	Sacrifice at D7: Allograft rejection, Peripheral blood Tregs Survival	↑ graft survival in the 3 groups with MSC 45-57D vs 21D No difference with MSC source (→ immune action independent of MHC) At D7: acute rejection ↓ + ↑ Tregs in MSC groups	Wang *et al.* [213] 2009
Rat n=14 /group Allo-LTx DA to Lewis	A) Saline B) MSC C) MSC lentivirus D) IL-10 engineered MSC	BM DD 2.5x10^5	none	IV	1 injection: 30 min post LTx	Sacrifice at D3, 5 and 7 + survival: Allograft rejection, Tregs (spleen), serum cytokines (IL-17, IL-23, IL-6, IL-10, IFN-γ, TNF-α, TGF-β1)	↑ Graft survival (MSC IL10>MSC-lenti/MSC>>>Saline) ↑ FoxP3 in IL-10-MSC (vs saline) ↑ Anti-inflammatory cytokines in IL-10 MSC (vs saline) ↓ Pro-inflammatory cytokines in IL-10-MSC (vs saline)	Niu *et al.* [214] 2014
Rat n=28/group Allo-LTx DA to Lewis	A) Ctl B) MSC / donor-KC inhibited C) MSC D) MSC overexpressing PGE2	BM 3p 5 x10^5	none	PV	1 injection: Directely after LTx	Sacrifice at D3, 5 and 7 Intragraft cytokines (IL10, IL-2, IFN-γ, TNF-α), allograft rejection Serum ALT, AST, TB Survival	MSC can reprogram KC (with PGE2) and conversely (with TNF-α) from M1 to M2 phenotype. ↑ Allograft survival in MSC-treated (43-90D vs 10D): group D>C>B>>A ↑ Serum IL-10 in MSC-treated group ↓ Serum pro-inflammatory cytokines (IL-2, IFN-γ, TNF-α)	You *et al.* [215] 2015
Rat n=14/group Allo-LTx Lewis to BN	A) Ctl B) TAC C) MSC + TAC	BM DD 1x10^6	TAC 2mg/kg/d im for 1M	PV	1 injection: Directly after LTx	Sacrifice at D7 (n=6): ALT, AST, TB, TGF-ß, IL10, IL12, allograft rejection, homing Survival	↑ Liver function in MSC+TAC ↑ Survival in MSC+TAC (63D) > TAC (44D) > Ctl (11D) ↓ Acute rejection in MSC+TAC < TAC ↑TGFß, IL10 ↓ IL12 Homing of MSC in the portal area	Sun *et al.* [216] 2015
Rat n=35/group Allo-LTx DA to Lewis	A) Saline B) LV-TGF (supernatant) C) MSC D) TGF/MSC (TGF ß1-secreting MSCs)	BM RD 5x10^6	none	PV	1 injection: Directly after LTx	Sacrifice at D1, 3, 5, 7: Allograft rejection, intragraft Tregs Serum ALT, TB, IL-10, IL1-ß, IL-6, IFN-γ Survival	↓ Acute rejection TGF/MSC < MSC ≅ LV-TGF < Saline ↑ Graft survival TGF/MSC (110.4D) > MSC (61.5D) ≅ LV-TGF (56.3D) > Saline (8.5 D) ↑ Intragraft induced Tregs (TGF/MSC > MSC ≅ LV-TGF > Saline) ↑ IL10 ↓ IL1-ß, IL-6, IFN-γ	Tang *et al.* [217] 2016

Table 4 (part 2). Preclinical studies using MSC therapy in model of liver allograft rejection and of GVHD.

Model	Groups	Source Dose	IS	Route of MSC injection	Timing of MSC injection	Time point of analysis	Outcomes	Ref Year
Rat n=15/group Allo-LTx Lewis to BN	A) MSC-allograft B) Allograft C) Isograft D) Sham-op	BM 3p (SD) $2x10^6$	none	PV	1 injection: Directly after LTx	D3: serum ALT,AST, TB Sacrifice at D7: allograft rejection Survival Coculture of BM-MSC with sera of different groups: PD-L1 expression (mRNA) in BM-MSCs	Better liver function and ↓acute rejection in MSC-treated vs group B ↑ Survival in group A (median 29D) vs B (13 D) BM-MSCs upregulate PD-L1 expression in "allograft environment" by downregulating miR-17-5p	Chen *et al.* [218] 2018
Rat n=14/group Allo-LTx Lewis to ACI	A) Saline B) MSC	?? DD 2.5×10^5	none	IV	1 injection: 30min after LTx	Sacrifice at D7: TGF-ß1 and FoxP3+ expression in liver/spleen, Allograft rejection Survival	↑ Survival in MSC-treated rats (105D) vs MSC-untreated (16.2D) ↓ Acute rejection ↑TGF-ß1, IL10, Tregs	Niu *et al.* [219] 2018
Rat n=40/group Allo-LTx Lewis to BN	A) MSC overexpressing HO-1 B) MSC C) HO-1 D) Saline	BM RD $5x10^6$	none	IV	1 injection: Directly after LTx	Sacrifice at D1, 3, 5, 7, 10, 14, 28: AST, ALT, TB Allograft rejection, apoptosis Survival	↑ median survival in MSC-treated (A: 77days > B: 61days >> C: 25 days > D: 21 days) ↓ AST, ALT, TB (A<B<<C<D) ↓ Histological signs of acute rejection (A<B<<C<D) ↓ Apoptosis (A<B<<C<D) ↑ Peripheral Tregs proportion	Wu *et al.* [220] 2016
Rat n=45/group Allo-RSLT Lewis to BN	A) Saline B) MSC C) MSC overexpressing HO-1	BM RD 1.10^7	none	IV	1 injection: Directly after LTx	Sacrifice at H0, 3, 6, 24, D3, 5, 7, 14: Acute rejection, serum IL-10, TGF-ß, IL-2, IL-17, IL-23, TNF-α, Tregs proportion (spleen), NK activity (blood) Survival	In MSC treated: ↑ Survival (C: 38D > B: 25D > A: 13D) ↓ Acute rejection, pro-inflammatory cytokines, NK cell viability (C>B) ↑ Peripheral Tregs ↓ Anti-inflammatory cytokines (C<B)	Shen *et al.* [221] 2018
Treatment/prevention of GVHD								
Rat n=8/group Allo-LTx **GVHD model** Lewis to F1(Lew+BN)	1) Ctl 2) MSC-DD 3) MSC-RD	BM DD / RD $5 x10^6$	none	IV	7 injections 1) D0 to D6 ---------- OR 2) delayed: D8 to 14	D8 and D16 (or at death) : Histology, serum ALT/ AST, blood Tregs Detection of MSC in recipient's tissues Survival	↑ Survival in MSC groups (75%) vs (0%) ↓ Symptoms of GVHD ↓ Histo-pathological features of GVHD No ≠ in serum ALT, AST ↑ Tregs in peripheral blood (X3) and in intestine No difference of efficacy between sources **Not effective when delayed MSC administration** (D8 to 14) No detection of MSC in targeted organs; in the skin at D1	Xia *et al.* [222] 2012

Legend: ALT, alanine aminotransferases; AST, aspartate aminotransferase; AT, adipose tissue; Allo-, allogeneic; BM, bone-marrow; BN, Brown-Norway rats; Ctl, control; D, day; DA, Dark Agouti rats; DD, donor-derived; histo, histology; IFN, interferon; GVHD, graft-versus-host disease; HO, heme oxygenase; IL, interleukine; IV, intravenous; KC, Kupffer cell; LTx, liver transplantation; LV, lentivirus; M, month; MSC, mesenchymal stromal cell; NK, natural killer; op, operated; PD-L1, Programmed death-ligand 1; PGE, prostaglandin E; PV, portal vein; RD, recipient-derived; RSLTx, reduced size liver transplantation; SD, Sprague-Dawley rats; TAC, tacrolimus; TB: total bilirubin; TGF, tumor growth factor; TNF, tumor necrosis factor; Treg, regulatory T cell; 3p, third-party;

Only a few studies have been published in LTx models (**Table 4).** Compared to a control group, adipose tissue-derived MSCs significantly decreased AR after orthotopic LTx in rats, based on serum rejection markers and on histological analysis on D7. Serum levels of IL-2 were reduced and those of IL-10 were increased. In this model, donor-derived MSCs were infused IV 7 days before and 3 days after LTx as well as during the LTx via the portal vein [212] (**Table 4).** Another group studied the ability of a BM-MSC infusion to inhibit acute graft rejection after allogeneic LTx in rats [213]. MSCs were derived from the recipient, the liver donor or a third party, and infused intravenously at the time of surgery as well as one daily for 3 days thereafter. MSC-treated recipients survived significantly longer compared to controls. Furthermore, there was no significant difference between the 3 groups receiving MSCs from various origins. Histological analysis showed severe acute graft rejection at day 7 in rats without MSC infusion, while acute graft rejection was significantly decreased in the other groups **(Table 4)**. These observations were associated with a marked increase in the number of Tregs in recipients receiving MSCs. This suggests an important role of Tregs in MSC-mediated immunosuppression. In a preclinical study published in 2014, 42 liver-transplanted Lewis rats were injected intravenously 30 minutes post-OLT with BM-MSCs (3 groups) derived from liver donors (DA) and compared with 14 transplanted rats injected with saline. One of the groups received IL-10 engineered MSCs (with lentiviral vector-mediated expression of IL-10). Graft survival was significantly higher in the MSC-treated group than in the saline group. Moreover, pro-inflammatory and anti-inflammatory cytokines were respectively decreased and increased in the IL-10 MSC group compared to the saline group, suggesting the potential important role of IL-10 secretion in MSC-mediated immunomodulation **(Table 4)** [214]. To underline the importance of the PGE2 secretion by MSCs and of the effect of MSCs on KCs, You *et al.* showed in a model of acute liver allograft rejection that MSCs could significantly prolong allograft survival and that this effect was increased by PGE2 overexpression in MSCs **(Table 4)**. They also showed *in vitro* that MSCs can reprogram KCs (through PGE2 secretion) from a M1 to a M2 phenotype and, conversely, that KCs (through TNF-α secretion) play a central role in the induction of MSC-mediated allograft tolerance [215]. In 2016, Tant et al. studied the effect of MSCs over-expressing TGF-ß1 (TGF/MSC) in inducing tolerance towards the liver in a model of AR. In this model, MSCs were injected through the portal vein directly after reperfusion. Four groups were compared: rats receiving saline, “conventional” MSCs,

TGF/MSCs, or supernatant of the latter **(Table 4)**. They found that TGF/MSCs prevented rejection and improved survival after LTx, more so than conventional MSCs or supernatants. This was also associated with more Tregs and fewer Th17 cells in the graft [217]. These two studies suggest that "modified" MSCs could be a key to improve MSC-mediated effects. The role of TGF-ß1 in MSC-mediated tolerogenic effects was also demonstrated in another paper in which a single MSC injection significantly prolonged graft survival with increased intragraft levels of TGF-ß1 and Tregs in a model of liver graft AR [219]. Another group showed that, in addition to treatment with tacrolimus, an infusion of MSCs through the portal vein in a liver allograft AR model significantly decreased allograft rejection and prolonged allograft survival when compared to rats transplanted with tacrolimus alone or without any immunosuppression. They also showed that MSCs injected through the portal vein were able to home to the portal area of the liver and were still detected 7 days after LTx [216] **(Table 4)**. Another paper showed that a single 3rd party BM-MSC injection through the portal vein after LTx significantly increased survival with a better liver function compared to rats not receiving MSCs. This study also showed that part of the immunoregulatory properties of MSCs in this allograft model could be partially mediated by upregulation of PDL-1 on MSCs **(Table 4)**. It was also shown earlier that cell-cell interaction of PDL-1 with its ligand PD-1 could activate inhibitory signals resulting in the blockade of T-cell proliferation/function and stimulate Treg generation, thereby promoting allograft tolerance [218]. As mentioned supra, HO-1 is one of the factors playing a role in MSC-mediated properties. HO-1 is an inducible enzyme implicated in the degradation of heme, which has been shown, both *in vitro* and *in vivo,* to have anti-inflammatory and anti-IRI properties by promoting the secretion of IL-10 and TGF-ß and increasing the immunomodulatory effects of Tregs. Two recent studies showed that transduction of HO-1 into MSCs could improve their tolerogenic properties in LTx models. It was demonstrated in a model of liver graft AR and in a model of AR associated with a reduced-sized LTx that engineered MSCs overexpressing HO-1 exerted enhanced activity and a longer duration of action compared to "conventional" MSCs. MSC-treated rats had a longer survival, better liver function, fewer histological signs of AR and higher levels of anti-inflammatory cytokines and peripheral Tregs compared to control groups **(Table 4)** [220, 221].

It has also been demonstrated in a rat model of GVHD after LTx that MSCs injected daily from D0 to D6 could mitigate the signs of GVHD and significantly increase survival

compared to controls. This effect was comparable in groups receiving donor- or recipient-derived MSCs. Interestingly a delayed administration of MSCs (from D8 to D16 after LTx) was not effective in preventing GVHD [222]. **(Table 4)**.

3.1.3 Effects of immunosuppressive drugs on MSCs

In clinical transplant studies (see infra), MSCs are systematically used concomitantly with ISDs. As MSCs and ISDs inhibit the same targets (essentially T cells), it is reasonable to consider that interactions between them can occur. Therefore, it is essential to know which ISD can (positively or negatively) affect MSC functions. *In vitro*, it was shown in a mixed lymphocyte reaction (MLR) comparing the inhibitory effects of human MSCs associated with various ISDs, that tacrolimus and rapamycin decreased MSC immunosuppressive properties, corticoids had no effect while MMF promoted them [223]. In culture conditions, it was shown that high doses of tacrolimus appeared to be toxic for MSCs, while MMF and rapamycin at therapeutic doses just inhibited MSC proliferation [224]. Conversely, others have demonstrated that preincubation of MSCs with tacrolimus could increase their inhibitory properties [224] and that CsA and MSCs could exert cumulative effects against activated lymphocytes [225].

In vivo, in a rat KTx model, CsA antagonized MSC efficacy, and their combination carried no advantage in terms of allograft survival rates compared to CsA alone. Nevertheless, this study has to be contrasted with other studies using various ISDs used together with CsA, in which the efficacy of MSC was not altered [226]. MMF and MSCs synergized to promote long-term allograft tolerance in a rat heart transplantation [227]. In contrast to what is observed *in vitro*, mTOR inhibitors and MSCs synergized as immunomodulators to promote heart graft survival. Moreover, in 2018, in a model of islet allograft in mice, Duan et al. found that treatment with MSCs alone or with low-dose mTOR inhibitors was not effective in treating acute rejection and prolonging graft survival, but that the combination of MSCs and low-dose rapamycin could significantly increase graft function and survival suggesting a synergistic effect of this association **(Table 5)**. However, this combination has not been tested in LTx.
The choice of concomitant immunosuppressive drugs is an important matter for debate, and more studies are needed to define which are the most effective drugs to use with MSCs.

Table 5. Preclinical studies evaluating the association of MSC + m-TOR inhibitors in allograft rejection.

Model	Groups	Source Dose	IS	Route and Timing of MSC injection	Time point of analysis	Outcomes	Ref Year
Mice n=5/group Allo-ITx	1) Ctl 2) MSC 3) Rapa 3) MSC + Rapa	RD or 3p BM $1x10^6$	Rapa 0,1mg/kg D0 to 7	3 IP injections D0 to D2	Sacrifice at D9-10 post ITx + survival	-MSC or rapa alone: nearly no effect -MSC + rapa: ↑ graft survival and graft function ↑ Treg in islet allograft and draining lymph nodes	Duan [228] 2018
Mice n=16/group Allo- HTx C57BL/6 to BALB/c	1) Ctl 2) Rapa 3) MSC-DD 4) MSC-DD + rapa 5) MSC-RD + rapa 6) MSC-3p + rapa	DD or 3p $1x10^6$	Rapa 2 mg/kg D0 to 13	1 IV, 24h after HTx	D 7, 30: Graft histology, immune profile, MSC tracking. +survival (n=8/group)	-MSC: ↓graft rejection, double graft survival (16.5d) -Rapa: ↓graft rejection, double graft survival -MSC+ rapa: normal histology, long term-survival (>100d) Independently of MSC origin ↑ Tregs in the spleen ↑ MSC in lymphoid organs and graft	Ge [206] 2009

Legend:
Allo, allogeneic; Ctl, control; D,day; DD, donor-derived; HTx, heart transplantation; inj, injection; IP, intra-peritoneal; ITx, islet transplantation; IV, intravenous; MSC, mesenchymal stromal cell; rapa, rapamycine; RD, recipient-derived; Treg, regulatory T cell; Tx, transplantation; 3p, third-party;

3.2 Clinical trials

3.2.1 Clinical trials using mesenchymal stromal cells in liver transplantation

Similar to KTx, convincing results in preclinical LTx models supported the launch of clinical trials (**Table 7**).

The *MISOT-I Study* aimed to investigate the safety and feasibility of multiple injections of MultiStem®, a commercial product (Athersys Inc., Cleveland, OH) of BM-derived multipotent adult progenitor cells (MAPCs), after LTx (**Table 7**). This trial also evaluated the

impact of MAPC administration on the time to first biopsy-proven acute rejection (BPAR) within the first 90 days *post* LTx. MAPCs belong to the family of MSCs and exhibit very similar properties [229]. The patients enrolled in this single-arm study were supposed to receive two doses of third-party MAPCs, at the time of LTx and 48h later. Cell dose escalation was scheduled for every third patient, in association with basiliximab, mycophenolic acid and steroids[230]. CNIs were used only after BPAR. In 2015, Soeder *et al.* published the first-in-man case of this phase I study [231]. The patient did not present any acute complication in connection with MAPC injections but experienced major adverse events within the first week, as well as an AR requiring CNI introduction. The leukocyte profile demonstrated an increased number of $CD4^+FoxP3^+CD127^{low}$ Tregs from post-operative day 3, with subsequent normalization at day 29. Two more cases were registered on ClinicalTrials.gov. These patients also developed major adverse events, but none of them was directly linked to MAPC. MAPCs associated with CNI-free IS could not replace the classical immunosuppressive regimen [232]. The *MISOT-I Study* was discontinued by the investigators (**Table 7**).

In 2017, Shi *et al.* published the results of their trial studying the effectiveness of UC-MSCs to treat liver allograft AR (**Table 7**) [233]. Twenty-seven LTRs under conventional immunosuppressive drugs who presented BPAR were enrolled in this study. These patients were randomly assigned either to (i) the MSC-group receiving conventional immunosuppressive treatment associated with a single (n=13) or multiple (n=1) injection(s) of UC-MSCs, or (ii) the control group treated with conventional IS drugs (n=13). In terms of safety, no complications were associated with the use of UC-MSCs in the first group at the 24-week follow-up. Furthermore, a significantly stronger decrease of aspartate aminotransferases, alanine aminotransferases and total bilirubin was observed in MSC-treated *versus* control patients. Nearly half the MSC-treated patients and none of the controls presented with histological improvements of the liver allograft 4 weeks after infusion. Four weeks after MSC infusion, MSC-treated patients displayed a significant increase of circulating Tregs and of the Treg/Th 17 ratio, while HLA-DR expression on $CD4^+$ T cells was significantly lower, which suggests an inhibition of $CD4^+$ T-cell activation. Soluble factors, i.e. PGE2 and TGF-ß1, were significantly increased in 86% of the MSC-treated patients after four weeks. Hence, a single MSC injection was safe and could possibly be effective in controlling liver AR. A repetition of MSC injections was feasible (n=1) in case of unresponsiveness to the first MSC

administration. Still, there was no conclusive information about the efficacy of repeated administrations of MSCs.

The results of a Chinese controlled, randomized study evaluating the use of MSC-based immune induction in ABO-incompatible LTx was recently published (**Table 7**) [234]. The investigations evaluated the safety and efficacy of MSC therapy, compared to Rituximab in the control group, as induction therapy. In total, 22 patients were enrolled (11 MSC-treated LTRs and 11 control LTRs). To prevent ABMR, all enrolled LTRs in both groups received steroids, basiliximab, tacrolimus, MMF and IV immunoglobulins. In addition, LTRs received UC-MSC injections ($1x10^6$/kg/injection) during LTx through the portal vein followed by 8 IV injections on post-operative week 1, 2, 4, 8, 12, 16, 20 and 24, or Rituximab (375 mg/m^2) in the control group. The primary endpoint was the safety of MSC injections and the incidence of allograft rejection in the two groups at 2-year follow-up. Secondary endpoints evaluated LTR and graft survivals and the incidence of postoperative complications. No MSC-related severe events were reported in this study. Although non-significant, MSC-therapy yielded better results than rituximab in reducing the incidence of acute rejection (9.1% vs 27.3%). The rate of biliary complications and infections was significantly lower in the MSC group. The 2-year graft and LTR survivals were similar in the two groups. The authors concluded that MSC therapy and Rituximab, in combination with steroids, basiliximab, tacrolimus, MMF and IV immunoglobulins, are equally effective for preventing ABMR after ABO-incompatible LTx. Furthermore, the findings suggest that MSCs might be used as a new immunosuppressive strategy for ABO-incompatible LT and that MSCs are more effective at preventing infections and biliary complications in LTRs.

Table 7. Clinical trials using MSCs in LTx

Trial **Patients**	**Dose** **Source** **Timing** **Route**	**IS regimen**	**Outcomes**	**Immune modulation**	**Ref** **Year**
NCT01841632 n=3 LD	2 injections $1.5x10^8$ 3p BM-MAPCs - during LTx (PV) - at D2 (IV)	*Initial:* Basiliximab (D0+D4) Steroids MMF *If needed:* +CNI	-No injection-related AE -Major AE in n=3 -Need for CNI -No malignancy	Transient increase of $CD4^+FoxP3^+CD127^{low}$	Soeder *et al.* (n=1) [231] 2015 + data from clinicaltrials.gov (n=2) 2018
NCT01690247 n=14 DCD LTR with AR	1 injection $1x10^6$/kg 3p UC-MSCs - 1 injection after BPAR (IV) (n=13) - + 2 injections (n=1)	tacrolimus MMF CS	-No AE -ALT, AST, and TB -Histological improvement	↑ Tregs and Treg/Th17 ratio ↓ $CD4^+$ T-cell activation ↑ TGF-ß1 and PGE2	Shi *et al.[233]* 2017
NCT02706132 n=22 ABO-I MSC (n=11) vs Rituximab (n=11)	9 injections 1 $x10^6$/kg 3p UC-MSC - during LTx (PV) - at W1, 2, 4, 8, 12, 16, 20, 24 (IV)	*Induction:* - Rituximab (control group) - IVIg - Basiliximab *Maintenance:* - tacrolimus - MMF - Steroids	In MSC-treated group - Less AR (9.1 vs 27.3%) - ↓biliary complications - ↓infections	N/A	Zhang *et al.*[234] 2021
NCT02223897 n=12 LTR with ITBL	6 injections 1 $x10^6$/kg 3p UC-MSC - at W1, 2, 4, 8, 12, 16 (IV)	*Conventional IS*	-No AE -↓ need for therapy for ITBL -↑ 1-yr survival	N/A	Zhang *et al.[235]* 2017

Legend: ABO-I, ABO incompatible; AE, adverse event; ALT, alanine aminotransferase; ALP, alkaline phosphatase; ABMR, antibody-mediated rejection; AST, aspartate aminotransferase; BM, bone marrow; BPAR, biopsy-proven acute rejection; CNI, calcineurin inhibitor; CS, corticosteroids; DCD, donation after circulatory death; DD, deceased donor; GGT, gamma-glutamyl transpeptidase; IS, immunosuppression; IV, intravenous; IVIg, intravenous immunoglobulin; ITBL, ischemic-type biliary lesions; LD, living donor; LTR, liver transplant recipient; LTx, liver transplantation; MAPC, multipotent adult progenitor cells; MMF, mycophenolate mofetil; MOF, multiple organ failure; PGE2, prostaglandin E2; PV, portal vein; TB, total bilirubin; TGF-ß1: Transforming Growth Factor Beta 1;Treg, regulatory T cells; UC, umbilical cord; W, week.

Besides immunomodulation and induction of tolerance, MSCs have also been tested for the treatment of ischemic-type biliary lesions (ITBL) (**Table 7**) [235]. Hence, 12 patients with ITBLs *post* LTx were treated with repeated injections (n=6) of UC-MSCs and were retrospectively matched to 70 "comparable" patients with ITBLs who had been "conventionally" treated. The need for interventional therapies was lower in the MSC-treated

group (33.3 *vs.* 64.3%, p<0.05) compared to the historical controls. The investigators also showed that 1-year graft survival was significantly higher in the MSC-treated group (72 *vs.* 100%, p<0.05)[235]. This study showed that multiple injections of UC-MSCs were safe and well tolerated and could potentially be effective in order to treat (or to prevent the worsening of) ITBLs in the context of LTx.

Moreover, a prospective pilot trial aiming to investigate the safety and feasibility, and their effects on immunomodulation, of donor-derived MSCs in pediatric living-donor LTx is in the pipeline (MYSTEP1, NCT02957552)[236]. Remuzzi *et al.* have also started a study evaluating the safety and tolerance of a single *post*induction injection of third-party MSCs in the context of LTx (NCT02260375).

Finally, we published the first monocentric, prospective, phase I-II controlled clinical study evaluating the feasibility, safety and tolerability of a single infusion of third-party MSCs in 10 LTR. The 1-year and long-term results of this study are presented in the **chapter II** of this work.

3.2.2 Mesenchymal stromal cells in other solid organ transplantation

Numerous studies report the use of MSC therapy in other SOT, mainly in KTx but also in lung and small bowel transplantation[137]. The results of these studies are summarized in the review of the literature in the **appendices** of the present thesis (Vandermeulen *et al., Transplantation,* 2020). In addition, we should also mention the recently published non-randomized phase Ib clinical trial studying 3rd party-MSC after KTx using a matching strategy to avoid repeated mismatches (the Neptune study). Ten kidney transplant recipients (KTRs) received two injections of 1.5 x10^{6}/kg 3rd party MSCs 6 months after KTx. After injections, tacrolimus was lowered (trough levels 3 ng/mL) in combination with EVR and prednisone [237]. Renal function remained stable and neither a BPAR loss nor graft loss occurred. No major alterations in leucocytes or cytokines were observed upon MSC infusion. The authors concluded that HLA-selected allogeneic MSCs combined with low dose tacrolimus, EVR and prednisone was safe 6 months after KTx.

4 Aims of this work

LTx currently represents the gold-standard treatment for most end-stage liver diseases, offering excellent short- and long-term outcomes. Nevertheless, the life-long need for immunosuppression hampers long-term outcomes because of the toxicities of commonly used immunosuppressive molecules, including malignancies, infections, metabolic syndrome, renal, cardiovascular and neurological complications. Moreover, the limited availability of donors imposes the use of expanded criteria grafts, which are more susceptible to IRI, with increased rates of graft loss due to dysfunction or ischemic cholangiopathy. To address these challenges and improve LTx outcomes, the development of novel strategies that modulate immunity while reducing the dependance on ISD and attenuating IRI are needed. MSCs, thanks to their immunomodulatory properties, show promise in achieving immunotolerance without excessive immunosuppression and in mitigating IRI to reach even higher success rates and thus avoid severe and life-threatening side effects or complications. While animal models have shown encouraging results in preventing IRI and treating or preventing allograft AR, the efficacy and, importantly, the safety of MSC therapy in humans need to be established before widespread implementation in LTx programs. Optimizing the potential efficacy of MSCs also involve determining the most effective combination with synergistic ISD.

The primary aim of this thesis was to evaluate the interest and safety of use of MSC-therapy after LTx. We therefore:

1) reviewed the literature on this topic (chapter I and appendices)
2) conducted the first prospective phase I-II clinical trial and published the 1-year (chapter II.1) and 5-year results (chapter II.2)
3) evaluated the association of MSCs and EVR in a rat model (chapter III)

CHAPTER II. INFUSION OF MESENCHYMAL STROMAL CELLS AFTER DECEASED LIVER TRANSPLANTATION: A PHASE I-II, OPEN-LABEL, CLINICAL STUDY

CHAPTER II. INFUSION OF MESENCHYMAL STROMAL CELLS AFTER DECEASED LIVER TRANSPLANTATION: A PHASE I-II, OPEN-LABEL, CLINICAL STUDY

1 One-year follow-up

Infusion of mesenchymal stromal cells after deceased liver transplantation: A phase I-II, open-label, clinical study

Authors

Olivier Detry[1,2], Morgan Vandermeulen[1,2], Marie-Hélène Delbouille[1], Joan Somja[3], Noella Bletard[3], Alexandra Briquet[4], Chantal Lechanteur[4], Olivier Giet[4], Etienne Baudoux[4], Muriel Hannon[5], Frederic Baron[5,6], Yves Beguin[5,6]

Affiliations:

1. Department of Abdominal Surgery and Transplantation, CHU Liege, University of Liege, (CHU ULg), Belgium;
2. Mesenchymal stromal cell In Solid Organ Transplantation (MISOT) consortium1;
3. Department of Pathology, CHU Liege, University of Liege, (CHU ULg), Belgium;
4. Laboratory of Cell and Gene Therapy (LTCG), CHU Liege, University of Liege, (CHU ULg), Belgium;
5. Interdisciplinary Cluster for Applied Genoproteomics (GIGA)-I3-haematology, University of Liege, Belgium;
6. Department of Haematology, CHU Liege, University of Liege, (CHU ULg), Belgium

Journal of Hepatology, 2017; 67(1):47-55

Summary

MSC infusion could be a mean to establish tolerance in solid organ recipients. The aim of this monocentric, prospective, controlled, phase-1 study was to evaluate the feasibility, safety and tolerability of a single infusion of MSCs in liver transplant recipients with 1 year of follow-up.

In this study, ten LTR under standard ISD received 1.5- 3x10^6/kg 3rd party unrelated MSCs on post-operative day 3±2, and were prospectively compared to a control group of 10 LTR. In addition, in patients from the MSC group who did not develop rejection and had normal graft function and month-6 graft biopsy, progressive weaning of immunosuppression was attempted. The primary end-points aimed at evaluating MSC infusional toxicity by recording body temperature, heart rate, mean arterial blood pressure and peripheral capillary oxygen saturation and signs of allergy before, during and after MSC infusion and at recording prospectively the incidence of any infectious (bacterial, viral, fungal) and cancerous complications until month 12 in both groups. As secondary end-points, patient and graft survivals and biopsy-proven graft rejection rates were prospectively recorded in both groups until month 12. Liver graft function, kidney function, tacrolimus levels and peripheral blood lymphocyte phenotyping were compared.

No statistical difference could be detected between the MSC and control groups concerning the characteristics of both liver graft donors and recipients. As primary end-points, no variation in vital parameters or cytokine release syndrome could be detected during and after MSC infusion. No MSC patient developed clinical signs of allergy or impairment of vital functions (including liver graft function) within the week following MSC infusion. At 1-year follow-up, no increased rate of opportunistic infection or *de novo* cancer was detected. As secondary end-points, there was no difference in overall rates of rejection or graft survival (90% at 1-year in both groups). Month-6 biopsies did not demonstrate a difference between groups in the evaluation of rejection according to the Banff criteria, in the fibrosis score or in immunohistochemistry (including Tregs). No difference in peripheral blood lymphocyte typing could be detected. Treg counts and phenotype (naive versus resting versus activated) were comparable in the 2 groups of patients at each time point. Furthermore, Treg as well as

conventional T cell proliferation (assessed by Ki67 expression) was also similar in the 2 groups of patients, as were the levels of phosphoSTAT5 in Tregs (the latter translating similar IL-2 signalling in Tregs). These combined observations suggest that a single MSC infusion had no impact on Treg count or phenotype in this study. The immunosuppression weaning in MSC recipients was not successful. One patient from the MSC group was excluded from immunosuppression withdrawal attempt due to hepatocarcinoma recurrence, but the nine others met the necessary criteria. In one patient, tacrolimus and MMF withdrawal was performed without rejection and she remained off immunosuppression for 12 months. In two patients, MMF monotherapy was achieved at month 9, but graft rejection occurred during MMF withdrawal and was successfully treated by tacrolimus reintroduction. In 6 patients, the transaminases significantly increased during tacrolimus withdrawal. In these cases, withdrawal was cancelled and liver tests normalized after increase of the tacrolimus dose.

In conclusion, this phase 1, prospective, controlled study is the first to evaluate the feasibility, safety and tolerability of MSC infusion in a series of 10 LTR under classical tacrolimus-based immunosuppression. In these patients, a post-transplantation intravenous 1.5-3x10^6/kg MSC infusion was well tolerated, without evidence of pulmonary dysfunction or of cytokine-release syndrome. These LTRs receiving MSC did not develop any evidence of impairment in vital organ functions, including the liver graft and the kidneys. In addition, they did not suffer from an increased susceptibility to infections. No *de novo* cancer was detected after one year of follow-up. For all these primary endpoints, the LTRs who received MSCs did not react differently compared to patients in the control group. The patients from the MSC group underwent unsuccessful progressive immunosuppression weaning and we were not able to show that a single infusion of MSCs at day 3 after deceased LTx could promote Treg expansion, Treg infiltration of the liver graft at biopsy at day 180, or operational tolerance. There are many shortcomings to this study. First, although 10 MSC-treated LTRs are not enough to prove the safety of MSC-therapy in LTx, this first study did not demonstrate any potential adverse effects of MSC infusion in this setting. These results will have to be confirmed by further studies in larger groups of SOT recipients. The absence of detectable effects of MSCs might be due to an insufficient sample size, to the tacrolimus-based immunosuppressive regimen or to an insufficient MSC dosing, which should possibly be increased or repeated. The timing (pre-, intra- or post-operative) and the infusion routes

(peripheral vein, portal vein or hepatic artery) of MSC infusion should also be evaluated. Different sources (BM, fat tissue, liver) or donors (organ donor, organ recipient) of MSCs might also be tested in further studies. This study opens the way for further MSC or Treg-based trials in LTx.

Research Article

JOURNAL OF HEPATOLOGY

Infusion of mesenchymal stromal cells after deceased liver transplantation: A phase I–II, open-label, clinical study

Olivier Detry[1,2,*], Morgan Vandermeulen[1,2], Marie-Hélène Delbouille[1], Joan Somja[3], Noella Bletard[3], Alexandra Briquet[4], Chantal Lechanteur[4], Olivier Giet[4], Etienne Baudoux[4], Muriel Hannon[5], Frederic Baron[5,6], Yves Beguin[5,6]

[1]*Department of Abdominal Surgery and Transplantation, CHU Liege, University of Liege, (CHU ULg), Belgium;* [2]*Mesenchymal stromal cell In Solid Organ Transplantation (MISOT) consortium*[1]*;* [3]*Department of Pathology, CHU Liege, University of Liege, (CHU ULg), Belgium;* [4]*Laboratory of Cell and Gene Therapy (LTCG), CHU Liege, University of Liege, (CHU ULg), Belgium;* [5]*Interdisciplinary Cluster for Applied Genoproteomics (GIGA)-I3-haematology, University of Liege, Belgium;* [6]*Department of Haematology, CHU Liege, University of Liege, (CHU ULg), Belgium*

See Editorial, pages 7–9

Background & Aims: Mesenchymal stromal cell (MSC) infusion could be a means to establish tolerance in solid organ recipients. The aim of this prospective, controlled, phase I study was to evaluate the feasibility, safety and tolerability of a single infusion of MSCs in liver transplant recipients.

Methods: Ten liver transplant recipients under standard immunosuppression received 1.5–3 $\times$ 10^6/kg third-party unrelated MSCs on postoperative day 3 ± 2, and were prospectively compared to a control group of ten liver transplant recipients. As primary endpoints, MSC infusion toxicity was evaluated, and infectious and cancerous complications were prospectively recorded until month 12 in both groups. As secondary endpoints, rejection rate, month-6 graft biopsies, and peripheral blood lymphocyte phenotyping were compared. Progressive immunosuppression weaning was attempted from month 6 to 12 in MSC recipients.

Results: No variation in vital parameters or cytokine release syndrome could be detected during and after MSC infusion. No patient developed impairment of organ functions (including liver graft function) following MSC infusion. No increased rate of opportunistic infection or *de novo* cancer was detected. As secondary endpoints, there was no difference in overall rates of rejection or graft survival. Month-6 biopsies did not demonstrate a difference between groups in the evaluation of rejection according to the Banff criteria, in the fibrosis score or in immunohistochemistry (including Tregs). No difference in peripheral blood lymphocyte typing could be detected. The immunosuppression weaning in MSC recipients was not successful.

Conclusions: No side effect of MSC infusion at day 3 after liver transplant could be detected, but this infusion did not promote tolerance. This study opens the way for further MSC or Treg-based trials in liver transplant recipients.

Lay summary: Therapy with mesenchymal stromal cells (MSCs) has been proposed as a means to improve results of solid organ transplantation. One of the potential MSC role could be to induce tolerance after liver transplantation, *i.e.* allowing the cessation of several medications with severe side effects. This study is the first-in-man use of MSC therapy in ten liver transplant recipients. This study did not show toxicity after a single MSC infusion but it was not sufficient to allow withdrawal of immunosuppression.

Clinical trial registration number: Eudract: # 2011-001822-81, ClinicalTrials.gov: # NCT 01429038.

Keywords: Stem cells; Mesenchymal stem cells; Cell therapy; Liver failure; Liver diseases; Hepatic insufficiency; Cirrhosis; Immune tolerance; Cancer; MISOT.

Received 12 October 2016; received in revised form 24 February 2017; accepted 1 March 2017; available online 9 March 2017

* Corresponding author. Address: Department of Abdominal Surgery and Transplantation, CHU Liege, University of Liege, Belgium. Tel.: +32 43667645; fax: +32 43667069.

E-mail address: olivier.detry@transplantation.be (O. Detry).

[1] www.misot.eu.

Introduction

Liver transplantation (LT) has become the gold standard treatment of many hepatic end-stage diseases. Long-term graft and patient survivals are now common after LT, but recipients are still subjected to life-long immunosuppression, which impairs quality of life and might reduce survival by promoting cancer development or by increasing the risks for infection, kidney function impairment and cardiovascular diseases. Therefore, there is a need for improvement in the immunosuppressive protocols after LT. Finding a way to establish donor-specific immunological tolerance without the need for non-specific immunosuppression remains one of the major goals in transplantation medicine [1].

Mesenchymal stromal cells (MSCs) are multipotent progenitors within the bone marrow, capable of differentiating into various cells and tissues, such as chondrocytes, osteoblasts and adipocytes [2]. MSCs can be isolated after *ex vivo* culture of the adherent mononuclear bone marrow cell fraction. In addition to

the bone marrow, MSCs reside in the connective tissues of many organs including the liver. After *ex vivo* expansion, human MSCs have a fibroblastic-like morphology, and are uniformly positive for SRC homology domains (SH)2, SH3, cluster of differentiation (CD)29, CD44, CD71, CD90, CD105, CD106, CD120a, CD124, and CD166, but are negative for common hematopoietic markers such as CD14, CD45 or CD34 [2]. Human MSCs express human leukocyte antigen (HLA)-class I and can be induced to express HLA-class II by interferon (IFN)γ. A large number of *in vitro* and *in vivo* studies have documented the anti-inflammatory and immunoregulatory properties of MSCs on both the adaptive and innate immune system [3], as well as a potential beneficial effect in ischaemia–reperfusion injury [4,5]. Specifically, MSCs have been shown to decrease effector T cell response while promoting the emergence of regulatory T cells (Treg) [6]. These MSC properties suggest that they could be particularly attractive in solid organ transplantation (SOT) [7,8], and a consortium of European academic centres studying this subject has been created (http://www.misot.eu). The first randomized controlled trial, evaluating the effects of autologous MSCs in living-related kidney transplantation has been performed in China [9]. In this study, MSCs significantly correlated with fewer acute rejections, a lower risk of opportunistic infections and a better renal function at 1 month. Furthermore, fewer adverse effects were seen in the MSC groups compared to the control group [9]. Compared to other transplanted organs, the liver graft is immunologically protected, and LT recipients are considered the ideal candidates for MSC therapy and for operative tolerance trials after SOT [10]. To date there has been no published trial evaluating MSC infusion in a series of LT patients [1].

Despite the absence of major adverse effects in the preliminary clinical trials evaluating MSC-based therapy to date [11], clinical infusion of MSCs might theoretically be complicated by impairment of pulmonary function due to MSC embolism in the lung vasculature [12] and by a cytokine release syndrome [13]. As MSCs are potentially immunosuppressive, another concern is the potential emergence of higher rates of opportunistic infections and induced cancers after MSC infusion in SOT recipients under immunosuppression. In a small European clinical series, MSC infusion in kidney recipients was associated with transient renal dysfunction [14] and opportunistic infections [15]. It is also possible that MSC injection promotes liver fibrosis [16]. Finally, *in vitro* MSC expansion and culture might generate genomic instability and chromosomal aberrations with a potential risk of MSC neoplastic transformation [17,18].

The aim of this study was to evaluate the feasibility, the safety and the tolerability of a single MSC infusion after LT in a first-in-man, prospective, controlled, phase I study. The primary endpoints were set to clinically detect potential side effects of MSC infusion, as well as the occurrence of infectious and malignant complications. As secondary endpoints, the potential immune-regulative effects of MSCs and the impact of MSCs on Treg counts and phenotype were analysed by comparison with a control group. In addition, progressive immunosuppressive withdrawal was attempted as a phase II study in stable patients who received MSCs, to evaluate if a single infusion of MSCs might induce operative tolerance after LT.

Materials and methods

Study design

This study was a monocentric, prospective, non-randomized, controlled, open-label trial. Protocol inclusion and exclusion criteria are presented in Table 1. Between March 2012 and February 2014, ten stable and low-risk LT recipients under standard immunosuppression received 1.5–3 × 10^6/kg third-party MSCs on postoperative day 3 ± 2 (MSC group). These patients were prospectively compared to a control group of ten LT recipients who fulfilled the study inclusion criteria, declined to receive MSCs, but accepted to be included in the trial as control patients during the same period (control group). In addition, in patients from the MSC group who did not develop rejection and had normal graft function and month 6 graft biopsy, progressive weaning of immunosuppression was attempted (Fig. 1). Weaning of immunosuppression was not considered in the control group as it is well established that early (<1 year) immunosuppression withdrawal is not possible and unethical in LT recipients under regular immunosuppression protocols. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local Ethics Committee and by the Belgian Federal Agency for Medicines and Health Products (Eudract #2011-001822-81). The study was registered at ClinicalTrials.gov (protocol # NCT 01429038). Written informed consent was obtained from each MSC donor and LT patient.

Liver transplant procedures and postoperative management

The following deceased liver graft donor characteristics were prospectively collected: age, gender, donation after brain or circulatory death, Eurotransplant donor risk index (ET-DRI) [19], cause of brain damage, terminal blood sodium level, terminal liver function tests, need for vasopressors, length of intensive care unit stay, body mass index (BMI), last 24 h diuresis, and past cardiopulmonary resuscitation.

Table 1. LT recipient inclusion and exclusion criteria.

Inclusion criteria	
Surgery	First whole liver deceased LT (DBD or DCD)
Age	Between 18 and 75 years
Graft	Functioning graft at time of MSC infusion Graft doppler ultrasonography confirming arterial and portal flows
Exclusion criteria	
Surgery	Re LT, partial LT, combined LT
Cancer	Past history of cancer in the donor or recipient, with the exception of hepatocarcinoma within Milan criteria
Infection	Active infection in the donor or recipient, including HIV and HCV EBV negative (recipient)
Miscellaneous	Auto-immune liver disease (recipient) Endotracheal intubation (recipient) Severe postoperative complications (recipient)

LT, Liver transplantation; DBD, donation after brain death; DCD, donation after circulatory death; MSC, Mesenchymal Stromal Cells; HIV, Human Immunodeficiency virus; HCV, Hepatitis C virus; EBV, Epstein-Barr virus.

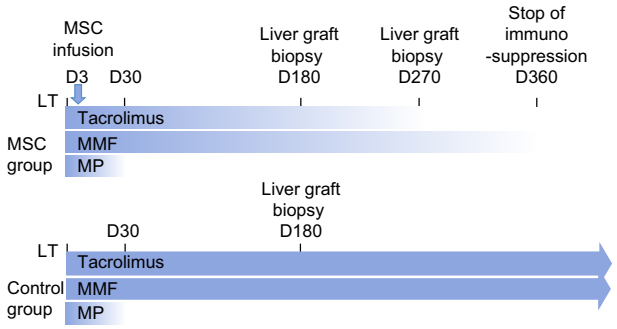


Fig. 1. Scheme of the study. MSC: Mesenchymal stromal cell; MMF: mycophenolate mofetil; MP: Methylprednisolone.

The LT procedures were regular deceased LT as performed in the authors' centre [20,21]. The following LT recipient characteristics were collected: age, gender, BMI, LT indication, and the laboratory model for end-stage liver disease (MELD) score at admission for transplantation. Cold and total graft ischaemic times were recorded. The immunosuppressive regimen consisted of a triple therapy of tacrolimus, mycophenolate mofetyl (MMF) and steroids. The tacrolimus dose was adapted according to through whole blood values (between 8 and 12 ng/ml the first 28 days and between 5 and 8 thereafter) until day 180 in both groups. In the MSC group, if a rejection episode had not been suspected based on the liver tests and month 6 biopsy, tacrolimus was progressively tapered from day 180 to be discontinued by day 270 in the absence of rejection (Fig. 1). A graft biopsy was performed at day 270 ± 15 in the MSC group. MMF was administered orally from day 1 through day 270 at the dose of 500 mg twice a day (b.i.d.) In the MSC group, if the patient did not develop rejection during tacrolimus withdrawal and at day 270 graft biopsy, MMF was progressively tapered and definitely discontinued by day 365 in the absence of rejection (Fig. 1). Steroid treatment consisted of administration of methylprednisolone 500 mg intravenously (i.v.) before liver graft reperfusion, followed by progressively decreasing daily doses until progressive withdrawal during month 1 (Fig. 1). Liver graft rejection was assessed according to standard criteria, including clinical symptoms, blood liver enzymes, and liver graft biopsy if needed. Therapy for rejection included an increase in tacrolimus administration, boluses of methylprednisolone 500 mg i.v. per day for 3 days, and anti-thymocyte globulins in steroid-resistant rejection, if needed.

Antibacterial and antiviral prophylaxis was standardized between groups including cefuroxime 3 × 1.5 g or piperacillin-tazobactam 4 × 4 g/d for 5 days, prevention of pneumocystis (co-trimoxazole 500 mg orally (p.o.) 1/d for three months) and of cytomegalovirus (CMV) infection if indicated (donor positive, recipient negative [D+, R−], 100 days of valgancyclovir 2 × 450 mg/d p.o.).

MSC donors

Inclusion criteria for MSC donors included: unrelated to the recipient; aged >18-years; no human leucocyte antigen (HLA) matching required; fulfilling generally accepted criteria for allogeneic hematopoietic stem cell donation; and informed consent given. Exclusion criteria were: known allergy to lidocaine; any risk factor for transmissible infectious diseases; meeting generally accepted exclusion criteria for allogeneic hematopoietic stem cell donation [22].

MSC production

MSC expansion cultures were performed and evaluated at the Laboratory of Cell and Gene Therapy (LTCG) of the University Hospital of Liege, CHU of Liege, as previously described [22,23]. Briefly, bone marrow (BM) (30–50 ml) was collected under local anaesthesia in sterile conditions, and put in sterile heparin-containing syringes. Mononuclear BM cells were isolated by Ficoll (GE Healthcare-Amersham Biosciences AB, Upsala, Sweden), seeded in sterile tissue culture flasks (BD Falcon, Bedford, MA), and cultured in Dulbecco's modified Eagles medium–low glucose (Invitrogen, Merelbeke, Belgium) with glutamate supplemented with 10% irradiated fetal bovine serum (Hyclone- Perbio Science, Merelbeke, Belgium) and antibiotics (penicillin/streptomycin, Lonza Bio Science, Verviers, Belgium). Cultures were maintained at 37°C in humidified atmosphere containing 5% CO_2 for a total of about 4 weeks. The medium was replaced twice a week and, after approximately 2 weeks, the cultures were near confluence (>70%). Cells were then detached by treatment with irradiated trypsin–EDTA (Invitrogen, Merelbeke, Belgium) and replated (passaged) at a lower density to allow further expansion. A second passage was performed when the cells reached confluence again (>70%). At confluence, the cells were harvested, washed, and re-suspended using phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA; Miltenyi Biotec, Utrecht, The Netherlands) and Human Serum Albumin (HSA) (CDF-CAF, Brussels, Belgium). The MSCs were then frozen in a medium containing 70% PBS, 20% human serum albumin (HSA), and 10% dimethyl sulfoxide (DMSO) (WAK-Chemie, Steinbach, Germany) using standard techniques. Before infusion, the MSCs were thawed and diluted in PBS, and then injected into the patients within 60 min. All reagents were certified sterile, and endotoxin-free, and had been used in other clinical trials in Europe. In addition, the batch of fetal bovine serum used was selected after extensive testing, and was irradiated to ensure removal of all potential viruses. The following analyses were performed as quality controls for each MSC expansion culture: nucleated cell count on a manual cell counter, flow cytometry analysis with determination of the % cells (out of total cells) positive for CD73, CD90, and CD105, and negative for HLA-DR, CD31, CD80, CD14, CD45, CD3, and CD34; cell viability using trypan blue exclusion; microbiology testing, including standard virology, bacterial culture, and search for mycoplasma; endotoxin detection using the limulus test; and cytogenetics. MSC potency was evaluated by determining the percentage inhibition of T cell proliferation in Mixed-Lymphocyte Reaction essay. Finally, MSC differentiation into adipocytes, osteocytes, and chondrocytes was validated in preliminary experiments [22].

MSC infusion

Third-party unrelated MSC infusion was performed on post-transplant day 3 ± 2 through a central intravenous line in fully monitored, stable, conscious and extubated patients who were receiving standard LT recipient care, after liver Doppler ultrasonography confirming arterial and portal flows. MSC infusion had to be performed within 60 min of thawing, with two investigators at the patients' bedside.

Primary endpoints

MSC infusional toxicity

The duration and volume of the MSC infusion were noted. To assess pulmonary and systemic toxicity of MSC infusion, tympanic body temperature, heart rate, mean arterial blood pressure and peripheral capillary oxygen saturation (SpO_2) were recorded 5 min before infusion, after 15 min and at the end of the MSC infusion. Clinical signs of allergy, such as skin reaction or anaphylactic shock, were also recorded.

MSC infectious and cancerous complications

The incidence, timing and severity of any infections (bacterial, viral, fungal) and any malignant diseases were prospectively recorded until month 12 in both groups.

Secondary endpoints

Patient and graft survivals and biopsy-proven graft rejection rates were prospectively recorded in both groups until month 12. Liver graft function (bilirubin, liver enzymes, international normalised ratio (INR)), kidney function (creatinine), C-reactive protein (CRP) and tacrolimus levels were compared using standard clinical blood tests at day 7 and months 1, 3, and 6. Blood immunoglobulin levels were compared at months 1 and 6.

Liver graft biopsy and immunohistochemistry

Month-6 formalin-fixed, paraffin-embedded graft biopsies were blindly analysed by two gastrointestinal pathologists (N.B., J.S), who described fibrosis and signs of graft rejection according to the Banff criteria [24]. Paraffin-embedded sections of liver biopsy specimens (4 μm thick) underwent immunostaining using an automated immunostainer (Ventana Medical Systems, Tucson, AZ) with antibodies directed against human CD3, CD4, CD8, CD20, CD138, CD68, CD1a and FoxP3. An amplification kit (Ventana Medical Systems) and a detection system including diaminobenzidine (Ventana Medical Systems) as a chromogen were used during the automated procedure. Archival lymph node sections were used as positive controls. For negative controls, the primary antibody was omitted. The mean

number of positive cells in each patient was calculated by counting these cells (original magnification, 400×) in the three most cellular microscopic fields, also called hot spots.

Peripheral blood lymphocyte immunophenotyping and CD4 phenotyping

Peripheral blood mononuclear cells were phenotyped on days 30, 90 and 180 using 4-color flow cytometry after treatment with a red blood cell lyzing solution as described [25]. The analysed cell subsets were T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+$ $CD4^+$ lymphocytes), $CD8^+$ T cells ($CD3^+$ $CD8^+$ lymphocytes), naïve $CD4^+$ T cells ($CD4^+$ $CD45RA^{high}$ lymphocytes), memory $CD4^+$ T cells ($CD4^+$ $CD45RO^+$ lymphocytes), natural killer (NK) cells ($CD3^-$ $CD56^+$ lymphocytes), as well as B cells ($CD19^+$ lymphocytes). The percentage of positive cells was measured relative to total nucleated cells, after subtraction of non-specific staining. Absolute counts were obtained by multiplying the percentages of positive cells by the white blood cell counts (Advia 120 haematology analyser, Bayer Technicon).

More detailed $CD4^+$ T cell phenotyping was performed on days 0 (before LT), 30 and 90 as previously reported [19]. Tregs were defined as $CD4^+$ $CD25^+$ $CD127^{dim}$ $FOXP3^+$ lymphocytes while remaining $CD4^+$ T cells were considered as conventional T cells (Tconvs). Naïve Tregs were defined as $CD45RA^+$ $HLA\text{-}DR^{neg}$ Tregs, and activated effector Tregs were defined as $CD45RA^{neg}$ $HLA\text{-}DR^+$ Tregs as previously reported [26]. T cell proliferation was assessed by Ki67 expression, and IL-2 signalling was estimated by quantifying the expression of phosphorylated STAT5 (phosphoSTAT5) [27]. The following antibodies specific for human epitopes were used: CD4-APC (RPA-T4), CD25-PeCy7 (BC96, Sony), CD127-biotin (eBioRDR5), CD45RA-BV510 (HI100, BD), HLA-DR-PE (L243), FOXP3-AlexaFluor488 (259 D, Biolegend, ImTech Antwerp, Belgium), posphoSTAT5-BV421 (pY694, BD), Ki67-PercPCy5.5 (B56, BD) and anti-streptavidin APCCy7 (all from eBioscience, unless otherwise indicated). Samples from patients were thawed and washed with staining buffer. One million cells of each sample were then incubated with surface antibodies for 20 min at 4°C in the dark and washed with staining buffer. This process was repeated for a 15 min period for the streptavidin staining step. Then, samples were permeabilized using the PerFix EXPOSE (Beckman Coulter, Brea, CA) according to the manufacturer's instructions and as previously reported [26]. Data were acquired using a fluorescence activated cell sorting (FACS) Canto II (Becton Dickinson) and were analysed with FlowJo v7.6.5 (Treestar Inc., San Carlos, CA).

Statistical analysis

Data are presented as median values and ranges, and the difference between groups was evaluated by the Mann–Whitney *U* test. Proportions were analysed using Fischer's test. Differences between repeated measures were evaluated by one-way ANOVA using the Friedman test as a *post-hoc* test. Survival rates were calculated with the Kaplan–Meier method and compared with the log-rank (Mantel-Cox) test. A value of $p < 0.05$ was considered significant. Data were analysed using Prism 6.0c software for Macintosh OSX (GraphPad Software, San Diego, CA).

For further details regarding the materials used, please refer to the CTAT table.

Results

Liver transplantation donor and recipient characteristics

No statistical difference could be detected between the MSC and control groups concerning the characteristics of both liver graft donors and recipients (Supplementary material).

Primary endpoints

MSC infusional toxicity

On day 3 (2–5), the 10 MSC patients received $2.1 \cdot 10^6$/kg (1.9–2.7) MSC, representing a perfusion volume of 341 ml (302–614). Median duration of infusion was 25 min (11–60). No variation in vital parameters or cytokine release syndrome could be clinically detected during and after MSC infusion (Table 2). No MSC patient developed clinical signs of allergy or impairment of vital functions (including liver graft function) within the week following MSC infusion.

Infectious and cancerous complications

No patient in either group developed life-threatening opportunistic infection or *de novo* cancer (including post-transplant lymphoproliferative disease) during follow-up. There was no difference in overall rates of infection between the two groups (Table 3). In the MSC group, two patients developed labial herpetic infections successfully treated by oral acyclovir. In addition, two MSC patients at high risk of CMV (D+, R−) developed asymptomatic CMV seroconversion under valganciclovir therapy. No patients developed CMV disease. Two patients transplanted for hepatocellular carcinoma (HCC) complicating cirrhosis had a pejorative pathology report and developed HCC recurrence: one MSC patient had a R1 LT with a HCC nodule invading the diaphragm (he died from HCC recurrence at month 10) and one control patient had an unsuspected neoplastic thrombus in a supra-hepatic vein at liver pathology (still alive at 5-year follow-up after HCC recurrence at month 23 and resection of pulmonary metastases).

Secondary endpoints

No patient required retransplantation during the first year of follow-up. One patient from the control group died at day 16 from a hypovolemic shock induced by a fistula between the hepatic artery and the bile duct, probably due to an infected pseudoaneurysm. Six-month graft and patient survivals were 100% and 90% in the MSC and control group, respectively (not significant [NS]). One year graft and patient survivals were 90% in both groups (NS). No patient in either group developed biopsy-proven rejection during the first 6 months of follow-up. Protocol month-6 biopsies did not demonstrate a difference between groups in the evaluation of the Banff criteria, the fibrosis score or the immunohistochemistry (Table 4; Figs. S1, S2). No difference could be detected in liver graft or kidney function between the two groups during the 6 months of comparison (Table 5; Fig. S3). No difference in peripheral blood lymphocyte phenotyping could be detected on day 30, 90 and 180 (Table 6; Fig. S4).

Impact of MSCs on peripheral blood CD4+ T cells (including Tregs)

The two groups of patients had similar counts of peripheral blood $CD4^+$ T cells and Tconvs on days 0, 30 and 90 after transplantation (Fig. 2A–B). As shown in Fig. 2C–F, Treg counts and phenotype (naïve *vs.* resting *vs.* activated) were comparable in the two groups of patients at each time point. Furthermore, Treg as well as Tconv proliferation (assessed by Ki67 expression) was also similar in the two groups of patients, as were the levels of phosphoSTAT5 in Tregs (the latter translating similar IL-2 signalling in Tregs). These combined observations suggest that a single MSC infusion had no impact on Treg count or phenotype in this study.

Immunosuppression withdrawal in the MSC group

One patient from the MSC group was excluded from immunosuppression withdrawal attempt due to HCC recurrence, but the nine others met the necessary criteria. In one patient, tacrolimus and

Table 2. Comparison of vital parameters before, during and after MSC infusion.

	Pre MSC infusion	After 15 min	End of MSC infusion	*p* value
Temperature (°C)	36.1 (35.4–37.7)	36.4 (35–36.9)	36.2 (35.5–37)	0.87
Mean arterial pressure (mmHg)	103 (87–124)	107 (84–120)	106 (94–115)	0.83
Heart rate (per min)	81 (65–102)	83 (65–102)	81 (68–101)	0.17
SpO_2 (%)	99 (93–100)	100 (92–100)	98 (93–100)	0.67

MSC, Mesenchymal stromal cells; SpO_2, peripheral capillary oxygen saturation.
p values were calculated using a one-way ANOVA and Friedman test for *post-hoc* analysis.

Table 3. Cancerous and infectious complications (1-year follow-up).

	MSC group (n = 10)	Control group (n = 9)	*p* value
Cancer			
Total	1	0	> 0.99
de novo	0	0	
HCC recurrence	1	0	
Infection			
Total	2	6	0.06
Fungal	0	0	
Viral	0	0	
CMV disease	2	0	
HSV	0	1	
VZV			
Other	0	1	
Wound	0	2	
Urinary	0	1	
Sinusitis	0	1	
Pulmonary			

MSC, Mesenchymal stromal cell; HCC, hepatocellular carcinoma; CMV, cytomegalovirus; HSV, herpes simplex virus; VZV, Varicella-zoster virus.
p values were calculated using a Fischer's test.

MMF withdrawal was performed without rejection and she remained off immunosuppression for 12 months. In two patients, MMF monotherapy was achieved at month 9, but graft rejection occurred during MMF withdrawal and was successfully treated by tacrolimus reintroduction. In six patients, the transaminases significantly increased during tacrolimus withdrawal. In these cases, withdrawal was cancelled and liver tests normalised after increase of the tacrolimus dose.

Discussion

This phase I, prospective, controlled study is the first to evaluate the feasibility, safety and tolerability of MSC infusion in a series of 10 LT patients under classical tacrolimus-based immunosuppression. In these patients, a post-transplantation intravenous $1.5–3 \times 10^6$/kg MSC infusion was well tolerated, without evidence of pulmonary dysfunction or of cytokine release syndrome. This dosing was chosen according to the authors' experiences with MSC infusion after hepatic stellate cell (HSC) transplantation [23,28]. These LT patients receiving MSC did not develop any evidence of impairment in vital organ functions, including the liver graft and the kidneys. In addition, they did not suffer from an increased susceptibility to infections. No *de novo* cancer was detected after one year of follow-up, and a HCC recurrence was observed in a patient with a very poor prognosis due to unexpected extra-hepatic HCC spread discovered during LT. For all these primary endpoints, the LT recipients who received MSCs did not react differently compared to patients in the control group. This finding is an important step in the evaluation of the potential role of MSCs in SOT recipients, and particularly after LT.

In the last decade, MSCs have been extensively studied both *in vitro* and *in vivo*. Their anti-inflammatory and immunoregulatory properties [29,3], added to potential beneficial effects on ischaemia/reperfusion injury [5], might select MSCs as a potential future therapy for SOT recipients in whom life-long immunosuppression and chronic allograft dysfunction still impair quality of life and graft survival. However, as the clinical use of MSCs is still under evaluation in preliminary trials in non-transplant patients, their potential secondary effects need to be carefully assessed in SOT recipients. Due to their size, MSCs are known to embolize within the pulmonary circulatory bed when they are infused in the peripheral or central venous circulation of mice [12]. There is therefore a theoretical risk of decreased pulmonary exchange after MSC infusion, but this complication has not been reported so far in the early phase clinical trials nor in the randomized study in living-related kidney transplantation performed in China [9]. As reported previously by our group, MSC infusion in hematopoietic stem cell transplant recipients has not been

Table 4. Histology and immunohistochemistry of D180 liver graft biopsies.

	MSC group (n = 10)	Control group (n = 9)	*p* value
Banff score	3 (0–6)	4 (0–7)	0.21
Fibrosis score	1 (0–2)	1 (0–3)	0.48
CD3	196 (95–334)	162 (93–590)	0.86
CD4	101 (54–212)	103 (17–496)	>0.99
CD8	69 (15–196)	85 (12–300)	0.49
CD68	28.5 (12–75)	40 (15–104)	0.58
CD1a	1 (0–3)	1 (0–3)	0.83
CD138	7.5 (4–38)	6 (2–44)	0.50
CD20	27 (3–95)	28 (10–163)	0.66
FoxP3	2 (0–16)	4 (0–33)	0.49

MSC, mesenchymal stromal cell.
Data are presented as median and ranges; *p* values were calculated using a Mann-Whitney *U* test.

Table 5. Post-operative laboratory tests.

	MSC group (n = 10)	Control group (n = 9)	*p* value
D7			
Total bilirubin (mg/L)	10.2 (4.6–26.8)	8.3 (3.7–20.7)	0.21
AST (U/L)	28.5 (19–101)	46 (30–105)	0.16
AP (U/L)	140 (43–475)	256 (172–590)	0.04
GGT (U/L)	218 (29–626)	368 (172–760)	0.24
INR	1.14 (1–1.21)	1.06 (1–1.26)	0.16
Creatinine (mg/L)	11.55 (5.7–36)	8.9 (5.9 – 16.9)	0.32
CRP (mg/L)	32.8 (8.4–50.1)	24.6 (12.8–144.3)	0.82
Tacrolimus (μg/L)	7.1 (3.1–9)	9 (2.1–11.7)	0.12
D30			
Total bilirubin (mg/L)	5.6 (3.4–11.6)	4.6 (1.3–7.5)	0.34
AST (U/L)	18 (11–51)	16 (9–61)	0.48
AP (U/L)	137.5 (53–554)	144 (103–857)	0.43
GGT (U/L)	101 (26–596)	112 (42–690)	0.82
INR	1.15 (0.97–1.26)	1.08 (1–1.19)	0.53
Creatinine (mg/L)	16.2 (5.3–24.4)	14.1 (8.2–27.6)	0.45
CRP (mg/L)	12.9 (4.8–62.2)	17.2 (3.5–73)	0.94
Tacrolimus (μg/L)	8.1 (2.4–10)	8 (5–16.3)	0.51
D90			
Total bilirubin (mg/L)	4.8 (3–19.8)	4.3 (2.3–7.5)	0.34
AST (U/L)	20 (14–31)	20 (11–58)	0.79
AP (U/L)	101.5 (56–1461)	119 (86–570)	0.54
GGT (U/L)	58.5 (15–695)	49 (14–332)	0.76
INR	1.1 (0.95–1.29)	1.13 (1.01–1.56)	0.65
Creatinine (mg/L)	12.05 (5–25.7)	13.4 (7–21.7)	0.92
CRP (mg/L)	3.1 (1–27.6)	6.8 (1.3–23.5)	0.20
Tacrolimus (μg/L)	7.7 (3.7–13)	6.4 (5.2–13.2)	0.61
D180			
Total bilirubin (mg/L)	6.6 (3.7–25.7)	4.6 (0.43–27)	0.27
AST (U/L)	25 (15–44)	24 (14–136)	0.64
AP (U/L)	143.5 (67–1,166)	186 (82–554)	0.26
GGT (U/L)	81 (22–978)	53 (12–2,064)	0.43
INR	1.1 (1–1.26)	1.07 (1–1.17)	0.23
Creatinine (mg/L)	11.6 (7.1–18.9)	10.1 (1.28–15.8)	0.30
CRP (mg/L)	3.5 (0.7–36.5)	5.6 (0.9–151)	0.23
Tacrolimus (μg/L)	4.9 (2.3–9.3)	7.4 (4.9–13)	0.02

MSC, mesenchymal stromal cells; D, day; AST, aspartate aminotransferase; AP, alkaline phosphatase; GGT, gamma glutamil transferase; INR, international normalised ratio; CRP, C-reactive protein.
Data are presented as median and ranges; *p* values were calculated using a Mann-Whitney *U* test.

associated with any infusional toxicity [23], nor with long-term impairment of lung function [28]. This was confirmed in the current trial, as our ten patients receiving MSCs did not develop any sign of pulmonary dysfunction. In addition, there was no suspicion of allergy or cytokine release syndrome observed in this study, or of any other possible complications concerning the liver graft or extra-hepatic organ function. In a preliminary evaluation in two kidney recipients, possible toxicity of MSC infusion on kidney graft function was suggested [14], but this "engraftment syndrome" was not detected in our cohort of LT recipients or in any other MSC clinical trial to date.

As MSCs are immunosuppressive, SOT recipients who receive MSCs in addition to standard immunosuppression could be over-immunosuppressed and develop higher rates of opportunistic infections [26]. Again, in a small series of kidney recipients, opportunistic infections were observed after MSC treatment [15]. On the contrary, in the largest experience reported so far of MSC infusion after living-related kidney transplantation, MSC recipients developed fewer infectious complications than controls [9]. In our series, the MSC patients did not develop life-threatening infections, and no difference could be detected in comparison with the control group.

It has been suggested in *in vitro* experiments that MSCs might carry a potential for cancerous degeneration [17]. This potential risk has so far not been demonstrated in the preliminary MSC clinical experiences in either SOT or in non-SOT patients, and no patient in our series had developed *de novo* cancer after one year follow-up. This important issue needs to be confirmed by further follow-ups of this series and by further experience in larger series. Furthermore, in the series described here, one patient died from early HCC recurrence after a R1 LT with a very bad prognosis. The authors do not consider that HCC within Milan criteria should be excluded for further MSC trials in LT, but the possibility of an increased risk of HCC recurrence after MSC infusion cannot be excluded by this preliminary phase I study.

As secondary endpoints, this study prospectively evaluated the possible effects of a single infusion of MSCs on LT recipient immunity by comparison with a control group. No difference could be detected between the MSC and control groups on graft rejection episodes, opportunistic infection rates, graft histology

Table 6. Peripheral blood lymphocyte counts.

	MSC group (n = 10)	Control group (n = 9)	*p* value
D30			
White blood cells (/μl)	6,630 (3,280–9,700)	5,190 (4,150–10,030)	0.67
Lymphocytes (/μl)	855 (380–1,690)	940 (300–1,550)	0.92
CD3 (/μl)	687 (288–1,406)	620 (200–1,336)	0.48
CD45RA (/μl)	119 (50–557)	147 (48–234)	0.70
CD45RO (/μl)	373 (179–516)	201 (79–609)	0.23
$CD3^+$ $CD4^+$ (/μl)	535 230–978)	349 (128–786)	0.30
$CD3^+$ $CD56^+$ (/μl)	27 (1–87)	42 (4–154)	0.35
$CD3^+$ $CD8^+$ (/μl)	115 (49–418)	142 (57–336)	0.76
CD19 (/μl)	144 (30–286)	99 (38–369)	0.70
CD56 (/μl)	109 (45–365)	188 (58–618)	0.27
D90			
White blood cells (/μl)	5,265 (970–8,160)	5,200 (2,470–7,030)	0.39
Lymphocytes (/μl)	875 (420–1,880)	760 (490–1,760)	0.82
CD3 (/μl)	767 (352–1,225)	553 (274–1,419)	0.30
CD45RA (/μl)	123 (51–389)	82 (54–259)	0.58
CD45RO (/μl)	381 (171–680)	179 (135–765)	0.23
$CD3^+$ $CD4^+$ (/μl)	516 (292–923)	285 (202–976)	0.27
$CD3^+$ $CD56^+$ (/μl)	21 (1–99)	34 (2–197)	0.76
$CD3^+$ $CD8^+$ (/μl)	202 (41–496)	228 (56–362)	0.94
CD19 (/μl)	93 (34–354)	100 (21–321)	0.76
CD56 (/μl)	154 (66–331)	119 (59–550)	0.82
D180			
White blood cells (/μl)	4,815 (4,200–8,150)	5,440 (2,680–11,430)	0.99
Lymphocytes (/μl)	1,250 (660–2,260)	1,000 (540–1,340)	0.23
CD3 (/μl)	880 (395–2,098)	592 (342–1,366)	0.27
CD45RA (/μl)	127 (76–364)	108 (61–298)	0.58
CD45RO (/μl)	396 (214–615)	267 (156–864)	0.20
$CD3^+$ $CD4^+$ (/μl)	623 (348–728)	359 (224–1,163)	0.20
$CD3^+$ $CD56^+$ (/μl)	31 (1–91)	36 (3–117)	0.54
$CD3^+$ $CD8^+$ (/μl)	238 (38–1,471)	210 (73–345)	0.70
CD19 (/μl)	99 (25–256)	192 (52–258)	0.27
CD56 (/μl)	191 (66–386)	210 (55–490)	> 0.99

Data are presented as median and ranges; *p* values were calculated using a Mann-Whitney *U* test.

and immunohistochemistry at day 180 and on peripheral blood $CD4^+$ T cell subsets. Particularly, no impact of MSC infusion on Tconv counts/proliferation was demonstrated, suggesting that MSCs did not impact T cell immunity while, in contrast to what has been observed in mice [30], MSC infusion did not influence Treg number, proliferation or phenotype in this cohort of patients. This finding might indicate that a single infusion of MSCs in LT patients receiving tacrolimus and MMF will not modify their immunity status. As MSCs and immunosuppressive drugs inhibit the same targets (essentially T cells), it is reasonable to consider that interactions between them can occur. The current standard of immunosuppression after LT is a triple therapy associating low-dose steroids, MMF and tacrolimus, with rapid steroid weaning. *In vitro*, some authors have shown that tacrolimus and rapamycin might decrease MSC immunosuppressive properties [31], and conversely, that MSCs might reduce the immunosuppressive capacities of tacrolimus and rapamycin. Such an effect has not been found with mycophenolic acid (MPA), an MMF metabolite. Moreover, a high dose of tacrolimus seems to be toxic for MSCs, while MPA and rapamycin at a therapeutic dose just inhibit MSC proliferation [32]. Furthermore, it has been demonstrated that MPA and MSCs have a synergistic immunosuppressive effect [32]. *In vivo*, MPA and MSCs also synergize to promote long-term allograft tolerance in rat heart transplantation [33]. As Tregs probably play an important role in MSC-mediated immunomodulatory effects, it is important to confirm that such a combination therapy is also favourable for Treg expansion. Hence, a recent study supported that mTOR inhibitor-based immunosuppression favours survival of Tregs after administration in a nonhuman primate model, whereas tacrolimus does not [34].

In addition, in a phase II part of this study, patients from the MSC group underwent unsuccessful progressive immunosuppression weaning. Induction of operational tolerance is a major goal in SOT and particularly in LT patients [1]. Operational tolerance is a rare phenomenon after LT [18]. Tregs have been proposed to be key in strategies aiming for tolerance and immunomodulation after SOT [35]. In a recent paper, Todo *et al.* demonstrated that a single Treg injection might promote operational tolerance after living-related LT [36]. Recently, it has been demonstrated both *in vitro* and *in vivo* that MSCs could promote Treg expansion by their effects on immature dendritic cells [37]. In this study, the authors were not able to show that a single infusion of MSCs at day 3 after deceased LT could promote Treg expansion, Treg infiltration of the liver graft at biopsy at day 180, or operational tolerance.

There are many shortcomings to this study. First, it is clear that this first study in ten LT recipients does not prove the safety

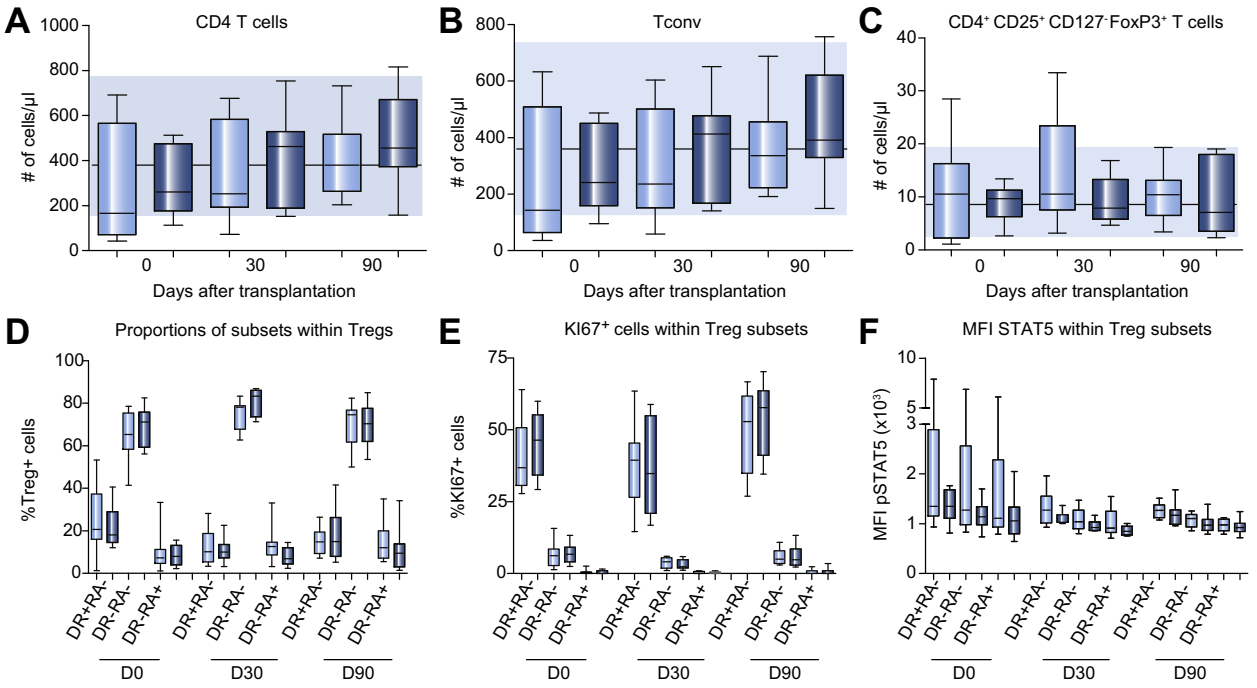


Fig. 2. Evolution of on peripheral blood CD4⁺ T cells (including Tregs) on days 0, 30 and 90 in the control (light blue boxes) or MSC (dark blue rectangles) groups. (A) Total CD4⁺ T cells; (B) Conventional CD4⁺ T cells (Tconv); (C) Regulatory T cells (Tregs); (D) Treg subsets; (E) Treg proliferation (assessed by Ki67 expression); (F) Treg IL-2 signalling (assessed through phosphoSTAT5 expression). HLA-DR^{pos} CD45RA^{neg} (DR+, RA−) Tregs refer to activated Tregs, HLA-DR^{neg} CD45RA^{neg} (DR+, RA−) Tregs refer to resting Tregs and HLA-DR^{neg} CD45RA^{pos} (DR−, RA+) Tregs refer to naïve Tregs. Plots display the median, 25th and 75th percentiles of the distribution (boxes), and whiskers extend to the 10th and 90th percentiles. Blue zones show normal ranges (from 5th to 95th percentiles) and horizontal lines the medians in 45 age-matched healthy controls. No statistical difference could be detected between the two. Mann-Whitney *U* test.

of MSC infusion in this setting. These results will have to be confirmed by further studies in larger groups of SOT recipients. The absence of detectable effects of MSCs might be due to an insufficient sample size, to the tacrolimus-based immunosuppressive regimen or to an insufficient MSC dosing, which should possibly be increased or repeated. The timing (pre-, intra- or postoperative) and the infusion routes (peripheral vein, portal vein or hepatic artery) of MSC infusion should also be evaluated. Different sources (BM, fat tissue, liver) or donors (organ donor, organ recipient) of MSCs might also be tested in further studies.

In summary, this study reported the first prospective controlled phase I clinical trial evaluating the toxicity of a MSC-based immune-regulating regimen in a series of deceased LT recipients receiving classical tacrolimus-based immunosuppression. In this trial, no side effects of MSC infusion at day 3 after transplantation could be detected. Even if no modification of the patient immunity and Treg expansion could be demonstrated, and even if immunosuppression weaning was not successful in this first series of ten patients, this study opens the way for further MSC or Treg-based trials in LT recipients.

Financial support

This study was supported by the Fonds d'investissement de recherche scientifique (FIRS) du CHU de Liege, the Fonds Léon Frédéricq of the Medical School of the University of Liege, and through a Senior Research Grant from the European Society for Organ Transplantation (ESOT). The LTCG is supported in part by the FNRS and the "Federal cancer plan – Belgium". MV is a PhD fellow sponsored by the FNRS. FB is a senior research associate of the FNRS, Belgium.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

OD designed the study, collected and analysed the data, and wrote the manuscript; MV and MD collected and analysed the data; JS and NB analysed the graft biopsies; AB, CL, OG, EB produced MSC in a GMP-compatible environnent MH and FB designed and performed the Treg subphenotyping analyses. YB designed the study, supervised the MSC production and co-wrote the manuscript. All authors approved the final version of the manuscript.

Acknowledgements

The authors value and thank all the members (physicians, nurses, coordinators) to the liver transplantation program of the CHU de Liege, who greatly contributed to this study, and the technicians

of the LTCG. The authors thank also Jacques Foguenne and André Gothot for the lymphocyte phenotyping.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2017.03.001.

References

Author names in bold designate shared co-first authorship

[1] Adams DH, Sanchez-Fueyo A, Samuel D. From immunosuppression to tolerance. J Hepatol 2015;62:S170–S185.

[2] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315–317.

[3] Crop M, Baan C, Weimar W, Hoogduijn M. Potential of mesenchymal stem cells as immune therapy in solid organ transplantation. Transpl Int 2009;22:365–376.

[4] Erpicum P, Detry O, Weekers L, Bonvoisin C, Lechanteur C, Briquet A, et al. Mesenchymal stromal cell therapy in conditions of renal ischaemia/reperfusion. Nephrol Dial Transplant 2014;29:1487–1493.

[5] Rowart P, Erpicum P, Detry O, Weekers L, Gregoire C, Lechanteur C, et al. Mesenchymal Stromal Cell Therapy in Ischaemia/Reperfusion Injury. J Immunol Res 2015;2015:602597.

[6] Casiraghi F, Perico N, Remuzzi G. Mesenchymal stromal cells to promote solid organ transplantation tolerance. Curr Opin Organ Transplant 2013;18:51–58.

[7] Franquesa M, Hoogduijn MJ, Reinders ME, Eggenhofer E, Engela AU, Mensah FK, et al. Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Fourth Meeting: lessons learned from first clinical trials. Transplantation 2013;96:234–238.

[8] English K, Wood KJ. Mesenchymal stromal cells in transplantation rejection and tolerance. Cold Spring Harb Perspect Med 2013;3:a015560.

[9] Tan J, Wu W, Xu X, Liao L, Zheng F, Messinger S, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. JAMA 2012;307:1169–1177.

[10] Vandermeulen M, Gregoire C, Briquet A, Lechanteur C, Beguin Y, Detry O. Rationale for the potential use of mesenchymal stromal cells in liver transplantation. World J Gastroenterol 2014;20:16418–16432.

[11] Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PLoS ONE 2012;7:e47559.

[12] Furlani D, Ugurlucan M, Ong L, Bieback K, Pittermann E, Westien I, et al. Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. Microvasc Res 2009;77:370–376.

[13] Dillmann J, Popp FC, Fillenberg B, Zeman F, Eggenhofer E, Farkas S, et al. Treatment-emergent adverse events after infusion of adherent stem cells: the MiSOT-I score for solid organ transplantation. Trials 2012;13:211.

[14] **Perico N**, **Casiraghi F**, **Introna M**, Gotti E, Todeschini M, Cavinato RA, et al. Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. Clin J Am Soc Nephrol 2011;6:412–422.

[15] Reinders ME, de Fijter JW, Roelofs H, Bajema IM, de Vries DK, Schaapherder AF, et al. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. Stem Cells Trans Med 2013;2:107–111.

[16] Baerschiger RM, Serre-Beinier V, Morel P, Bosco D, Peyrou M, Clement S, et al. Fibrogenic potential of human multipotent mesenchymal stromal celles in injured liver. PLoS ONE 2009;4:e6657.

[17] Haarer J, Johnson CL, Soeder Y, Dahlke MH. Caveats of mesenchymal stem cell therapy in solid organ transplantation. Transpl Int 2015;28:1–9.

[18] Barkholt L, Flory E, Jekerle V, Lucas-Samuel S, Ahnert P, Bisset L, et al. Risk of tumorigenicity in mesenchymal stromal cell-based therapies–bridging scientific observations and regulatory viewpoints. Cytotherapy 2013;15:753–759.

[19] **Braat AE**, **Blok JJ**, Putter H, Adam R, Burroughs AK, Rahmel AO, et al. The Eurotransplant donor risk index in liver transplantation: ET-DRI. Am J Transplant 2012;12:2789–2796.

[20] Detry O, Deroover A, Meurisse N, Hans MF, Delwaide J, Lauwick S, et al. Donor age as a risk factor in donation after circulatory death liver transplantation in a controlled withdrawal protocol programme. Br J Surg 2014;101:784–792.

[21] Belghiti J, Noun R, Sauvanet A. Temporary portocaval anastomosis with preservation of caval flow during orthotopic liver transplantation. Am J Surg 1995;169:277–279.

[22] Lechanteur C, Briquet A, Giet O, Delloye O, Baudoux E, Beguin Y. Clinical-scale expansion of mesenchymal stromal cells: a large banking experience. J Transl Med 2016;14:145.

[23] **Baron F**, **Lechanteur C**, Willems E, Bruck F, Baudoux E, Seidel L, et al. Cotransplantation of mesenchymal stem cells might prevent death from graft-*vs.*-host disease (GVHD) without abrogating graft-*vs.*-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. Biol Blood Marrow Transplant 2010;16:838–847.

[24] Banff schema for grading liver allograft rejection. An international consensus document. Hepatology 1997;25:658–663.

[25] Hannon M, Beguin Y, Ehx G, Servais S, Seidel L, Graux C, et al. Immune Recovery after Allogeneic Hematopoietic Stem Cell Transplantation Following Flu-TBI *vs.* TLI-ATG Conditioning. Clin Cancer Res 2015;21:3131–3139.

[26] Balan A, Lucchini G, Schmidt S, Schneider A, Tramsen L, Kuci S, et al. Mesenchymal stromal cells in the antimicrobial host response of hematopoietic stem cell recipients with graft-vs.-host disease–friends or foes? Leukemia 2014;28:1941–1948.

[27] **Ehx G**, **Hannon M**, Beguin Y, Humblet-Baron S, Baron F. Validation of a multicolor staining to monitor phosphoSTAT5 levels in regulatory T cell subsets. Oncotarget 2015;6:43255–43266.

[28] Moermans C, Lechanteur C, Baudoux E, Giet O, Henket M, Seidel L, et al. Impact of cotransplantation of mesenchymal stem cells on lung function after unrelated allogeneic hematopoietic stem cell transplantation following non-myeloablative conditioning. Transplantation 2014;98:348–353.

[29] Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. Front Immunol 2014;5:148.

[30] Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. J Immunol 2008;181:3933–3946.

[31] Buron F, Perrin H, Malcus C, Hequet O, Thaunat O, Kholopp-Sarda MN, et al. Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: an *in vitro* study using human cells. Transplant Proc 2009;41:3347–3352.

[32] Hoogduijn MJ, Crop MJ, Korevaar SS, Peeters AM, Eijken M, Maat LP, et al. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. Transplantation 2008;86:1283–1291.

[33] **Popp FC**, **Eggenhofer E**, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol 2008;20:55–60.

[34] Singh K, Stempora L, Harvey RD, Kirk AD, Larsen CP, Blazar BR, et al. Superiority of rapamycin over tacrolimus in preserving nonhuman primate Treg half-life and phenotype after adoptive transfer. Am J Transplant 2014;14:2691–2703.

[35] **Taubert R**, **Danger R**, Londono MC, Christakoudi S, Martinez-Picola M, Rimola A, et al. Hepatic Infiltrates in Operational Tolerant Patients After Liver Transplantation Show Enrichment of Regulatory T Cells Before Proinflammatory Genes Are Downregulated. Am J Transplant 2016;16:1285–1293.

[36] **Todo S**, **Yamashita K**, Goto R, Zaitsu M, Nagatsu A, Oura T, et al. A Pilot Study of Operational Tolerance with a Regulatory T Cell-Based Cell Therapy in Living Donor Liver Transplantation. Hepatology 2016;64:632–643.

[37] Choi YS, Jeong JA, Lim DS. Mesenchymal stem cell-mediated immature dendritic cells induce regulatory T cell-based immunosuppressive effect. Immunol Invest 2012;41:214–229.

2 Five-year follow-up

Infusion of allogeneic mesenchymal stromal cells after liver transplantation: a 5-year follow-up

Authors

Morgan Vandermeulen[1,2], Maleyko Mohamed-Wais[2], Pauline Erpicum[2,4], Marie-Hélène Delbouille[1], Chantal Lechanteur[5], Alexandra Briquet[5], Gianni Maggipinto[3], François Jouret[2,4], Yves Beguin[5,6,7], Olivier Detry[1,2]

Affiliations:

1. Department of Abdominal Surgery and Transplantation, University of Liege Hospital (CHU ULiege), University of Liege, Belgium
2. Centre de Recherche et de Developpement du Departement de Chirurgie (CREDEC), Interdisciplinary Cluster for Applied Genoproteomics (GIGA) Cardiovascular sciences, University of Liege, Belgium
3. Division of Immuno-Hematology, CHU ULiege, University of Liege, Belgium
4. Department of Nephrology, CHU ULiege, University of Liege, Belgium
5. Laboratory of Cell and Gene Therapy (LTCG), CHU ULiege, University of Liege, Belgium GIGA-I3-hematology, University of Liege, Belgium
6. Department of Hematology, CHU ULiege, University of Liege, Belgium

Liver Transplantation, 2022; 28: 636-646

Summary

In this article, we report the long-term results of a prospective, controlled, and first-in-human phase 1 study evaluating the safety of a single MSC infusion after LTx after 5 years of follow-up. A total of 10 LTR treated with standard immunosuppression received 1.5-3 × 10^6/kg 3rd party unrelated MSCs on postoperative day 3 and were prospectively compared with a control group of 10 LTR. No attempt was made to match HLAs between liver graft donors and recipients on one hand and MSC donors (3rd party MSCs) on the other.

Primary endpoints were set to prospectively detect potentially delayed adverse effects of MSC infusion, particularly the occurrence of infections and cancers. Secondary endpoints of liver graft and patient survival, graft rejection and function, occurrence of bile duct complications, and development of DSAs against liver (liver-DSAs) or MSC (MSC-DSAs) donors were studied.

No patient had been lost to follow-up, that was fixed at March 21st, 2021. Median follow-up was 85 months with a follow-up of at least 5 years in all surviving patients. From transplantation to year 5, there was no significant difference in infection rates between groups. There was no difference in the rates of cancer diagnosis between groups. The 5-year graft and patient survival rates were 70% and 80% in the MSC and control groups, respectively. No differences could be detected in liver graft, kidney function, or tacrolimus levels between the 2 groups at years 1, 3, or 5. No patient in either group developed biopsy-proven acute rejection requiring bolus steroid therapy during the whole follow-up. A total of 13 patients underwent liver graft biopsies (7 and 6 in the MSC and control groups, respectively). The median Banff scores were 3 (1-5) and 1.5 (0-3) in the MSC and control groups, respectively (NS). The median fibrosis scores were 0 (0-3) and 0 (0-1) in the MSC and control groups, respectively (NS). Regarding immunosuppression at the 5-year follow-up, 6 patients in each group were on tacrolimus. A total of 5 and 6 patients were on mycophenolate mofetil in the MSC and control groups, respectively, and a total of 1 and 2 patients were on everolimus in the MSC and control groups, respectively.

Concerning HLA mismatches and DSAs, total number of HLA mismatches between recipients and donors was 72 in both the control and MSC group. In the MSC group, the total

number of HLA mismatches between recipients and MSC donors was 79. In the control group, 3 patients developed 1 *de novo* liver-DSA class I during the first 6 months after LTx. Another recipient developed 2 *de novo* liver-DSAs class II (both with MFI >5000) more than 2 years after transplantation. In total, 5 *de novo* liver-DSAs were detected in 4 control recipients during follow-up. In the MSC group, 6 patients developed at least 1 *de novo* liver-DSA (all but 1 were HLA class II antibodies of which 3 were with MFI>5000) during the first year of follow-up. Among these 6 patients, 3 developed 1 *de novo* liver-DSA and 3 developed 2 *de novo* liver-DSAs. A total of 4 patients with *de novo* liver-DSAs class II were with MFI >5000. The prevalence of *de novo* liver-DSA was 6.9% (n=5) and 12.5% (n=9) of HLA mismatches in the control and MSC groups, respectively (P=0.4). The prevalence of *de novo* liver-DSA for class I HLA mismatches was 7% (n=9) and 2.2% (n=5) in the control and MSC groups, respectively (P=0.36). The prevalence of *de novo* liver-DSA for class II HLA mismatches was 6.9% (n=2) and 29.6% (n=8) in the control and MSC groups, respectively (P=0.04). In the MSC group, 3 patients developed at least 1 *de novo* MSC-DSA with a total of 4 detected MSC-DSAs. One patient developed 1 class I MSC-DSA detected from month 1 (MFI>5000) to the end of follow-up, and 1 class II MSC-DSA at month 12. Two *de novo* MSC-DSAs class II were also detected in 2 other patients, 1 at month 1 (MFI>5000) and 1 at month 6. All of the *de novo* MSC-DSAs class II were linked to a shared HLA mismatch between the liver and MSC donors. Considering the 5 HLA class II shared mismatches, 3 (60%) led to *de novo* liver+MSC-DSA detection in the MSC group. The development of anti-HLA antibodies against an MSC donor should be further evaluated, especially in cases of shared HLA mismatches between graft and MSC donors, despite the fact that no deleterious effect has been detected.

In conclusion, this first prospective clinical trial investigating the safety of injecting allogeneic MSCs after deceased donor LTx did not demonstrate potential adverse effects, particularly no increased rate of opportunistic infections and cancers. Injecting allogeneic MSCs after deceased donor LTx may promote liver-DSA class II emergence in LTR. This subject deserves further investigation. The potential benefits of MSC injections in the context of organ transplantation have yet to be demonstrated.

Infusion of Allogeneic Mesenchymal Stromal Cells After Liver Transplantation: A 5-Year Follow-Up

Morgan Vandermeulen,[1,2] Maleyko Mohamed-Wais,[2] Pauline Erpicum,[2,3] Marie-Hélène Delbouille,[1] Chantal Lechanteur,[4] Alexandra Briquet,[4] Gianni Maggipinto,[5] François Jouret,[2,3] Yves Beguin,[4,6,7] and Olivier Detry [1,2]

[1]Department of Abdominal Surgery and Transplantation, University of Liege Hospital (CHU ULiege), University of Liege, Liege, Belgium; [2]Centre de Recherche et de Developpement du Departement de Chirurgie, Interdisciplinary Cluster for Applied Genoproteomics (GIGA) Cardiovascular Sciences, University of Liege, Liege, Belgium; [3]Department of Nephrology, CHU ULiege, University of Liege, Liege; [4]Laboratory of Cell and Gene Therapy, CHU ULiege, University of Liege, Liege; [5]Division of Immuno-Hematology, CHU ULiege, University of Liege, Liege, Belgium; [6]Interdisciplinary Cluster for Applied Genoproteomics (GIGA)-I3-Hematology, University of Liege, Liege, Belgium; and [7]Department of Hematology, CHU ULiege, University of Liege, Liege, Belgium

Various properties of mesenchymal stromal cells (MSCs) might be particularly of interest after liver transplantation (LT). In this article, we report the long-term results of a prospective, controlled, and first-in-human phase 1 study evaluating the safety of a single MSC infusion after LT. A total of 10 LT recipients treated with standard immunosuppression received 1.5 to 3 × 10^6/kg third-party unrelated MSCs on postoperative day 3 and were prospectively compared with a control group of 10 LT recipients. Primary endpoints were set to prospectively detect potentially delayed adverse effects of MSC infusion, particularly the occurrence of infections and cancers. Secondary endpoints of liver graft and patient survival, graft rejection and function, occurrence of bile duct complications, and development of donor-specific anti–human leukocyte antigen (HLA) antibodies (DSA) against liver or MSC donors were studied. The median follow-up was 85 months. There was no difference in overall rates of infection or cancer at 5 years of follow-up between the 2 groups. There was also no difference in secondary endpoints. The prevalence of de novo liver DSAs related to HLA mismatches was twice as high in the MSC group compared with the control group. All of the de novo class II HLA antibodies against MSCs were linked to a shared HLA mismatch between the liver and MSCs. This study confirms the safety of a single MSC infusion after LT. The potential benefits of MSC injections in the context of organ transplantation have yet to be demonstrated by larger prospective studies. The development of anti-HLA antibodies against an MSC donor should be further evaluated, especially in cases of shared HLA mismatches between graft and MSC donors, despite the fact that no deleterious effect has been detected.

Liver Transplantation 28 636–646 2022 AASLD.
Received July 9, 2021; accepted September 22, 2021.

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells capable of differentiating into various cells and tissues, such as chondrocytes, osteoblasts, and adipocytes.[1] In addition, some MSC characteristics might be particularly of interest in solid organ transplantation, such as their capacity to attenuate ischemia/reperfusion injury, their potential role in tissue regeneration or repair, and their immunomodulation properties.[2-6] Promising preclinical results, including the demonstration of the ability of MSCs to inhibit T cell proliferation and dendritic cell maturation and to induce $CD4^+CD25^+FoxP3^+$ T regulator lymphocyte expansion,[7] prompted clinical trials using MSC-based therapy after living related or deceased donor transplantation, particularly in kidney transplantation (KT) and in liver transplantation (LT).[2]

Our group initiated 2 prospective clinical trials investigating the safety of injecting allogeneic third-party

Abbreviations: allo-MSC, allogeneic mesenchymal stromal cell; AS, anastomotic stricture; AST, aspartate aminotransferase; BM, bone marrow; CMV, cytomegalovirus; CRP, C-reactive protein; DSA, donor-specific anti-HLA antibodies; GGT, gamma glutamyltransferase; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; HLA-A, HLA antigen A; HLA-B, HLA antigen B; HLA-C, HLA antigen C; HLA-DRB1, HLA antigen DRB1; HLA-DQB1, HLA antigen DQB1; HSV, herpes simplex virus; INR, international normalized

MSCs after deceased donor KT and LT.[8,9] In theory, among the potential adverse effects, intravenous MSC infusion could be complicated by an immediate toxicity, by a cytokine-release syndrome,[10] and by MSC embolism in the pulmonary vasculature.[11] In addition, as MSCs are potentially immunosuppressive, concerns remain about the potential increased rates of opportunistic infections and cancers after MSC infusion in transplant recipients already receiving standard immunosuppression.[12] The 1-year reports of our 2 studies did not demonstrate an increase in such complication rates either in the KT or the LT cohort,[8,9] adding evidence of MSC short-term safety.[12]

Long-term safety of the clinical use of MSCs has still to be confirmed. In addition to their immunologic risk, it has been suggested that in vitro MSC expansion and culture might generate genomic instability and chromosomal aberrations with a potential risk of MSC neoplastic transformation.[13,14] Another potential long-term adverse effect of MSC injection could be the induction of liver fibrosis.[15] Furthermore, the question of MSC immunogenicity remains debated.[8] Preclinical data suggest that allogeneic MSCs (allo-MSCs) could promote an antidonor immune response in the host.[16] Thus, clinical administration of allo-MSCs could induce the development of anti-MSC donor human leukocyte antigen (HLA) antibodies that potentially could promote rejection, especially in cases of common HLAs between MSCs and graft donors, and could harbor potential issues in cases of the need for retransplantation, particularly in KT.[8,17]

In this article, we report the long-term results of a prospective, controlled, and first-in-human phase 1 study evaluating the safety of a single third-party allo-MSC infusion after LT, the 1-year data of which has been previously published elsewhere.[9] The primary endpoints of this study were set to prospectively detect potential delayed adverse effects of MSC infusion, particularly the occurrence of opportunistic infections and cancers. As secondary endpoints, liver graft and patient survival, graft rejection and function, occurrence of bile duct complications, and development of de novo donor-specific anti-HLA antibodies (DSA) against both liver and MSC donors were studied.

ratio; KT, kidney transplantation; $_{liver}$DSA, DSA against liver donor; $_{liver+MSC}$DSA, DSA against both liver and MSC donors; LT, liver transplantation; LTCG, Laboratory of Cell and Gene Therapy; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MSC, mesenchymal stromal cell; $_{MSC}$DSA, DSA against MSC; NAS, nonanastomotic stricture; NS, not significant; SSO, sequence-specific oligonucleotides; VZV, varicella-zoster virus.

Address reprint requests to Morgan Vandermeulen, M.D., Department of Abdominal Surgery and Transplantation, CHU ULiege, University of Liege, Sart Tilman B35, B4000 Liege, Belgium. Telephone: +3243667645; FAX: +3243667517; E-mail: m.vandermeulen@chuliege.be

This study was supported by the Fonds d'Investissement de la Recherche Scientifique at the University of Liege (CHU ULiege), the Fonds Leon Fredericq of the Medical School of the University of Liege, and through a Senior Research Grant from the European Society for Organ Transplantation. The Laboratory of Cell and Gene Therapy is supported in part by the Fonds National pour la Recherche Scientifique (FNRS) and the "Federal Cancer Plan–Belgium." Morgan Vandermeulen is a Ph.D. fellow sponsored by the FNRS. Pauline Erpicum is a fellow of the FNRS.

Additional supporting information may be found in the online version of this article.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/lt.26323

Potential conflict of interest: Nothing to report.

Patients and Methods

STUDY DESIGN

This study was a monocentric, prospective, nonrandomized, controlled, open-label trial.[9] In summary, between March 2012 and February 2014, 10 stable and low-risk LT recipients treated with standard immunosuppression received 1.5-3 × 10^6/kg third-party bone marrow (BM) MSCs on postoperative day 3 ± 2 (MSC group). The protocol of MSC isolation and expansion has been detailed elsewhere.[9,18] MSC donors were unrelated to the recipient and fulfilled generally accepted criteria for allogeneic hematopoietic stem cell donation. MSC expansion cultures were performed and evaluated at the Laboratory of Cell and Gene Therapy (LTCG) of the University Hospital of Liege, CHU ULiège. Briefly, BM was collected in sterile conditions under local anesthesia and put in sterile heparinized syringes. Mononuclear BM cells were then isolated, seeded in sterile tissue culture flasks, cultured in specific medium, and maintained at 37℃ in a humidified atmosphere containing 5% CO_2 for a total of approximately 4 weeks. After 2 passages, cells were harvested, washed, and resuspended and then frozen. Before infusion, the MSCs were thawed and diluted

in phosphate-buffered saline, and then injected into the patients within 60 minutes. As quality controls, for each MSC expansion culture we performed flow cytometry analysis to confirm the identity of the MSCs, an evaluation of cell viability using trypan blue exclusion, and microbiology testing. MSC potency was evaluated by determining the percentage inhibition of T cell proliferation in a mixed-lymphocyte reaction essay. MSC differentiation into osteocytes, chondrocytes, and adipocytes was validated in preliminary experiments.[18] No attempt was made to match HLAs between liver graft donors and recipients on one hand and MSC donors on the other.

These patients were prospectively compared with a control group of 10 LT recipients who fulfilled the study inclusion criteria (control group). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, was approved by the local Ethics Committee and by the Belgian Federal Agency for Medicines and Health Products (Eudract no. 2011-001822-81), and was registered at ClinicalTrials.gov (protocol no. NCT 01429038). Written informed consent was obtained from each MSC donor and LT recipient. No organs from executed prisoners were used.

POSTTRANSPLANT MANAGEMENT AND IMMUNOSUPPRESSION

In the phase 2 part of the trial, recipients from the MSC group, who did not develop rejection and had normal graft biopsy, underwent an unsuccessful attempt of immunosuppression withdrawal.[9] All patients were therefore treated according to the local immunosuppression protocol, consisting of low dose tacrolimus (trough levels of between 3 and 6 ng/mL) and mycophenolate mofetil 500 mg with adaptation according to adverse effects. All patients underwent lifelong transplant follow-up and regular outpatient visits with routine blood analyses. No patient had been lost to follow-up, that was fixed at March 21st, 2021. Median follow-up was 85 months with a follow-up of at least 5 years in all surviving patients.

PRIMARY ENDPOINTS: INFECTIONS AND CANCERS

The incidence, timing, and severity of any infection (bacterial, viral, fungal) and any malignant diseases were prospectively recorded in both groups.

SECONDARY ENDPOINTS

Patient and graft survival and biopsy-proven graft rejection rates were prospectively recorded in both groups. Liver graft function (bilirubin, liver enzymes, international normalized ratio [INR]), kidney function (creatinine), C-reactive protein (CRP), and tacrolimus levels were compared at months 12, 36, and 60.

No scheduled long-term graft biopsies were performed, according to the study protocol and to local clinical management. Liver graft biopsies were only performed if clinically indicated (per-cause biopsies) or when a patient underwent unrelated abdominal surgery to repair incisional hernias or hepaticojejunostomy (passage biopsies). These biopsies were blindly compared for fibrosis and for rejection according to the Banff criteria.[19]

Magnetic resonance cholangiopancreatography was performed during the patient follow-up when clinically indicated. Biliary strictures were defined as any stricture requiring endoscopic, percutaneous, or surgical management. Anastomotic stricture (AS) was defined as a stenosis located at the bile duct anastomosis. Non-AS (NAS) was defined as biliary stenosis located in the intrahepatic or extrahepatic bile ducts at least 1 cm above the anastomosis and characterized as extrahepatic if located in the donor's common bile duct or hepatic ducts up to 2 cm above the bifurcation and intrahepatic if located above this level.

Each LT recipient and each liver or MSC donor was genotyped for HLA antigen A (HLA-A), HLA antigen B (HLA-B), HLA antigen C (HLA-C), HLA antigen DRB1 (HLA-DRB1), and HLA antigen DQB1 (HLA-DQB1) based on low/medium resolution molecular typing (Luminex Corp. [Austin, TX]/ Immucor sequence-specific oligonucleotides (SSO) [Immucor Inc., Norcross, GA]); ambiguous results were resolved by means of sequence-specific primer molecular typing (Olerup, Stockholm, Sweden). DSA against MSC ($_{MSC}$DSA) and DSA against liver donor ($_{liver}$DSA) detection and identification were performed using Luminex solid-phase antibody detection technology (Luminex Corp. [Austin, TX] / Immucor LSA [Immucor Inc., Norcross, GA]). HLA antibodies were considered as positive when mean fluorescence intensity (MFI) was >1500 and in accordance with the manufacturer's recommendations. An antibody was considered de novo if not detected before transplantation. An identical

HLA mismatch between the liver recipient and both the MSC and liver donors was considered a shared HLA mismatch. Sera were tested before transplantation and at months 1, 3, 6, and 12 and then at long-term after transplantation. One patient in the control group who died from hemorrhage before month 1 was not included in the mismatch and DSA analyses.

STATISTICAL ANALYSES

Data are presented as median values and ranges, and the differences between groups were evaluated using the Mann-Whitney U test. Proportions were compared using Fisher's exact test. Survival rates were calculated with the Kaplan-Meier curve method and compared with the log-rank (Cox-Mantel) test. A P value <0.05 was considered significant. Data were analyzed using Prism 9.1.0 software for Macintosh OS (GraphPad Software, San Diego, CA).

Results

PRIMARY ENDPOINTS

From transplantation to year 5, there was no significant difference in infection rates between groups (Table 1). In addition to the infections previously described,[9] in the MSC group, 2 patients developed *Clostridium colitis*, 2 others developed biliary infections requiring antibiotics, and 1 suffered from herpetic keratitis. In the control group, 1 patient developed pneumonia and later died from sepsis, 1 suffered from biliary infection requiring antibiotics, and 1 suffered from a resistant *Escherichia coli* urinary tract infection.

There was no difference in the rates of cancer diagnosis between groups (Table 1). In each group, 1 patient developed hepatocellular carcinoma (HCC) recurrence and ultimately died from this recurrence.[9] In the MSC group, 1 patient developed non–small cell lung carcinoma that caused death at posttransplantation month 90. In the control group, 1 patient was diagnosed with prostate adenocarcinoma at month 78, 1 developed a T2 basal skin cancer operated on at month 78, and another died from pulmonary adenocarcinoma at month 21.

SECONDARY ENDPOINTS

The 5-year graft and patient survival rates were 70% and 80% in the MSC and control groups, respectively (not significant [NS]; Supporting Fig. 1). At follow-up, 6 and 5 patients had died in the MSC and control groups, respectively (NS). The cuses of death were malignant diseases in 4 patients (2 in each group), recurrence of primary liver disease in 4 patients (3 in the MSC group and 1 in the control group), septic complications in 2 patients (1 in each group), and 1 patient in the control group died from abdominal hemorrhage. No differences could be detected in liver graft, kidney function, or tacrolimus levels between the 2 groups at years 1, 3, or 5 (Table 2).

No patient in either group developed biopsy-proven acute rejection requiring bolus steroid therapy during the whole follow-up. A total of 13 patients underwent liver graft biopsies (7 and 6 in the MSC and control groups, respectively [passage biopsies, n = 10; per-cause biopsies, n = 3]). The median Banff scores were 3 (1-5) and 1.5 (0-3) in the MSC and control groups, respectively (NS). The median fibrosis scores were 0 (0-3) and 0 (0-1) in the MSC and control groups, respectively (NS). Regarding biliary complications, 6 MSC patients and 3 control patients developed AS that required invasive management by endoscopic dilatation and stenting in 7 patients and by hepaticojejunostomy

TABLE 1. Primary Endpoints

Variables	MSC Group (n = 10)	Control Group (n = 9)	*P* Value
Infection			
Overall	7	9	NS
Fungal	0	0	
Viral			
CMV disease	0	0	
HSV	3	0	
VZV	0	1	
Bacterial			
Wound	0	1	
Urinary	0	3	
Sinusitis	0	1	
Pulmonary	0	2	
Digestive	2	0	
Biliary	2	1	
Cancer			
Overall	2	4	NS
HCC recurrence	1	1	
Lung	1	1	
Prostate	0	1	
Skin	0	1	

NOTE: Fisher's exact test.

TABLE 2. Laboratory Tests

Variables	MSC Group	Control Group	*P* Value
Month 12			
Total bilirubin, mg/dL	0.56 (0.4-0.8)	0.48 (0.28-0.74)	0.22
AST, U/L	31.5 (18-141)	21 (16-55)	0.03
Alkaline phosphatase, U/L	157 (93-253)	140 (83-284)	0.71
GGT, U/L	144 (46-810)	81 (12-183)	0.06
INR	1.06 (0.98-1.28)	1 (0.92-1.18)	0.21
Creatinine, mg/dL	1.04 (0.5-2.4)	1.17 (0.5-1.7)	0.98
CRP, mg/L	6.5 (2.2-25.8)	5.2 (1.2-22)	0.47
Tacrolimus, µg/L	7.5 (1.4-9.5)	7.8 (3.7-13.8)	0.69
Month 36			
Total bilirubin, mg/dL	0.89 (0.34-2.4)	0.56 (0.37-0.77)	0.02
AST, U/L	30 (16-64)	27 (16-34)	0.84
Alkaline phosphatase, U/L	106 (59-214)	98.5 (65-206)	0.86
GGT, U/L	152 (16-447)	44 (14-497)	0.28
INR	1.03 (1-1.14)	1.01 (1-1.06)	0.58
Creatinine, mg/dL	0.99 (0.83-1.78)	1.01 (0.74-1.71)	0.71
CRP, mg/L	5.8 (1.5-27.5)	3.7 (1.4-15.6)	0.44
Tacrolimus, µg/L	7 (4-10.6)	5.4 (1.5-7)	0.18
Month 60			
Total bilirubin, mg/dL	0.72 (0.34-8.58)	0.69 (0.35-0.78)	0.87
AST, U/L	40 (14-112)	20 (12-48)	0.24
Alkaline phosphatase, U/L	158 (64-598)	126 (71-150)	0.20
GGT, U/L	116 (11-580)	44 (25-161)	0.16
INR	0.99 (0.87-1.3)	1 (0.98-1.1)	0.66
Creatinine, mg/dL	1.2 (0.85-8.34)	1.07 (0.65-2.47)	0.59
CRP, mg/L	4.7 (0.8-15.5)	9.6 (2.9-35.8)	0.29
Tacrolimus, µg/L	6.3 (2.7-15.9)	3.9 (1.8-6.2)	0.08

NOTE: Data are presented as median (range; Mann-Whitney *U* test).

after failure of endoscopic treatment in 2 patients. In the MSC group, 1 patient developed NAS requiring retransplantation after failure of hepaticojejunostomy and ultimately died from septic complications.

Regarding immunosuppression at the 5-year follow-up, 6 patients in each group were on tacrolimus. A total of 5 and 6 patients were on mycophenolate mofetil in the MSC and control groups, respectively, and a total of 1 and 2 patients were on everolimus in the MSC and control groups, respectively.

HLA MISMATCHES

Considering the 5 evaluated HLA loci (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1), the total number of HLA mismatches between recipients and donors was 72 in both the control (n = 9 patients alive at month 1) and MSC group (n = 10 patients alive at month 1). The median number of HLA mismatches was 9 (5 for class I and 3 class II loci) and 7 (4.5 for class I and 3 for class II loci) in the control and MSC groups, respectively (Table 3 and Fig. 1).

In the MSC group, the total number of HLA mismatches between recipients and MSC donors was 79. The median number of HLA mismatches was 8 (4.5 for class I and 3 for class II loci; Table 2). A total of 9 patients presented at least 1 shared HLA mismatch between the liver and MSC donors. The total number of shared HLA mismatches was 14 (9 for class I and 5 for class II loci; Table 3 and Fig. 2).

PREFORMED DSA

In each group, 1 patient presented with preformed class I $_{liver}$DSA before LT. In the MSC group, the preformed $_{liver}$DSA (HLA*B55:01) persisted up to month 6 with a decreasing intensity (MFI 7500 before LT to 2500 at month 6). In the control group, preformed $_{liver}$DSA (HLA*A25:01) were cleared from month 1. In the MSC group, 1 patient presented $_{MSC}$DSA before LT (HLA*A25:01), which persisted up to the month 6 evaluation with a decreasing intensity (MFI 7500 before LT and 1600 at month 6).

DE NOVO $_{LIVER}$DSA

In the control group, 3 patients developed 1 de novo $_{liver}$DSA class I during the first 6 months after LT. Another recipient developed 2 de novo $_{liver}$DSA class II (both with MFI >5000) more than 2 years after transplantation. In total, 5 de novo $_{liver}$DSA were detected in 4 control recipients during follow-up (Table 2 and Fig. 3A). In the MSC group, 6 patients developed at least 1 de novo $_{liver}$DSA (all but 1 were HLA class II antibodies) during the first year of follow-up. Among these 6 patients, 3 developed 1 de novo $_{liver}$DSA and 3 developed 2 de novo $_{liver}$DSA (Fig. 3A and Table 3). A total of 4 patients with de novo $_{liver}$DSA class II were with MFI >5000 (Fig. 3A and Table 3).

The prevalence of de novo $_{liver}$DSA was 6.9% (n = 5) and 12.5% (n = 9) of HLA mismatches in the control and MSC groups, respectively (P = 0.4). The prevalence of de novo $_{liver}$DSA for class I HLA mismatches

TABLE 3. HLA Mismatches and Donor-Specific Anti-HLA Antibodies

	Number of HLA Mismatches			Month 1		Month 3		Month 6		Month 12		>Month 12	
Group and Patient No.	Liver n (class I /II)	MSC n (class I/II)	Shared n (class I/II)	$_{liver}$DSA	$_{MSC}$DSA	$_{liver}$DSA	$_{MSC}$DSA	$_{liver}$DSA	$_{MSC}$DSA	$_{liver}$DSA	$_{MSC}$DSA	$_{liver}$DSA	$_{MSC}$DSA
MSC													
1	9 (6/3)	7 (3/4)	2 (1/1)					DRB1*07 :01 DRB1*11 :01	DRB1*07 :01				
2	10 (6/4)	9 (6/3)	1 (1/0)					B*44:02					
3	5 (3/2)	9 (6/3)	1 (1/0)		**B*57:01**		B*57:01		B*57:01	DQB1*03:01	B*57:01 DQB1*03:01		B*57:01
4	7 (5/2)	8 (4/4)	3 (3/0)										
5	4 (3/1)	6 (4/2)	2 (0/2)										
6	7 (6/1)	9 (6/3)	1 (1/0)										
7	7 (3/4)	6 (4/2)	2 (0/2)	DQB1*06:04	DQB1*06:04							**DQB1*03:01**	
8	9 (5/4)	9 (5/4)	1 (1/0)										
9	7 (4/3)	8 (6/2)	0	DQB1*03:01 DQB1*02:01				**DQB1*03:01** **DQB1*02:01**		**DQB1*03:01** **DQB1*02:01** DRB1*11 :04		**DQB1*03:01** **DQB1*02:01**	
10	7 (4/3)	8 (5/3)	1 (1/0)										
Total	72 (45/27)	79 (49/30)	14 (9/5)										
Control													
1	5 (3/2)	/	/		/		/		/		/		/
2	10 (6/4)	/	/	A*24:02	/		/		/		/		/
3	7 (4/3)	/	/		/		/		/		/		/
5	9 (5/4)	/	/		/		/		/		/	**DQB1*05:01** **DQB1*06:03**	/
6	5 (3/2)	/	/		/		/		/		/		/
7	9 (6/3)	/	/	C*04:01	/	C*04:01	/	C*04:01	/		/		/
8	8 (5/3)	/	/		/		/		/		/		/
9	10 (6/4)	/	/		/	A*24:02	/		/		/		/
10	9 (5/4)	/	/		/		/		/		/		/
Total	72 (43/29)	/	/										

NOTE: Control group patient 4 died before month 1 and was not included in this analysis. Underlined DSA in cases of $_{liver+MSC}$DSA. Bold DSA in cases of MFI >5000.

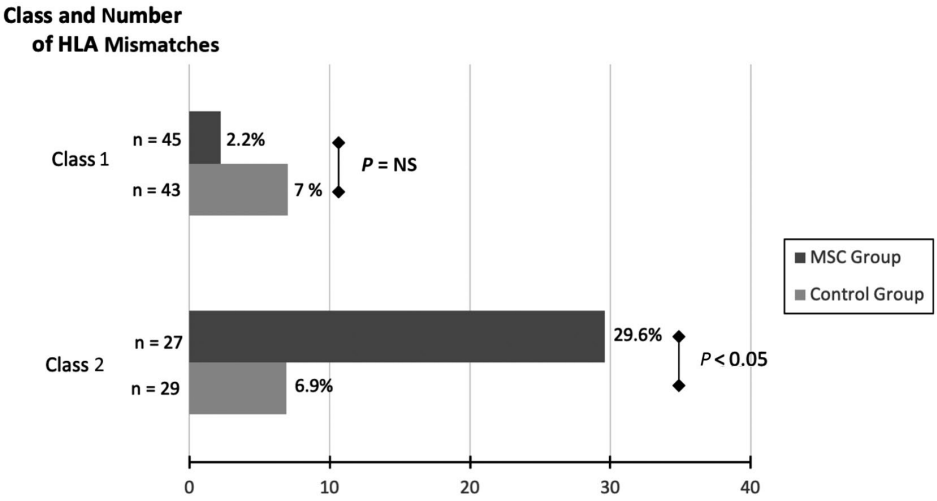


FIG. 1. Prevalence of locus-specific de novo $_{liver}$DSA relative to the number of HLA mismatches in the MSC and control groups (%; Fisher's exact test).

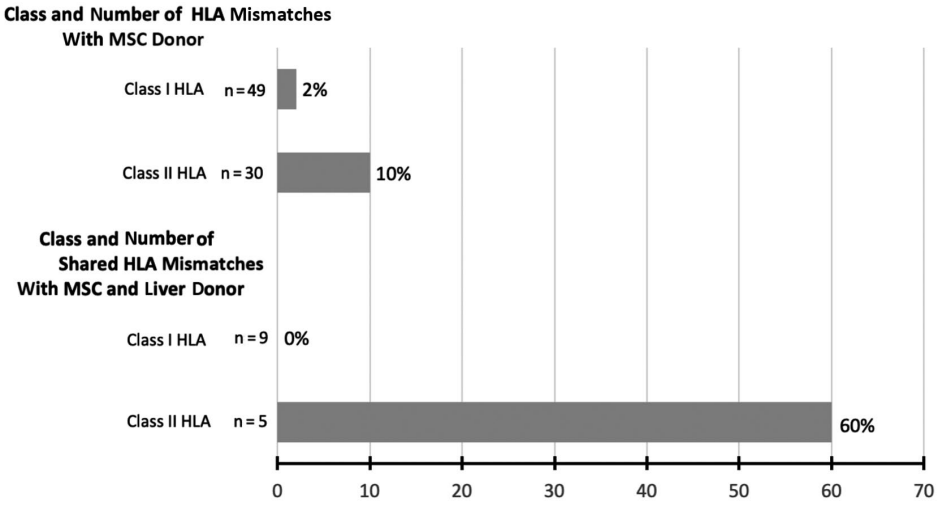


FIG. 2. Prevalence of locus-specific de novo $_{MSC}$DSA relative to the number of HLA mismatches with MSC donors (upper) and prevalence of locus-specific de novo $_{liver+MSC}$DSA relative to the number of shared HLA mismatches (lower).

was 2.2% (n = 5) and 7% (n = 9) in the control and MSC groups, respectively ($P = 0.36$). The prevalence of de novo $_{liver}$DSA for class II HLA mismatches was 6.9% (n = 2) and 29.6% (n = 8) in the control and MSC groups, respectively ($P = 0.04$; Figs. 1 and 3A and Table 3).

DE NOVO $_{MSC}$DSA

In the MSC group, 3 patients developed at least 1 de novo $_{MSC}$DSA. Patient 3 developed 1 class I $_{MSC}$DSA (B57:01), detected from month 1 (with MFI>5000) to the end of follow-up, and 1 class II $_{MSC}$DSA at month

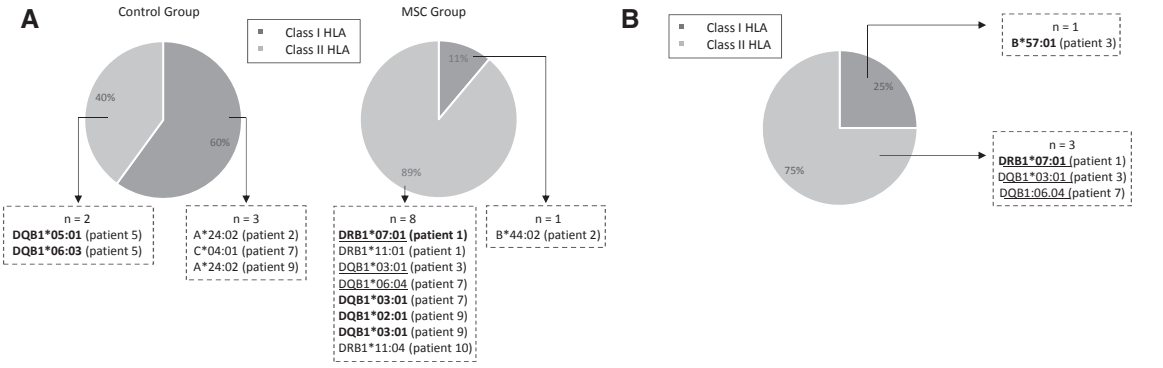


FIG. 3. Class and identity of de novo(A) $_{\text{Liver}}$DSA and (B) $_{\text{MSC}}$DSA. Underlined DSA in cases of $_{\text{liver+MSC}}$DSA. Bold DSA in cases of MFI >5000.

12. De novo $_{\text{MSC}}$DSA class II was detected in patient 7 and patient 1 (MFI >5000) at month 1 and month 6, respectively (Table 3). All of the de novo $_{\text{MSC}}$DSA class II were linked to a shared HLA mismatch between the liver and MSC donors (Table 3 and Fig. 3B). Considering the 5 HLA class II shared mismatches, 3 (60%) led to de novo $_{\text{liver+MSC}}$DSA detection in the MSC group (Figs. 2 and 3B and Table 3).

Discussion

We report the long-term results of the first clinical phase 1, prospective, controlled study aiming to evaluate the feasibility, safety, and tolerability of MSC infusion in 10 LT recipients. The 1-year results showed that a posttransplantation intravenous infusion of 1.5 to 3 × 10^6/kg MSC was well tolerated without evidence of pulmonary dysfunction or cytokine-release syndrome and without short-term toxicity.[9] These short-term safety results were recently confirmed in a study evaluating the effect of third-party MSC injection before LT.[20] After a median follow-up of 85 months, this study did not detect any toxicity attributed to this MSC infusion, particularly no increased rate of infection or cancer in LT recipients who received MSCs. If the short-term safety of MSC infusion has already been proven by numerous clinical studies providing early data, the present study adds new important information on the absence of the long-term deleterious clinical adverse effects of MSC infusion in this particular patient population receiving immunosuppressive drugs after LT.

In this small series, the potential advantages of MSC infusion were evaluated as secondary endpoints. With regard to the different studied parameters, a single allo-MSC posttransplant infusion did not appear to provide a clear clinical benefit to LT recipients in the long-term: there was no detected difference in liver graft survival or function. In addition, the overall rate of biliary complications was not lower in the patients treated with MSCs compared with the control group. There was no detected difference in fibrosis or Banff scores in the performed graft biopsies, and no patient from either group suffered from acute graft rejection requiring treatment. In some trials by Chinese groups, MSCs have been proposed as possibly playing a role in the management of the acute rejection of liver grafts,[21] in the prevention of antibody-mediated rejection after ABO-incompatible LT,[22] and in the management of ischemic-type biliary lesions.[23] In this study of 20 LTs, 1 recipient who had received MSCs developed NAS that ultimately required retransplantation after failure of surgical management. Biliary ASs were also detected in both groups and were not less frequent in the MSC patients. These issues deserve to be specifically analyzed in further studies on larger cohorts of patients.

This study also confirmed the preliminary data from our group[8] and others[2] on the potential immunogenicity of MSCs, an issue that might be particularly important in the field of organ transplantation. In LT, the impact of DSAs is not yet clearly understood. So far, donor and recipient HLA matching is not routinely recommended in deceased donor LT

because of the tolerogenic properties of the liver contributing to its resistance to antibody-mediated injuries.[24] Nevertheless, recent articles have shown that the appearance of de novo DSAs could be linked to an increased risk of rejection and lower graft and patient survival rates.[25,26]

Considering that MSC immunomodulatory properties may decrease immune responses against liver HLAs and the formation of de novo $_{liver}$DSA, the comparison of the prevalence of $_{liver}$DSA in the MSC and control groups is of particular importance. The appearance of $_{MSC}$DSA is also relevant to determine if MSCs promote an MSC-directed immune response in the host. In vitro, MSCs classically do not express class II HLA nor costimulatory molecules such as CD40, B7-1, or B7-2, but do express low levels of class I HLA. Because of these characteristics, MSCs were initially considered as minimally immunogenic and thus "immune privileged." However, the upregulation of both major histocompatibility complex (MHC)–I and MHC-II antigens on MSCs after interferon γ exposure, in addition to preclinical evidence of an immune response against MSCs, have brought into question that notion of low immunogenicity.[27,28] These concerns are particularly relevant in the field of solid organ transplantation. Indeed, in the case of a shared mismatch between third-party MSC donor and graft donor, MSCs could theoretically promote an immune response leading to the production of additional DSAs with their inherent risks to the graft.

There is in fact limited data on sensitization by MSCs. In 2019, Avivar-Valderas et al. reported that of 63 patients treated with allogenic adipose-derived MSCs used for perineal fistulas of Crohn's disease, 23 developed class I $_{MSC}$DSA 12 weeks after injection, and none against class II HLA, with no consequences on efficacy.[29] In an article reporting the use of allogeneic BM-MSCs for rheumatological diseases in 2 clinicals trials, de novo $_{MSC}$DSA could be detected only in 2 of 23 treated patients during the 2-year follow-up.[30] An article showing greater efficacy in allogeneic (versus autologous) MSCs for the treatment of nonischemic dilated cardiomyopathy reported $_{MSC}$DSA in only 1 patient without clinical impact.[31] This was approximately the same incidence as previous studies employing MSCs for cardiac diseases.[32,33] In 2019, our team published the results of a phase I/II trial reporting the use of third-party MSCs after KT in which MSCs were randomly assigned to KT recipients without HLA matching with kidney recipients or donors. De novo $_{MSC}$DSA class I with MFI >1500 were detected in only 1 patient. A total of 2 more de novo $_{MSC}$DSA (classes I and II) and 1 shared de novo kidney/MSC DSA (class II) with MFI <1500 were also detected.[8] However, one may question the clinical relevance of these $_{MSC}$DSA data given their low MFI values and the stability of the graft function during follow-up.[8]

Considering that MSCs could be immunogenic and cause sensitization against the graft, another team recently published a study in which a matching strategy protocol to prevent repeated mismatches between MSCs and kidney donors in 10 KT patients was used.[34] In this study, selected third-party BM MSCs were injected 6 months after KT. No de novo MSCs nor kidney DSAs were detected during the 6-month follow-up after MSC infusion.

In the present study, because all of the class II $_{MSC}$DSA were also $_{liver}$DSA because of a shared mismatch, it is difficult to differentiate sensitization caused by the liver graft or by the MSC HLA class II antigen recognition by the host immune system. Nevertheless, the high prevalence (60%) of HLA antibody detection in cases of shared mismatches in class II loci in our study might suggest that this combination could promote immunogenicity. In other reported studies evaluating this issue, most of the detected $_{MSC}$DSA after MSC infusion were against class I HLA. Nevertheless, it seems that in cases of shared class II HLA mismatches between MSCs and the graft, class II DSA could also be promoted. These observations could potentially urge caution by avoiding repeated mismatches between third-party MSCs and graft donors at least for HLA class II or by using autologous MSCs, especially for KT, but this needs to be investigated further with larger cohorts. These observations may lead to reconsidering the risk of development of $_{MSC}$DSA as well as the necessity of a matching strategy. However, the absence of impact on long-term allograft outcomes in this study is in line with previous data in non-transplant settings regarding the clinical significance of $_{MSC}$DSA.[16]

There are many shortcomings to this study. First, it is clear that this first study in 10 LT recipients does not prove the long-term safety of MSC infusion after transplantation. These results will have to be confirmed by further studies in larger groups of liver recipients, focusing particularly on the potential immunogenicity of MSCs. The absence of detectable effects of MSCs

might be attributed to an insufficient sample size, to the immunosuppressive regimen, or to an insufficient MSC dosing, which should possibly be increased or repeated. The timing (preoperative, intraoperative, or postoperative) and the infusion routes (peripheral vein, portal vein, or hepatic artery) of MSC infusion should also be evaluated. Different MSC sources (BM, fat tissue, liver) or donors (organ donor, organ recipient) should also be tested in further studies.

In conclusion, this first prospective clinical trial investigating the safety of injecting allogeneic MSCs after deceased donor LT did not demonstrate potential adverse effects, particularly no increased rate of opportunistic complications. Injecting allogeneic MSCs after deceased donor LT may promote $_{liver}$DSA class II emergence in LT recipients. This subject deserves further investigation. The potential benefits of MSC injections in the context of organ transplantation have yet to be demonstrated.

Acknowledgments: The authors value and thank all of the members of the liver transplantation program of the University of Liege Hospital (physicians, nurses, coordinators, etc.), who contributed extensively to this study, and the LTCG.

REFERENCES

1) Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.
2) Vandermeulen M, Erpicum P, Weekers L, Briquet A, Lechanteur C, Detry O, et al. Mesenchymal stromal cells in solid organ transplantation. Transplantation 2020;104:923-936.
3) Erpicum P, Detry O, Weekers L, Bonvoisin C, Lechanteur C, Briquet A, et al. Mesenchymal stromal cell therapy in conditions of renal ischaemia/reperfusion. Nephrol Dial Transplant 2014;29:1487-1493.
4) Rowart P, Erpicum P, Detry O, Weekers L, Grégoire C, Lechanteur C, et al. Mesenchymal stromal cell therapy in ischemia/reperfusion injury. J Immunol Res 2015;2015:1-8.
5) Grégoire C, Lechanteur C, Briquet A, Baudoux É, Baron F, Louis E, Beguin Y. Review article: mesenchymal stromal cell therapy for inflammatory bowel diseases. Aliment Pharmacol Ther 2017;45:205-221.
6) Servais S, Baron F, Lechanteur C, Seidel L, Selleslag D, Maertens J, et al. Infusion of bone marrow derived multipotent mesenchymal stromal cells for the treatment of steroid-refractory acute graft-versus- host disease: a multicenter prospective study. Oncotarget 2018;9:20590-20604.
7) Negi N, Griffin MD. Effects of mesenchymal stromal cells on regulatory T cells: current understanding and clinical relevance. Stem Cells 2020;38:596-605.
8) Erpicum P, Weekers L, Detry O, Bonvoisin C, Delbouille M-H, Grégoire C, et al. Infusion of third-party mesenchymal stromal cells after kidney transplantation: a phase I-II, open-label, clinical study. Kidney Int 2019;95:693-707.
9) Detry O, Vandermeulen M, Delbouille M-H, Somja J, Bletard N, Briquet A, et al. Infusion of mesenchymal stromal cells after deceased liver transplantation: a phase I-II, open-label, clinical study. J Hepatol 2017;67:47-55.
10) Dillmann J, Popp FC, Fillenberg B, Zeman F, Eggenhofer E, Farkas S, et al. Treatment-emergent adverse events after infusion of adherent stem cells: the MiSOT-I score for solid organ transplantation. Trials 2012;13:211.
11) Furlani D, Ugurlucan M, Ong LL, Bieback K, Pittermann E, Westien I, et al. Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. Microvasc Res 2009;77:370-376.
12) Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PLoS ONE 2012;7:1-21.
13) Haarer J, Johnson CL, Soeder Y, Dahlke MH. Caveats of mesenchymal stem cell therapy in solid organ transplantation. Transpl Int 2015;28:1-9.
14) Barkholt L, Flory E, Jekerle V, Lucas-Samuel S, Ahnert P, Bisset L, et al. Risk of tumorigenicity in mesenchymal stromal cell-based therapies—bridging scientific observations and regulatory viewpoints. Cytotherapy 2013;15:753-759.
15) Kisseleva T, Brenner DA. The phenotypic fate and functional role for bone marrow-derived stem cells in liver fibrosis. J Hepatol 2012;56:965-972.
16) Lohan P, Treacy O, Griffin M, Ritter T, Ryan AE. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells and their extracellular vesicles: are we still learning? Front Immunol 2017;8:1.
17) Alagesan S, Griffin MD. Autologous and allogeneic mesenchymal stem cells in organ transplantation: what do we know about their safety and efficacy? Curr Opin Organ Transplant 2014;19:65-72.
18) Lechanteur C, Briquet A, Giet O, Delloye O, Baudoux E, Beguin Y. Clinical-scale expansion of mesenchymal stromal cells: a large banking experience. J Transl Med 2016;14:1-15.
19) Demetris AJ, Batts KP, Dhillon AP, Ferrell L, Fung J, Geller SA, et al. Banff schema for grading liver allograft rejection: an International Consensus Document. Hepatology 1997;25:658-663.
20) Casiraghi F, Perico N, Podestà MA, Todeschini M, Zambelli M, Colledan M, et al. Third-party bone marrow—derived mesenchymal stromal cell infusion before liver transplantation: a randomized controlled trial. Am J Transplant 2021;21:2795-2809.
21) Shi M, Liu Z, Wang Y, Xu R, Sun Y, Zhang M, et al. A pilot study of mesenchymal stem cell therapy for acute liver allograft rejection. Stem Cells Transl Med 2017;6:2053-2061.
22) Zhang Y, Zhang J, Yi H, Zheng J, Cai J, Chen W, et al. A novel MSC-based immune induction strategy for ABO-incompatible liver transplantation: a phase I/II randomized, open-label, controlled trial. Stem Cell Res Ther 2021;12:244.
23) Zhang Y-C, Liu W, Fu B-S, Wang G-Y, Li H-B, Yi H-M, et al. Therapeutic potentials of umbilical cord–derived mesenchymal stromal cells for ischemic-type biliary lesions following liver transplantation. Cytotherapy 2017;19:194-199.
24) Cheng EY. The role of humoral alloreactivity in liver transplantation: lessons learned and new perspectives. J Immunol Res 2017;2017:1-9.
25) Kaneku H, O'Leary JG, Banuelos N, Jennings LW, Susskind BM, Klintmalm GB, et al. De novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients. Am J Transplant 2013;13:1541-1548.

26) O'Leary JG, Demetris AJ, Friedman LS, Gebel HM, Halloran PF, Kirk AD, et al. The role of donor-specific HLA alloantibodies in liver transplantation. Am J Transplant 2014;14:779-787.
27) Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: immune evasive, not immune privileged. Nat Biotechnol 2014;32:252-260.
28) Berglund AK, Fortier LA, Antczak DF, Schnabel LV. Immunoprivileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells. Stem Cell Res Ther 2017;8:288.
29) Avivar-Valderas A, Martín-Martín C, Ramírez C, Del Río B, Menta R, Mancheño-Corvo P, et al. Dissecting allo-sensitization after local administration of human allogeneic adipose mesenchymal stem cells in perianal fistulas of Crohn's disease patients. Front Immunol 2019;10:1-15.
30) García-sancho J, Sánchez A, Vega A, Noriega DC, Nocito M. Influence of HLA matching on the efficacy of allogeneic mesenchymal stromal cell therapies for osteoarthritis and degenerative disc disease. Transpl Direct 2017;3:2-5.
31) Hare JM, DiFede DL, Rieger AC, Florea V, Landin AM, El-Khorazaty J, et al. Randomized comparison of allogeneic versus autologous mesenchymal stem cells for nonischemic dilated cardiomyopathy: POSEIDON-DCM trial. J Am Coll Cardiol 2017;69:526-537.
32) Ascheim DD, Gelijns AC, Goldstein D, Lemuel A, Smedira N, Lee S, et al. Mesenchymal precursor cells as adjunctive therapy in recipients of contemporary LVADs. Circulation 2015;129:2287-2296.
33) Hare JM, Fishman JE, Gerstenblith G, DiFede Velazquez DL, Zambrano JP, Suncion VY, et al. Comparison of allogeneic vs autologous bone marrow–derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. JAMA 2012;308:2369-2379.
34) Dreyer GJ, Groeneweg KE, Heidt S, Roelen DL, van Pel M, Roelofs H, et al. Human leukocyte antigen selected allogeneic mesenchymal stromal cell therapy in renal transplantation: the Neptune study, a phase I single-center study. Am J Transplant 2020;20:2905-2915.

CHAPTER III. EFFECT OF THE COMBINATION OF EVEROLIMUS AND MESENCHYMAL STROMAL CELLS ON REGULATORY T CELLS IN A LIVER TRANSPLANT REJECTION MODEL IN RATS

CHAPTER III. EFFECT OF THE COMBINATION OF EVEROLIMUS AND MESENCHYMAL STROMAL CELLS ON REGULATORY T CELLS LEVELS AND IN A LIVER TRANSPLANT REJECTION MODEL IN RATS

Authors

Morgan Vandermeulen [1,2,3], Pauline Erpicum [3,4], Noella Bletard [5], Laurence Poma[3], François Jouret [3,4], Olivier Detry [1,2]

Affiliations :

1. Department of Abdominal Surgery and Transplantation, University of Liege Hospital (CHU ULiege), Liege, Belgium
2. *Centre de Recherche et de Développement du Département de Chirurgie* (CREDEC), Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA), Cardiovascular Sciences, University of Liege (ULiege), Liege, Belgium
3. Laboratory of Translational Research in Nephrology (LTRN), Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA), Cardiovascular Sciences, University of Liege (ULiege), Liege, Belgium
4. Department of Nephrology, University of Liege Hospital (CHU ULiege), Liege, Belgium
5. Department of Pathology, University of Liege Hospital (CHU ULiege), Liege, Belgium

Frontiers in immunology, 2022; 13: 877953

Summary

MSCs have particular properties that are of interest in organ transplantation, including the expansion of Tregs, a key factor in transplant tolerance induction. The ISD, necessarily combined with MSC infusion, probably explains in part the controversial observations of MSC therapy in SOT and the most effective ISD to associate with MSCs has yet to be defined. The impact of the association of EVR with MSCs on Treg expansion, and on the induction of liver graft tolerance, has never been studied. The aim of this study was to evaluate the effects of MSCs in combination, or not, with EVR on Treg expansion and in a model of rejection after LTx in the rat.

The first part of the experiment aimed at studying the efficiency of MSCs and of the combination of MSCs with EVR in promoting Tregs. Twenty-four 7-week-old male Lewis rats were randomly assigned to 4 groups (n=6 in each group) receiving intravenous MSCs or saline injection at day (D)9 with/without subcutaneous EVR from D0 to D14. A fluorescence-activated cell sorting analysis of circulating Tregs was performed at D0, D14 and D28. In a second set of experiment, 30 Lewis rats were randomized in 3 groups 48 hours after LTx with a DA rat liver: EVR (subcutaneous for 14 days), MSCs (intravenous injection at post-operative day 2 and 9), or both EVR and MSCs. Rejection of the liver graft was assessed by repeated liver tests, histology and survival. Three rats of each group were sacrificed at D9 for histological analysis and At POD23 the living remaining rats of each group were sacrificed for laboratory tests and histological analysis.

In the first part of this study, the percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs amongst $CD4^+$ $CD25^+$ cells measured in each group was not statistically different. In the Control group, the percentage of $FoxP3^+$ Tregs at D14 and D28 remained stable without statistical difference between these timepoints. In both the MSC Group and the MSC+EVR group, the percentage of $FoxP3^+$ Tregs had significantly increased by D14 from their baseline rate and this increase was even greater at D28 in both groups. In the EVR Group, $FoxP3^+$ Treg percentage showed a slight but significant increase at D14 but not at D28. Individually, MSC infusion and EVR promoted Treg expansion in rats, and EVR had no negative impact on Treg expansion in combination with MSCs. In the second part of this work, survival of liver transplanted rats

receiving EVR with (LTx-MSC+EVR) or without (LTx-EVR) was 100% and 86% respectively at D23. Rats treated with MSCs alone (LTx-MSC group) showed a high mortality rate, with no surviving rats at the end of the experiment, and with a median survival of 15 days [12- 15] ($P<0.001$). Pre-operative laboratory kidney and liver tests were similar in the three LT-groups. At POD9, rats from the LTx-MSC group showed signs of acute rejection with major cytolysis. Rats from the LTx-EVR group and LTx-MSC+EVR group had only slightly increased liver test results. On D16 only one rat from the LTx-MSC group remained alive for biological analysis and revealed extremely high cytolysis. AST and ALT levels were slightly increased in the LTx-EVR and LTx-MSC+EVR groups on D16 and on D23 without any significant differences between these groups. On D23 no rat remained alive in the LTx-MSC group. On D9 the rats in the groups receiving EVR (LTx-EVR and LTx-MSC+EVR groups) showed histological signs of moderate acute rejection with both a median Banff score of 5/9 and mild fibrosis (F1), while conversely, those treated with MSCs alone (LTx-MSC group) showed histological signs of severe acute rejection with a median Banff score of 9/9 and severe fibrosis (F3). At D23 the median Banff score was similar in the sacrificed rats of the LT-EVR and LT-MSC +EVR groups (5/9). Liver fibrosis was also similar, with a median lower than F1 in both groups. On D9, rats receiving MSC (LTx-MSC and LTx-MSC+EVR groups) seemed to have a higher, but not statistically significant, count of $FoxP3^+$ cells in the graft when compared to rats of the LTx- EVR group. At D23 the number of Tregs in the liver graft was comparable in the LTx-EVR and in the LTx-MSC+EVR groups ($P>0.99$).

In conclusion, despite a significant increase of the percentage of circulating Tregs after one injection of MSCs, MSCs were not effective in preventing severe acute rejection in our LTx model. In addition, no effect of the association of MSCs with EVR was found, when compared to EVR alone. EVR alone could significantly alleviate acute rejection, showing a stable level of rejection even one week after its final administration. Furthermore, the addition of MSCs had no observable positive synergistic impact on acute rejection treatment, or prevention, when compared to EVR alone. However, it is possible that MSC injections closer to LTx time, and a longer follow-up, would reveal some differences in graft survival and tolerance induction. Given these results, the association of EVR and MSCs in SOT deserves further investigation.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 10 June 2022
doi: 10.3389/fimmu.2022.877953

Effect of the Combination of Everolimus and Mesenchymal Stromal Cells on Regulatory T Cells Levels and in a Liver Transplant Rejection Model in Rats

Morgan Vandermeulen[1,2,3]*, *Pauline Erpicum*[3,4], *Noella Bletard*[5], *Laurence Poma*[3], *François Jouret*[3,4] and *Olivier Detry*[1,2]

[1] Department of Abdominal Surgery and Transplantation, University of Liege Hospital [Centre Hospitalier Universitaire (CHU) ULiege], Liege, Belgium, [2] Centre de Recherche et de Développement du Département de Chirurgie (CREDEC), Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA), Cardiovascular Sciences, University of Liege (ULiege), Liege, Belgium, [3] Laboratory of Translational Research in Nephrology (LTRN), Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA), Cardiovascular Sciences, University of Liege (ULiege), Liege, Belgium, [4] Division of Nephrology, University of Liege Hospital [Centre Hospitalier Universitaire (CHU) ULiege], Liege, Belgium, [5] Department of Pathology, University of Liege Hospital [Centre Hospitalier Universitaire (CHU) ULiege], Liege, Belgium

OPEN ACCESS

Edited by:
Federica Casiraghi, Mario Negri Pharmacological Research Institute (IRCCS), Italy

Reviewed by:
Marcella Franquesa, Germans Trias i Pujol Health Science Research Institute (IGTP), Spain
Ross Francis, Princess Alexandra Hospital, Australia

***Correspondence:**
Morgan Vandermeulen
m.vandermeulen@chuliege.be

Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *17 February 2022*
Accepted: *09 May 2022*
Published: *10 June 2022*

Citation:
Vandermeulen M, Erpicum P, Bletard N, Poma L, Jouret F and Detry O (2022) Effect of the Combination of Everolimus and Mesenchymal Stromal Cells on Regulatory T Cells Levels and in a Liver Transplant Rejection Model in Rats. Front. Immunol. 13:877953. doi: 10.3389/fimmu.2022.877953

Introduction: Mesenchymal stromal cells (MSCs) have particular properties that are of interest in organ transplantation, including the expansion of regulatory T cells (Tregs), a key factor in transplant tolerance induction. However, the most effective immunosuppressive drug to associate with MSCs has yet to be defined. Additionally, the impact of the association of everolimus with MSCs on Treg expansion, and on the induction of liver graft tolerance, has never been studied. The aim of this study was to evaluate the effects of MSCs in combination, or not, with everolimus on Treg expansion and in a model of rejection after liver transplantation (LT) in the rat.

Methods: Firstly, 24 Lewis rats were assigned to 4 groups (n=6 in each group) receiving intravenous MSCs or saline injection at day (D)9 with/without subcutaneous everolimus from D0 to D14. Analysis of circulating Tregs was performed at D0, D14 and D28. In a second set of experiment, 30 Lewis rats were randomized in 3 groups 48hours after LT with a Dark Agouti rat liver: everolimus (subcutaneous for 14 days), MSCs (intravenous injection at post-operative day 2 and 9), or both everolimus and MSCs. Rejection of the liver graft was assessed by liver tests, histology and survival.

Results: Individually, MSC infusion and everolimus promoted Treg expansion in rats, and everolimus had no negative impact on Treg expansion in combination with MSCs. However, in the LT model, injections of MSCs two and nine days following LT were not effective at preventing acute rejection, and the combination of MSCs with everolimus failed to show any synergistic effect when compared to everolimus alone.

Conclusion: Everolimus may be used in association with MSCs. However, in our model of LT in the rat, post-transplant MSC injections did not prevent acute rejection, and the association of MSCs with everolimus did not show any synergistic effect.

Keywords: experimental model, cell-therapy, stem cells, mTOR inhibitors, graft tolerance

1 INTRODUCTION

Mesenchymal stromal cells (MSCs) are a heterogeneous population of fibroblast-like cells which have been defined by the International Society for Cellular Therapy (1). They can be isolated from various tissues, including bone marrow (2, 3). MSCs have been used in many fields, including solid organ transplantation (SOT), in which they are considered to be a promising cell therapy. *In vitro* and *in vivo* experiments have demonstrated that MSCs have interesting immunomodulatory properties (4–6), combined with beneficial effects on ischemia-reperfusion injury and on tissue/organ repair (7). One of the most favorable effects of MSCs in SOT could be their capacity of inducing the expansion of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Tregs) (6, 8). Indeed, increased levels of circulating Tregs in peripheral blood have been identified as a key factor in inducing transplant tolerance after SOT (9). Still, despite encouraging pre-clinical data, preliminary clinical studies using MSC therapy in SOT patients have shown inconsistent results, including after liver transplantation (LT) (4, 10, 11). The immunosuppressive drugs (ISDs), necessarily combined with MSC infusion, probably explain in part the controversial observations of MSC therapy in SOT (10). *In vitro*, high doses of calcineurin inhibitors (CNIs) have been shown to be toxic for MSCs and to antagonize their immunomodulatory properties (12). *In vivo*, CNI effects on MSCs have been variable, sometimes promoting, sometimes antagonizing MSC function (13), while Treg function and proliferation have been inhibited by CNIs both *in vitro* and *in vivo* (14, 15). In a recent clinical trial our group failed to find any difference in Treg levels in LT recipients receiving MSCs when compared to a control group (16). Amongst the other available ISDs that are clinically used in SOT, mammalian/mechanistic target of rapamycin (mTOR) inhibitors, *i.e.* rapamycin (sirolimus) and everolimus, inhibit mTOR complexes. This leads to the blockade of T-cell progression in response to growth factors or cytokines, thereby limiting T-cell proliferation (17). *In vitro*, rapamycin inhibits MSC proliferation and antagonizes its inhibitory effect (18). However, in contrast to what has been observed *in vitro*, the *in vivo* combination of MSCs and rapamycin has been shown to promote graft tolerance in cardiac or islet allograft models (19, 20). However, such an immunosuppressive association has never been tested in a LT model.

The "ideal" ISD to be associated with MSCs in SOT has not yet been defined, and the impact of everolimus in association with MSCs on Treg expansion and on the establishment of liver graft tolerance has never previously been studied. Given that both MSCs and everolimus appear to have a positive impact on Tregs, their association deserves to be investigated. The aim of this study was to evaluate the effects of MSC injections in combination, or not, with everolimus, on Treg expansion and to test the hypothesis that this association prevents acute rejection in a LT model in the rat.

2 MATERIAL AND METHODS

All experimental procedures were approved by the Animal Ethics Committee of the University of Liege, Belgium (Protocol number #1957). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The authors followed the institutional and national guidelines for the care and use of laboratory animals. All rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France). All invasive procedures were performed under anesthetic gas (isoflurane), and prevention of post-operative pain was achieved with a dose of buprenorphine (0.05 mg/kg) at the end of each procedure. Rats had access to water and food *ad libitum* throughout the duration of the experiment.

2.1 Everolimus Administration

Everolimus (E-4040, LC Laboratories, MA) was administered through a subcutaneous (sc) osmotic pump (#2002, Alzet, Cupertino, CA). It had previously been dissolved in a mixture containing 50% dimethyl sulfoxide (Sigma-Aldrich, MO), 40% propylene glycol (Fagron, Rotterdam, The Netherlands) and 10% absolute ethanol (VWR, PA), to obtain a solution with an everolimus concentration of 4.17mg/mL. 250µL of this solution was then used to fill each osmotic pump. After sc implantation the pump delivered the solution at a constant flow of 0.5µL/h for 14 days, as defined by the manufacturer's specifications, corresponding to a dose of everolimus of 0.25mg/kg/day. In the groups not receiving everolimus, the pumps were filled with the same mixture but without the addition of everolimus (placebo). To implant the pump a shaved and washed site posterior to the scapula was incised, and a sc pocket was created with the aid of a hemostatic clamp before insertion of the filled pump. The wound was then closed with sutures.

2.2 Isolation, *In Vitro* Expansion and Identification of MSCs

The protocol of isolation, culture and identification of bone marrow-derived MSCs from Lewis rats was performed according to our previously reported protocol (21). In brief, bone marrow was collected by flushing femurs and tibias of 10-week-old inbred Lewis rats. The bone marrow was then homogenized in Phosphate-Buffered Saline (PBS, Lonza) + 2% fetal bovine serum (FBS, Lonza) before being filtered and centrifuged twice. Cells were cultured in $75cm^2$ culture flask (Falcon) with DMEM supplemented with 10% FBS, 1% penicillin (Lonza) and 1% L-Glutamin (Lonza). Cells were cryopreserved before the third passage. When needed, cells from 3 donors were thawed in a water-bath at 37°C, centrifuged and re-suspended together in DMEM culture medium. MSC were cultured at 37°C in a humidified 5%CO_2 incubator. Supplemented DMEM was changed twice a week. Cells were split in two culture flasks when they reached 80% of confluency. Phenotyping was performed twice: before cryopreservation and before i.v. injection. The cells were adherent to plastic and presented fibroblast-like morphology. MSC positive markers were evidenced by flow-cytometry, using APC-conjugated anti-rat CD90 (BD Pharmingen) antibody and AlexaFluor-conjugated anti-rat CD29 antibody (BD Pharmingen). MSC were negative for V450-conjugated anti-rat CD45 (BD Horizon) antibody,

MSC were negative for PE-conjugated anti-CD79a (abcam) antibody and for FITC-conjugated anti-rat CD11b (BD Pharmingen) antibody. The ability of MSC to differentiate into adipocytes, osteoblasts and chondroblasts lineages was demonstrated by positive staining for Oil Red O, Alizarin Red, and toluidine blue, respectively. In addition, the differentiation status into adipogenic (FABP4/DAPI), chondrogenic (Aggrecan/DAPI) and osteogenic (Osteocalcin/DAPI) lineages by immunofluorescence was confirmed using a commercial kit (R&D systems, #SC020, USA) (**Supplementary Figure 1**).

2.3 MSC Administration

Trypsin - Ethylenediaminetetraacetic acid (EDTA) was used to detach MSCs from the culture flasks. Cells were then centrifuged in DMEM cell culture media at 500G for 5 minutes (min) and counted using a Thoma chamber. MSC were used only before the fifth passage. The required number of cells was resuspended in 1ml of pre-warmed saline and slowly injected through the penile vein of the rat. Rats not treated with MSCs received an equal volume of pre-warmed saline.

2.4 Experimental Design

The first part of the experiment aimed at studying the efficiency of MSCs and of the combination of MSCs with everolimus in promoting Tregs. Twenty-four 7-week-old male Lewis rats were randomly assigned to 4 groups (**Figure 1**): *MSC+EVR group* (n=6), received the association of sc everolimus from D0 to D14 and intravenous (iv) MSC injection (3.10^6cells/kg) at D9; *MSC group* (n=6), received placebo administration sc from D0 to D14 and iv MSC injection (3.10^6cells/kg) at D9; *EVR group* (n=6), received everolimus sc from D0 to D14 and iv saline injection at D9; *Control group* (n=6), received placebo sc from D0 to D14, and iv saline injection at D9.

A fluorescence-activated cell sorting (FACS) analysis of circulating Tregs was performed at D0, D14 and D28. 250µL of whole blood was obtained in an EDTA tube (BD Microtainer K2E tubes, 365975) at D7, D14, D21 and D28 post-pump implantation to monitor everolimus blood levels (**Figure 1**). Samples were analyzed using an UHPLC-MS/MS Acquity® analyzer (Waters, #186002350).

In a second part, the association of MSCs and everolimus was tested in a rat LT model. Accordingly, 10-week-old Dark Agouti male rats and 8-week-old Lewis male rats were used as liver donors and recipients, respectively. A non-arterialized orthotopic LT model in a rat was established using a modified Kamada's two-cuff technique with adapted 3D-printed instruments and cuffs generated with a *Form2* 3-D printer (Formlabs, MA, USA) (22, 23). Directly following surgery, the rats were kept during 1 hour in an intensive care unit (Vetario, UK) with an O_2-, temperature- and humidity- controlled environment. 48 hours after LT the rats were randomized in one of the three experimental groups (**Figure 2**) as follows:

- *LT-EVR group* (n=10): sc everolimus (0.25mg/kg/day) from post-operative day (POD) 2 to POD16 and iv saline injection (1ml through the penile vein) at POD2 and POD9.
- *LT-MSC group* (n=10): sc placebo from POD2 to POD16 and iv MSC injection (3.10^6 cells/kg) at POD2 and POD9.
- *LT-MSC+EVR* group (n=10): sc everolimus from POD2 to POD16 and iv MSC injection (3.10^6 cells/kg) at POD 2 and POD9.

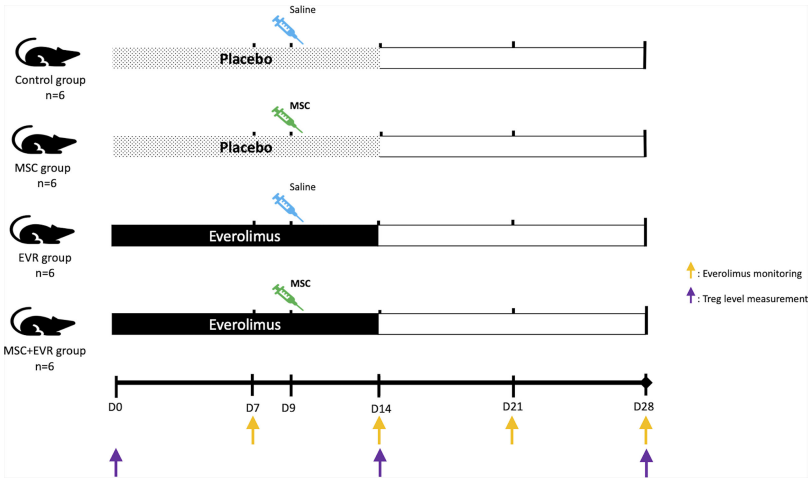


FIGURE 1 | Evaluation of the effect of MSCs and everolimus on Treg proliferation (experimental design). Pumps were implanted at D0 and functioned for 14 days. One injection of MSCs or saline was performed at D9. Everolimus blood levels were monitored once a week (orange arrows); Treg blood levels were measured by flow cytometry on D0, D14 and D28 (purple arrows). D, day; EVR, everolimus; MSC, mesenchymal stromal cell; Treg, regulatory T cells.

2.5 Liver and Renal Function Measurements and Histological Analysis

In each LT-group (**Figure 2**), blood samples were taken through the rat tail vein at 4 time points: just before LT (D0), POD9, 16 and 23, in order to measure the serum levels of creatinine, alanine aminotransferase (ALT), aminotransferase (AST) and total bilirubin.

At POD 9, 3 rats from each group were sacrificed for liver histological analysis. At POD23 the living remaining rats of each group were sacrificed for laboratory tests and histological analysis. Liver tissue was obtained for pathological examination after animal sacrifice (**Figure 2**). To determine Banff score and fibrosis score (METAVIR), samples were fixed in 10% formalin, embedded in paraffin, sliced into 5µm-thick sections and stained with Hematoxylin and Eosin (H&E) or Masson's trichrome. A liver pathologist (N.B.) blindly examined the H&E-stained slices and trichrome-stained slices for Banff score and for fibrosis score (METAVIR) grading, respectively (24). To visualize FoxP3 positive cells in the graft, liver samples were fixed in paraformaldehyde 4% for 24 hours and then embedded in paraffin. Sections (5µm) were dewaxed and then gradually hydrated before immunohistochemistry and were subjected to antigen retrieval EDTA buffer (Dako, #S2367). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (Merck 30%, #107209) for 20 min at RT. Non-specific binding was constrained by incubation for 10min with protein block reagent (Cell Signaling Technology, #15019L). Then, sections were incubated for 60min at room temperature with primary antibodies: Rabbit anti FoxP3 (ABCAM# 215206 1/250 in diluent Dako#S3022). After washing, sections were incubated for 30 min with secondary antibodies (Envision anti-Rabbit/HRP, Dako #K4003), and subsequently washed and exposed to horseradish peroxidase for 30min. Immunoreactivity was detected using 3,3′-Diaminobenzidine (Dako #K3468). N.B. blindly counted the number of FoxP3+ cells at 40x magnification in three high power fields from the graft sections.

2.6 Treg Flow Cytometry Analysis

Cells were harvested from whole blood samples and FACS was performed using 3 fluorochrome-conjugated antibodies: i) fluorescein-conjugated anti-CD4 (R&D, #967211) ii) Rα PE-conjugated anti-CD25 (R&D, #968436) iii) Alexa Fluor-conjugated anti-FoxP3 (R&D, #968435). Beforehand, a red blood cells lysis buffer (eBioscience, #00433357) was used to remove Red Blood cells from the whole blood samples. A FoxP3/transcription factor permeabilization buffer (R&D, #43481) was used for nuclear permeabilization before incubation with anti-FoxP3 antibody. Flow cytometry was performed on an FACS Canto II flow cytometer to evaluate cell fluorescence. Obtained data were analyzed using FACS Diva software (BD, San Jose, CA). Percentages of FoxP3+/CD4+CD25+ cells and of FoxP3+/CD4+ total cells were calculated. Fluorescence Minus One Control was defined for FoxP3 using an isotype control. Gating strategy is given in **Supplementary Figure 2**.

2.7 Statistical Analysis

Data are presented as median [P25-P75]. GraphPad Prism version 7.00 Software (San Diego, CA) was used for statistical analysis. A p-value <0.05 was considered as statistically significant. Kruskal-Wallis test, Mann-Whitney test or Wilcoxon test was used when appropriate. Graft survival was analyzed with the Kaplan-Meier method using the log-rank (Mantel-Cox) test.

3 RESULTS

3.1 Everolimus Can be Efficiently Delivered in Rats *via* a Subcutaneous Osmotic Pump

In the *EVR group* and *MSC+EVR group*, everolimus was efficiently delivered in all rats (with no significant difference between these groups) (**Figure 3**). With a sc delivery of 0.25mg/kg/day, the concentration of everolimus measured in the blood at

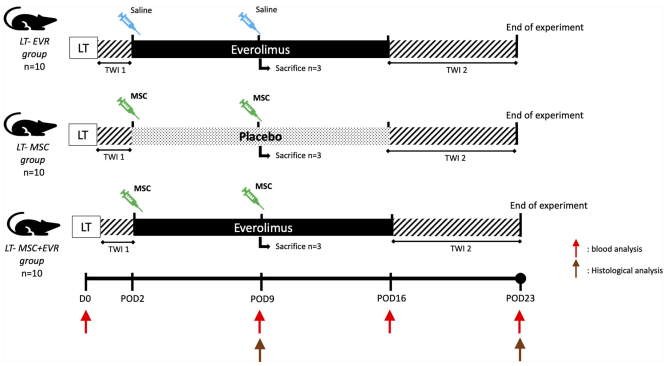


FIGURE 2 | Evaluation of the association of MSCs and everolimus in a liver transplantation model (experimental design). (EVR, everolimus; LT, liver transplantation; MSC, mesenchymal stromal cell; POD, post-operative day; TWI, time without immunosuppression -1: first post-operative 48h, -2: from POD16 to POD23 (end of experiment); Red arrow, blood analysis; Brown arrow, histological analysis).

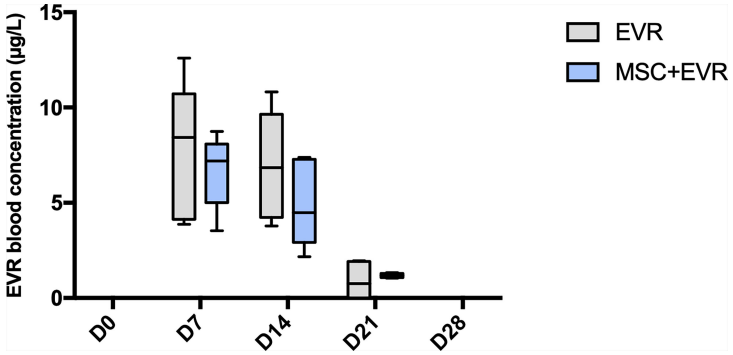


FIGURE 3 | Evolution of everolimus blood concentration. In the 2 groups (n = 6/group) receiving everolimus with osmotic pumps, the drug could be efficiently delivered with a statistically increased concentration during 14 days. Plots display the median (25th and 75th percentiles) of the distributions (boxes), and whiskers extend to the minimal and maximal values. There was no statistical difference between the groups at any time point (Mann-Whitney test). (D, Day; EVR, Everolimus ; MSC, mesenchymal stromal cells).

D7 was 8.43µg/L [4.13-10.73] in the *Evero group* and 7.19 µg/L [5.01-8.08] in the *MSC+Evero group* (P= 0.39). At D14 it was 6.84 µg/L [4.24-9.64] and 4.49 µg/L [2.92-7.28] in the *Evero group* and in the *MSC+Evero group*, respectively (*P*= 0.24). At D21 the blood concentration of everolimus was close to 0 µg/L in all rats (*Evero group*: 0.76 µg/L [0-1.95]; *MSC+Evero group*: 1.18 µg/L [1.07-1.29]; *P* >.99) and at 0 µg/L in all rats at D28. Blood levels of everolimus were undetectable at all timepoints in the placebo group.

3.2 MSC-Induced Treg Expansion Is Not Inhibited by Everolimus Adjunction

The percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs amongst $CD4^+$ $CD25^+$ cells measured in each group was not statistically different in the 4 groups at D0 with 1.97% [1.33-6.47] in the *Control group*, 0.84% [0.24-3.33] in the *MSC group*, 0.89% [0.58-2.16] in the *Evero group* and 0.34% [0.24-1.26] in the *MSC+Evero group*; (*p=0.08*). In the *Control group*, the percentage of FoxP3+ Tregs at D14 (1.01% [0.68-2.49]) and D28 (3.74%[2.03-6.07]) remained stable without statistical difference between these timepoints. In both the *MSC Group* and the *MSC+Evero Group*, the percentage of FoxP3+ Tregs had significantly increased by D14 from their base rate (6.79% [5.06-7.21] and 1.91% [1.52-3.39] respectively) and this increase was even greater at D28 in both groups (15%[13.1-15.8] and 20% [19.2-23.1] respectively). In the *Evero Group*, FoxP3+ Treg percentage showed a slight but significant increase at D14 (2.41% [1.08-3.75] but not at D28 (1.25% [0.76-2.58]) (**Figure 4**).

3.3 MSCs Alone Are Not Effective in Preventing Acute Rejection

3.3.1 Survival Time of Transplant Recipients

Thirty liver-transplanted rats were randomized in 3 groups (n=10/group) on POD2 (**Figure 2**). The weight of rat donors and recipients, operative- and clamping-times in addition to total ischemia time were comparable in the three groups (**Supplementary Table 1**).

As presented in **Figure 5**, 100% and 86% of the rats of the *LT-MSC+EVR* and *LT-EVR groups*, respectively, were alive and euthanized at POD23 (after exclusion of the rats sacrificed on POD9). Rats treated with MSCs alone (*LT-MSC group*) showed a high mortality rate, with no surviving rats at the end of the experiment, and with a median survival of 15 days [12-15] (*P*<0.001).

3.3.2 Liver Tests and Histological Observations of Transplanted Rats

Pre-operative laboratory kidney and liver tests were similar in the three LT-groups (**Figure 6**). At POD9, rats from the *LT-MSC group* showed signs of acute rejection with major cytolysis: AST and ALT levels of 793 U/L [403.5-1425] and 210 U/L [138-343.5], respectively (**Figure 6**). Rats from the *LT-EVR group* and *LT- MSC+EVR group* had only slightly increased liver test results with AST levels of 130 U/L [118-164] and 126 U/L [109.5-219], respectively, and ALT levels at 58 U/L [55-84.5] and 65 U/L [50-74.5], respectively (**Figure 6**). On POD16 only one rat from the *LT-MSC* group remained alive for biological analysis and revealed extremely high cytolysis (AST and ALT at 8702 and 2162 U/L, respectively). AST and ALT levels were slightly increased in the *LT-EVR* and *LT- MSC+EVR* groups on POD 16 (AST: 206 U/L [156.5-504] and 155U/L [126-180] respectively, P=0.06; ALT: 83 U/L [58.2-153.5] and 68 U/L [48-84] respectively, P= 0.29) and on POD 23 (AST: 191.5 U/L [134.8-247.5] and 170 U/L [123-217] respectively, P=0.8; ALT: 86 U/L [57.2-110.3] and 71 U/L [68-97] respectively, P= 0.73) without any significant differences between these groups. On POD23 no rat remained alive in *the LT- MSC group*.

Three rats from each group were sacrificed on POD9 in addition to all the living rats of each group on POD23, in order to evaluate the severity of acute rejection and fibrosis (**Figure 7**) as well as the presence of Treg cells in liver allografts (**Figure 8**). On

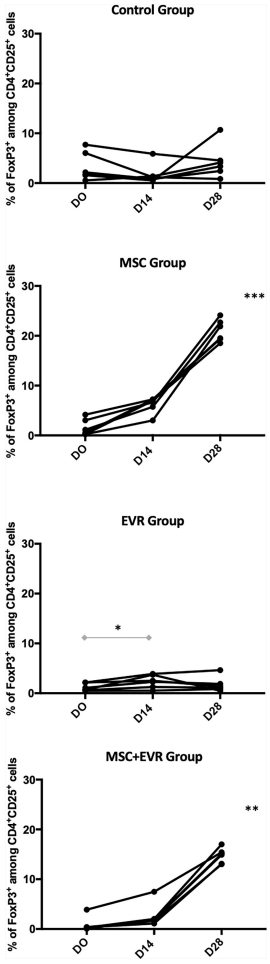


FIGURE 4 | Evolution of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg percentage. Evolution of the $FoxP3^+$ cells among $CD4^+CD25^+$ percentages in each group (each line represents a rat). Rats of the *Control group* kept a stable percentage of circulating Tregs until D28 (insignificant, NS, *P=0.43*) as well as *EVR group* (*P=0.052*). An important and significant expansion of FoxP3 percentage until D28 was observed in the rats of both the *MSC group* (***, *P*= 0.0001) and of the *MSC+EVR group* (**, *P*= 0.0017). The Friedman test was used for statistical analysis. Of note, when compared to D0, a slight but significant increase in the FoxP3+ Tregs percentage was observed in the *EVR group* at D14 (*, *P=0.03, Wilcoxon test*). (D: Day; EVR: Everolimus; MSC: mesenchymal stromal cells).

POD9 the rats in the groups receiving everolimus (*LT-EVR* and *LT- MSC+EVR* groups) showed histological signs of moderate acute rejection with both a median Banff score of 5/9 (**Figures 7A, B**) and mild fibrosis (F1) (**Figures 7C, D**), while conversely, those treated with MSCs alone (*LT-MSC group*) showed histological signs of severe acute rejection with a median Banff score of 9/9 (**Figures 7A, B**) and severe fibrosis (F3) (**Figures 7C, D**). At POD23 the median Banff score was similar in the sacrificed rats of the *LT-EVR* (n=6) and *LT-MSC +EVR* (n=6) groups (5/9) (**Figures 7E, F**). Liver fibrosis was also similar, with a median lower than F1 in both groups (**Figures 7G, H**). On POD9, rats receiving MSC (LT-MSC and LT MSC+EVR groups) seemed to have a higher, but not statistically significant, count of FoxP3+ cells in the graft (76 [35-49] and 81 [17-82] FoxP3+ cells/3HPF respectively) when compared to rats of the LT-EVR group (46 [35-49] FoxP3+ cells/3HPF) (**Figure 8A**). At POD23 the number of Tregs in the liver graft was comparable in the LT-EVR (89.5 [35-117] FoxP3+ cells/3HPF) and in the LT-MSC+EVR groups (92 [76-128] FoxP3+ cells/3HPF) (P>0.99) (**Figure 8B**).

4 DISCUSSION

MSCs, thanks to their immunomodulatory properties including their positive effect on Treg cells, have given rise to a therapy that is a promising approach in the field of SOT (6). In this study we confirmed that MSC infusion promotes Treg expansion in rats, and we additionally showed that everolimus might have a transient positive impact *per se* on the circulating Treg percentage. More interestingly, we found that everolimus has no negative impact on Treg expansion when combined with MSCs. Nevertheless, in a LT model in the rat we observed that MSC injections alone were not effective in preventing acute rejection, and additionally that combining MSCs and everolimus failed to show any synergistic effect when compared to everolimus alone.

Our team has recently reported on two phase I-II clinical trials investigating the effect of MSCs in kidney and liver transplantations (11, 16, 25). In these two studies, and in other reported clinical trials, the effect of MSCs as an immunosuppressive therapy are encouraging but the results remain inconstant and thus not yet clinically relevant (6). A predominant role among the various factors incriminated in this inconstancy may be the alteration of MSC and Treg functions due to the associated ISDs. In this perspective, it is important to note that the majority of clinical trials investigating the effect of MSCs in SOT have used a concomitant "classical immunosuppressive regimen" with CNIs as the central ISDs (4). The dilemma that has emerged relates to the probable CNI toxicity against Treg proliferation and function. Interestingly, it has been reported in an animal model that Tregs injected after LT could induce tolerance (26), and many clinical trials studying Treg injections after SOT have shown encouraging data pointing to the same conclusion - even if these studies were focused on safety (9). It therefore seems logical to try to reduce the ISDs that could impair the immunoregulatory functions of Tregs. Furthermore, CNIs may impair MSC functions (12–15). Therefore, MSC-based therapy requires additional pre-clinical investigations to define the best synergistic "MSC-compatible" ISDs. Meanwhile, mTOR inhibitors are an alternative ISD regularly used after SOT to replace or minimize CNIs, especially in cases of malignancies or of CNI-related renal toxicity (27). In contrast to what has been observed with CNIs, mTOR inhibitors have been shown to have a positive effect on

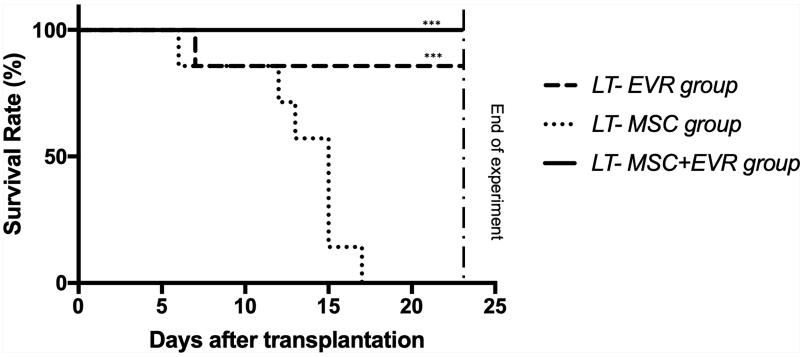


FIGURE 5 | The Kaplan-Meier survival curve of liver transplant recipients. Kaplan-Meier survival curves for the LT-EVR, LT-MSC and LT-MSC+EVR groups. The survival rates for the *LT-EVR* and *LT-MSC+EVR groups* were significantly higher than those of the *LT-MSC group*. All rats of the *LT-MSC group* died between post-operative day 6 and 17; Graft survival was analyzed with the Kaplan-Meier method using the log-rank (Mantel-Cox) test. (***P < 0.001; LT, liver transplanted rat; EVR, Everolimus; MSC, mesenchymal stromal cells).

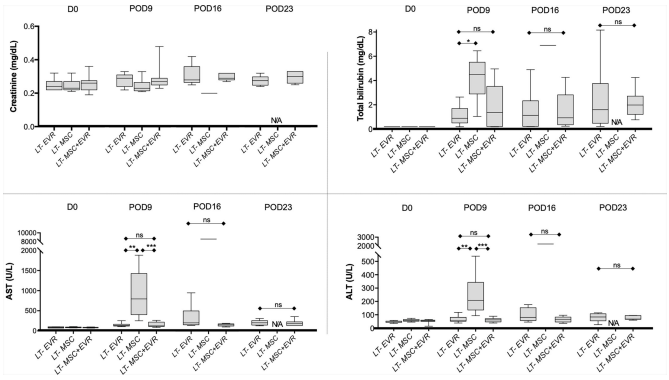


FIGURE 6 | Levels of creatinine, total bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood at D0, post-operative 9, 16 and 23 in recipients after liver transplantation. Plots display the median (25th and 75th percentiles) of the distributions (boxes), and whiskers extend to the minimal and maximal values. The Kruskal-Wallis test followed by Dunn's multiple was used when comparing three groups, and the Mann-Whitney test was carried out when comparing just two groups at a timepoint. At D0 n = 10 per group, at D9 n = 9/9/10, at D16 n = 9/1/10 and at D23 n = 9/0/10 for *LT-EVR/LT-MSC/LT-MSC+EVR groups*, respectively. (*P < 0.05, **P < 0.01 ***P < 0.001; ns, P > 0.05; ALT, alanine aminotransferase; AST, aminotransferase; D, Day; EVR, Everolimus; MSC, mesenchymal stromal cells; POD, post-operative day).

Treg function/proliferation in pre-clinical and clinical studies (14, 15, 28, 29) including in LT (30). Nevertheless, some *in vitro* data have suggested that rapamycin could be deleterious for MSC proliferation and immunomodulatory properties, while *in vivo* MSCs and rapamycin synergize to promote graft tolerance in allograft rejection models (19, 20).

In this work, our first aim was to observe the effect of a single dose of MSCs with EVR on the percentage of Tregs in blood and subsequently weigh up the effectiveness of this association in preventing acute rejection in a LT model.

Firstly, we were able to demonstrate that EVR, despite its very poor solubility in water, could be efficiently and reproducibly delivered through a sc osmotic pump to rats. As EVR is metabolized by the liver, levels of EVR in the blood of LT rats are probably different than those of non-LT rats. However, the specific purpose of the first part of this work was to confirm the effectiveness of EVR delivery with osmotic pumps, not to compare pharmacokinetics in these two populations, which would have meant adding blood sampling in liver transplanted rats.

Our study further confirmed that MSCs significantly increase circulating $CD4^+CD25^+FoxP3^+$ Treg percentage in Lewis rats. This effect was observed 5 days after the injection and persisted for more than 2 weeks thereafter. This increment in Treg percentage after MSC administration has already been

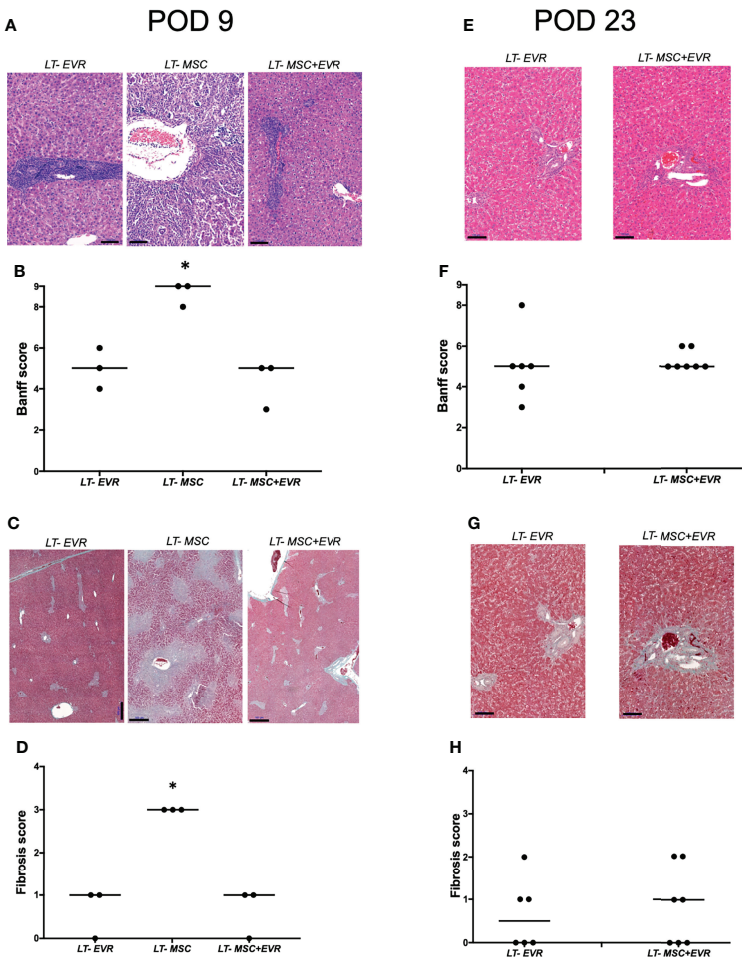


FIGURE 7 | Histological analysis.**(A)** H&E staining of liver graft and, **(B)** histological grading of liver graft acute rejection severity using Banff schema at D9 post-LT; **(C)** Masson's trichrome staining highlighting liver graft fibrosis lesions **(D)** Histological grading of liver graft fibrosis severity with METAVIR score at D9 post-LT; **(E)** H&E staining of liver graft and, **(F)** histological grading of the severity of acute liver graft rejection using Banff schema at D23 post-LT; **(G)** Masson's trichrome staining highlighting liver graft fibrosis lesions and **(D)** histological grading of liver graft fibrosis severity with METAVIR score at D23 post-LT. [*$P < 0.05$; **(B, D)** Kruskal-Wallis test; **(F, H)** Mann Whitney test; scale bars = 100 µM **(A, E, G)**, 500µM **(C)**].

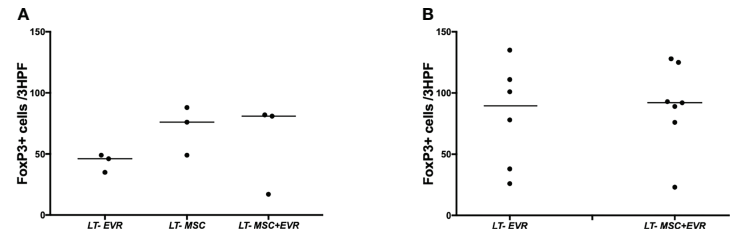


FIGURE 8 | Treg infiltration in the liver graft. Number of FoxP3+ cells at 40x magnification (per three high power fields) by examining graft section of each group after immunohistochemistry on POD 9 **(A)** and at the end of the experiment **(B)**.

documented in other pre-clinical studies (6, 31). In addition, rats from the *MSC+Evero group* showed a comparable increase in Treg percentage. Concurrently, everolimus alone had a positive impact on Treg expansion by 14 days. This finding suggests that everolimus may have a beneficial effect on Treg proliferation, but only while it is being administered, since the percentage of circulating Tregs returned to baseline at D28. Gedaly et al. have recently shown that mTOR inhibitors could be used to expand *ex vivo* functionally competent Tregs for clinical use (32). On the same subject, in clinical kidney transplantation it has been observed that recipients under rapamycin maintenance have increased circulating Treg levels compared to patients receiving cyclosporine (29). Another team recently confirmed these observations in LT recipients who showed increased Treg levels after being switched from tacrolimus to mTOR inhibitors (30). Additionally, a recent paper reported the results of a phase Ib trial studying the effect of 2 MSC infusions 6 months after kidney transplantation followed by a lowering of tacrolimus in combination with everolimus and prednisolone, but no effect on Treg levels or function could be found in this study (33). The mechanisms through which everolimus interacts with MSCs and Tregs was not investigated in this study. Importantly, it is not possible in this study to identify whether the MSC or everolimus are causing expansion of Tregs or deletion of other T cells as we evaluated Treg percentages rather than absolute number. Of note, it was shown that rapamycin does not directly promote Treg expansion, and that the positive effects of mTOR inhibitors on Tregs was probably due to selective inhibition having a greater action on conventional T-cells rather than on Tregs. This consequently leads to a "Treg-favoring" effect with an increase in circulating Tregs (14). Nonetheless, the different mechanisms for MSC-mediated effects on Tregs are likely to be complex and remain incompletely understood (6).

Based on these primary findings, we then tested the association of MSCs with everolimus in a LT model of acute rejection. Insufficient data are available in pre-clinical transplantation models studying the association of mTOR and MSCs. Studies have been carried out in heart (19) and in pancreas islet (20) transplantation models but none in a LT model. In this study we used a well-described model of acute rejection of liver graft in rats using Dark Agouti as donors and Lewis rats as recipients. Circulating levels of Treg in liver transplant recipient were not assessed in the present study. Nevertheless, in another pre-clinical study in rats, Wang et al. showed that peripheral Treg percentages were significantly higher in liver transplant recipient after MSC injections when compared to recipient not injected with MSC. Although not significant, we observed that the number of Tregs in the grafts at 7 days after the first injection tended to be greater in those of rats receiving MSCs, but this was not confirmed at the end of the experiment. The evaluation of the circulating and intragraft levels of other immune cells subsets known to play an important role in MSC-mediated immunoregulatory properties (such as NK cells, CD8+ T cells, B cells, etc.) would have probably been interesting in the present study. Furthermore, two post-operative MSC injections in the *LT-MSC group* (without ISDs) were not an effective treatment to prevent severe acute rejection, and in this group all the rats died prematurely in comparison to rats receiving everolimus. In contrast to our results, some other studies using the same LT model have shown that MSCs alone could significantly prolong graft survival, and prevent rejection when compared to the survival of recipients not receiving any treatment (34, 35). In these studies injected MSC doses were either higher (36) or lower (34) than in our protocol, but more relevantly, MSCs were injected through the portal vein during the transplantation procedure whereas we injected cells intravenously through the penile vein on POD2 and 9. This highlights the fact that the timing and site of the injection are probably key points in achieving MSC efficiency, and that injection closer to the time of transplantation and of ischemia-reperfusion injuries is perhaps needed in order to obtain superior immunomodulatory effects.

On the other hand, liver transplanted rats treated with everolimus alone, or with everolimus in combination with MSC injections, had significantly improved survival rates and less biological or histological damage than the rats of the *LT-MSC group*. No clinical, biological of histological difference was found between these two groups and thus no effective beneficial effect of MSC adjunction with everolimus in our model. Interestingly, levels of acute rejection were stable even 7 days after everolimus withdrawal. In 2018, in a model of islet allograft in mice, Duan et al. also found, that treatment with MSCs alone or with low-dose mTOR inhibitors was not effective in treating acute rejection and prolonging graft survival, but that the combination of MSCs and low-dose rapamycin could significantly increase graft function and survival (20). Nevertheless, in that experiment the survival of mice treated only with rapamycin was similar to those treated with MSCs alone, while in our study everolimus alone was effective in significantly lessening acute rejection when compared to MSCs alone. This might suggest that our "low dose everolimus protocol" led to a sufficient concentration of everolimus to alleviate acute rejection and thus conceal the potential beneficial effect of MSC adjunction. An additional group studied the combination of rapamycin (2mg/kg/d from POD 0 to 13) with MSCs (1 iv injection of $1x10^6$ cells at POD 1) in a heart allograft transplantation model (19) and found that MSCs alone led to a reduction of graft rejection and doubled graft survival compared to a control group. Furthermore, a combination of MSCs and rapamycin was even able to induce a tolerance of the graft, with long-term survival and normal histology.

In our LT model, MSCs alone were not effective in preventing acute rejection, and no beneficial effect of associating MSCs with everolimus was found. Nevertheless, the synergistic effect of MSCs with everolimus could potentially be revealed if the timeframe from transplantation and ISD withdrawal was extended further than in our present protocol. When comparing other LT models to our own, MSC injections were systematically performed earlier than in our case, but our choice to inject MSCs at D2 was carried out in order to reproduce our previous clinical data. Indeed, MSC injections before POD2 might be logistically difficult in a program of deceased LT (16).

In conclusion, despite a significant increase of the percentage of circulating Tregs after one injection of MSCs, MSCs were not effective in preventing severe acute rejection in our LT model. In addition, no effect of the association of MSCs with everolimus was found, when compared to everolimus alone. Everolimus alone could significantly alleviate acute rejection, showing a stable level of rejection even one week after its final administration. Furthermore, the addition of MSCs had no observable positive synergistic impact on acute rejection treatment, or prevention, when compared to everolimus alone. However, it is possible that MSC injections closer to LT time, and a longer follow-up, would reveal some differences in graft survival and tolerance induction. Given these results, the association of everolimus and MSCs in SOT deserves further investigation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Commission Ethique Animale ULiège.

AUTHOR CONTRIBUTIONS

Conception and design of the work: MV, OD, and FJ. Data collection: MV and LP. Data analysis and interpretation: MV, PE, NB, OD, and FJ. Drafting the article: MV and OD. Critical revision of the article: PE and FJ. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants with no role in the conduct of the study. MV, PE, and FJ are Fellows of the *Fonds National de la Recherche Scientifique* (FNRS) and received support from the FNRS (Research Credit 2013/2015) and from the University of Liege (Fonds Spéciaux à la Recherche - Fondation Léon Frédéricq (ACiiRT)). This study was supported by 2 *Crédits classiques* from the University Hospital of Liege and Awards from the Fondation Léon Frédéricq, University of Liege, (2017 and 2018).

ACKNOWLEDGMENTS

We would like to thank Stephan Raafat and Céline Vanwinge for their help with the Flow Cytometry analysis (GIGA – Flow Cytometry Platform) and Jean-Paul Cheramy-Bien for his assistance with animal care and management.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.877953/full#supplementary-material

REFERENCES

1. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement. *Cytotherapy* (2006) 8(4):315–7. doi: 10.1080/14653240600855905
2. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human Adipose Tissue Is a Source of Multipotent Stem Cells. *Mol Biol Cell* (2002) 13 (12):4279–95. doi: 10.1091/mbc.e02-02-0105
3. Majore I, Moretti P, Stahl F, Hass R, Kasper C. Growth and Differentiation Properties of Mesenchymal Stromal Cell Populations Derived From Whole Human Umbilical Cord. *Stem Cell Rev Rep* (2011) 7(1):17–31. doi: 10.1007/s12015-010-9165-y
4. Vandermeulen M, Erpicum P, Weekers L, Briquet A, Lechanteur C, Detry O, et al. Mesenchymal Stromal Cells in Solid Organ Transplantation. *Transplantation* (2020) 104(5):923–36. doi: 10.1097/TP.0000000000003077
5. Casiraghi F, Noris M, Remuzzi G. Immunomodulatory Effects of Mesenchymal Stromal Cells in Solid Organ Transplantation. *Curr Opin Organ Transplant* (2010) 15(6):731–7. doi: 10.1097/MOT.0b013e328340172c
6. Negi N, Griffin MD. Effects of Mesenchymal Stromal Cells on Regulatory T Cells : Current Understanding and Clinical Relevance. *Stem Cells* (2020) 38 (5):596–605. doi: 10.1002/stem.3151
7. Erpicum P, Detry O, Weekers L, Bonvoisin C, Lechanteur C, Briquet A, et al. Mesenchymal Stromal Cell Therapy in Conditions of Renal Ischaemia/Reperfusion. *Nephrol Dial Transplant* (2014) 29(8):1487–93. doi: 10.1093/ndt/gft538
8. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human Leukocyte Antigen-G5 Secretion by Human Mesenchymal Stem Cells Is Required to Suppress T Lymphocyte and Natural Killer Function and to Induce CD4 + CD25 High FOXP3 + Regulatory T Cells. *Stem Cells* (2008) 26(1):212–22. doi: 10.1634/stemcells.2007-0554
9. Atif M, Conti F, Gorochov G, Oo YH, Miyara M. Regulatory T Cells in Solid Organ Transplantation. *Clin Transl Immunol* (2020) 9(2):1–15. doi: 10.1002/cti2.1099
10. Reinders MEJ, van Kooten C, Rabelink TJ, de Fijter JW. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. *Transplantation* (2018) 102 (1):35–43. doi: 10.1097/TP.0000000000001879
11. Vandermeulen M, Mohamed-Wais M, Erpicum P, Delbouille MH, Lechanteur C, Briquet A, et al. Infusion of Allogeneic Mesenchymal Stromal Cells After Liver Transplantation: A 5-Year Follow-Up. *Liver Transplant* (2021) 67(1):47–55. doi: 10.1002/lt.26323
12. Hoogduijn MJ, Crop MJ, Korevaar SS, Peeters AMA, Eijken M, Maat LPWM, et al. Susceptibility of Human Mesenchymal Stem Cells to Tacrolimus, Mycophenolic Acid, and Rapamycin. *Transplantation* (2008) 86(9):1283–91. doi: 10.1097/TP.0b013e31818aa536
13. Vandermeulen M, Grégoire C, Briquet A, Lechanteur C, Beguin Y, Detry O. Rationale for the Potential Use of Mesenchymal Stromal Cells in Liver Transplantation. *World J Gastroenterol* (2014) 20(44):16418–32. doi: 10.3748/wjg.v20.i44.16418
14. Furukawa A, Wisel SA, Tang Q. Impact of Immune-Modulatory Drugs on Regulatory T Cell. *Transplantation* (2016) 100(11):2288–300. doi: 10.1097/TP.0000000000001379
15. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, et al. Inhibition of CD4+CD25+ Regulatory T-Cell Function by Calcineurin-Dependent Interleukin-2 Production. *Blood* (2006) 108(1):390–9. doi: 10.1182/blood-2006-01-0329

16. Detry O, Vandermeulen M, Delbouille MH, Somja J, Bletard N, Briquet A, et al. Infusion of Mesenchymal Stromal Cells After Deceased Liver Transplantation: A Phase I–II, Open-Label, Clinical Study. *J Hepatol* (2017) 67(1):47–55. doi: 10.1016/j.jhep.2017.03.001
17. Mondino A, Mueller DL. mTOR at the Crossroads of T Cell Proliferation and Tolerance. *Semin Immunol* (2007) 19(3):162–72. doi: 10.1016/j.smim.2007.02.008
18. Buron F, Perrin H, Malcus C, Héquet O, Thaunat O, Kholopp-Sarda MN, et al. Human Mesenchymal Stem Cells and Immunosuppressive Drug Interactions in Allogeneic Responses: An *In Vitro* Study Using Human Cells. *Transplant Proc* (2009) 41(8):3347–52. doi: 10.1016/j.transproceed.2009.08.030
19. Ge W, Jiang J, Baroja ML, Arp J, Zassoko R, Liu W, et al. Infusion of Mesenchymal Stem Cells and Rapamycin Synergize to Attenuate Alloimmune Responses and Promote Cardiac Allograft Tolerance. *Am J Transplant* (2009) 9(8):1760–72. doi: 10.1111/j.1600-6143.2009.02721.x
20. Duan W, Yu X, Ma D, Yang B, Li Y, Huang L, et al. Mesenchymal Stem Cells in Combination With Low-Dose Rapamycin Significantly Prolong Islet Allograft Survival Through Induction of Regulatory T Cells. *Biochem Biophys Res Commun* (2018) 506(3):619–25. doi: 10.1016/j.bbrc.2018.10.070
21. Erpicum P, Rowart P, Poma L, Krzesinski J-M, Detry O, Jouret F. Administration of Mesenchymal Stromal Cells Before Renal Ischemia/Reperfusion Attenuates Kidney Injury and may Modulate Renal Lipid Metabolism in Rats. *Sci Rep* (2017) 7(1):8687. doi: 10.1038/s41598-017-08726-z
22. Oldani G, Lacotte S, Morel P, Mentha G, Toso C. Orthotopic Liver Transplantation in Rats. *J Vis Exp* (2012) 65):1–7. doi: 10.3791/4143
23. Oldani G, Lacotte S, Orci LA, Delaune V, Slits F, Gex Q, et al. Efficient Nonarterialized Mouse Liver Transplantation Using 3-Dimensional–Printed Instruments. *Liver Transplant* (2016) 22(12):1688–96. doi: 10.1002/lt.24637
24. Demetris AJ, Batts KP, Dhillon AP, Ferrell L, Fung J, Geller SA, et al. Banff Schema for Grading Liver Allograft Rejection: An International Consensus Document. *Hepatology* (1997) 25(3):658–63. doi: 10.1002/hep.510250328
25. Erpicum P, Weekers L, Detry O, Bonvoisin C, Delbouille MH, Grégoire C, et al. Infusion of Third-Party Mesenchymal Stromal Cells After Kidney Transplantation: A Phase I-II, Open-Label, Clinical Study. *Kidney Int* (2019) 95(3):693–707. doi: 10.1016/j.kint.2018.08.046
26. Pu LY, Wang XH, Zhang F, Li XC, Yao AH, Yu Y, et al. Adoptive Transfusion of Ex Vivo Donor Alloantigen-Stimulated CD4+CD25+ Regulatory T Cells Ameliorates Rejection of DA-To-Lewis Rat Liver Transplantation. *Surgery* (2007) 142(1):67–73. doi: 10.1016/j.surg.2007.02.014
27. De Simone P, Fagiuoli S, Cescon M, De Carlis L, Tisone G, Volpes R, et al. Use of Everolimus in Liver Transplantation: Recommendations From a Working Group. *Transplantation* (2017) 101(2):239–51. doi: 10.1097/TP.0000000000001438
28. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin Selectively Expands CD4+CD25+FoxP3 + Regulatory T Cells. *Blood* (2005) 105:4743–8. doi: 10.1182/blood-2004-10-3932
29. Ruggenenti P, Perico N, Gotti E, Cravedi P, D'Agati V, Gagliardini E, et al. Sirolimus Versus Cyclosporine Therapy Increases Circulating Regulatory T Cells, But Does Not Protect Renal Transplant Patients Given Alemtuzumab Induction From Chronic Allograft Injury. *Transplantation* (2007) 84(8):956–64. doi: 10.1097/01.tp.0000284808.28353.2c
30. Ghazal K, Stenard F, Dahlqvist G, Barjon C, Aoudjehane L, Scatton O, et al. Treatment With mTOR Inhibitors After Liver Transplantation Enables a Sustained Increase in Regulatory T-Cells While Preserving Their Suppressive Capacity. *Clin Res Hepatol Gastroenterol* (2018) 42(3):237–44. doi: 10.1016/j.clinre.2017.10.001
31. Di Ianni M, Del Papa B, De Ioanni M, Moretti L, Bonifacio E, Cecchini D, et al. Mesenchymal Cells Recruit and Regulate T Regulatory Cells. *Exp Hematol* (2008) 36(3):309–18. doi: 10.1016/j.exphem.2007.11.007
32. Gedaly R, De Stefano F, Turcios L, Hill M, Mitov MI, Alstott MC, et al. mTOR Inhibitor Everolimus in Regulatory T Cell Expansion for Clinical Application in Transplantation. *Transplantation* (2019) 103(4):705–15. doi: 10.1097/TP.0000000000002495
33. Dreyer GJ, Groeneweg KE, Heidt S, Roelen DL, van Pel M, Roelofs H, et al. Human Leukocyte Antigen Selected Allogeneic Mesenchymal Stromal Cell Therapy in Renal Transplantation: The Neptune Study, a Phase I Single-Center Study. *Am J Transplant* (2020) 20(10):2905–15. doi: 10.1111/ajt.15910
34. You Y, Zhang J, Gong J, Chen Y, Li Y, Yang K, et al. Mesenchymal Stromal Cell-Dependent Reprogramming of Kupffer Cells Is Mediated by TNF-Alpha and and PGE2 and Is Crucial for Liver Transplant Tolerance. *Immunol Res* (2015) 62(3):292–305. doi: 10.1007/s12026-015-8660-2
35. Tang J, Yang R, Lv L, Yao A, Pu L, Yin A, et al. Transforming Growth Factor-β-Expressing Mesenchymal Stem Cells Induce Local Tolerance in a Rat Liver Transplantation Model of Acute Rejection. *Stem Cells* (2016) 34(11):2681–92. doi: 10.1002/stem.2437
36. Sun Z, Li T, Wen H, Wang H, Ji W, Ma Y. Immunological Effect Induced by Mesenchymal Stem Cells in a Rat Liver Transplantation Model. *Exp Ther Med* (2015) 10(2):401–6. doi: 10.3892/etm.2015.2551

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

CHAPTER IV. GENERAL DISCUSSION

CHAPTER IV. GENERAL DISCUSSION

MSCs, thanks to their immunosuppressive properties, could have the potential to achieve adequate immunotolerance without excessive immunosuppression as well as to reduce IRI after LTx. Although animal models have shown promising results in preventing IRI and treating or preventing AR, the safety and efficacy of MSC therapy in humans need further validation before being implemented on a larger scale in LTx programs. To optimize their potential efficacy, careful consideration of the best combination with synergistic ISDs is required and need to be further evaluated in preclinical settings.

The primary aim of this thesis was to evaluate the interest and safety of MSC therapy in LTx.

In the first part of these translational investigations, we initiated the "first-in-man" prospective phase I-II, open label, controlled clinical study evaluating the use of MSCs after LTx. This study was performed at the University Hospital of Liege from 2012 to 2021 and resulted from a collaboration between the Department of Abdominal Surgery and Transplantation and the Department of Hematology. A single MSC injection after deceased donor LTx did not demonstrate potential adverse effects, particularly there was no increase in the rate of opportunistic infections and cancers. However, injecting allogeneic MSCs after deceased donor LTx may promote the emergence of MSC-DSAs and liver-DSAs class II in LTR. This topic, also described after MSC injection in KTx, deserves further investigation since our study included a limited number of patients. Hence, the potential benefits of MSC injections in the context of LTx deserve further investigations before application in clinical routine.

Considering that the use of CNI might be deleterious for MSCs in clinical trials, the second part of our project, back to bench, aimed to evaluate the potential synergy of EVR on the immunoregulatory properties of MSC. To the best of our knowledge, the association of MSCs with an mTOR inhibitor had never been tested in a LTx model. We showed that a single MSC injection ($3x10^6$cells/kg) was able to expand circulating Tregs in rats and that this effect was preserved when associated with EVR. However, in our LTx rat model, two MSC injections ($3x10^6$cells/kg each) could not prevent AR. The association of EVR with MSCs did not bring an additional positive impact compared to EVR alone.

****

MSC in liver transplantation – Safety

On the basis of a reasonable number of clinical trials in different fields of SOT, one may claim that MSC administration in transplant recipients is feasible and safe, at least on the short term[137]. Concerning the potential **infusional toxicity**, our study did not show any change in vital parameters or cytokine release syndrome during and after MSC infusion. In the week following MSC infusion, no patient developed clinical signs of allergy or impairment of vital functions (including lung, kidney and liver graft function) [238]. This is in line with other published data in SOT and with a meta-analysis evaluating the safety of MSCs in general, including in nontransplant indications [137, 239]. Nevertheless, it should be noted that a very small uncontrolled study reported adverse events potentially linked to MSC infusion, including engraftment syndrome in 2 KTRs [240]. Although such an acute kidney injury post–MSC injection has not been observed in larger cohorts, one should remain cautious concerning the timing of MSC infusion in SOT. Similarly, a transient decrease of peripheral oxygen saturation directly after IV MSC infusion has been reported in lung transplant recipients, which putatively suggests infusion-related lung toxicity [241].

One of the main theoretical safety concerns of MSC therapy is an increased **risk of opportunistic infections and neoplasms**. To date, the vast majority of published studies has not found any difference in terms of opportunistic **infections** in SOT recipients exposed to MSCs. Furthermore, MSCs associated with low-dose CNIs significantly reduced the rate of opportunistic infections in a Chinese study [242]. In the Italian experience, no increased susceptibility to infections was observed in 4 KTRs at 5/7 years post–MSC infusion [243]. By contrast, a Dutch team reported that 3 among 6 MSC-treated KTRs developed opportunistic infections [244]. In our study, no evidence of increased risk of infection was found [238, 245]. The theoretical increased risk of **malignancies** in transplanted patients may result from neoplastic transformation of infused MSCs, or from an increased risk of malignant tumors under enhanced immunosuppression of the recipient. Indeed, it has been shown that murine MSCs could, after long-term *in vitro* culture, transform into malignant cells but this has not

been found with human MSCs [246]. In addition to the fact that human MSCs appear to show a very stable genome, each batch of MSCs is evaluated for genomic stability according to Good Manufacturing Practice before clinical use [197, 247]. To our knowledge, no evidence of neoplastic transformation after clinical use of MSCs has been described to date. Concerning the risk of enhancing growth of *de novo* cancer or of recurrence (e.g. hepatocarcinoma in LTR) in patients exposed to MSC therapy, the different studies in SOT have not generated any increase in the incidence of cancer [137]. At 5 years of follow-up, despite the limited number of patients exposed to MSCs, our trial did not show any increased risk of cancer in patients treated with MSCs compared to controls [245]. Recently, Casiraghi *et al.* published the results of their randomized open-label phase Ib/IIa clinical trial in which 20 LTR were randomly assigned (1:1) to receive either a single pre-transplant 3rd party BM-MSC IV infusion or standard of care alone. Out of the 19 patients who completed the study, none of those who received MSCs experienced any complications related to the infusion. The incidence of both serious and non-serious adverse events was similar between the two groups at 1-year follow-up [248]. This is in line with another study in KTR given MSCs in which the incidence of cancer was not enhanced [243]. In total, on the basis of a reasonable number of clinical trials in different areas of SOT, one may claim that MSC administration in SOT recipients is feasible and safe with regards to the risks of infections and cancer.

MSC - Impact on immune cells

It has been shown both *in vitro* and *in vivo* that MSCs highly depend on their interactions with many immune cells to mediate their immunomodulatory properties. With regards to the innate immune system, MSCs were for example able to influence macrophage differentiation, favoring a shift toward the immunosuppressive, anti-inflammatory M2 phenotype[138, 156], to inhibit NK cell cytotoxicity and proliferation[158] and to reprogram DCs into a pro-tolerogenic DC phenotype [161]. With regards to the adaptive immune system, MSCs were shown to suppress T-cell proliferation triggered by allogeneic stimuli and to inhibit T-cell differentiation and cytotoxicity [161], as well as B-cell proliferation [173]. One of the potential major actors mediating MSC immunoregulatory properties is probably Tregs which

are known to be stimulated by MSCs. Tregs are a particular T-cell subpopulation inhibiting immunological responses and maintaining immune homeostasis and (self-)tolerance, which makes them of particular interest in the field of SOT to control allogeneic responses. Both in humans and in rodents, the classical phenotype characterizing Tregs is $CD4^+$, $CD25^+$ (IL-2 receptor) and $FoxP3^+$. In humans only, low levels of CD127 can also distinguish Tregs from other T cells [95, 96]. Recently, the development of modern immunophenotyping methods allows to classify Tregs in many subsets. For example, Treg subpopulations include HLA-DR^- $CD45RA^+$ $FoxP3^{low}$ Tregs, representing resting/naive Tregs, $CD45RA^-FoxP3^{high}$ Tregs, representing activated/effector Tregs, and HLA-$DR^+CD45RA^-FoxP3^{low}$ Tregs that are not immunosuppressive and do not secrete cytokines [97]. In mice, increased numbers of Tregs ($CD4^+CD25^+FoxP3^+$) with enhanced CTLA4 expression facilitated tolerance towards MHC-mismatched allografts [91]. Clinically, it has been shown that increased Tregs and FoxP3 transcripts are associated with patients developing operational tolerance or showing low rejection rates [95, 99]. Monitoring of Tregs is thus of particular importance clinical trials evaluating the immunomodulatory properties of MSC therapy.

In our clinical study, no difference was observed concerning white blood cell counts (including NK cells) between the two groups (MSC group vs control group). LTRs in the two groups had similar peripheral blood counts of $CD4^+$ T cells on days 0, 30 and 90 after transplantation. Treg counts and phenotype (naive versus resting versus activated) were also comparable in the 2 groups. Additionally, Treg and conventional T-cell proliferations (measured by KI67 expression) were also comparable in the 2 groups, as were the levels of phosphoSTAT5 in Tregs (which indicate similar IL-2 signaling in Tregs) [238]. These findings collectively imply that T-cell and Treg counts or phenotypes were not affected by a single MSC infusion in our study. Among the few studies in LTx, two described a transient increase in circulating Tregs [231, 233] with a downregulation of Th17 cells [233]. In a recent randomized Italian study evaluating pretransplant MSC infusion in LTx, a slight increase in circulating Tregs, memory Tregs, and a tolerant subset of $CD56^{bright}$ NK cells has been observed in MSC-treated patients compared to baseline but these changes were not statistically significant when compared to the control group [248]. In phase I-II trials in KTx, most studies also observed an increase of the Treg/conventional T-cell ratios whereas the counts of NK cells were either the same, lower or higher [137].

In conclusion, our study in LTx provided no evidence that a single injection of MSCs in SOT recipients affects immune cells. The parameters influencing MSC effects on immune cells (timing, doses, origins of MSCs, associated ISDs) are still poorly understood. One possible explanation for inconsistency among studies is the choice of associated ISDs. Even if the beneficial effect of MSCs on Tregs is validated by a wealth of preclinical data, the *in vivo* influence of ISDs on this relationship between MSCs and Tregs remains to be better defined[249]. Indeed, in all SOT trials studying MSC therapy, CNIs were used as the main ISD for maintenance. Given that there is some evidence suggesting that CNIs may be toxic to both Tregs and MSCs, it would be interesting to consider a better ISD combination in order to obtain a synergistic effect and better survival/function of Tregs and MSCs in future clinical studies.

MSC – immunogenicity and DSA

MSCs were initially considered as "immune-privileged", *i.e.* not activating allogeneic lymphocytes [192, 194], essentially because of their low expression of MHC class I and the absence of MHC class II and costimulatory molecules *in vitro*. Nevertheless, it has been demonstrated that, when stimulated (e.g. with IFN-γ), MSCs can act as APCs and express both MHC class I and class II molecules on their surface. The absolute immune-privileged status of MSCs was then questioned [195, 196]. The monitoring of MSC-DSAs after MSC therapy in SOT is thus of major importance in phase I clinical trials.

The importance and impact of DSAs in the field of KTx is now well established. DSAs are recognized as a reliable biomarker for ABMR, which is the main reason for graft loss following KTx [250]. On the opposite, the impact of DSAs in LTx is still not fully understood. Due to the tolerogenic characteristics of the liver, which contribute to its resistance to antibody-mediated injuries, donor and recipient HLA matching is not currently routinely recommended in deceased donor LTx [251]. Nevertheless, recent data seem to suggest that the presence of DSAs, particularly *de novo* DSAs, influences liver graft outcomes with an increased risk of rejection and poorer graft and patient survival [42, 43]. Since class II antigen expression on the liver endothelium, hepatocytes, and bile ducts occurs after an initial injury to the allograft, class II DSAs are likely to have the greatest impact on clinical outcomes. Pre-

formed class I DSAs typically disappear after LTx, but pre-formed class II DSAs frequently persist and have been associated with worse outcomes [51]. It was shown that around 8% of LTRs experienced *de novo* DSAs, almost all of them anti-class II DSAs, at 1 year *post*transplant. *De novo* DSAs have been shown to be an independent risk factor for graft loss and death and are associated with higher risks of AR and graft fibrosis [52, 53].

The question of MSC immunogenicity in SOT is of great importance, *a fortiori* in KTx, but also in LTx. Comparing the prevalence of liver-DSAs in the MSC and control groups is of particular importance because MSC immunomodulatory properties may reduce immune responses against liver HLAs and thus the formation of *de novo* liver DSAs. On the opposite, to ascertain whether MSCs encourage an MSC-directed immune response in the host, the appearance of MSC-DSAs is also important because 1) it could indicate "rejection" and rapid destruction of MSCs by the host 2) it could be deleterious for the host in case of shared mismatch between MSC and graft donors with production of additional graft DSAs. In KTx, probably because of the uncertainties regarding the risk of MSCs being the cause of *de novo* MSC-DSA production (and therefore potentially of kidney-DSAs), many clinical studies have chosen to administer autologous MSCs (derived from the recipient). In LTx, the choice of 3rd party MSCs is justified by their more rapid availability for a deceased donor LTx program in which it would be difficult to plan the thawing and expansion of autologous MSCs in the peritransplant period and the uncertainties of finding a suitable donor within the required time frame would result to the waste of numerous MSC products. Moreover, as MSC availability and function may be limited by the patient's condition, MSCs from patients with end-stage liver disease (autologous) might not be suitable [252]. In the literature on MSC therapy, despite more than 3,000 patients have been treated for various diseases, only a few studies report on the development of DSAs against MSC antigens. In non-transplant settings, various trials studying 3rd-party MSC therapy for type II diabetes, diabetic nephropathy, Crohn's disease, rheumatological or cardiac diseases reported MSC-DSAs, all of class I, in 0-37% of more than 100 patients, without clinical impact and, importantly, without impacting MSC efficacy [253-255].

In KTx, our team recently reported *de novo* MSC-DSAs class I in only 1 out of 10 MSC-treated recipients. Two more *de novo* MSC-DSAs (classes I and II) and 1 shared *de novo* kidney/MSC DSA (class II) with MFI <1500 were also detected. Nevertheless, the clinical

relevance of low MFI DSA is probably very low and still a matter for debate [4]. Another team elaborated a protocol with a matching strategy to avoid repeated mismatches between MSCs and kidney donors in 10 KT patients. In this study, no *de novo* MSCs nor kidney-DSAs were detected during the 6-month follow-up after MSC infusion, contrasting with our study where MSC-DSAs could be detected in 3 out of 10 LTR during follow-up for a total of 4 MSC-DSAs detected (1 of class I, 3 of class II). Of note, all anti-class II DSAs were against a shared mismatch (MSC+Liver), representing immunization against 60% of shared HLA class II mismatches. DSA appearance was not linked to long-term outcomes in our cohort [245].

In conclusion, the available data suggest that MSCs could elicit MSC-DSA production essentially of class I. Our study suggests also that in case of HLA mismatch in class II loci, immunogenicity could be promoted and lead to anti-class II DSAs. These observations may lead to reconsidering the risk of developing MSC-DSAs, as well as the necessity for a matching strategy, particularly in KTx but also potentially in LTx. This needs to be further investigated with larger cohorts. One potential solution to circumvent these immunogenicity concerns could be to use a cell-free strategy such as MSC-derived EVs. Nevertheless, it has also been mentioned from research with DCs that EVs might also carry immunogenic proteins such as MHC molecules and thus potentially elicit immune responses but this remains unclear at this stage [256].

4.1.1 MSC efficacy

Operational tolerance is a rare phenomenon after LTx and can only be seen in a minority of LTRs after a few years[69]. The potential induction of “early” operational tolerance in SOT and particularly in LTx is a major hope of MSC therapy. In different strategies aiming for tolerance and immunomodulation after SOT, it has been proposed that Tregs probably play a central role. Clinical studies have demonstrated a correlation between elevated numbers of Tregs or elevated FoxP3 transcripts and operational tolerance or low rejection rates [80, 84]. A study published in 2016 showed that donor-derived exogenous Treg injection was effective for ISD weaning and induction of operational tolerance in a majority of LTR from living donors [114]. Others suggested that donor alloantigen-reactive Tregs could produce graft-specific immunosuppression and allow weaning ISD [115]. Nevertheless, exogenous

Tregs are very difficult to manufacture, limiting the possibilities for large-scale patient treatment.

MSCs, which can be easily isolated from many sources, have been shown to have particular tolerogenic properties, including their beneficial effect on Tregs demonstrated both *in vitro* and *in vivo* [249]. Many LTx preclinical models have shown that MSC were able to decrease allograft rejection and to induce tolerance [137, 217, 219].

In KTx trials, while the largest study demonstrated a lower BPAR rate in MSC-treated patients, most studies did not find any difference in AR rate between MSC-treated vs control KTR. Although not systematically, an increase in Tregs was nevertheless observed [137]. In LTx studies, Shi et al. published the results of their trial comparing the effectiveness conventional ISDs with or without UC-MSCs for the treatment of liver allograft AR in LTR. MSC-treated patients presented signs of biological and histological improvement 4 weeks after infusion compared to controls. Circulating Tregs and Treg/Th17 ratios were significantly increased in MSC-treated LTR. In 2015, Soeder et al. published the first case of injection of MAPC at D0 and D2 after living donor transplantation associated with a CNI-free ISD regimen. They observed a transient increase of circulating Tregs but because of AR, CNI had to be reintroduced. Two more LTR were treated with MAPC (data from clinicaltrials.gov, not published), but, because of major events, the study was interrupted [231]. In the second part of our clinical study, we were unable to demonstrate any clear efficacy from MSC therapy. Ten LTR under standard ISD received 1.5-3x10^6/kg 3rd party MSCs on post-operative day 3±2, and were prospectively compared to a control group of 10 LTR not receiving MSC. In the MSC group, we could not observe any Treg expansion in the blood nor Treg infiltration of the liver graft at the month 6 biopsy, and our attempt at gradual weaning patients from immunosuppression remained unsuccessful except in 1 patient [238].

Many hypotheses could be drawn for explaining the failure of MSC to improve outcomes. First of all, the first aim of this phase I trial was to investigate the safety of MSC in the settings of LTx, and the study was not powered to demonstrate efficacy. The absence of detectable effects might be due to an insufficient sample size but also to the dose used (which could possibly be increased or repeated), timing (which could instead be, preoperative, intraoperative, or sooner or later postoperatively) and infusion route (which could be in the portal vein or hepatic artery) of the MSC infusion, MSCs being potentially trapped in the lung

after IV infusion [137, 182]. The choice of associated ISDs is probably of major importance too. It is important to underline that nearly all trials evaluating MSCs in SOT were performed in association with CNI-based immunosuppression, which has been shown to be potentially toxic for both MSCs and Tregs.

****

The second part of our work aimed to assess the potential synergy between EVR and MSCs in terms of immunoregulatory properties. We demonstrated that a single MSC injection ($3x10^6$ cells/kg) effectively increased circulating Tregs in a rat model of LTx, and that this effect was maintained when combined with EVR. However, in our allograft rejection model, two MSC injections ($3x10^6$ cells/kg each) were unable to prevent AR after LTx. Furthermore, the addition of MSCs to EVR did not provide any additional positive impact compared to EVR alone.

First of all, our model confirmed that MSCs are able to expand Tregs. This effect of MSCs on Tregs has already been documented in many other preclinical studies [249, 257]. *In vitro*, it was shown that, when cocultured with MSCs, $CD4^+CD25^+$FoxP3+ Tregs maintained a sustainable phenotype and function over time [257]. In our model, Treg expansion after a single injection of MSCs was significant and persisted more than 2 weeks thereafter. *In vivo*, it has also been suggested in a KTx model that Treg expansion was due to the homing of the MSCs into the spleen where they promoted Treg expansion, especially when injected before transplantation [208]. In our model, however, we did not label MSCs to monitor their distribution after IV injection. *In vitro*, *in vivo* and in clinical practice, it has been shown that Tregs appear to be promoted by mTOR inhibitors. For example, KTRs showed increased Treg numbers under rapamycin maintenance compared to those under CNIs [112]. These findings were subsequently corroborated by a different team in LTR who displayed elevated Tregs following conversion from tacrolimus to a mTOR inhibitor [113]. Because of their respective beneficial effect on Tregs, mTOR inhibitors and MSCs would have been expected to be effective (or even synergistic) in preventing allograft rejection. In our model, two injections of MSCs 2 and 9 days after LTx were not effective in treating/preventing severe allograft rejection. Nevertheless, in contrast to our observations, other studies using the same allograft

rejection model showed that MSCs alone may significantly prevent rejection and lengthen graft survival [214, 215, 217]. While injected doses of MSCs were either higher or lower than in our study, the difference in efficacy could be explained, in part, by the fact that, as opposed to our protocol, MSCs were systematically injected through the portal vein during the LTx. This underlines the likely crucial importance of the timing and route of MSC injection for their efficacy. It might thus be necessary to administer the injection closer to the time of the transplant and/or IRI to achieve the best immunomodulatory effects. In our model, we had chosen to perform repeated injections from D2 to reproduce our previous clinical experience (injection at D2) in which an earlier injection would be logistically difficult in a program of deceased donor [238].

In our hands, the association of MSCs and EVR did not confer any benefit when compared to EVR alone. This association had never been tested in a LTx model, but in a heart transplantation model, the association of MSCs with low-dose rapamycin significantly prolonged allograft tolerance while MSCs or rapamycin alone had only a comparable, minor effect, in increasing allograft survival [206]. The same observation was made in an islet allograft transplantation model [258]. The absence of a demonstrated synergistic effect in our protocol might be due to the fact that our "low-dose" EVR protocol led to a concentration of EVR that was high enough to prevent AR while masking any potential advantage of the adjunction of MSCs. Moreover, we suggest that the expected synergistic effect with development of a tolerogenic state could potentially be revealed if the timeframe from ISD withdrawal was extended further than in our present protocol.

CHAPTER V. CONCLUSION & PERSPECTIVES

CHAPTER V. CONCLUSION & PERSPECTIVES

The present thesis studied the use of MSCs after LTx both in a prospective clinical trial and in a rodent model. This first prospective, controlled, trial clinical investigating the safety of injecting allogeneic MSCs after deceased donor LTx did not demonstrate potential adverse effects, particularly there was no increased rate of opportunistic infections or cancers. Injecting allogeneic MSCs after deceased donor LTx may promote the emergence of liver-DSA and MSC-DSA after LTx. This topic deserves further investigation with close monitoring of DSAs after LTx. The potential benefits of MSC injections in the context of organ transplantation have yet to be demonstrated in large randomized studies. The choice of associated ISDs is probably a crucial point to take into account. Moving from bed to bench-side, we showed *in vivo* that MSC and the association of EVR and MSCs could be beneficial for Treg expansion. However, in our LTx model, MSCs could not prevent severe AR. Additionally, when compared to EVR alone, there was no beneficial effect of the association of MSCs with EVR. EVR could significantly reduce the risk of AR, which remained stable 1 week after the end of its administration, suggesting its potential tolerogenic effect. Furthermore, when compared to EVR alone, the addition of MSCs had no observable beneficial synergistic effect on the treatment or prevention of AR. However, one might consider that a longer follow-up and MSC injections closer to the LTx and through the portal vein may reveal some enhancement in graft survival and tolerance induction. In conclusion, the present contribution on MSC therapy for LTx will hopefully open up new research perspectives in this field. The association of EVR and MSCs could be a good regimen for further study given the potential beneficial effect of each therapy on Tregs even if MSCs were not effective on AR. Better understanding the mechanisms of action of MSCs will probably help to design more efficient clinical studies in SOT.

****

The perspectives of this work are multiple and many projects could originate from it. The first project would be to study the potential effect of MSCs on reducing IRI for marginal grafts. Victim of its success, the increasing number of medical indications for LTx and of potential candidates with a critical shortage of deceased liver donors leads to the utilization of more and more high-risk grafts, such as steatotic or/and DCD liver, particularly in Belgium. Cold static preservation, the current standard for liver preservation, insufficiently preserves these high-risk grafts as they are more vulnerable to IRI with higher rates of graft loss due to ischemic dysfunction or cholangiopathy. Recent evidence suggests that normothermic machine perfusion (NMP) prevents IRI and cholangiopathy in DCD livers compared to cold storage [259]. It has also been recently demonstrated that cells can be delivered directly into the liver during NMP without compromising perfusion and that MSC infusion during NMP was proposed as part of an "ischemia conditioning" strategy [260]. MSCs, in addition to their potential effect on AR/tolerance induction are known to have potential beneficial effects on IRI [199, 261]. The main objectives of this project would be to confirm the higher risks associated with DCD grafts in the LTx cohort at the University Hospital of Liege, and to test the hypothesis that infusing MSCs before/during/after transplantation of high-risk liver grafts could improve graft perfusion and function by reducing IRI. This hypothesis will be tested in 2 preclinical models of NMP of high-risk liver grafts, using first a porcine DCD model, and then human DCD grafts or steatotic livers rejected for clinical transplantation. These porcine and human livers will be surgically divided in two, and the two parts will be simultaneously perfused with NMP at 37°C for 6 to 24 h, one being used as a control and the other as a MSC-treated graft, thereby allowing pairwise comparison of control (control group) and experimental (MSC group) grafts **(Figure 8)**. To this aim, our preliminary work involved splitting pig livers and performing portal angiography to confirm the vascular possibility of splitting the liver **(Figure 9)**. The next step will be to perform cholangiography and then to test split livers on the NMP. In parallel, thanks to a future collaboration with the University of Torino, we will soon develop an EV technology and a NMP in a rodent model[262]. This will allow us to study the effect of EV both on IRI and in a LTx model. MSC-derived EV therapy have many potential advantages in terms of production, preservation and administration protocols and is less likely to induce allosensitization than whole MSCs [262]. Finally, a new clinical trial is currently being considered to evaluate the efficacy of MSC therapy after LTx in association

with a CNI-free (or minimal level of tacrolimus) immunosuppression protocol associating EVR + MMF to favor MSC and Treg proliferation and try to create a tolerogenic environment and try achieve the ("prope") tolerance of the transplanted liver.

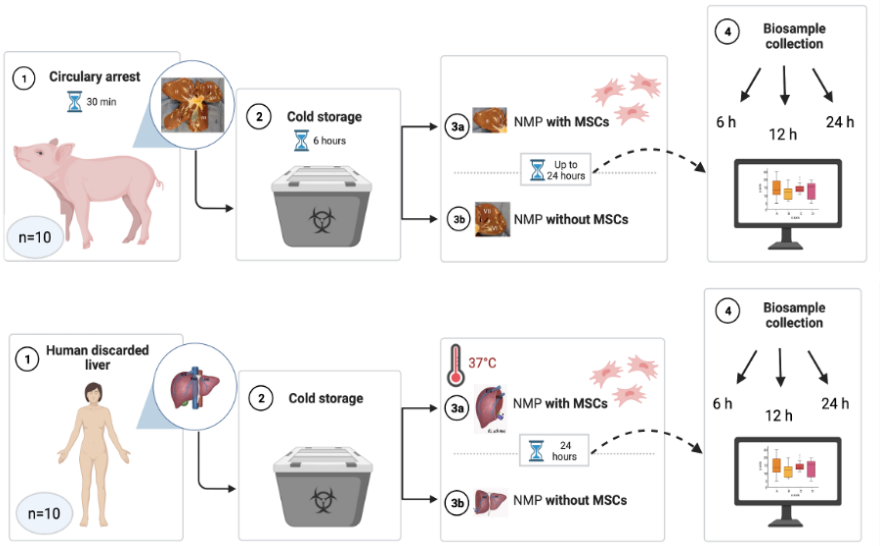


Figure 8. Upper part: Illustration of the NMP of pig split livers with or without pMSCs comparing simultaneous normothermic perfusion of 2 different segments of pig liver with (pMSC group) or without (control group) pig MSCs from the local pMSC bank already built in the surgical laboratory of ULiege. **Lower part**: Illustration of the NMP comparing the simultaneous normothermic perfusions of 2 different human split livers with (hMSC group) or without (control group) human clinical-grade MSCs that are available in the LTCG of the CHU of Liege (NMP of human split livers with or without hMSCs).
h, hours; hMSCs, human-MSCs; min, minutes; MSC, mesenchymal stromal cell; NMP, normothermic machine perfusion; pMSCs, pig-MSCs

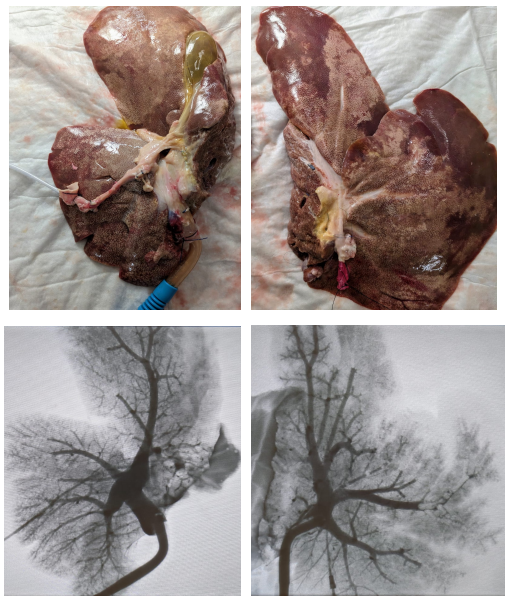


Figure 9. Upper part: Picture of the anatomical split of a pig's liver in 2 parts**; Lower part:** Portal angiography of the split liver.

PERSONAL CONTRIBUTIONS

Original articles

VANDERMEULEN M., ERPICUM P., BLETARD N., POMA L., JOURET F., & DETRY O. Effect of the Combination of Everolimus and Mesenchymal Stromal Cells on Regulatory T Cells Levels and in a Liver Transplant Rejection Model in Rats. *Frontiers in Immunology* 2022, 13, 877953.

VANDERMEULEN M., MOHAMED-WAIS M., ERPICUM P., DELBOUILLE M.-H., LECHANTEUR C., BRIQUET A., MAGGIPINTO G., JOURET F., BEGUIN Y., & DETRY O. Infusion of allogeneic mesenchymal stromal cells after liver transplantation: Five-year follow-up. *Liver Transplantation* 2022*,* 28, 636-646.

DETRY O., **VANDERMEULEN M.,** DELBOUILLE M.-H., SOMJA J., BLETARD N., BRIQUET A., LECHANTEUR C., GIET O., BAUDOUX E., HANNON M., BARON F., & BEGUIN Y. Infusion of mesenchymal stromal cells after deceased liver transplantation: A phase I-II, open-label, clinical study. *Journal of Hepatology* 2017, 67 (1), 47-55.

Review articles

VANDERMEULEN M.*, ERPICUM P.*, WEEKERS L., BRIQUET A., LECHANTEUR C., DETRY O., BEGUIN Y., & JOURET F. Mesenchymal Stromal Cells in Solid Organ Transplantation. *Transplantation* 2020, 104 (5), 923-936. (*these authors contributed equally to this work)

VANDERMEULEN M., GREGOIRE C., BRIQUET A., LECHANTEUR C., BEGUIN Y., & DETRY O.
Rationale for the potential use of mesenchymal stromal cells in liver transplantation. *World Journal of Gastroenterology* 2014, 20 (44), 16418-32.

DETRY O., JOURET F., **VANDERMEULEN M**., ERPICUM P., DELENS L., GREGOIRE C., BRIQUET A., WEEKERS L., BAUDOUX E., LECHANTEUR C., & BEGUIN Y. Cellules stromales mésenchymateuses et transplantation d'organes. *Revue Médicale de Liège* 2014, 69, 53-56.

Posters and presentations

VANDERMEULEN M.
Utilisation de cellules souches mésenchymateuses en transplantation hépatique. **Oral communication**. Invited Speaker for the local "World Liege Liver Day" symposium, April 2023, Liege, Belgium

VANDERMEULEN M., ERPICUM P., BLETARD N., POMA L., DRION P., JOURET F., DETRY O. Mesenchymal stromal cells combined with everolimus promote T Reg expansion but do not synergize in a rat transplant rejection model. Transplant International, 2021 Vol. 34 (Suppl. 1), 5–404. **Oral communication**, ESOT congress, August 2021, Milan, Italy

VANDERMEULEN M., MOHAMED WAISS M., DELBOUILLE MH, MAGGIPINTO G., BEGUIN Y., DETRY O. Long-term follow-up for liver transplant recipients after allogeneic mesenchymal stromal cell infusion. Transplant International, 2021 Vol. 34 (Suppl. 1), 5–404. Full **oral communication**, ESOT congress, August 2021, Milan, Italy

VANDERMEULEN M., ERPICUM P., POMA L., DETRY O., JOURET F. Impact of mesenchymal stromal cells and/or everolimus on T-reg lymphocyte expansion in rats. Accepted for **oral communication**, annual BTS Congress, March 2020, Antwerp, Belgium

VANDERMEULEN M., MAGGINPINTO G., JOURET F., BEGUIN Y., DETRY O. Do mesenchymal stromal cells promote HLA specific HLA antibodies formation after infusion in liver transplant recipient? **Poster**, ESOT congress, September 2017, Barcelona, Spain

DETRY O., **VANDERMEULEN M.**, DELBOUILLE MH., DE ROOVER A., SOMJA J., BLETARD N., BRIQUET A., LECHANTEUR C., BEGUIN Y. Infusion of third-party mesenchymal stromal cells after liver transplantation: a phase 1, open-label, clinical study. **Poster,** *22nd Annual Meeting of the Belgian Transplantation Society*, March 2015, Brussels, Belgium

REFERENCES

1. Durand, F. and C. Francoz, *Liver Transplantation: Prevention and Treatment of Rejection*, in *Evidence-based Gastroenterology and Hepatology 4e*. 2019. p. 729-743.
2. Ronca, V., et al., *The Immunological Basis of Liver Allograft Rejection.* Front Immunol, 2020. **11**: p. 2155.
3. McKenna, G. and G. Klintmalm, *The History of Liver Transplantation* in *Transplantation of the liver*, R.W. Busuttil, Editor. 2015, Elsevier. p. 2-22.
4. Erpicum, P., et al., *Infusion of third-party mesenchymal stromal cells after kidney transplantation: a phase I-II, open-label, clinical study.* Kidney Int, 2019. **95**(3): p. 693-707.
5. Calne, R.Y., et al., *Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organs: 32 kidneys, 2 pancreases, and 2 livers.* Lancet, 1979. **2**(8151): p. 1033-6.
6. Starzl, T.E., et al., *Liver transplantation with use of cyclosporin a and prednisone.* N Engl J Med, 1981. **305**(5): p. 266-9.
7. Hong, J.C., R.W. Busuttil, and G. Klintmalm, *Outcome predictors in transplantation*, in *Transplantation of the liver*, R.W. Busuttil, Editor. 2015, Elsevier. p. 1366-1378.
8. Thomson, A.W., J. Vionnet, and A. Sanchez-Fueyo, *Understanding, predicting and achieving liver transplant tolerance: from bench to bedside.* Nat Rev Gastroenterol Hepatol, 2020. **17**(12): p. 719-739.
9. Ruiz, R. and A.D. Kirk, *Long-term toxicity of immunosuppressive therapy*, in *Transplantation of the liver*, R.W. Busuttil, Editor. 2015, Elsevier. p. 1354-1363.
10. Dar, W.A., et al., *Ischaemia reperfusion injury in liver transplantation: Cellular and molecular mechanisms.* Liver Int, 2019. **39**(5): p. 788-801.
11. Monbaliu, D., J. Pirenne, and D. Talbot, *Liver transplantation using Donation after Cardiac Death donors.* J Hepatol, 2012. **56**(2): p. 474-85.
12. Rampes, S. and D. Ma, *Hepatic ischemia-reperfusion injury in liver transplant setting: mechanisms and protective strategies.* J Biomed Res, 2019. **33**(4): p. 221-234.
13. Abu-Amara, M., et al., *Liver ischemia/reperfusion injury: processes in inflammatory networks--a review.* Liver Transpl, 2010. **16**(9): p. 1016-32.
14. Peralta, C., M.B. Jiménez-Castro, and J. Gracia-Sancho, *Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal milieu.* J Hepatol, 2013. **59**(5): p. 1094-106.
15. Gehrau, R.C., et al., *Donor Hepatic Steatosis Induce Exacerbated Ischemia-Reperfusion Injury Through Activation of Innate Immune Response Molecular Pathways.* Transplantation, 2015. **99**(12): p. 2523-33.
16. Meurisse, N., et al., *Effect of a Combined Drug Approach on the Severity of Ischemia-Reperfusion Injury During Liver Transplant: A Randomized Clinical Trial.* JAMA Netw Open, 2023. **6**(2): p. e230819.
17. Sumpter, T.L., et al., *Dendritic cells, the liver, and transplantation.* Hepatology, 2007. **46**(6): p. 2021-31.

18. Martinez, O.M. and H.R. Rosen, *Basic concepts in transplant immunology.* Liver Transpl, 2005. **11**(4): p. 370-81.
19. Demetris, A.J., et al., *2016 Comprehensive Update of the Banff Working Group on Liver Allograft Pathology: Introduction of Antibody-Mediated Rejection.* Am J Transplant, 2016. **16**(10): p. 2816-2835.
20. Taner, T., *Liver transplantation: Rejection and tolerance.* Liver Transpl, 2017. **23**(S1): p. S85-s88.
21. Shetty, S., D.H. Adams, and S.G. Hubscher, *Post-transplant liver biopsy and the immune response: lessons for the clinician.* Expert Rev Clin Immunol, 2012. **8**(7): p. 645-61.
22. Lovinfosse, P., et al., *Re: The role of FDG PET in detecting rejection after liver transplantation.* Surgery, 2019. **165**(4): p. 853-858.
23. Eksteen, B., et al., *Immune-mediated liver injury.* Semin Liver Dis, 2007. **27**(4): p. 351-66.
24. Golubovskaya, V. and L. Wu, *Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy.* Cancers (Basel), 2016. **8**(3).
25. Jiang, S., O. Herrera, and R.I. Lechler, *New spectrum of allorecognition pathways: implications for graft rejection and transplantation tolerance.* Curr Opin Immunol, 2004. **16**(5): p. 550-7.
26. Lin, S.Z., et al., *Emodin attenuates acute rejection of liver allografts by inhibiting hepatocellular apoptosis and modulating the Th1/Th2 balance in rats.* Clin Exp Pharmacol Physiol, 2010. **37**(8): p. 790-4.
27. Barbara, J.A., et al., *Islet allograft rejection can be mediated by CD4+, alloantigen experienced, direct pathway T cells of TH1 and TH2 cytokine phenotype.* Transplantation, 2000. **70**(11): p. 1641-9.
28. Xie, X.J., et al., *Th17 promotes acute rejection following liver transplantation in rats.* J Zhejiang Univ Sci B, 2010. **11**(11): p. 819-27.
29. Jhun, J., et al., *Serial Monitoring of Immune Markers Being Represented Regulatory T Cell/T Helper 17 Cell Ratio: Indicating Tolerance for Tapering Immunosuppression after Liver Transplantation.* Front Immunol, 2018. **9**: p. 352.
30. Heeger, P.S., et al., *Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes.* J Immunol, 1999. **163**(4): p. 2267-75.
31. Sánchez-Fueyo, A. and T.B. Strom, *Immunologic basis of graft rejection and tolerance following transplantation of liver or other solid organs.* Gastroenterology, 2011. **140**(1): p. 51-64.
32. Hartigan, C.R., H. Sun, and M.L. Ford, *Memory T-cell exhaustion and tolerance in transplantation.* Immunol Rev, 2019. **292**(1): p. 225-242.
33. Li, J., et al., *The Evolving Roles of Macrophages in Organ Transplantation.* J Immunol Res, 2019. **2019**: p. 5763430.
34. Scozzi, D., et al., *The Role of Neutrophils in Transplanted Organs.* Am J Transplant, 2017. **17**(2): p. 328-335.
35. Pillay, J., et al., *A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1.* J Clin Invest, 2012. **122**(1): p. 327-36.
36. de Groen, P.C., et al., *The eosinophil as an effector cell of the immune response during hepatic allograft rejection.* Hepatology, 1994. **20**(3): p. 654-62.

37. Roitt, I., J. Brostoff, and D. Male, *Immunology*. Sitxth Edition ed. 2001: Harcourt Publishers
38. van der Touw, W. and J.S. Bromberg, *Natural killer cells and the immune response in solid organ transplantation.* Am J Transplant, 2010. **10**(6): p. 1354-8.
39. Lee, B.T., M.I. Fiel, and T.D. Schiano, *Antibody-mediated rejection of the liver allograft: An update and a clinico-pathological perspective.* J Hepatol, 2021. **75**(5): p. 1203-1216.
40. O'Leary, J.G., et al., *Antibody-mediated rejection as a contributor to previously unexplained early liver allograft loss.* Liver Transpl, 2014. **20**(2): p. 218-27.
41. Cuadrado, A., et al., *Clinical significance of donor-specific human leukocyte antigen antibodies in liver transplantation.* World J Gastroenterol, 2015. **21**(39): p. 11016-26.
42. Kaneku, H., et al., *De novo donor-specific HLA antibodies decrease patient and graft survival in liver transplant recipients.* Am J Transplant, 2013. **13**(6): p. 1541-8.
43. O'Leary, J.G., et al., *The role of donor-specific HLA alloantibodies in liver transplantation.* Am J Transplant, 2014. **14**(4): p. 779-87.
44. Kim, P.T., A.J. Demetris, and J.G. O'Leary, *Prevention and treatment of liver allograft antibody-mediated rejection and the role of the 'two-hit hypothesis'.* Curr Opin Organ Transplant, 2016. **21**(2): p. 209-18.
45. Grafals, M. and J.M. Thurman, *The Role of Complement in Organ Transplantation.* Front Immunol, 2019. **10**: p. 2380.
46. Valenzuela, N.M., J.T. McNamara, and E.F. Reed, *Antibody-mediated graft injury: complement-dependent and complement-independent mechanisms.* Curr Opin Organ Transplant, 2014. **19**(1): p. 33-40.
47. Del Bello, A., et al., *Prevalence, incidence and risk factors for donor-specific anti-HLA antibodies in maintenance liver transplant patients.* Am J Transplant, 2014. **14**(4): p. 867-75.
48. O'Leary, J.G., et al., *Proposed Diagnostic Criteria for Chronic Antibody-Mediated Rejection in Liver Allografts.* Am J Transplant, 2016. **16**(2): p. 603-14.
49. O'Leary, J.G., et al., *The Influence of Immunosuppressive Agents on the Risk of De Novo Donor-Specific HLA Antibody Production in Solid Organ Transplant Recipients.* Transplantation, 2016. **100**(1): p. 39-53.
50. Charco, R., et al., *Influence of anti-HLA antibodies and positive T-lymphocytotoxic crossmatch on survival and graft rejection in human liver transplantation.* J Hepatol, 1996. **24**(4): p. 452-9.
51. O'Leary, J.G., et al., *Preformed class II donor-specific antibodies are associated with an increased risk of early rejection after liver transplantation.* Liver Transpl, 2013. **19**(9): p. 973-80.
52. Vandevoorde, K., et al., *Prevalence, Risk Factors, and Impact of Donor-Specific Alloantibodies After Adult Liver Transplantation.* Liver Transpl, 2018. **24**(8): p. 1091-1100.
53. Feng, S., et al., *Evidence of Chronic Allograft Injury in Liver Biopsies From Long-term Pediatric Recipients of Liver Transplants.* Gastroenterology, 2018. **155**(6): p. 1838-1851.e7.
54. Del Bello, A., et al., *Outcome of Liver Transplant Patients With Preformed Donor-Specific Anti-Human Leukocyte Antigen Antibodies.* Liver Transpl, 2020. **26**(2): p. 256-267.

55. Demetris, A.J., et al., *Functional Immune Anatomy of the Liver-As an Allograft.* Am J Transplant, 2016. **16**(6): p. 1653-80.
56. Taner, T., et al., *Decreased chronic cellular and antibody-mediated injury in the kidney following simultaneous liver-kidney transplantation.* Kidney Int, 2016. **89**(4): p. 909-17.
57. Olausson, M., et al., *Successful combined partial auxiliary liver and kidney transplantation in highly sensitized cross-match positive recipients.* Am J Transplant, 2007. **7**(1): p. 130-6.
58. Wong, T.W., et al., *Liver Allograft Provides Immunoprotection for the Cardiac Allograft in Combined Heart-Liver Transplantation.* Am J Transplant, 2016. **16**(12): p. 3522-3531.
59. Abu-Elmagd, K.M., et al., *Five hundred intestinal and multivisceral transplantations at a single center: major advances with new challenges.* Ann Surg, 2009. **250**(4): p. 567-81.
60. Furukawa, A., S.A. Wisel, and Q. Tang, *Impact of Immune-Modulatory Drugs on Regulatory T Cell.* Transplantation, 2016. **100**(11): p. 2288-2300.
61. Shaked, A., et al., *Outcomes of immunosuppression minimization and withdrawal early after liver transplantation.* Am J Transplant, 2019. **19**(5): p. 1397-1409.
62. Delgoffe, G.M., et al., *The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2.* Nat Immunol, 2011. **12**(4): p. 295-303.
63. Sarbassov, D.D., et al., *Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB.* Mol Cell, 2006. **22**(2): p. 159-68.
64. Boudjema, K., et al., *Reduced-dose tacrolimus with mycophenolate mofetil vs. standard-dose tacrolimus in liver transplantation: a randomized study.* Am J Transplant, 2011. **11**(5): p. 965-76.
65. Sterneck, M., et al., *Everolimus and early calcineurin inhibitor withdrawal: 3-year results from a randomized trial in liver transplantation.* Am J Transplant, 2014. **14**(3): p. 701-10.
66. Saliba, F., et al., *Efficacy and Safety of Everolimus and Mycophenolic Acid With Early Tacrolimus Withdrawal After Liver Transplantation: A Multicenter Randomized Trial.* Am J Transplant, 2017. **17**(7): p. 1843-1852.
67. Boillot, O., et al., *Reversal of early acute rejection with increased doses of tacrolimus in liver transplantation: a pilot study.* Transplantation, 1998. **66**(9): p. 1182-5.
68. Feng, S. and J. Bucuvalas, *Tolerance after liver transplantation: Where are we?* Liver Transpl, 2017. **23**(12): p. 1601-1614.
69. Adams, D.H., A. Sanchez-Fueyo, and D. Samuel, *From immunosuppression to tolerance.* J Hepatol, 2015. **62**(1 Suppl): p. S170-85.
70. Calne, R.Y., *Prope tolerance--the future of organ transplantation from the laboratory to the clinic.* Int Immunopharmacol, 2005. **5**(1): p. 163-7.
71. You, Q., et al., *Mechanism of T cell tolerance induction by murine hepatic Kupffer cells.* Hepatology, 2008. **48**(3): p. 978-90.
72. Breous, E., et al., *Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver.* Hepatology, 2009. **50**(2): p. 612-21.
73. Baratelli, F., et al., *Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells.* J Immunol, 2005. **175**(3): p. 1483-90.

74. Shetty, S., P.F. Lalor, and D.H. Adams, *Liver sinusoidal endothelial cells - gatekeepers of hepatic immunity.* Nat Rev Gastroenterol Hepatol, 2018. **15**(9): p. 555-567.
75. Kruse, N., et al., *Priming of CD4+ T cells by liver sinusoidal endothelial cells induces CD25low forkhead box protein 3- regulatory T cells suppressing autoimmune hepatitis.* Hepatology, 2009. **50**(6): p. 1904-13.
76. Schildberg, F.A., et al., *Liver sinusoidal endothelial cells veto CD8 T cell activation by antigen-presenting dendritic cells.* Eur J Immunol, 2008. **38**(4): p. 957-67.
77. Jiang, G., et al., *Hepatic stellate cells preferentially expand allogeneic CD4+ CD25+ FoxP3+ regulatory T cells in an IL-2-dependent manner.* Transplantation, 2008. **86**(11): p. 1492-502.
78. Sumpter, T.L., et al., *Hepatic stellate cells undermine the allostimulatory function of liver myeloid dendritic cells via STAT3-dependent induction of IDO.* J Immunol, 2012. **189**(8): p. 3848-58.
79. Thomson, A.W. and P.A. Knolle, *Antigen-presenting cell function in the tolerogenic liver environment.* Nat Rev Immunol, 2010. **10**(11): p. 753-66.
80. Subleski, J.J., et al., *TCR-dependent and -independent activation underlie liver-specific regulation of NKT cells.* J Immunol, 2011. **186**(2): p. 838-47.
81. Akbulut, S., M. Yilmaz, and S. Yilmaz, *Graft-versus-host disease after liver transplantation: a comprehensive literature review.* World J Gastroenterol, 2012. **18**(37): p. 5240-8.
82. Kamada, N., *The immunology of experimental liver transplantation in the rat.* Immunology, 1985. **55**(3): p. 369-89.
83. Qian, S., et al., *Murine liver allograft transplantation: tolerance and donor cell chimerism.* Hepatology, 1994. **19**(4): p. 916-24.
84. Murase, N., et al., *Suppression of allograft rejection with FK506. I. Prolonged cardiac and liver survival in rats following short-course therapy.* Transplantation, 1990. **50**(2): p. 186-9.
85. Kobayashi, E., et al., *Prevention by liver transplantation of the graft-versus-host reaction and allograft rejection in a rat model of small bowel transplantation.* Transplantation, 1994. **57**(2): p. 177-81.
86. Kamada, N., H.S. Davies, and B. Roser, *Reversal of transplantation immunity by liver grafting.* Nature, 1981. **292**(5826): p. 840-2.
87. Demetris, A.J., N. Murase, and T.E. Starzl, *Donor dendritic cells after liver and heart allotransplantation under short-term immunosuppression.* Lancet, 1992. **339**(8809): p. 1610.
88. Kreisel, D., et al., *The role of passenger leukocyte genotype in rejection and acceptance of rat liver allografts.* Transplantation, 2002. **73**(9): p. 1501-7.
89. Sharland, A., et al., *Kinetics of intragraft cytokine expression, cellular infiltration, and cell death in rejection of renal allografts compared with acceptance of liver allografts in a rat model: early activation and apoptosis is associated with liver graft acceptance.* Transplantation, 1998. **65**(10): p. 1370-7.
90. Bishop, G.A., et al., *Spontaneous acceptance of liver transplants in rodents: evidence that liver leucocytes induce recipient T-cell death by neglect.* Immunol Cell Biol, 2002. **80**(1): p. 93-100.
91. Li, W., et al., *CTLA4 engagement is required for induction of murine liver transplant spontaneous tolerance.* Am J Transplant, 2005. **5**(5): p. 978-86.

92. Mele, T.S., et al., *IFN-gamma is an absolute requirement for spontaneous acceptance of liver allografts.* Am J Transplant, 2003. **3**(8): p. 942-51.
93. Morita, M., et al., *Rejection triggers liver transplant tolerance: Involvement of mesenchyme-mediated immune control mechanisms in mice.* Hepatology, 2015. **62**(3): p. 915-31.
94. Ye, Y., et al., *Galectin-1 prolongs survival of mouse liver allografts from Flt3L-pretreated donors.* Am J Transplant, 2013. **13**(3): p. 569-79.
95. Atif, M., et al., *Regulatory T cells in solid organ transplantation.* Clin Transl Immunology, 2020. **9**(2): p. e01099.
96. Liu, W., et al., *CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells.* J Exp Med, 2006. **203**(7): p. 1701-11.
97. Miyara, M., et al., *Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor.* Immunity, 2009. **30**(6): p. 899-911.
98. Weaver, C.T. and R.D. Hatton, *Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective.* Nat Rev Immunol, 2009. **9**(12): p. 883-9.
99. Pons, J.A., et al., *FoxP3 in peripheral blood is associated with operational tolerance in liver transplant patients during immunosuppression withdrawal.* Transplantation, 2008. **86**(10): p. 1370-8.
100. Shevach, E.M., *Mechanisms of foxp3+ T regulatory cell-mediated suppression.* Immunity, 2009. **30**(5): p. 636-45.
101. Zeiser, R., et al., *Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production.* Blood, 2006. **108**(1): p. 390-9.
102. Miroux, C., et al., *In vitro effects of cyclosporine A and tacrolimus on regulatory T-cell proliferation and function.* Transplantation, 2012. **94**(2): p. 123-31.
103. Satake, A., et al., *Differential targeting of IL-2 and T cell receptor signaling pathways selectively expands regulatory T cells while inhibiting conventional T cells.* J Autoimmun, 2013. **44**: p. 13-20.
104. Abadja, F., et al., *Impact of mycophenolic acid and tacrolimus on Th17-related immune response.* Transplantation, 2011. **92**(4): p. 396-403.
105. Demirkiran, A., et al., *Conversion from calcineurin inhibitor to mycophenolate mofetil-based immunosuppression changes the frequency and phenotype of CD4+FOXP3+ regulatory T cells.* Transplantation, 2009. **87**(7): p. 1062-8.
106. Tuckermann, J.P., et al., *Molecular mechanisms of glucocorticoids in the control of inflammation and lymphocyte apoptosis.* Crit Rev Clin Lab Sci, 2005. **42**(1): p. 71-104.
107. Bereshchenko, O., et al., *GILZ promotes production of peripherally induced Treg cells and mediates the crosstalk between glucocorticoids and TGF-β signaling.* Cell Rep, 2014. **7**(2): p. 464-475.
108. Seissler, N., et al., *Methylprednisolone treatment increases the proportion of the highly suppressive HLA-DR(+)-Treg-cells in transplanted patients.* Transpl Immunol, 2012. **27**(4): p. 157-61.
109. Basu, S., et al., *Cutting edge: Foxp3-mediated induction of pim 2 allows human T regulatory cells to preferentially expand in rapamycin.* J Immunol, 2008. **180**(9): p. 5794-8.
110. Battaglia, M., A. Stabilini, and M.G. Roncarolo, *Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells.* Blood, 2005. **105**(12): p. 4743-8.

111. Gedaly, R., et al., *mTOR Inhibitor Everolimus in Regulatory T Cell Expansion for Clinical Application in Transplantation.* Transplantation, 2019. **103**(4): p. 705-715.
112. Ruggenenti, P., et al., *Sirolimus versus cyclosporine therapy increases circulating regulatory T cells, but does not protect renal transplant patients given alemtuzumab induction from chronic allograft injury.* Transplantation, 2007. **84**(8): p. 956-64.
113. Ghazal, K., et al., *Treatment with mTOR inhibitors after liver transplantation enables a sustained increase in regulatory T-cells while preserving their suppressive capacity.* Clin Res Hepatol Gastroenterol, 2018. **42**(3): p. 237-244.
114. Todo, S., et al., *A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation.* Hepatology, 2016. **64**(2): p. 632-43.
115. Tang, Q., et al., *Selective decrease of donor-reactive T(regs) after liver transplantation limits T(reg) therapy for promoting allograft tolerance in humans.* Sci Transl Med, 2022. **14**(669): p. eabo2628.
116. da Silva Meirelles, L., A.I. Caplan, and N.B. Nardi, *In search of the in vivo identity of mesenchymal stem cells.* Stem Cells, 2008. **26**(9): p. 2287-99.
117. Najar, M., et al., *Mesenchymal stromal cells and immunomodulation: A gathering of regulatory immune cells.* Cytotherapy, 2016. **18**(2): p. 160-71.
118. Moroso, V., et al., *NK cells can generate from precursors in the adult human liver.* Eur J Immunol, 2011. **41**(11): p. 3340-50.
119. García de la Garza, R., et al., *Immune monitoring of immunosuppression withdrawal of liver transplant recipients.* Transpl Immunol, 2015. **33**(2): p. 110-6.
120. Lee, S., et al., *A technique for orthotopic liver transplantation in the rat.* Transplantation, 1973. **16**(6): p. 664-9.
121. Kamada, N. and R.Y. Calne, *Orthotopic liver transplantation in the rat. Technique using cuff for portal vein anastomosis and biliary drainage.* Transplantation, 1979. **28**(1): p. 47-50.
122. Kamada, N. and R.Y. Calne, *A surgical experience with five hundred thirty liver transplants in the rat.* Surgery, 1983. **93**(1 Pt 1): p. 64-9.
123. Czigány, Z., et al., *Improving Research Practice in Rat Orthotopic and Partial Orthotopic Liver Transplantation: A Review, Recommendation, and Publication Guide.* Eur Surg Res, 2015. **55**(1-2): p. 119-38.
124. Hori, T., et al., *Comprehensive and innovative techniques for liver transplantation in rats: a surgical guide.* World J Gastroenterol, 2010. **16**(25): p. 3120-32.
125. Oldani, G., et al., *Efficient nonarterialized mouse liver transplantation using 3-dimensional-printed instruments.* Liver Transpl, 2016. **22**(12): p. 1688-1696.
126. Kamada, N., *Animal models of hepatic allograft rejection.* Semin Liver Dis, 1992. **12**(1): p. 1-15.
127. Gong, W., *Rodent Transplantation Medicine*, in *Rodent Transplant Medicine*, W. Gong, Editor. 2015, Springer. p. 1-9.
128. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.
129. Caplan, A.I., *Mesenchymal stem cells.* J Orthop Res, 1991. **9**(5): p. 641-50.
130. Zuk, P.A., et al., *Human adipose tissue is a source of multipotent stem cells.* Mol Biol Cell, 2002. **13**(12): p. 4279-95.

131. Majore, I., et al., *Growth and differentiation properties of mesenchymal stromal cell populations derived from whole human umbilical cord.* Stem Cell Rev Rep, 2011. **7**(1): p. 17-31.
132. Wang, H.S., et al., *Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord.* Stem Cells, 2004. **22**(7): p. 1330-7.
133. Strioga, M., et al., *Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells.* Stem Cells Dev, 2012. **21**(14): p. 2724-52.
134. Hass, R., et al., *Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC.* Cell Commun Signal, 2011. **9**: p. 12.
135. Phinney, D.G. and L. Sensebé, *Mesenchymal stromal cells: misconceptions and evolving concepts.* Cytotherapy, 2013. **15**(2): p. 140-5.
136. Grégoire, C., et al., *Comparison of Mesenchymal Stromal Cells From Different Origins for the Treatment of Graft-vs.-Host-Disease in a Humanized Mouse Model.* Front Immunol, 2019. **10**: p. 619.
137. Vandermeulen, M., et al., *Mesenchymal Stromal Cells in Solid Organ Transplantation.* Transplantation, 2020. **104**(5): p. 923-936.
138. Li, N. and J. Hua, *Interactions between mesenchymal stem cells and the immune system.* Cell Mol Life Sci, 2017. **74**(13): p. 2345-2360.
139. Gao, F., et al., *Mesenchymal stem cells and immunomodulation: current status and future prospects.* Cell Death Dis, 2016. **7**(1): p. e2062.
140. Shi, Y., et al., *How mesenchymal stem cells interact with tissue immune responses.* Trends Immunol, 2012. **33**(3): p. 136-43.
141. Ren, G., et al., *Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression.* Stem Cells, 2009. **27**(8): p. 1954-62.
142. Meisel, R., et al., *Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation.* Blood, 2004. **103**(12): p. 4619-21.
143. Ge, W., et al., *Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression.* Transplantation, 2010. **90**(12): p. 1312-20.
144. Selmani, Z., et al., *Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells.* Stem Cells, 2008. **26**(1): p. 212-22.
145. Nasef, A., et al., *Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G.* Transplantation, 2007. **84**(2): p. 231-7.
146. Aggarwal, S. and M.F. Pittenger, *Human mesenchymal stem cells modulate allogeneic immune cell responses.* Blood, 2005. **105**(4): p. 1815-22.
147. Sensebé, L. and P. Bourin, *Mesenchymal stem cells for therapeutic purposes.* Transplantation, 2009. **87**(9 Suppl): p. S49-53.
148. Spees, J.L., R.H. Lee, and C.A. Gregory, *Mechanisms of mesenchymal stem/stromal cell function.* Stem Cell Res Ther, 2016. **7**(1): p. 125.
149. Chabannes, D., et al., *A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells.* Blood, 2007. **110**(10): p. 3691-4.

150. Vandermeulen, M., et al., *Rationale for the potential use of mesenchymal stromal cells in liver transplantation.* World J Gastroenterol, 2014. **20**(44): p. 16418-32.
151. Harrell, C.R., et al., *Molecular Mechanisms Responsible for Therapeutic Potential of Mesenchymal Stem Cell-Derived Secretome.* Cells, 2019. **8**(5).
152. Farzamfar, S., et al., *Extracellular micro/nanovesicles rescue kidney from ischemia-reperfusion injury.* J Cell Physiol, 2019. **234**(8): p. 12290-12300.
153. Di Trapani, M., et al., *Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions.* Sci Rep, 2016. **6**: p. 24120.
154. Kusuma, G.D., et al., *Effect of the Microenvironment on Mesenchymal Stem Cell Paracrine Signaling: Opportunities to Engineer the Therapeutic Effect.* Stem Cells Dev, 2017. **26**(9): p. 617-631.
155. Matula, Z., et al., *The Role of Extracellular Vesicle and Tunneling Nanotube-Mediated Intercellular Cross-Talk Between Mesenchymal Stem Cells and Human Peripheral T Cells.* Stem Cells Dev, 2016. **25**(23): p. 1818-1832.
156. Cho, D.I., et al., *Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages.* Exp Mol Med, 2014. **46**(1): p. e70.
157. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* J Clin Invest, 2012. **122**(3): p. 787-95.
158. Spaggiari, G.M., et al., *Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2.* Blood, 2008. **111**(3): p. 1327-33.
159. Crop, M.J., et al., *Human mesenchymal stem cells are susceptible to lysis by CD8(+) T cells and NK cells.* Cell Transplant, 2011. **20**(10): p. 1547-59.
160. Reinders, M.E. and M.J. Hoogduijn, *NK Cells and MSCs: Possible Implications for MSC Therapy in Renal Transplantation.* J Stem Cell Res Ther, 2014. **4**(2): p. 1000166.
161. Zhao, Z.G., et al., *Immunomodulatory function of regulatory dendritic cells induced by mesenchymal stem cells.* Immunol Invest, 2012. **41**(2): p. 183-98.
162. Liu, X., et al., *Mesenchymal stem/stromal cells induce the generation of novel IL-10-dependent regulatory dendritic cells by SOCS3 activation.* J Immunol, 2012. **189**(3): p. 1182-92.
163. Li, Y.P., et al., *Human mesenchymal stem cells license adult CD34+ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway.* J Immunol, 2008. **180**(3): p. 1598-608.
164. Zhang, B., et al., *Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population.* Blood, 2009. **113**(1): p. 46-57.
165. Bartholomew, A., et al., *Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo.* Exp Hematol, 2002. **30**(1): p. 42-8.
166. Plumas, J., et al., *Mesenchymal stem cells induce apoptosis of activated T cells.* Leukemia, 2005. **19**(9): p. 1597-604.
167. Ménard, C. and K. Tarte, *Immunoregulatory properties of clinical grade mesenchymal stromal cells: evidence, uncertainties, and clinical application.* Stem Cell Res Ther, 2013. **4**(3): p. 64.

168. Rasmusson, I., et al., *Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells.* Transplantation, 2003. **76**(8): p. 1208-13.
169. Maccario, R., et al., *Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype.* Haematologica, 2005. **90**(4): p. 516-25.
170. Duffy, M.M., et al., *Mesenchymal stem cell effects on T-cell effector pathways.* Stem Cell Res Ther, 2011. **2**(4): p. 34.
171. Braza, F., et al., *Regulatory T Cells in Kidney Transplantation: New Directions?* Am J Transplant, 2015. **15**(9): p. 2288-300.
172. Beyth, S., et al., *Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness.* Blood, 2005. **105**(5): p. 2214-9.
173. Corcione, A., et al., *Human mesenchymal stem cells modulate B-cell functions.* Blood, 2006. **107**(1): p. 367-72.
174. Krampera, M., *Mesenchymal stromal cell 'licensing': a multistep process.* Leukemia, 2011. **25**(9): p. 1408-14.
175. Phinney, D.G. and D.J. Prockop, *Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views.* Stem Cells, 2007. **25**(11): p. 2896-902.
176. Khubutiya, M.S., et al., *Paracrine mechanisms of proliferative, anti-apoptotic and anti-inflammatory effects of mesenchymal stromal cells in models of acute organ injury.* Cytotherapy, 2014. **16**(5): p. 579-85.
177. Sordi, V., *Mesenchymal stem cell homing capacity.* Transplantation, 2009. **87**(9 Suppl): p. S42-5.
178. Mundra, V., I.C. Gerling, and R.I. Mahato, *Mesenchymal stem cell-based therapy.* Mol Pharm, 2013. **10**(1): p. 77-89.
179. Bi, B., et al., *Stromal cells protect against acute tubular injury via an endocrine effect.* J Am Soc Nephrol, 2007. **18**(9): p. 2486-96.
180. Geng, Y., et al., *Mesenchymal stem cells ameliorate rhabdomyolysis-induced acute kidney injury via the activation of M2 macrophages.* Stem Cell Res Ther, 2014. **5**(3): p. 80.
181. Schrepfer, S., et al., *Stem cell transplantation: the lung barrier.* Transplant Proc, 2007. **39**(2): p. 573-6.
182. Eggenhofer, E., et al., *Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion.* Front Immunol, 2012. **3**: p. 297.
183. Duffield, J.S., et al., *Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells.* J Clin Invest, 2005. **115**(7): p. 1743-55.
184. Prockop, D.J., *"Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs).* Clin Pharmacol Ther, 2007. **82**(3): p. 241-3.
185. Potapova, I.A., et al., *Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro.* Stem Cells, 2007. **25**(7): p. 1761-8.
186. Tang, Y.L., et al., *Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium.* Regul Pept, 2004. **117**(1): p. 3-10.

187. Tögel, F., et al., *Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury.* Am J Physiol Renal Physiol, 2007. **292**(5): p. F1626-35.
188. Islam, M.N., et al., *Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury.* Nat Med, 2012. **18**(5): p. 759-65.
189. Perico, L., et al., *Human mesenchymal stromal cells transplanted into mice stimulate renal tubular cells and enhance mitochondrial function.* Nat Commun, 2017. **8**(1): p. 983.
190. Erpicum, P., et al., *Administration of mesenchymal stromal cells before renal ischemia/reperfusion attenuates kidney injury and may modulate renal lipid metabolism in rats.* Sci Rep, 2017. **7**(1): p. 8687.
191. Alagesan, S. and M.D. Griffin, *Autologous and allogeneic mesenchymal stem cells in organ transplantation: what do we know about their safety and efficacy?* Curr Opin Organ Transplant, 2014. **19**(1): p. 65-72.
192. Le Blanc, K., et al., *HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells.* Exp Hematol, 2003. **31**(10): p. 890-6.
193. Ankrum, J.A., J.F. Ong, and J.M. Karp, *Mesenchymal stem cells: immune evasive, not immune privileged.* Nat Biotechnol, 2014. **32**(3): p. 252-60.
194. Tse, W.T., et al., *Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation.* Transplantation, 2003. **75**(3): p. 389-97.
195. Schu, S., et al., *Immunogenicity of allogeneic mesenchymal stem cells.* J Cell Mol Med, 2012. **16**(9): p. 2094-103.
196. Chan, W.K., et al., *MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge.* Exp Hematol, 2008. **36**(11): p. 1545-55.
197. Lechanteur, C., et al., *Clinical-scale expansion of mesenchymal stromal cells: a large banking experience.* J Transl Med, 2016. **14**(1): p. 145.
198. Souidi, N., M. Stolk, and M. Seifert, *Ischemia-reperfusion injury: beneficial effects of mesenchymal stromal cells.* Curr Opin Organ Transplant, 2013. **18**(1): p. 34-43.
199. Jin, G., et al., *Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats.* Int J Mol Med, 2013. **31**(6): p. 1395-401.
200. Sun, C.K., et al., *Systemic administration of autologous adipose-derived mesenchymal stem cells alleviates hepatic ischemia-reperfusion injury in rats.* Crit Care Med, 2012. **40**(4): p. 1279-90.
201. Kanazawa, H., et al., *Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model.* PLoS One, 2011. **6**(4): p. e19195.
202. Du, Z., et al., *Mesenchymal stem cell-conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation.* J Surg Res, 2013. **183**(2): p. 907-15.
203. Wang, W., et al., *Mesenchymal stem cells promote liver regeneration and prolong survival in small-for-size liver grafts: involvement of C-Jun N-terminal kinase, cyclin D1, and NF-κB.* PLoS One, 2014. **9**(12): p. e112532.
204. Sasajima, H., et al., *Cytoprotective Effects of Mesenchymal Stem Cells During Liver Transplantation From Donors After Cardiac Death in Swine.* Transplant Proc, 2020. **52**(6): p. 1891-1900.

205. Zhou, H.P., et al., *Administration of donor-derived mesenchymal stem cells can prolong the survival of rat cardiac allograft.* Transplant Proc, 2006. **38**(9): p. 3046-51.
206. Ge, W., et al., *Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance.* Am J Transplant, 2009. **9**(8): p. 1760-72.
207. Casiraghi, F., et al., *Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells.* J Immunol, 2008. **181**(6): p. 3933-46.
208. Casiraghi, F., et al., *Localization of mesenchymal stromal cells dictates their immune or proinflammatory effects in kidney transplantation.* Am J Transplant, 2012. **12**(9): p. 2373-83.
209. Zhang, W., C. Qin, and Z.M. Zhou, *Mesenchymal stem cells modulate immune responses combined with cyclosporine in a rat renal transplantation model.* Transplant Proc, 2007. **39**(10): p. 3404-8.
210. De Martino, M., et al., *Mesenchymal stem cells infusion prevents acute cellular rejection in rat kidney transplantation.* Transplant Proc, 2010. **42**(4): p. 1331-5.
211. Eggenhofer, E., et al., *Features of synergism between mesenchymal stem cells and immunosuppressive drugs in a murine heart transplantation model.* Transpl Immunol, 2011. **25**(2-3): p. 141-7.
212. Wan, C.D., et al., *Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model.* Hepatobiliary Pancreat Dis Int, 2008. **7**(1): p. 29-33.
213. Wang, Y., et al., *Bone marrow-derived mesenchymal stem cells inhibit acute rejection of rat liver allografts in association with regulatory T-cell expansion.* Transplant Proc, 2009. **41**(10): p. 4352-6.
214. Niu, J., et al., *Prevention of acute liver allograft rejection by IL-10-engineered mesenchymal stem cells.* Clin Exp Immunol, 2014. **176**(3): p. 473-84.
215. You, Y., et al., *Mesenchymal stromal cell-dependent reprogramming of Kupffer cells is mediated by TNF-α and PGE2 and is crucial for liver transplant tolerance.* Immunol Res, 2015. **62**(3): p. 292-305.
216. Sun, Z., et al., *Immunological effect induced by mesenchymal stem cells in a rat liver transplantation model.* Exp Ther Med, 2015. **10**(2): p. 401-406.
217. Tang, J., et al., *Transforming growth factor-β-Expressing Mesenchymal Stem Cells Induce Local Tolerance in a Rat Liver Transplantation Model of Acute Rejection.* Stem Cells, 2016. **34**(11): p. 2681-2692.
218. Chen, Q., et al., *Bone marrow mesenchymal stromal cells attenuate liver allograft rejection may via upregulation PD-L1 expression through downregulation of miR-17-5p.* Transpl Immunol, 2018. **51**: p. 21-29.
219. Niu, J., et al., *Mesenchymal stem cells prolong the survival of orthotopic liver transplants by regulating the expression of TGF-β1.* Turk J Gastroenterol, 2018. **29**(5): p. 601-609.
220. Wu, B., et al., *Improvement of Liver Transplantation Outcome by Heme Oxygenase-1-Transduced Bone Marrow Mesenchymal Stem Cells in Rats.* Stem Cells Int, 2016. **2016**: p. 9235073.

221. Shen, Z.Y., et al., *Immunomodulatory effects of bone marrow mesenchymal stem cells overexpressing heme oxygenase-1: Protective effects on acute rejection following reduced-size liver transplantation in a rat model.* Cell Immunol, 2017. **313**: p. 10-24.
222. Xia, X., et al., *Mesenchymal stem cells administered after liver transplantation prevent acute graft-versus-host disease in rats.* Liver Transpl, 2012. **18**(6): p. 696-706.
223. Buron, F., et al., *Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: an in vitro study using human cells.* Transplant Proc, 2009. **41**(8): p. 3347-52.
224. Hoogduijn, M.J., et al., *Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin.* Transplantation, 2008. **86**(9): p. 1283-91.
225. Maccario, R., et al., *Human mesenchymal stem cells and cyclosporin a exert a synergistic suppressive effect on in vitro activation of alloantigen-specific cytotoxic lymphocytes.* Biol Blood Marrow Transplant, 2005. **11**(12): p. 1031-2.
226. Sbano, P., et al., *Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model.* Arch Dermatol Res, 2008. **300**(3): p. 115-24.
227. Popp, F.C., et al., *Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate.* Transpl Immunol, 2008. **20**(1-2): p. 55-60.
228. Doğan, S.M., et al., *Mesenchymal stem cell therapy in patients with small bowel transplantation: single center experience.* World J Gastroenterol, 2014. **20**(25): p. 8215-20.
229. Reyes, M. and C.M. Verfaillie, *Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells.* Ann N Y Acad Sci, 2001. **938**: p. 231-3; discussion 233-5.
230. Popp, F.C., et al., *Safety and feasibility of third-party multipotent adult progenitor cells for immunomodulation therapy after liver transplantation--a phase I study (MISOT-I).* J Transl Med, 2011. **9**: p. 124.
231. Soeder, Y., et al., *First-in-Human Case Study: Multipotent Adult Progenitor Cells for Immunomodulation After Liver Transplantation.* Stem Cells Transl Med, 2015. **4**(8): p. 899-904.
232. Johnson, C.L., Y. Soeder, and M.H. Dahlke, *Mesenchymal stromal cells for immunoregulation after liver transplantation: the scene in 2016.* Curr Opin Organ Transplant, 2016. **21**(6): p. 541-549.
233. Shi, M., et al., *A Pilot Study of Mesenchymal Stem Cell Therapy for Acute Liver Allograft Rejection.* Stem Cells Transl Med, 2017. **6**(12): p. 2053-2061.
234. Zhang, Y., et al., *A novel MSC-based immune induction strategy for ABO-incompatible liver transplantation: a phase I/II randomized, open-label, controlled trial.* Stem Cell Res Ther, 2021. **12**(1): p. 244.
235. Zhang, Y.C., et al., *Therapeutic potentials of umbilical cord-derived mesenchymal stromal cells for ischemic-type biliary lesions following liver transplantation.* Cytotherapy, 2017. **19**(2): p. 194-199.
236. Hartleif, S., et al., *Safety and Tolerance of Donor-Derived Mesenchymal Stem Cells in Pediatric Living-Donor Liver Transplantation: The MYSTEP1 Study.* Stem Cells Int, 2017. **2017**: p. 2352954.

237. Dreyer, G.J., et al., *Human leukocyte antigen selected allogeneic mesenchymal stromal cell therapy in renal transplantation: The Neptune study, a phase I single-center study.* Am J Transplant, 2020. **20**(10): p. 2905-2915.
238. Detry, O., et al., *Infusion of mesenchymal stromal cells after deceased liver transplantation: A phase I-II, open-label, clinical study.* J Hepatol, 2017. **67**(1): p. 47-55.
239. Lalu, M.M., et al., *Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials.* PLoS One, 2012. **7**(10): p. e47559.
240. Perico, N., et al., *Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility.* Clin J Am Soc Nephrol, 2011. **6**(2): p. 412-22.
241. Chambers, D.C., et al., *Mesenchymal Stromal Cell Therapy for Chronic Lung Allograft Dysfunction: Results of a First-in-Man Study.* Stem Cells Transl Med, 2017. **6**(4): p. 1152-1157.
242. Tan, J., et al., *Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial.* Jama, 2012. **307**(11): p. 1169-77.
243. Perico, N., et al., *Long-Term Clinical and Immunological Profile of Kidney Transplant Patients Given Mesenchymal Stromal Cell Immunotherapy.* Front Immunol, 2018. **9**: p. 1359.
244. Reinders, M.E., et al., *Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study.* Stem Cells Transl Med, 2013. **2**(2): p. 107-11.
245. Vandermeulen, M., et al., *Infusion of Allogeneic Mesenchymal Stromal Cells After Liver Transplantation: A 5-Year Follow-Up.* Liver Transpl, 2022. **28**(4): p. 636-646.
246. Casiraghi, F., et al., *Multipotent mesenchymal stromal cell therapy and risk of malignancies.* Stem Cell Rev Rep, 2013. **9**(1): p. 65-79.
247. Tarte, K., et al., *Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation.* Blood, 2010. **115**(8): p. 1549-53.
248. Casiraghi, F., et al., *Third-party bone marrow-derived mesenchymal stromal cell infusion before liver transplantation: A randomized controlled trial.* Am J Transplant, 2021. **21**(8): p. 2795-2809.
249. Negi, N. and M.D. Griffin, *Effects of mesenchymal stromal cells on regulatory T cells: Current understanding and clinical relevance.* Stem Cells, 2020. **38**(5): p. 596-605.
250. Zhang, R., *Donor-Specific Antibodies in Kidney Transplant Recipients.* Clin J Am Soc Nephrol, 2018. **13**(1): p. 182-192.
251. Cheng, E.Y., *The Role of Humoral Alloreactivity in Liver Transplantation: Lessons Learned and New Perspectives.* J Immunol Res, 2017. **2017**: p. 3234906.
252. Yang, X., et al., *Mesenchymal stem cell therapy for liver disease: full of chances and challenges.* Cell Biosci, 2020. **10**: p. 123.
253. Avivar-Valderas, A., et al., *Dissecting Allo-Sensitization After Local Administration of Human Allogeneic Adipose Mesenchymal Stem Cells in Perianal Fistulas of Crohn's Disease Patients.* Front Immunol, 2019. **10**: p. 1244.
254. García-Sancho, J., et al., *Influence of HLA Matching on the Efficacy of Allogeneic Mesenchymal Stromal Cell Therapies for Osteoarthritis and Degenerative Disc Disease.* Transplant Direct, 2017. **3**(9): p. e205.

255. Hare, J.M., et al., *Randomized Comparison of Allogeneic Versus Autologous Mesenchymal Stem Cells for Nonischemic Dilated Cardiomyopathy: POSEIDON-DCM Trial.* J Am Coll Cardiol, 2017. **69**(5): p. 526-537.
256. Lohan, P., et al., *Anti-Donor Immune Responses Elicited by Allogeneic Mesenchymal Stem Cells and Their Extracellular Vesicles: Are We Still Learning?* Front Immunol, 2017. **8**: p. 1626.
257. Di Ianni, M., et al., *Mesenchymal cells recruit and regulate T regulatory cells.* Exp Hematol, 2008. **36**(3): p. 309-18.
258. Duan, W., et al., *Mesenchymal stem cells in combination with low-dose rapamycin significantly prolong islet allograft survival through induction of regulatory T cells.* Biochem Biophys Res Commun, 2018. **506**(3): p. 619-625.
259. Durán, M., et al., *Machine perfusion and the prevention of ischemic type biliary lesions following liver transplant: What is the evidence?* World J Gastroenterol, 2023. **29**(20): p. 3066-3083.
260. Laing, R.W., et al., *The Delivery of Multipotent Adult Progenitor Cells to Extended Criteria Human Donor Livers Using Normothermic Machine Perfusion.* Front Immunol, 2020. **11**: p. 1226.
261. Rowart, P., et al., *Mesenchymal Stromal Cell Therapy in Ischemia/Reperfusion Injury.* J Immunol Res, 2015. **2015**: p. 602597.
262. De Stefano, N., et al., *Human liver stem cell-derived extracellular vesicles reduce injury in a model of normothermic machine perfusion of rat livers previously exposed to a prolonged warm ischemia.* Transpl Int, 2021. **34**(9): p. 1607-1617.

APPENDICES

List of appendices:

World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v20.i44.16418

World J Gastroenterol 2014 November 28; 20(44): 16418-16432
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Rationale for the potential use of mesenchymal stromal cells in liver transplantation

Morgan Vandermeulen, Céline Grégoire, Alexandra Briquet, Chantal Lechanteur, Yves Beguin, Olivier Detry

Morgan Vandermeulen, Olivier Detry, Department of Abdominal Surgery and Transplantation, CHU Liege, University of Liege, B4000 Liege, Wallonia, Belgium
Céline Grégoire, Yves Beguin, Department of Hematology, CHU Liege, University of Liege, B4000 Liege, Wallonia, Belgium
Alexandra Briquet, Chantal Lechanteur, Yves Beguin, Laboratory of Cell and Gene Therapy, CHU Liege, University of Liege, Sart Tilman B35, B4000 Liege, Wallonia, Belgium
Author contributions: Vandermeulen M performed the literature review and wrote the manuscript under the supervision of Beguin Y and Detry O; Grégoire C, Briquet A and Lechanteur C are responsible for the culture and production of the mesenchymal stromal cells used in the clinical study on the use of the aforementioned in liver transplantation performed at the Liege center; all authors actively reviewed the manuscript, and approved its last version.
Supported by CHU Liege, the Fonds Léon Frédéricq of the Medical School of the University of Liege, and through a Senior Research Grant from the European Society for Organ Transplantation
Correspondence to: Olivier Detry, Professor, Department of Abdominal Surgery and Transplantation, CHU Liege, University of Liege, Sart Tilman B35, B4000 Liege, Wallonia, Belgium. olivier.detry@transplantation.be
Telephone: +32-43-667645 Fax: +32-43-667069
Received: May 5, 2014 Revised: June 4, 2014
Accepted: August 13, 2014
Published online: November 28, 2014

Abstract

Mesenchymal stromal cells (MSCs) are multipotent and self-renewing cells that reside essentially in the bone marrow as a non-hematopoietic cell population, but may also be isolated from the connective tissues of most organs. MSCs represent a heterogeneous population of adult, fibroblast-like cells characterized by their ability to differentiate into tissues of mesodermal lineages including adipocytes, chondrocytes and osteocytes. For several years now, MSCs have been evaluated for their *in vivo* and *in vitro* immunomodulatory and 'tissue reconstruction' properties, which could make them interesting in various clinical settings, and particularly in organ transplantation. This paper aims to review current knowledge on the properties of MSCs and their use in pre-clinical and clinical studies in solid organ transplantation, and particularly in the field of liver transplantation. The first available clinical data seem to show that MSCs are safe to use, at least in the medium-term, but more time is needed to evaluate the potential adverse effects of long-term use. Many issues must be resolved on the correct use of MSCs. Intensive *in vitro* and pre-clinical research are the keys to a better understanding of the way that MSCs act, and to eventually lead to clinical success.

Key words: Mesenchymal stem cells; Organ transplantation; Complication; Immunosuppression; Tolerance

Core tip: For several years now, mesenchymal stromal cells (MSC) have been evaluated for their *in vivo* and *in vitro* immunomodulatory and 'tissue reconstruction' properties which could make them interesting in various clinical settings, and particularly in organ transplantation. This paper aims to review current knowledge on the properties of MSCs and their use in pre-clinical and clinical studies, and particularly in the field of liver transplantation.

Vandermeulen M, Grégoire C, Briquet A, Lechanteur C, Beguin Y, Detry O. Rationale for the potential use of mesenchymal stromal cells in liver transplantation. *World J Gastroenterol* 2014; 20(44): 16418-16432 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i44/16418.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i44.16418

INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent and self-renewing cells that reside essentially in the bone marrow as a non-hematopoietic cell population. MSCs represent a heterogeneous population of adult, fibroblast-like cells characterized by their ability to differentiate into tissues of mesodermal lineages including adipocytes, chondrocytes and osteocytes. In addition to the bone marrow, MSCs have been isolated from various other tissues such as adipose tissue[1], skin[2], heart and spleen[3], placenta[4], umbilical cord blood[5] as well as lung and liver[6,7], and it appears that MSCs reside in the connective tissues of most organs[8].

No specific marker for MSCs has yet been found. Presently, MSCs are identified using a number of features defined by the International Society for Cellular Therapy which states three minimal criteria[9]: (1) adhesion to plastic in standard culture conditions; (2) expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and Human Leukocyte Antigen (HLA)-DR surface molecules; and (3) *in vitro* differentiation into osteoblasts, adipocytes and chondroblasts.

For several years now, MSCs have been evaluated for their *in vivo* and *in vitro* immunomodulatory and "tissue reconstruction" properties that could make them interesting in various clinical settings such as organ transplantation. This paper aims to review current knowledge on the properties of MSCs and their use in pre-clinical and clinical studies in solid organ transplantation, and particularly in the field of liver transplantation.

IMMUNOMODULATORY EFFECTS OF MSCS

A large number of *in vitro* and *in vivo* studies have documented the anti-inflammatory and immunoregulatory properties of MSCs on both the adaptive and innate immune system. However, there is strong evidence that MSCs are not constitutively immunosuppressive, they have to be "activated" or primed by local inflammatory conditions. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β and interferon (IFN)-γ are the key cytokines to allow MSC immunomodulation by regulating their immunophenotype[10,11]. The high dependence on environment settings could also explain conflicting data in some *in vitro* and *in vivo* studies. These settings must be further studied and considered in clinical trials.

MSC immunogenicity

Both human MSCs (hMSCs) and murine MSCs (mMSCs) show low immunogenicity and do not lead to alloreactive T lymphocyte-mediated immune response *in vitro*. Indeed, under normal conditions, MSC membranes express low levels of human leukocyte antigen (HLA) class I molecules and do not express HLA class Ⅱ (major histocompatibility complex (MHC)-Ⅱ) nor co-stimulatory molecules[12,13]. MSCs were thus considered as immune privileged cells. However, more recent data with mMSCs has suggested that MHC-I on MSCs could present antigen to CD8+ T cells[14]. In addition, a narrow window of IFN-γ could induce MSCs to upregulate MHC-I and MHC-Ⅱ and thus, induces an "antigen presenting cell-like" function. This finding has been observed with both mMSCs and hMSCs[10,15-17]. Furthermore, it has been demonstrated in an animal model of bone marrow[18] and skin transplantation[19] that donor-derived MSCs could be immunogenic and could promote graft rejection.

MSC interaction with immune cells

It is important to highlight that, in some experimental conditions, effects of mMSCs and hMSCs have been evaluated on murine immune cells. Results are not always transposable to human clinical conditions, especially as it is well known that tolerance is more easily achieved in animal models than in humans.

It has been demonstrated *in vitro* and *in vivo*, that MSCs may exert their immunomodulatory effects by acting on many types of immune cells including T cells, B cells and natural killer (NK) cells. The ability of MSCs to inhibit T cell proliferation has been shown in various experimental settings both with mMSCs and hMSCs. *In vitro*, hMSCs highly inhibit proliferation and cytokine production[20] as well as the development of human cytotoxic CD8+ T cells in mixed-lymphocyte reactions (MLRs)[21,22]. Moreover, it has been observed that MSCs promote human T cell anergy and inhibit alloreactive T cells through a T_H2 pathway[23]. Nevertheless, it appears that the effect of MSCs on T cells is dependent on the dose used. While a high MSC/T cell ratio exert strong inhibitory effects, low MSC/T cell ratios enhance T cell proliferation[24].

MSC-induced T-regulatory (T-reg) cell recruitment and generation probably play an important role in MSC-mediated immunomodulatory effects. This has been observed both *in vitro*[25,26] and *in vivo*[27,28] both on murine and human immune cells. Additionally, previous studies have shown that T-reg induced production requires cell contact and some MSC released factors such as prostaglandin (PG)-E2 and tumor growth factor (TGF)-β1[29] or HLA-G[30,31]. It has been suggested that this effect could also be partially mediated by an interaction between MSC chemokine (c-c motif) ligand 1 (CCL1) and its receptor on T cells, chemokine (c-c motif) receptor 8 (CCR8)[23]. More recently, it has been demonstrated that mMSCs could promote T-reg expansion by their effects on immature dendritic cells[32].

Published results on the effects of hMSCs on B cells and NK cells are contradictory. Some studies have demonstrated that MSCs could inhibit the proliferation and immunoglobulin secretion of B cells[33-35] while others have found no effect of MSCs on human B cell proliferation[11,21]. Some researchers have even found that MSCs could stimulate human B cell proliferation and antibody secretion[36,37]. MSCs have shown an ability to inhibit the proliferation of IL-2 or IL-15 stimulated human NK

cells[38,39] and their IFN-γ production[38]. The effects of MSCs on the cytotoxic activity of NK cells are even more controverted. While some studies failed to find such an effect[40] (especially in freshly isolated NK cells[41]), others have demonstrated that MSCs could inhibit NK-cell cytotoxicity[30,39]. As MSCs express HLA-1 antigens, even at a low level, it appears that they may be vulnerable to activated NK-cell lysis[42].

Many studies have shown that MSCs can prevent the differentiation, maturation and functions of antigen-presenting cells (APCs), such as human or murine dendritic cells (DC)[17,43,44], and thus indirectly modulate T and B cell functions. In addition, it was shown that mMSCs may induce murine mature DC into a Jagged-2-dependent regulatory DC population[45]. MSCs may also exert effects on innate immune cells, for example through increased IL-10 secretion by macrophages in mice[46].

Mechanisms

The mechanisms of immunosuppression by MSCs remain unclear. Whereas MSCs exert their effect by direct cell contact *via* the expression of adhesion molecules, it has also been shown that the immunomodulatory and anti-inflammatory properties of MSCs mainly involve the production of secreted soluble factors. It has been observed that MSCs are still immunosuppressive without cell contact[22]. It should be noted that the mechanisms of MSC-mediated immunosuppression seems to vary from one species to another[47].

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyses the degradation of tryptophan. The resulting depletion of tryptophan and the accumulation of its metabolites have shown strong inhibitory properties on immune cells, including human T cells[48], activated B cells[11] and NK cells[39]. MSCs do not constitutively express IDO, but IDO can be upregulated under inflammatory conditions, for example after exposure to IFN-γ, TNF-α and IL-1[47,48]. IDO could play an important role regarding transplantation given that it has been shown to partially inhibit allo-responses of T cells *in vitro*, and to enhance tolerance towards the graft and allogeneic T cell transfer *in vivo*[49,50]. IDO seems to be predominant in human MSC-mediated immunomodulatory properties[47]. However IDO does not seem to be the only mechanism implicated as in some conditions where MSCs do not express IDO they keep their immunomodulatory properties[51]. A high concentration of nitric oxide (NO) is known to inhibit the immune response in both *in vitro* and *in vivo* studies. It has been shown to inhibit the proliferation of T cells in murine models. NO is synthetized by the inducible NO synthase (iNOS) that is induced in murine MSCs by interaction with CD4+ or CD8+ lymphocytes in inflammatory conditions involving IFN-γ and TNF-α or IL-1[52,53]. As in the case of IDO for human MSCs, iNOS appears to play a major role in murine MSC-mediated immunomodulation[47,52]. Both tryptophan depletion and NO are expected to have an exclusively local action[54,55].

The HLA-G protein is a non-classical human MHC-I molecule. Initially found in trophoblasts, where it plays a crucial role in maternal-fetal tolerance[56], HLA-G has recently been involved in immunomodulation by MSCs[57]. HLA-G has shown tolerogenic properties *inter alia* due to its interactions with inhibitory receptors on dendritic cells, NK, and T cells. Selmani *et al*[30] have demonstrated that hMSCs, by secreting the soluble isoform HLA-G5, are capable of inhibiting human allo-activated T lymphocytes, NK-cell cytolysis and IFN-gamma secretion, and of promoting the expansion of $CD4^+CD25^{high}FoxP3^+$ regulatory T cells. Likewise, HLA-G can promote $CD3^+CD4^{low}$ and $CD3^+$ $CD8^{low}$ immunosuppressive T cells. It seems that HLA-G expression is IL-10-dependent and needs close cell contact with alloreactive T cells[30]. It has been suggested that co-injection of HLA-G and MSCs could be used to prevent rejection in organ transplantation.

Another candidate mechanism involves the role of PGE2 (Prostaglandin E2) secreted by MSCs. It appears that MSC-derived PGE2 is involved in MSC-mediated immunomodulation by acting on murine and human T cells (in both TH1 and TH2 responses), NK cells and macrophages[46,58]. Prostaglandins have a short half-life. This suggests that they play their role using a paracrine or autocrine action mechanism. Furthermore, it has been observed in human MSCs that IDO and PGE2 have a synergistic inhibitory effect on T cell proliferation, and on the proliferation and cytotoxicity of NK cells[39,59]. However, other studies suggest that PGE2 could in fact have an immunostimulatory role by facilitating TH1 cell differentiation and TH17 cell expansion[60].

IL-10 plays an important role in MSC-mediated immunosuppression through the induction of IL-10 production in APCs[61]. Nevertheless, no direct secretion of IL-10 by MSCs has yet been proven.

Blocking each of these factors alone does not restore immune cell function and proliferation, indicating that multiple factors are involved.

Other factors are also secreted: TGF-β and Hepatocyte growth factor (HGF)[20] (inhibition T-lymphocyte proliferation), IL-1 receptor Antagonist[62] (anti-inflammatory), Peptide LL-37[63] (anti-inflammatory and antibacterial), Matrix Metalloproteinase (MMP) 3, MMP9[64] (acting on neoangiogenesis), angiopoietin-1[65] (acting on protein permeability). TNF-α and insulin-like growth factor-binding proteins[51] also seem to be implicated.

On the other hand, MSCs also have the ability to secrete pro-inflammatory chemokines and cytokines, such as monocyte chemo-attractant protein 1 (MCP-1 or CCL2)[66], IL-6, IL-8, soluble ICAM-1, Interferon gamma-induced protein 10 (IP-10 or CXCL10) and MCP-2 (or CCL8). The secretion of these factors is dependent on inflammatory conditions and could enhance immune response *via* immune cell attraction[67]. Therefore, MSCs appear to have a dual immunomodulatory capacity depending on the above-identified secreted factors.

The mechanisms involved in the immunomodula-

tory capacity of MSCs are complex and remain largely unknown. Their properties seem to be highly dependent on many parameters in which local immunologic conditions seem to play a crucial role. Finally, it is important to know that there is currently no single standard method to isolate MSCs. It is thus conceivable that changes in the culture medium used to increase and select MSC population may influence their properties.

TISSUE REPAIR/"ORGAN RECONSTRUCTION" EFFECT

In addition to their ability to differentiate into cells of the mesenchymal lineage, it has been demonstrated that MSCs can also differentiate *in vitro* into other cells such as neurons[68], cardiomyocytes[69], tubular epithelial cells in kidneys and hepatocytes[70-72]. They are also capable of differentiating and engrafting into many tissues, especially if an inflammatory signal is present[73]. These data have motivated further research in the field of MSCs as potential "tissue repairers". Cultured MSCs have shown strong evidence of "tissue repair" properties in response to tissue injury or disease in many animal models with myocardial infarction[74], kidney disease[75,76], lung injury or some neurological disorders[64]. In clinical trials, MSCs have been used successfully to treat bone and cartilage diseases[77] (*e.g.*, osteogenesis imperfecta), as well as acute and chronic myocardial infarction[78-80].

MSCs have shown the ability to home in on injured tissue after intravenous infusion. It has been demonstrated that MSCs can express several chemokine receptors such as CCR1, CCR7, CXCR4, CXCR6, CX3CR1[81], CCR4, CCR10, CXCR5[82], c-Kit, c-Met[83], VEGF receptors[84] and PDGF receptors[85]. This variety of receptors and the chemotactic migration they have shown in response to the stimulating chemokines and cytokines could partially explain their ability to migrate to sites of inflammation. This hypothesis assumes that the injured tissue also expresses specific receptors facilitating the adhesion and migration of MSCs. However, the exact mechanism of homing in on injured tissue remains largely unknown.

Nevertheless, many studies have observed that MSCs are significantly trapped in the lung after intravenous infusion[86,87]. Despite their ability to migrate to inflammation sites and to differentiate into many tissues, MSCs exhibit very low and transient levels of engraftment *in vivo*[86,88]. For example, in a mouse model of acute myocardial infarction, a significant improvement of myocardial function was observed after human MSC injection, while no donor cell could be detected 3 wk after infusion. In a rat model, no MSC could be found in the liver within 7 d after injection of syngeneic rat MSCs in recipient livers through the portal vein[89]. Contradictorily, in a clinical trial treating myocardial infarction with intracoronary injection of MSCs, the MSCs were still viable 3 mo after transplantation[90]. In another study, MSCs were detected in various tissues of baboons 19 mo after intravenous injection[88].

In fact, it is thought that MSCs are likely to act through the secretion of soluble factors and change of the tissue microenvironment with paracrine interactions, rather than through their transdifferentiation capacity[91,92]. However, current *in vivo* data are not sufficient to define the exact mechanism. It has been demonstrated that MSCs could facilitate tissue repair by stimulating angiogenesis[93] and inhibiting apoptosis, as well as fibrosis, in the site of injury[94].

Furthermore, there is much evidence supporting the protective effect of MSCs in acute kidney injury models[95]. It appears that MSCs could increase the proliferation of tubular cells and reduce apoptosis[96,97]. There is a lack of data on the treatment of liver injury with MSCs, but their properties and regenerative potential mentioned above have encouraged researchers and clinicians to investigate further in this field. They could play a therapeutic role in the replacement of diseased hepatocytes, and the stimulation of their regeneration through the action of trophic molecules[98].

In a study on acute liver injury, rats were successfully treated with MSC infusion, with a decrease of biochemical markers of liver injury and an improved survival rate. Hepatocyte replication was enhanced while apoptosis decreased by 90%[98]. Similarly, it has been demonstrated that MSCs are efficient in treating fulminant hepatic failure in rats[99]. Otherwise, it has been suggested that MSCs could only be efficient in a therapeutic window, indicating that higher doses could paradoxically be inefficient or even induce liver fibrosis[98].

Although it is hoped that MSCs could potentially be an alternative to liver transplantation in end-stage liver disease, or a potential temporary solution to maintaining liver conditions of patients waiting for a graft, MSCs have been tried in only a small number of clinical trials to treat cirrhosis.

In a phase I - II trial, 8 patients with end-stage liver cirrhosis were treated with the infusion of autologous MSCs *via* a peripheral or portal vein. The treatment was well tolerated, with no significant adverse effects and the liver function was significantly improved[100]. A randomized placebo-controlled trial using MSCs to treat decompensated cirrhosis has recently been published[101]. Out of 27 patients, 15 received autologous bone marrow MSCs *via* a peripheral vein and 12 received a placebo. The results were evaluated using the Model for End-Stage Liver Disease (MELD) score, Child-Pugh score, liver function tests and liver volume. In this study, there was no beneficial effect of MSC infusion in cirrhotic patients. It is clear that other studies with larger cohorts are necessary to clarify the therapeutic potential of MSCs in cirrhosis.

ANTI-OXIDATIVE EFFECT/TREATMENT OF ISCHEMIA REPERFUSION INJURY

Ischemia reperfusion injury (IRI) is caused by the blood supply returning into a tissue after an ischemic period.

This sudden reperfusion and oxygenation paradoxically impairs the endothelium with a dilatation in arterioles, increased fluid filtration and plasma protein extravasation from post-capillary venules, as well as an increased production of oxygen radicals and a reduction of nitric oxide generation. This imbalance leads to the release of inflammatory mediators (*e.g.*, TNF, platelet activating factor) and the expression of adhesion molecules that cause leukocyte adhesion to the endothelium[102]. This results in the stimulation of both innate and adaptive immune responses with an accumulation of immune cells, followed by organ damage. The release of danger-associated molecular patterns (DAMPs) and the complement system are also implicated[103].

Solid organ transplantation is impacted by IRI, which contributes to acute graft rejection, delayed graft function and enhanced immunogenicity. IRI represents a major concern in liver transplantation, and use of MSCs in IRI has been studied for solid organ transplantation in animal models and in clinical trials.

MSCs seem to be recruited by hypoxic and injured tissues that express adhesion molecules and a SDF-1 gradient stimulating CXCR4 and CXCR7 on these cells[104]. Furthermore, it has been demonstrated that MSCs can transmigrate through TNF-alpha activated endothelium to join the inflamed tissue[105]. Lately, Pan *et al*[106] found that the inactivation of the MEK/ERK signalling pathway by MSCs plays a major role in the improvement of hepatic IRI in rats.

Prevention and treatment of liver IRI in animal models

MSCs have shown therapeutic effects for the treatment of IRI in the kidney, heart and lung in a significant number of studies[107]. Only a few studies have been published for IRI in the liver, and the exact role of MSCs has not yet been defined.

Jin *et al*[108] recently evaluated the effect of allogeneic bone marrow (BM)-derived MSCs to attenuate IRI in rats during the first 24 h after liver reperfusion. In their model partial ischemia was obtained by vascular clamping during 60 min. BM-MSCs were injected through the portal vein. Injury severity, oxidative stress response and apoptosis of the liver was regularly evaluated during the first 24 h and compared to a sham-transplanted control group. The conclusion of this study is that allogeneic BM-MSCs partially protect the liver from IRI when injected *via* the portal vein due to their ability to suppress oxidative stress and to inhibit apoptosis. Another related model using adipose-derived MSC injections *via* a peripheral vein in mice also showed a significant protective effect against liver IRI[109].

In addition to liver IRI, research has also focused on the potential beneficial effect of MSCs in partial liver transplantation. In a recent study 50% reduced-size liver transplantations in rats were used to examine whether MSC-conditioned medium (MSC-CM) could protect hepatocytes and sinusoidal endothelial cells (SEC) and enhance their regeneration[110]. MSC-CM was injected in rats *via* a peripheral vein directly after orthotopic partial liver transplantation. Compared with the control group, the MSC-CM group showed a significantly lower release of liver injury biomarkers and a clear survival benefit. More proliferating hepatocytes and SECs, and less apoptosis were observed. Many inflammatory cytokine levels and the infiltration by neutrophils and Kupffer cell activation were decreased. VEGF and MMP-9 expression was increased in the graft. All these facts suggest that MSC-CM could have potential in prevention of liver injury, and to enhance its regeneration in partial liver transplant. Kanazawa *et al*[111] also found in a model of IRI with major hepatectomy that MSCs protected the liver from IRI and that liver regeneration was enhanced.

However, it has been demonstrated in a liver IRI model that intravenously injected MSCs are short-lived, that viable MSCs do not go beyond the lungs, and that they remain in the circulation for a very limited period[112]. It has thus been suggested that other cells should be implicated to mediate the powerful immunomodulatory and regenerative properties of MSCs on target organs.

POTENTIAL USE OF MSCS IN LIVER TRANSPLANTATION

Liver transplantation represents the unavoidable treatment of end-stage liver diseases. Despite satisfactory long-term results, transplantation success mostly relies on immunotolerance, *via* acceptable graft-host immune matches and immunosuppressive measures. The latter unfortunately exposes the patient to the classical consequences of a down-regulated immune system, such as opportunistic infections and the typical outbreak of neoplasms. Due to their immunomodulatory properties, MSCs could prove highly effective in obtaining sufficient immunotolerance to reach even higher success rates while avoiding excessive immunosuppression, and thus severe and life-threatening side effects.

MSCs as immunomodulation therapy in transplantation

MSCs for graft-*vs*-host disease after hematopoietic cell transplantation: A clinical success? Graft-*vs*-host disease (GVHD) is a major complication frequently observed after hematopoietic cell transplantation (HCT), resulting from the attack of recipient organs by donor lymphocytes. MSCs might play a role in the treatment of GVHD through their immunomodulatory effects rather than their regenerative properties. Although pre-clinical studies for the prevention or treatment of GVHD by MSCs gave rise to conflicting results, MSCs have shown a clear efficacy in clinical trials, especially in steroid-resistant GVHD[113]. In a phase Ⅱ study, 68% of patients with acute steroid-resistant GVHD showed a complete response to MSC infusion with a significant decrease in mortality[114]. A series of other studies have shown similar results with varying degrees of GVHD, suggesting that MSCs have a serious potential future in GVHD management[115-117].

MSCs in solid-organ transplantation

Animal models: MSC infusion has shown the ability to prolong graft survival in heart[118-120], skin[121] and kidney[122-124] animal transplantation models. However, one group found no effect of MSCs alone on heart allograft survival in a mouse model[125], and another group found that MSCs infused after kidney transplantation could cause premature graft dysfunction[122].

Only a few studies have been published in liver transplantation models. In one such study, it was demonstrated that adipose-derived MSCs significantly decreased acute rejection after orthotopic liver transplantation in rats[126], based on serum rejection markers and on hepatocyte apoptosis. Serum levels of IL-2 were reduced and those of IL-10 were increased. In this model, MSC were infused intravenously 7 d before and 3 d after liver transplantation as well as during the operation *via* the portal vein. MSCs also played a role in a discordant liver xenotransplant model by alleviating acute rejection[127].

Another group studied the ability of BM-MSC infusion to inhibit acute graft rejection after allogeneic liver transplantation in rats[128]. MSCs were derived from the recipient, the liver donor or a third party, and infused intravenously at the time of surgery as well as once daily for 3 d thereafter. MSC-treated recipients survived significantly longer compared with the control group. Furthermore, there was no significant difference between the 3 groups receiving MSCs from various origins. Histological analysis showed severe acute graft rejection at day 7 in rats without MSC infusion, while acute graft rejection was significantly decreased in the other groups. These observations were associated with a marked increase in the number of T-reg cells in recipients receiving MSCs. This suggests an important role of T-reg cells in MSC-mediated immunosuppression.

Available data in humans (kidney transplantation)

Results of a phase I clinical trial studying the treatment of allograft rejection after kidney transplantation by autologous BM-MSCs, have recently been published[129]. The MSC-based treatment was well-tolerated and no related serious adverse effects were reported. Two MSC infusions were performed after a biopsy-proven rejection or interstitial fibrosis/tubular atrophy (IF/TA). In this study, MSCs showed their ability to reduce IF/TA. In addition, a donor-specific down-regulation of the peripheral blood mononuclear cell proliferation was shown. However, a potentially increased susceptibility to opportunistic infections was observed, with the development of viral infections in 3 out of 6 MSC-treated patients.

In a randomized controlled trial in living donor kidney transplantation, Tan *et al*[130] demonstrated that, in comparison with antibody induction therapy, induction by autologous MSCs significantly correlated with fewer acute rejections, a lower risk of opportunistic infections and a better renal function at 1 mo. Furthermore, fewer adverse effects were seen in both autologous MSC groups compared to the control group. This study was conducted on 156 patients recruited from February 2008 to May 2009 and divided into 3 groups (group 1 and 2 received MSCs at kidney reperfusion and two weeks later, plus a standard dose or low dose of calcineurin inhibitors (CNIs), respectively. The control group received anti-IL-2 receptor antibody plus standard-dose CNIs.

In a pilot study, Perico *et al*[131] injected autologous BM-MSC in 2 living-related kidney transplant recipients at day 7 post-transplant, after induction therapy with basiliximab/low-dose thymoglobulin. The peripheral blood showed a progressive increase of the T-reg population and a strong inhibition of memory/effector CD8 T cell function/expansion, promoting a long-term tolerogenic environment compared with the control group. However, a few days after MSC infusion transient renal dysfunction was observed. A biopsy excluded graft rejection but revealed a focal inflammatory infiltrate with neutrophil and MSC recruitment as well as a complement-C3 deposition.

The same group also investigated pre-transplant infusion of autologous BM-MSCs in 2 living-related kidney transplant recipients[132]. No renal dysfunction was observed while MSC immunomodulatory properties were preserved. In addition, it was observed that the avoidance of basiliximab in induction therapy did not facilitate further T-reg expansion.

In another recent pilot study, six patients transplanted with living-donor related kidneys received 2 donor-derived BM-MSC infusions (the first at the time of transplantation, the second one month later) in combination with sparing doses of tacrolimus[133]. Six other patients were used as a control group and received standard doses of tacrolimus and no MSCs. The MSC-treated group had stable renal function 12 mo post-transplant despite reduced tacrolimus compared with the control group. No acute rejection occurred, except for one in the control group. Significantly increased B cell levels were observed in the MSC-treated group 3 mo after transplantation. No toxic side effects were associated with MSC infusion.

Ongoing clinical trials in liver transplantation

MSC Liege study: Taking advantage of our expertise and experience concerning the use of MSCs in the HCT context[115], and using an already functioning good manufacturing practice (GMP)-compliant laboratory able to produce clinical-grade MSCs, we initiated a first trial in 2011 exploring the safety and tolerability of third-party MSC infusions after kidney or liver transplantation in a prospective phase I - II study (NCT01429038).

In this study, after successful transplantation, 10 liver and 10 kidney transplant recipients under standard immunosuppressive treatment (tacrolimus, mycophenolate mofetil (MMF) and steroids) receive an intravenous infusion of 1.5×10^6/kg-3×10^6/kg of third-party MSCs on post-operative day 3 ± 2. These patients are prospectively compared to the same number of liver or kidney transplant recipients who meet inclusion criteria but have not not received MSC infusion. Safety is assessed by recording side effects, including opportunistic infections and

cancers. The immunosuppressive potential of MSCs will be evaluated by the rate of rejection episodes, graft/patient survivals, immunohistology of 3-mo (kidney) and 6-mo (liver) graft biopsies and *in vitro* evaluation of patient immune functions. In a second step, reduction (kidney) and progressive weaning (liver) of immunosuppression will be attempted in recipients who received MSCs. Final results are expected by the end of 2014. The next step will be to assert the immunosuppressive potential of MSCs after organ transplantation, and the opportunity to develop larger, randomised and controlled phase III trials.

"Mesenchymal stem cells in solid organ transplantation"-1 study: In a mesenchymal stem cells in solid organ transplantation phase I study (MiSOT-I) started in April 2013, the safety of MultiStem® infusion for immunomodulation after liver transplantation has been evaluated (NCT01841632). MultiStem is a new biological product derived from multipotent adult progenitor cells (MAPCs) which belong to the family of MSCs. Patients, divided into four cohorts, will receive 2 doses of MultiStem (first intraportal at liver transplantation, second at day 3 post-transplant) in addition to immunosuppression (calcineurin-inhibitor-free 'bottom-up' immunosuppressive regimen with basiliximab, mycophenolic acid, and steroids). From cohort 1 to 4, an increasing dose escalation is performed (3-6 patients in each group). The primary outcome will be infusional and acute toxicity (intraportal, pulmonary and systemic). The secondary outcomes will be biopsy-proven acute rejection, whether MultiStem promotes malignant transformationnor tumor growth, and the long-term safety of MultiStem administration (up to 6 years). Final results are expected in 2016.

The Beijing study

A third study is ongoing. This phase I study will include a total of 50 patients randomly assigned to two groups; in the first group, patients will receive conventional immunosuppressive agents plus umbilical cord (UC-) MSCs at the day of liver transplantation and then once every 4 wk, at a dose of 1×10^6 UC-MSCs/kg for 12 wk (NCT01690247). In the second group patients will receive conventional treatment plus a placebo. Both groups will be followed for 48 wk. The study will evaluate the incidence of acute rejection and early liver function recovery, as well as patient and graft survival rates, and the prevalence of adverse events as secondary outcomes.

VARIABLES TO BE CONSIDERED/ISSUES TO BE RESOLVED

At present many questions remain unanswered in the field of MSCs therapy in solid-organ transplantation. These issues could explain the conflicting data obtained in previous studies. Further *in vitro* investigations and pre-clinical studies could help to define the settings of future clinical trials through a better understanding of the mechanisms of action of MSCs.

Dosage and sources of MSCs

The ideal amount of MSCs necessary to achieve some clinical effect has not yet been studied, and additionally, the ideal source of MSCs in the setting of organ transplantation has not been determined. Usually isolated from the bone marrow, MSCs can now be isolated from other more easily accessible human tissues such as adipose tissue or cord-blood. Compared with BM-derived MSCs, adipose- and cord- derived MSCs have comparable phenotypical and immunomodulatory properties[134]. Nevertheless, it seems that many genes are differentially expressed in MSCs depending on their tissue origin[135]. These differences could alter the function of MSCs in clinical use.

Although not quite clear, it should be noted that MSCs derived from adipose tissue seem to be more likely to develop chromosomal abnormalities than BM-derived MSCs, after many passages in culture[136,137]. High-passage MSCs should thus be avoided for clinical applications.

Origin of MSCs- autologous vs allogeneic

MSCs can be isolated from the organ recipient (autologous) or from the organ donor, or from a third party (allogeneic).

While some have suggested that allogeneic MSCs may be more efficient as immunosuppressors[138], others have shown in animal models that donor-derived MSCs could be preferable[139]. In a recent study, it has been demonstrated that both autologous and allogeneic MSCs were able to inhibit alloreactivity and had comparable efficacy[22,127].

In terms of alloreactivity, MSCs appear to bear low immunogenicity (see above). In a clinical case of osteogenesis imperfecta, no sign of alloreactivity was observed in the recipient after infusion of fully mismatched allogeneic MSCs[140]. Yet some papers have reported the induction of memory T cell responses and immune rejection after allogeneic MSC infusion[18,141]. One cannot exclude that donor-derived MSCs could induce alloreactivity and accelerate graft rejection. Nevertheless, in the field of kidney transplantation, Crop *et al*[22] have demonstrated that donor-derived MSCs are not immune-rejected and are even able to inhibit alloreactivity in kidney transplant patients when infused before transplantation.

MSC interaction with immunosuppressive drugs

In clinical transplant studies, MSCs are used concomitantly with immunosuppressive drugs. As MSCs and immunosuppressive drugs inhibit the same targets (essentially T cells), it is reasonable to consider that interactions between them can occur. Therefore, it is essential to know which drugs can (positively or negatively) affect MSC function.

In vitro, some have shown that tacrolimus (a calcineurin inhibitor) and rapamycin (a mTOR inhibitor) decrease MSCs immunosuppressive properties[142], and conversely, that MSCs reduce the immunosuppressive capacities of tacrolimus and rapamycin. Such an effect has not been

found with mycophenolic acid (MPA). Moreover, a high dose of tacrolimus seems to be toxic for MSCs, while MPA and rapamycin at a therapeutic dose just inhibit MSC proliferation[143]. Nevertheless, others have shown that cyclosporine A (CsA) (another calcineurin inhibitor) and MSCs exert cumulative effects against alloactivated lymphocytes[138]. Furthermore, it has been demonstrated that MPA and MSCs have a synergistic immunosuppressive effect[143].

In vivo, MPA and MSCs also synergize to promote long-term allograft tolerance in rat heart transplantation[144]. In contrast to what is observed *in vitro*, rapamycin and MSCs synergize as immunomodulators to promote cardiac allograft long-term survival[119]. Moreover, in a rat renal transplantation model, it has been shown that CsA antagonizes MSC efficacy, and that this combination has no advantage in terms of allograft survival rates compared with CsA alone[122]. Nevertheless, this study has to be contrasted with other studies using various immunosuppressive drug used together with CsA in which MSC efficacy was not altered[19,110]. The choice of concomitant immunosuppressive drugs is an important matter for debate, and more studies are needed to define which are the most effective drugs to use with MSCs.

Timing of administration of MSCs

MSCs can be injected before, during or after transplantation, and with single or repeated injection(s). Timing of administration is another important point for discussion. It has been shown *in vivo* that pre-transplant infusion could be more effective than peri-transplant infusion in preventing graft rejection in a murine heart transplantation model[120]. On the other hand, it has been demonstrated that MSCs are effective in the treatment of steroid-resistant GVHD[113], so at the peak of the disease. In a clinical trial, Perico *et al*[131] observed that early post-transplant infusion of MSCs could induce a transient renal dysfunction. This group is now investigating pre-transplant infusions[132].

Protocols investigating timings of administration will probably have to be defined according to expected effects and drugs used concomitantly. Regarding liver transplantation, our group infuses MSCs at day 3 post-liver transplantation, while the MiSOT group performs 2 injections of MSCs at day 0 (intra operatively) and day 3 post-transplantation. In the Beijing study, an injection is performed on the day of liver transplantation and then once every 4 wk during a 12-wk period.

Administration route

In case of liver transplantation, MSCs can be injected through a peripheral vein or through intraportal infusion during surgery, or a combination of both. Intraportal infusion could be helpful in increasing the amount of MSCs homing to the liver. On the other hand, MSC homing behaviour to the inflammation site[69] could potentially concentrate them in the liver when intravenously infused after hepatic transplantation. However, some studies have observed that MSCs could be trapped in the lung after intravenous infusion[86,87]. Whatever the case, it is clear that to define the best route of administration, it is necessary to better understand the homing capacity of MSCs, and whether MSCs really require close contact with the target organ in order to be effective.

MSC side effects and safety

To date, no major adverse effects have been reported in the mid-term in the significant number of clinical trials using MSC-based therapy, for example in the context of BMT[113-117], solid-organ transplantation[129-133] and in many completed clinical trials for various therapeutic applications[145]. Only some studies have shown mild and transient adverse effects around the time of injection[145]. More experience is needed in order to confirm the long-term safety of MSCs.

To reach a sufficient number of cells for MSC-based therapy, *in vitro* expansion is needed. In this context, one of the major concerns is the potential risk of a neoplastic transformation of MSCs[122]. The occurrence of chromosomal aberrations is not uncommon after *in vitro* culture of mMSC, especially after long-term culture. It has been shown *in vivo* that these chromosomally unstable cells could transform into malignant cells with generation of tumors *in vivo*[146-148].

Contrary to mMSCs, *in vitro* expansion of hMSC seems to be far more stable and does not seem to generate genomic instability in these cells even after long-term culture. They do not transform into malignant cells after transplantation in mice[149,150]. Nevertheless, a French study observed the occurrence *in vitro* of transient chromosomal aberrations (aneuploidy) in twenty preparations of BM-MSCs obtained under GMP with two different culture processes. However these cells showed the same senescence as "normal" MSCs and did not lead to tumoral process after injection in immunocompromised mice[151]. Another study has found a high rate of human MSCs spontaneously transformed in malignant cells *in vivo*[152] but this has been strongly controverted suggesting a cross-contamination with cancerous cells[153]. Moreover, in two recent reviews analysing numerous studies, no evidence was found to affirm the potential of human MSCs for malignant transformation and so far, no risk of malignant transformation has been found in clinical use of hMSCs[149,154].

As MSCs are used as immunosuppressors, another concern is the potential emergence of opportunistic infections and induced cancers. In the case of solid organ transplantation with MSC-based immunosuppression, no increase risk of viral opportunistic infections has been observed so far-one group having even observed a decrease[130]. Nevertheless, another group reported viral opportunistic infections in three patients[129].

Interestingly, the MiSOT study group recently established a system to objectively score the potential emerging adverse effects related to MSC infusions (intravenous or intraportal infusion) after liver transplantation[155]. This

score is calculated using three parameters (pulmonary toxicity, intraportal-infusional toxicity and systemic toxicity), each of them receiving a score of 0 (no adverse events) to 3 (severe adverse events). It has been retrospectively validated on a cohort of 187 liver-transplanted patients not receiving MSCs as a control population. It has been suggested that this new tool could be helpful in assessing the safety of MSC use in solid organ transplantation.

CONCLUSION

The accumulating evidence shows that MSCs have immunosuppressive and reparative capacities *in vivo* and *in vitro*, as well as a potential beneficial effect in ischemia-reperfusion injury. These three principal properties suggest that MSCs could be interesting in liver transplantation to prevent or treat IRI, allograft dysfunction and graft rejection by inducing a durable tolerogenic environment. Using MSCs, and thereby removing or reducing the need for immunosuppressive drugs could avoid the serious side effects associated with these drugs.

Currently available data in clinic show that MSCs are safe to use, at least in the medium-term, but more time is needed to evaluate their potential adverse effects on the long-term. Caution is therefore recommended. Even if encouraging, the results of MSC use *in vitro* and *in vivo* (animals and humans) are sometimes contradictory. Nevertheless, negative results do not necessarily mean that MSCs are not effective in solid-organ transplantation, but rather that a countless number of still unknown (or poorly known) parameters may influence their effectiveness. At the same time, many issues must be resolved to optimize their use. Intensive *in vitro* and pre-clinical research is certainly the key to a better understanding of the way that MSCs act, and to eventually lead to clinical success.

REFERENCES

1 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13:** 4279-4295 [PMID: 12475952 DOI: 10.1091/mbc.E02-02-0105]

2 **Toma JG**, Akhavan M, Fernandes KJ, Barnabé-Heider F, Sadikot A, Kaplan DR, Miller FD. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001; **3:** 778-784 [PMID: 11533656 DOI: 10.1038/ncb0901-778]

3 **Hoogduijn MJ**, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, Weimar W, Baan CC. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. *Stem Cells Dev* 2007; **16:** 597-604 [PMID: 17784833 DOI: 10.1089/scd.2006.0110]

4 **Zhang Y**, Li C, Jiang X, Zhang S, Wu Y, Liu B, Tang P, Mao N. Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood CD34+ cells. *Exp Hematol* 2004; **32:** 657-664 [PMID: 15246162 DOI: 10.1016/j.exphem.2004.04.001]

5 **Erices A**, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000; **109:** 235-242 [PMID: 10848804]

6 **Fan CG**, Tang FW, Zhang QJ, Lu SH, Liu HY, Zhao ZM, Liu B, Han ZB, Han ZC. Characterization and neural differentiation of fetal lung mesenchymal stem cells. *Cell Transplant* 2005; **14:** 311-321 [PMID: 16052912]

7 **Campagnoli C**, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* 2001; **98:** 2396-2402 [PMID: 11588036]

8 **Young HE**, Mancini ML, Wright RP, Smith JC, Black AC, Reagan CR, Lucas PA. Mesenchymal stem cells reside within the connective tissues of many organs. *Dev Dyn* 1995; **202:** 137-144 [PMID: 7734732 DOI: 10.1002/aja.1002020205]

9 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8:** 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

10 **Marigo I**, Dazzi F. The immunomodulatory properties of mesenchymal stem cells. *Semin Immunopathol* 2011; **33:** 593-602 [PMID: 21499984 DOI: 10.1007/s00281-011-0267-7]

11 **Krampera M**, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006; **24:** 386-398 [PMID: 16123384 DOI: 10.1634/stemcells.2005-0008]

12 **Majumdar MK**, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, Mosca JD. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci* 2003; **10:** 228-241 [PMID: 12595759 DOI: 68710]

13 **Le Blanc K**, Tammik C, Rosendahl K, Zetterberg E, Ringdén O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003; **31:** 890-896 [PMID: 14550804]

14 **François M**, Romieu-Mourez R, Stock-Martineau S, Boivin MN, Bramson JL, Galipeau J. Mesenchymal stromal cells cross-present soluble exogenous antigens as part of their antigen-presenting cell properties. *Blood* 2009; **114:** 2632-2638 [PMID: 19654411 DOI: 10.1182/blood-2009-02-207795]

15 **Romieu-Mourez R**, François M, Boivin MN, Stagg J, Galipeau J. Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-gamma, TGF-beta, and cell density. *J Immunol* 2007; **179:** 1549-1558 [PMID: 17641021]

16 **Chan WK**, Lau AS, Li JC, Law HK, Lau YL, Chan GC. MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge. *Exp Hematol* 2008; **36:** 1545-1555 [PMID: 18715686 DOI: 10.1016/j.exphem.2008.06.008]

17 **Stagg J**, Pommey S, Eliopoulos N, Galipeau J. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2006; **107:** 2570-2577 [PMID: 16293599 DOI: 10.1182/blood-2005-07-2793]

18 **Nauta AJ**, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* 2006; **108:** 2114-2120 [PMID: 16690970 DOI: 10.1182/blood-2005-11-011650]

19 **Sbano P**, Cuccia A, Mazzanti B, Urbani S, Giusti B, Lapini I, Rossi L, Abbate R, Marseglia G, Nannetti G, Torricelli F, Miracco C, Bosi A, Fimiani M, Saccardi R. Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model. *Arch Dermatol Res* 2008; **300:** 115-124 [PMID: 18259766 DOI: 10.1007/s00403-007-0827-9]

20 **Di Nicola M**, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone

marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; **99**: 3838-3843 [PMID: 11986244]

21 **Angoulvant D**, Clerc A, Benchalal S, Galambrun C, Farre A, Bertrand Y, Eljaafari A. Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. *Biorheology* 2004; **41**: 469-476 [PMID: 15299278]

22 **Crop MJ**, Baan CC, Korevaar SS, Ijzermans JN, Alwayn IP, Weimar W, Hoogduijn MJ. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. *Transplantation* 2009; **87**: 896-906 [PMID: 19300194 DOI: 10.1097/TP.0b013e31819b3d72]

23 **Batten P**, Sarathchandra P, Antoniw JW, Tay SS, Lowdell MW, Taylor PM, Yacoub MH. Human mesenchymal stem cells induce T cell anergy and downregulate T cell allo-responses via the TH2 pathway: relevance to tissue engineering human heart valves. *Tissue Eng* 2006; **12**: 2263-2273 [PMID: 16968166 DOI: 10.1089/ten.2006.12.2263]

24 **Liu XJ**, Zhang JF, Sun B, Peng HS, Kong QF, Bai SS, Liu YM, Wang GY, Wang JH, Li HL. Reciprocal effect of mesenchymal stem cell on experimental autoimmune encephalomyelitis is mediated by transforming growth factor-beta and interleukin-6. *Clin Exp Immunol* 2009; **158**: 37-44 [PMID: 19737229 DOI: 10.1111/j.1365-2249.2009.03995.x]

25 **Di Ianni M**, Del Papa B, De Ioanni M, Moretti L, Bonifacio E, Cecchini D, Sportoletti P, Falzetti F, Tabilio A. Mesenchymal cells recruit and regulate T regulatory cells. *Exp Hematol* 2008; **36**: 309-318 [PMID: 18279718 DOI: 10.1016/j.exphem.2007.11.007]

26 **Ye Z**, Wang Y, Xie HY, Zheng SS. Immunosuppressive effects of rat mesenchymal stem cells: involvement of CD4+CD25+ regulatory T cells. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 608-614 [PMID: 19073406]

27 **Patel SA**, Meyer JR, Greco SJ, Corcoran KE, Bryan M, Rameshwar P. Mesenchymal stem cells protect breast cancer cells through regulatory T cells: role of mesenchymal stem cell-derived TGF-beta. *J Immunol* 2010; **184**: 5885-5894 [PMID: 20382885 DOI: 10.4049/jimmunol.0903143]

28 **Madec AM**, Mallone R, Afonso G, Abou Mrad E, Mesnier A, Eljaafari A, Thivolet C. Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. *Diabetologia* 2009; **52**: 1391-1399 [PMID: 19421731 DOI: 10.1007/s00125-009-1374-z]

29 **English K**, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP. Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. *Clin Exp Immunol* 2009; **156**: 149-160 [PMID: 19210524 DOI: 10.1111/j.1365-2249.2009.03874.x]

30 **Selmani Z**, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, Borg C, Saas P, Tiberghien P, Rouas-Freiss N, Carosella ED, Deschaseaux F. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. *Stem Cells* 2008; **26**: 212-222 [PMID: 17932417 DOI: 10.1634/stemcells.2007-0554]

31 **Rouas-Freiss N**, Naji A, Durrbach A, Carosella ED. Tolerogenic functions of human leukocyte antigen G: from pregnancy to organ and cell transplantation. *Transplantation* 2007; **84**: S21-S25 [PMID: 17632407 DOI: 10.1097/01.tp.0000269117.32179.1c]

32 **Choi YS**, Jeong JA, Lim DS. Mesenchymal stem cell-mediated immature dendritic cells induce regulatory T cell-based immunosuppressive effect. *Immunol Invest* 2012; **41**: 214-229 [PMID: 22017637 DOI: 10.3109/08820139.2011.619022]

33 **Comoli P**, Ginevri F, Maccario R, Avanzini MA, Marconi M, Groff A, Cometa A, Cioni M, Porretti L, Barberi W, Frassoni F, Locatelli F. Human mesenchymal stem cells inhibit antibody production induced in vitro by allostimulation. *Nephrol Dial Transplant* 2008; **23**: 1196-1202 [PMID: 18029377 DOI: 10.1093/ndt/gfm740]

34 **Corcione A**, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V, Uccelli A. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006; **107**: 367-372 [PMID: 16141348 DOI: 10.1182/blood-2005-07-2657]

35 **Tabera S**, Pérez-Simón JA, Díez-Campelo M, Sánchez-Abarca LI, Blanco B, López A, Benito A, Ocio E, Sánchez-Guijo FM, Cañizo C, San Miguel JF. The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. *Haematologica* 2008; **93**: 1301-1309 [PMID: 18641017 DOI: 10.3324/haematol.12857]

36 **Traggiai E**, Volpi S, Schena F, Gattorno M, Ferlito F, Moretta L, Martini A. Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. *Stem Cells* 2008; **26**: 562-569 [PMID: 18024418 DOI: 10.1634/stemcells.2007-0528]

37 **Rasmusson I**, Le Blanc K, Sundberg B, Ringdén O. Mesenchymal stem cells stimulate antibody secretion in human B cells. *Scand J Immunol* 2007; **65**: 336-343 [PMID: 17386024 DOI: 10.1111/j.1365-3083.2007.01905.x]

38 **Sotiropoulou PA**, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006; **24**: 74-85 [PMID: 16099998 DOI: 10.1634/stemcells.2004-0359]

39 **Spaggiari GM**, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008; **111**: 1327-1333 [PMID: 17951526 DOI: 10.1182/blood-2007-02-074997]

40 **Rasmusson I**, Ringdén O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation* 2003; **76**: 1208-1213 [PMID: 14578755 DOI: 10.1097/01.TP.0000082540.43730.80]

41 **Sotiropoulou PA**, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells* 2006; **24**: 462-471 [PMID: 16109759 DOI: 10.1634/stemcells.2004-0331]

42 **Spaggiari GM**, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 2006; **107**: 1484-1490 [PMID: 16239427 DOI: 10.1182/blood-2005-07-2775]

43 **Ramasamy R**, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F. Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation* 2007; **83**: 71-76 [PMID: 17220794 DOI: 10.1097/01.tp.0000244572.24780.54]

44 **Nauta AJ**, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. *J Immunol* 2006; **177**: 2080-2087 [PMID: 16887966]

45 **Zhang B**, Liu R, Shi D, Liu X, Chen Y, Dou X, Zhu X, Lu C, Liang W, Liao L, Zenke M, Zhao RC. Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population. *Blood* 2009; **113**: 46-57 [PMID: 18832657 DOI: 10.1182/blood-2008-04-154138]

46 **Németh K**, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; **15**: 42-49 [PMID: 19098906 DOI: 10.1038/nm.1905]

47 **Ren G**, Su J, Zhang L, Zhao X, Ling W, L'huillie A, Zhang J, Lu Y, Roberts AI, Ji W, Zhang H, Rabson AB, Shi Y. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells* 2009; **27**: 1954-1962 [PMID: 19544427 DOI: 10.1002/stem.118]

48 **Meisel R**, Zibert A, Laryea M, Göbel U, Däubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004; **103**: 4619-4621 [PMID: 15001472 DOI: 10.1182/blood-2003-11-3909]

49 **Hainz U**, Jürgens B, Heitger A. The role of indoleamine 2,3-dioxygenase in transplantation. *Transpl Int* 2007; **20**: 118-127 [PMID: 17239019 DOI: 10.1111/j.1432-2277.2006.00370.x]

50 **Bauer TM**, Jiga LP, Chuang JJ, Randazzo M, Opelz G, Terness P. Studying the immunosuppressive role of indoleamine 2,3-dioxygenase: tryptophan metabolites suppress rat allogeneic T-cell responses in vitro and in vivo. *Transpl Int* 2005; **18**: 95-100 [PMID: 15612990 DOI: 10.1111/j.1432-2277.2004.00031.x]

51 **Gieseke F**, Schütt B, Viebahn S, Koscielniak E, Friedrich W, Handgretinger R, Müller I. Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFNgammaR1 signaling and IDO expression. *Blood* 2007; **110**: 2197-2200 [PMID: 17522338 DOI: 10.1182/blood-2007-04-083162]

52 **Sato K**, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, Muroi K, Ozawa K. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 2007; **109**: 228-234 [PMID: 16985180 DOI: 10.1182/blood-2006-02-002246]

53 **Ren G**, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008; **2**: 141-150 [PMID: 18371435 DOI: 10.1016/j.stem.2007.11.014]

54 **Mellor AL**, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 2004; **4**: 762-774 [PMID: 15459668 DOI: 10.1038/nri1457]

55 **Ignarro LJ**, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987; **84**: 9265-9269 [PMID: 2827174]

56 **Carosella ED**, Moreau P, Le Maoult J, Le Discorde M, Dausset J, Rouas-Freiss N. HLA-G molecules: from maternal-fetal tolerance to tissue acceptance. *Adv Immunol* 2003; **81**: 199-252 [PMID: 14711057]

57 **Nasef A**, Mathieu N, Chapel A, Frick J, François S, Mazurier C, Boutarfa A, Bouchet S, Gorin NC, Thierry D, Fouillard L. Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation* 2007; **84**: 231-237 [PMID: 17667815 DOI: 10.1097/01.tp.0000267918.07906.08]

58 **Aggarwal S**, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; **105**: 1815-1822 [PMID: 15494428 DOI: 10.1182/blood-2004-04-1559]

59 **Matysiak M**, Orlowski W, Fortak-Michalska M, Jurewicz A, Selmaj K. Immunoregulatory function of bone marrow mesenchymal stem cells in EAE depends on their differentiation state and secretion of PGE2. *J Neuroimmunol* 2011; **233**: 106-111 [PMID: 21354631 DOI: 10.1016/j.jneuroim.2010.12.004]

60 **Yao C**, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, Narumiya S. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. *Nat Med* 2009; **15**: 633-640 [PMID: 19465928 DOI: 10.1038/nm.1968]

61 **Yang SH**, Park MJ, Yoon IH, Kim SY, Hong SH, Shin JY, Nam HY, Kim YH, Kim B, Park CG. Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10. *Exp Mol Med* 2009; **41**: 315-324 [PMID: 19307751 DOI: 10.3858/emm.2009.41.5.035]

62 **Ortiz LA**, Dutreil M, Fattman C, Pandey AC, Torres G, Go K, Phinney DG. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci USA* 2007; **104**: 11002-11007 [PMID: 17569781 DOI: 10.1073/pnas.0704421104]

63 **Krasnodembskaya A**, Song Y, Fang X, Gupta N, Serikov V, Lee JW, Matthay MA. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010; **28**: 2229-2238 [PMID: 20945332 DOI: 10.1002/stem.544]

64 **Kim Y**, Kim H, Cho H, Bae Y, Suh K, Jung J. Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. *Cell Physiol Biochem* 2007; **20**: 867-876 [PMID: 17982269 DOI: 10.1159/000110447]

65 **Fang X**, Neyrinck AP, Matthay MA, Lee JW. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. *J Biol Chem* 2010; **285**: 26211-26222 [PMID: 20554518 DOI: 10.1074/jbc.M110.119917]

66 **Shi C**, Jia T, Mendez-Ferrer S, Hohl TM, Serbina NV, Lipuma L, Leiner I, Li MO, Frenette PS, Pamer EG. Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. *Immunity* 2011; **34**: 590-601 [PMID: 21458307 DOI: 10.1016/j.immuni.2011.02.016]

67 **Hoogduijn MJ**, Popp F, Verbeek R, Masoodi M, Nicolaou A, Baan C, Dahlke MH. The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. *Int Immunopharmacol* 2010; **10**: 1496-1500 [PMID: 20619384 DOI: 10.1016/j.intimp.2010.06.019]

68 **Tropel P**, Platet N, Platel JC, Noël D, Albrieux M, Benabid AL, Berger F. Functional neuronal differentiation of bone marrow-derived mesenchymal stem cells. *Stem Cells* 2006; **24**: 2868-2876 [PMID: 16902198 DOI: 10.1634/stemcells.2005-0636]

69 **Rose RA**, Keating A, Backx PH. Do mesenchymal stromal cells transdifferentiate into functional cardiomyocytes? *Circ Res* 2008; **103**: e120 [PMID: 18948624 DOI: 10.1161/CIRCRESAHA.108.186908]

70 **Taléns-Visconti R**, Bonora A, Jover R, Mirabet V, Carbonell F, Castell JV, Gómez-Lechón MJ. Human mesenchymal stem cells from adipose tissue: Differentiation into hepatic lineage. *Toxicol In Vitro* 2007; **21**: 324-329 [PMID: 17045453 DOI: 10.1016/j.tiv.2006.08.009]

71 **Seo MJ**, Suh SY, Bae YC, Jung JS. Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. *Biochem Biophys Res Commun* 2005; **328**: 258-264 [PMID: 15670778 DOI: 10.1016/j.bbrc.2004.12.158]

72 **Schwartz RE**, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, Lenvik T, Johnson S, Hu WS, Verfaillie CM. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* 2002; **109**: 1291-1302 [PMID: 12021244 DOI: 10.1172/JCI15182]

73 **Sordi V**. Mesenchymal stem cell homing capacity. *Transplantation* 2009; **87**: S42-S45 [PMID: 19424004 DOI: 10.1097/TP.0b013e3181a28533]

74 **Minguell JJ**, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. *Exp Biol Med (Maywood)* 2006; **231**: 39-49 [PMID: 16380643]

75 **Kunter U**, Rong S, Djuric Z, Boor P, Müller-Newen G, Yu D, Floege J. Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. *J Am Soc Nephrol* 2006; **17**: 2202-2212 [PMID: 16790513 DOI: 10.1681/ASN.2005080815]

76 **Herrera MB**, Bussolati B, Bruno S, Fonsato V, Romanazzi GM, Camussi G. Mesenchymal stem cells contribute to the

renal repair of acute tubular epithelial injury. *Int J Mol Med* 2004; **14:** 1035-1041 [PMID: 15547670]

77 **Horwitz EM**, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999; **5:** 309-313 [PMID: 10086387 DOI: 10.1038/6529]

78 **Zeinaloo A**, Zanjani KS, Bagheri MM, Mohyeddin-Bonab M, Monajemzadeh M, Arjmandnia MH. Intracoronary administration of autologous mesenchymal stem cells in a critically ill patient with dilated cardiomyopathy. *Pediatr Transplant* 2011; **15:** E183-E186 [PMID: 20880092 DOI: 10.1111/j.1399-3046.2010.01366.x]

79 **Yang Z**, Zhang F, Ma W, Chen B, Zhou F, Xu Z, Zhang Y, Zhang D, Zhu T, Wang L, Wang H, Ding Z, Zhang Y. A novel approach to transplanting bone marrow stem cells to repair human myocardial infarction: delivery via a noninfarct-relative artery. *Cardiovasc Ther* 2010; **28:** 380-385 [PMID: 20337639 DOI: 10.1111/j.1755-5922.2009.00116.x]

80 **Katritsis DG**, Sotiropoulou PA, Karvouni E, Karabinos I, Korovesis S, Perez SA, Voridis EM, Papamichail M. Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. *Catheter Cardiovasc Interv* 2005; **65:** 321-329 [PMID: 15954106 DOI: 10.1002/ccd.20406]

81 **Sordi V**, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, Belmonte N, Ferrari G, Leone BE, Bertuzzi F, Zerbini G, Allavena P, Bonifacio E, Piemonti L. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 2005; **106:** 419-427 [PMID: 15784733 DOI: 10.1182/blood-2004-09-3507]

82 **Von Lüttichau I**, Notohamiprodjo M, Wechselberger A, Peters C, Henger A, Seliger C, Djafarzadeh R, Huss R, Nelson PJ. Human adult CD34- progenitor cells functionally express the chemokine receptors CCR1, CCR4, CCR7, CXCR5, and CCR10 but not CXCR4. *Stem Cells Dev* 2005; **14:** 329-336 [PMID: 15969628 DOI: 10.1089/scd.2005.14.329]

83 **Forte G**, Minieri M, Cossa P, Antenucci D, Sala M, Gnocchi V, Fiaccavento R, Carotenuto F, De Vito P, Baldini PM, Prat M, Di Nardo P. Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. *Stem Cells* 2006; **24:** 23-33 [PMID: 16100005 DOI: 10.1634/stemcells.2004-0176]

84 **Ball SG**, Shuttleworth CA, Kielty CM. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J Cell Biol* 2007; **177:** 489-500 [PMID: 17470632 DOI: 10.1083/jcb.200608093]

85 **Fiedler J**, Röderer G, Günther KP, Brenner RE. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. *J Cell Biochem* 2002; **87:** 305-312 [PMID: 12397612 DOI: 10.1002/jcb.10309]

86 **Barbash IM**, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH, Kloner RA, Leor J. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* 2003; **108:** 863-868 [PMID: 12900340 DOI: 10.1161/01.CIR.0000084828.50310.6A]

87 **Mahmood A**, Lu D, Lu M, Chopp M. Treatment of traumatic brain injury in adult rats with intravenous administration of human bone marrow stromal cells. *Neurosurgery* 2003; **53:** 697-702; discussion 702-3 [PMID: 12943585]

88 **Devine SM**, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T, Stock W, Sher D, Weissman S, Ferrer K, Mosca J, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol* 2001; **29:** 244-255 [PMID: 11166464]

89 **Popp FC**, Slowik P, Eggenhofer E, Renner P, Lang SA, Stoeltzing O, Geissler EK, Piso P, Schlitt HJ, Dahlke MH. No contribution of multipotent mesenchymal stromal cells to liver regeneration in a rat model of prolonged hepatic injury. *Stem Cells* 2007; **25:** 639-645 [PMID: 17110617 DOI: 10.1634/stemcells.2006-0515]

90 **Chen SL**, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, Zhang JJ, Chunhua RZ, Liao LM, Lin S, Sun JP. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* 2004; **94:** 92-95 [PMID: 15219514 DOI: 10.1016/j.amjcard.2004.03.034]

91 **Prockop DJ**. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). *Clin Pharmacol Ther* 2007; **82:** 241-243 [PMID: 17700588 DOI: 10.1038/sj.clpt.6100313]

92 **Iso Y**, Spees JL, Serrano C, Bakondi B, Pochampally R, Song YH, Sobel BE, Delafontaine P, Prockop DJ. Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. *Biochem Biophys Res Commun* 2007; **354:** 700-706 [PMID: 17257581 DOI: 10.1016/j.bbrc.2007.01.045]

93 **Kachgal S**, Putnam AJ. Mesenchymal stem cells from adipose and bone marrow promote angiogenesis via distinct cytokine and protease expression mechanisms. *Angiogenesis* 2011; **14:** 47-59 [PMID: 21104120 DOI: 10.1007/s10456-010-9194-9]

94 **Caplan AI**. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009; **217:** 318-324 [PMID: 19023885 DOI: 10.1002/path.2469]

95 **Erpicum P**, Detry O, Weekers L, Bonvoisin C, Lechanteur C, Briquet A, Beguin Y, Krzesinski JM, Jouret F. Mesenchymal stromal cell therapy in conditions of renal ischaemia/reperfusion. *Nephrol Dial Transplant* 2014; **29:** 1487-1493 [PMID: 24516234 DOI: 10.1093/ndt/gft538]

96 **Tögel FE**, Westenfelder C. Mesenchymal stem cells: a new therapeutic tool for AKI. *Nat Rev Nephrol* 2010; **6:** 179-183 [PMID: 20186233 DOI: 10.1038/nrneph.2009.229]

97 **Donizetti-Oliveira C**, Semedo P, Burgos-Silva M, Cenedeze MA, Malheiros DM, Reis MA, Pacheco-Silva A, Câmara NO. Adipose tissue-derived stem cell treatment prevents renal disease progression. *Cell Transplant* 2012; **21:** 1727-1741 [PMID: 22305061 DOI: 10.3727/096368911X623925]

98 **van Poll D**, Parekkadan B, Cho CH, Berthiaume F, Nahmias Y, Tilles AW, Yarmush ML. Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. *Hepatology* 2008; **47:** 1634-1643 [PMID: 18395843 DOI: 10.1002/hep.22236]

99 **Parekkadan B**, van Poll D, Suganuma K, Carter EA, Berthiaume F, Tilles AW, Yarmush ML. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. *PLoS One* 2007; **2:** e941 [PMID: 17895982 DOI: 10.1371/journal.pone.0000941]

100 **Kharaziha P**, Hellström PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, Telkabadi M, Atashi A, Honardoost M, Zali MR, Soleimani M. Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I-II clinical trial. *Eur J Gastroenterol Hepatol* 2009; **21:** 1199-1205 [PMID: 19455046 DOI: 10.1097/MEG.0b013e32832a1f6c]

101 **Mohamadnejad M**, Alimoghaddam K, Bagheri M, Ashrafi M, Abdollahzadeh L, Akhlaghpoor S, Bashtar M, Ghavamzadeh A, Malekzadeh R. Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis. *Liver Int* 2013; **33:** 1490-1496 [PMID: 23763455 DOI: 10.1111/liv.12228]

102 **Carden DL**, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol* 2000; **190:** 255-266 [PMID: 10685060 DOI: 10.1002/(SICI)1096-9896(200002)190]

103 **Eltzschig HK**, Eckle T. Ischemia and reperfusion--from

renal repair of acute tubular epithelial injury. *Int J Mol Med* 2004; **14:** 1035-1041 [PMID: 15547670]

77 **Horwitz EM**, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999; **5:** 309-313 [PMID: 10086387 DOI: 10.1038/6529]

78 **Zeinaloo A**, Zanjani KS, Bagheri MM, Mohyeddin-Bonab M, Monajemzadeh M, Arjmandnia MH. Intracoronary administration of autologous mesenchymal stem cells in a critically ill patient with dilated cardiomyopathy. *Pediatr Transplant* 2011; **15:** E183-E186 [PMID: 20880092 DOI: 10.1111/j.1399-3046.2010.01366.x]

79 **Yang Z**, Zhang F, Ma W, Chen B, Zhou F, Xu Z, Zhang Y, Zhang D, Zhu T, Wang L, Wang H, Ding Z, Zhang Y. A novel approach to transplanting bone marrow stem cells to repair human myocardial infarction: delivery via a noninfarct-relative artery. *Cardiovasc Ther* 2010; **28:** 380-385 [PMID: 20337639 DOI: 10.1111/j.1755-5922.2009.00116.x]

80 **Katritsis DG**, Sotiropoulou PA, Karvouni E, Karabinos I, Korovesis S, Perez SA, Voridis EM, Papamichail M. Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. *Catheter Cardiovasc Interv* 2005; **65:** 321-329 [PMID: 15954106 DOI: 10.1002/ccd.20406]

81 **Sordi V**, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, Belmonte N, Ferrari G, Leone BE, Bertuzzi F, Zerbini G, Allavena P, Bonifacio E, Piemonti L. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 2005; **106:** 419-427 [PMID: 15784733 DOI: 10.1182/blood-2004-09-3507]

82 **Von Lüttichau I**, Notohamiprodjo M, Wechselberger A, Peters C, Henger A, Seliger C, Djafarzadeh R, Huss R, Nelson PJ. Human adult CD34- progenitor cells functionally express the chemokine receptors CCR1, CCR4, CCR7, CXCR5, and CCR10 but not CXCR4. *Stem Cells Dev* 2005; **14:** 329-336 [PMID: 15969628 DOI: 10.1089/scd.2005.14.329]

83 **Forte G**, Minieri M, Cossa P, Antenucci D, Sala M, Gnocchi V, Fiaccavento R, Carotenuto F, De Vito P, Baldini PM, Prat M, Di Nardo P. Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. *Stem Cells* 2006; **24:** 23-33 [PMID: 16100005 DOI: 10.1634/stemcells.2004-0176]

84 **Ball SG**, Shuttleworth CA, Kielty CM. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *J Cell Biol* 2007; **177:** 489-500 [PMID: 17470632 DOI: 10.1083/jcb.200608093]

85 **Fiedler J**, Röderer G, Günther KP, Brenner RE. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. *J Cell Biochem* 2002; **87:** 305-312 [PMID: 12397612 DOI: 10.1002/jcb.10309]

86 **Barbash IM**, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH, Kloner RA, Leor J. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* 2003; **108:** 863-868 [PMID: 12900340 DOI: 10.1161/01.CIR.0000084828.50310.6A]

87 **Mahmood A**, Lu D, Lu M, Chopp M. Treatment of traumatic brain injury in adult rats with intravenous administration of human bone marrow stromal cells. *Neurosurgery* 2003; **53:** 697-702; discussion 702-3 [PMID: 12943585]

88 **Devine SM**, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T, Stock W, Sher D, Weissman S, Ferrer K, Mosca J, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells are capable of homing to the bone marrow of non-human primates following systemic infusion. *Exp Hematol* 2001; **29:** 244-255 [PMID: 11166464]

89 **Popp FC**, Slowik P, Eggenhofer E, Renner P, Lang SA, Stoeltzing O, Geissler EK, Piso P, Schlitt HJ, Dahlke MH. No contribution of multipotent mesenchymal stromal cells to liver regeneration in a rat model of prolonged hepatic injury. *Stem Cells* 2007; **25:** 639-645 [PMID: 17110617 DOI: 10.1634/stemcells.2006-0515]

90 **Chen SL**, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, Zhang JJ, Chunhua RZ, Liao LM, Lin S, Sun JP. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* 2004; **94:** 92-95 [PMID: 15219514 DOI: 10.1016/j.amjcard.2004.03.034]

91 **Prockop DJ**. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). *Clin Pharmacol Ther* 2007; **82:** 241-243 [PMID: 17700588 DOI: 10.1038/sj.clpt.6100313]

92 **Iso Y**, Spees JL, Serrano C, Bakondi B, Pochampally R, Song YH, Sobel BE, Delafontaine P, Prockop DJ. Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. *Biochem Biophys Res Commun* 2007; **354:** 700-706 [PMID: 17257581 DOI: 10.1016/j.bbrc.2007.01.045]

93 **Kachgal S**, Putnam AJ. Mesenchymal stem cells from adipose and bone marrow promote angiogenesis via distinct cytokine and protease expression mechanisms. *Angiogenesis* 2011; **14:** 47-59 [PMID: 21104120 DOI: 10.1007/s10456-010-9194-9]

94 **Caplan AI**. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009; **217:** 318-324 [PMID: 19023885 DOI: 10.1002/path.2469]

95 **Erpicum P**, Detry O, Weekers L, Bonvoisin C, Lechanteur C, Briquet A, Beguin Y, Krzesinski JM, Jouret F. Mesenchymal stromal cell therapy in conditions of renal ischaemia/reperfusion. *Nephrol Dial Transplant* 2014; **29:** 1487-1493 [PMID: 24516234 DOI: 10.1093/ndt/gft538]

96 **Tögel FE**, Westenfelder C. Mesenchymal stem cells: a new therapeutic tool for AKI. *Nat Rev Nephrol* 2010; **6:** 179-183 [PMID: 20186233 DOI: 10.1038/nrneph.2009.229]

97 **Donizetti-Oliveira C**, Semedo P, Burgos-Silva M, Cenedeze MA, Malheiros DM, Reis MA, Pacheco-Silva A, Câmara NO. Adipose tissue-derived stem cell treatment prevents renal disease progression. *Cell Transplant* 2012; **21:** 1727-1741 [PMID: 22305061 DOI: 10.3727/096368911X623925]

98 **van Poll D**, Parekkadan B, Cho CH, Berthiaume F, Nahmias Y, Tilles AW, Yarmush ML. Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and in vivo. *Hepatology* 2008; **47:** 1634-1643 [PMID: 18395843 DOI: 10.1002/hep.22236]

99 **Parekkadan B**, van Poll D, Suganuma K, Carter EA, Berthiaume F, Tilles AW, Yarmush ML. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. *PLoS One* 2007; **2:** e941 [PMID: 17895982 DOI: 10.1371/journal.pone.0000941]

100 **Kharaziha P**, Hellström PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, Telkabadi M, Atashi A, Honardoost M, Zali MR, Soleimani M. Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I-II clinical trial. *Eur J Gastroenterol Hepatol* 2009; **21:** 1199-1205 [PMID: 19455046 DOI: 10.1097/MEG.0b013e32832a1f6c]

101 **Mohamadnejad M**, Alimoghaddam K, Bagheri M, Ashrafi M, Abdollahzadeh L, Akhlaghpoor S, Bashtar M, Ghavamzadeh A, Malekzadeh R. Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis. *Liver Int* 2013; **33:** 1490-1496 [PMID: 23763455 DOI: 10.1111/liv.12228]

102 **Carden DL**, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol* 2000; **190:** 255-266 [PMID: 10685060 DOI: 10.1002/(SICI)1096-9896(200002)190]

103 **Eltzschig HK**, Eckle T. Ischemia and reperfusion--from

mechanism to translation. *Nat Med* 2011; **17**: 1391-1401 [PMID: 22064429 DOI: 10.1038/nm.2507]
104 **Liu H**, Liu S, Li Y, Wang X, Xue W, Ge G, Luo X. The role of SDF-1-CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stem cells for renal ischemia/reperfusion injury. *PLoS One* 2012; **7**: e34608 [PMID: 22511954 DOI: 10.1371/journal.pone.0034608]
105 **Teo GS**, Ankrum JA, Martinelli R, Boetto SE, Simms K, Sciuto TE, Dvorak AM, Karp JM, Carman CV. Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells via both leukocyte-like and novel mechanisms. *Stem Cells* 2012; **30**: 2472-2486 [PMID: 22887987 DOI: 10.1002/stem.1198]
106 **Pan GZ**, Yang Y, Zhang J, Liu W, Wang GY, Zhang YC, Yang Q, Zhai FX, Tai Y, Liu JR, Zhang Q, Chen GH. Bone marrow mesenchymal stem cells ameliorate hepatic ischemia/reperfusion injuries via inactivation of the MEK/ERK signaling pathway in rats. *J Surg Res* 2012; **178**: 935-948 [PMID: 22658855 DOI: 10.1016/j.jss.2012.04.070]
107 **Souidi N**, Stolk M, Seifert M. Ischemia-reperfusion injury: beneficial effects of mesenchymal stromal cells. *Curr Opin Organ Transplant* 2013; **18**: 34-43 [PMID: 23254704 DOI: 10.1097/MOT.0b013e32835c2a05]
108 **Jin G**, Qiu G, Wu D, Hu Y, Qiao P, Fan C, Gao F. Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats. *Int J Mol Med* 2013; **31**: 1395-1401 [PMID: 23589072 DOI: 10.3892/ijmm.2013.1340]
109 **Sun CK**, Chang CL, Lin YC, Kao YH, Chang LT, Yen CH, Shao PL, Chen CH, Leu S, Yip HK. Systemic administration of autologous adipose-derived mesenchymal stem cells alleviates hepatic ischemia-reperfusion injury in rats. *Crit Care Med* 2012; **40**: 1279-1290 [PMID: 22336724 DOI: 10.1097/CCM.0b013e31823dae23]
110 **Du Z**, Wei C, Cheng K, Han B, Yan J, Zhang M, Peng C, Liu Y. Mesenchymal stem cell-conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation. *J Surg Res* 2013; **183**: 907-915 [PMID: 23522455 DOI: 10.1016/j.jss.2013.02.009]
111 **Kanazawa H**, Fujimoto Y, Teratani T, Iwasaki J, Kasahara N, Negishi K, Tsuruyama T, Uemoto S, Kobayashi E. Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. *PLoS One* 2011; **6**: e19195 [PMID: 21559442 DOI: 10.1371/journal.pone.0019195]
112 **Eggenhofer E**, Benseler V, Kroemer A, Popp FC, Geissler EK, Schlitt HJ, Baan CC, Dahlke MH, Hoogduijn MJ. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol* 2012; **3**: 297 [PMID: 23056000 DOI: 10.3389/fimmu.2012.00297]
113 **Baron F**, Storb R. Mesenchymal stromal cells: a new tool against graft-versus-host disease? *Biol Blood Marrow Transplant* 2012; **18**: 822-840 [PMID: 21963621 DOI: 10.1016/j.bbmt.2011.09.003]
114 **Le Blanc K**, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008; **371**: 1579-1586 [PMID: 18468541 DOI: 10.1016/S0140-6736(08)60690-X]
115 **Baron F**, Lechanteur C, Willems E, Bruck F, Baudoux E, Seidel L, Vanbellinghen JF, Hafraoui K, Lejeune M, Gothot A, Fillet G, Beguin Y. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. *Biol Blood Marrow Transplant* 2010; **16**: 838-847 [PMID: 20109568 DOI: 10.1016/j.bbmt.2010.01.011]
116 **Fang B**, Song Y, Liao L, Zhang Y, Zhao RC. Favorable response to human adipose tissue-derived mesenchymal stem cells in steroid-refractory acute graft-versus-host disease. *Transplant Proc* 2007; **39**: 3358-3362 [PMID: 18089385 DOI: 10.1016/j.transproceed.2007.08.103]
117 **Prasad VK**, Lucas KG, Kleiner GI, Talano JA, Jacobsohn D, Broadwater G, Monroy R, Kurtzberg J. Efficacy and safety of ex vivo cultured adult human mesenchymal stem cells (Prochymal™) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. *Biol Blood Marrow Transplant* 2011; **17**: 534-541 [PMID: 20457269 DOI: 10.1016/j.bbmt.2010.04.014]
118 **Zhou HP**, Yi DH, Yu SQ, Sun GC, Cui Q, Zhu HL, Liu JC, Zhang JZ, Wu TJ. Administration of donor-derived mesenchymal stem cells can prolong the survival of rat cardiac allograft. *Transplant Proc* 2006; **38**: 3046-3051 [PMID: 17112896 DOI: 10.1016/j.transproceed.2006.10.002]
119 **Ge W**, Jiang J, Baroja ML, Arp J, Zassoko R, Liu W, Bartholomew A, Garcia B, Wang H. Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance. *Am J Transplant* 2009; **9**: 1760-1772 [PMID: 19563344 DOI: 10.1111/j.1600-6143.2009.02721.x]
120 **Casiraghi F**, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, Cavinato RA, Todeschini M, Solini S, Sonzogni A, Perico N, Remuzzi G, Noris M. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. *J Immunol* 2008; **181**: 3933-3946 [PMID: 18768848]
121 **Bartholomew A**, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; **30**: 42-48 [PMID: 11823036]
122 **Casiraghi F**, Azzollini N, Todeschini M, Cavinato RA, Cassis P, Solini S, Rota C, Morigi M, Introna M, Maranta R, Perico N, Remuzzi G, Noris M. Localization of mesenchymal stromal cells dictates their immune or proinflammatory effects in kidney transplantation. *Am J Transplant* 2012; **12**: 2373-2383 [PMID: 22642544 DOI: 10.1111/j.1600-6143.2012.04115.x]
123 **Zhang W**, Qin C, Zhou ZM. Mesenchymal stem cells modulate immune responses combined with cyclosporine in a rat renal transplantation model. *Transplant Proc* 2007; **39**: 3404-3408 [PMID: 18089393 DOI: 10.1016/j.transproceed.2007.06.092]
124 **De Martino M**, Zonta S, Rampino T, Gregorini M, Frassoni F, Piotti G, Bedino G, Cobianchi L, Dal Canton A, Dionigi P, Alessiani M. Mesenchymal stem cells infusion prevents acute cellular rejection in rat kidney transplantation. *Transplant Proc* 2010; **42**: 1331-1335 [PMID: 20534294 DOI: 10.1016/j.transproceed.2010.03.079]
125 **Eggenhofer E**, Renner P, Soeder Y, Popp FC, Hoogduijn MJ, Geissler EK, Schlitt HJ, Dahlke MH. Features of synergism between mesenchymal stem cells and immunosuppressive drugs in a murine heart transplantation model. *Transpl Immunol* 2011; **25**: 141-147 [PMID: 21704160 DOI: 10.1016/j.trim.2011.06.002]
126 **Wan CD**, Cheng R, Wang HB, Liu T. Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 29-33 [PMID: 18234635]
127 **Wang JW**, Liu YB, Xu B, Li JT, Qian HR, Zhang M, Peng SY. [The study on immunomodulation of donor mesenchymal stem cells on discordant liver xenotransplantation]. *Zhonghua Wai Ke Zazhi* 2005; **43**: 1254-1258 [PMID: 16271223]
128 **Wang Y**, Zhang A, Ye Z, Xie H, Zheng S. Bone marrow-derived mesenchymal stem cells inhibit acute rejection of rat liver allografts in association with regulatory T-cell expansion. *Transplant Proc* 2009; **41**: 4352-4356 [PMID: 20005397 DOI: 10.1016/j.transproceed.2009.08.072]
129 **Reinders ME**, de Fijter JW, Roelofs H, Bajema IM, de Vries

DK, Schaapherder AF, Claas FH, van Miert PP, Roelen DL, van Kooten C, Fibbe WE, Rabelink TJ. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl Med* 2013; **2:** 107-111 [PMID: 23349326 DOI: 10.5966/sctm.2012-0114]

130 **Tan J**, Wu W, Xu X, Liao L, Zheng F, Messinger S, Sun X, Chen J, Yang S, Cai J, Gao X, Pileggi A, Ricordi C. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA* 2012; **307:** 1169-1177 [PMID: 22436957 DOI: 10.1001/jama.2012.316]

131 **Perico N**, Casiraghi F, Introna M, Gotti E, Todeschini M, Cavinato RA, Capelli C, Rambaldi A, Cassis P, Rizzo P, Cortinovis M, Marasà M, Golay J, Noris M, Remuzzi G. Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol* 2011; **6:** 412-422 [PMID: 20930086 DOI: 10.2215/CJN.04950610]

132 **Perico N**, Casiraghi F, Gotti E, Introna M, Todeschini M, Cavinato RA, Capelli C, Rambaldi A, Cassis P, Rizzo P, Cortinovis M, Noris M, Remuzzi G. Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation. *Transpl Int* 2013; **26:** 867-878 [PMID: 23738760 DOI: 10.1111/tri.12132]

133 **Peng Y**, Ke M, Xu L, Liu L, Chen X, Xia W, Li X, Chen Z, Ma J, Liao D, Li G, Fang J, Pan G, Xiang AP. Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: a clinical pilot study. *Transplantation* 2013; **95:** 161-168 [PMID: 23263506 DOI: 10.1097/TP.0b013e3182754c53]

134 **Kern S**, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006; **24:** 1294-1301 [PMID: 16410387 DOI: 10.1634/stemcells.2005-0342]

135 **Wagner W**, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* 2005; **33:** 1402-1416 [PMID: 16263424 DOI: 10.1016/j.exphem.2005.07.003]

136 **Rubio D**, Garcia-Castro J, Martín MC, de la Fuente R, Cigudosa JC, Lloyd AC, Bernad A. Spontaneous human adult stem cell transformation. *Cancer Res* 2005; **65:** 3035-3039 [PMID: 15833829 DOI: 10.1158/0008-5472.CAN-04-4194]

137 **Meza-Zepeda LA**, Noer A, Dahl JA, Micci F, Myklebost O, Collas P. High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. *J Cell Mol Med* 2008; **12:** 553-563 [PMID: 18419597 DOI: 10.1111/j.1582-4934.2007.00146.x]

138 **Maccario R**, Moretta A, Cometa A, Montagna D, Comoli P, Locatelli F, Podestà M, Frassoni F. Human mesenchymal stem cells and cyclosporin a exert a synergistic suppressive effect on in vitro activation of alloantigen-specific cytotoxic lymphocytes. *Biol Blood Marrow Transplant* 2005; **11:** 1031-1032 [PMID: 16338626 DOI: 10.1016/j.bbmt.2005.08.039]

139 **Inoue S**, Popp FC, Koehl GE, Piso P, Schlitt HJ, Geissler EK, Dahlke MH. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. *Transplantation* 2006; **81:** 1589-1595 [PMID: 16770249 DOI: 10.1097/01.tp.0000209919.90630.7b]

140 **Le Blanc K**, Götherström C, Ringdén O, Hassan M, McMahon R, Horwitz E, Anneren G, Axelsson O, Nunn J, Ewald U, Nordén-Lindeberg S, Jansson M, Dalton A, Aström E, Westgren M. Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. *Transplantation* 2005; **79:** 1607-1614 [PMID: 15940052]

141 **Eliopoulos N**, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood* 2005; **106:** 4057-4065 [PMID: 16118325 DOI: 10.1182/blood-2005-03-1004]

142 **Buron F**, Perrin H, Malcus C, Héquet O, Thaunat O, Kholopp-Sarda MN, Moulin FT, Morelon E. Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: an in vitro study using human cells. *Transplant Proc* 2009; **41:** 3347-3352 [PMID: 19857747 DOI: 10.1016/j.transproceed.2009.08.030]

143 **Hoogduijn MJ**, Crop MJ, Korevaar SS, Peeters AM, Eijken M, Maat LP, Balk AH, Weimar W, Baan CC. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. *Transplantation* 2008; **86:** 1283-1291 [PMID: 19005411 DOI: 10.1097/TP.0b013e31818aa536]

144 **Popp FC**, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, Geissler EK, Piso P, Schlitt HJ, Dahlke MH. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. *Transpl Immunol* 2008; **20:** 55-60 [PMID: 18762258 DOI: 10.1016/j.trim.2008.08.004]

145 **Otto WR**, Wright NA. Mesenchymal stem cells: from experiment to clinic. *Fibrogenesis Tissue Repair* 2011; **4:** 20 [PMID: 21902837 DOI: 10.1186/1755-1536-4-20]

146 **Zhou YF**, Bosch-Marce M, Okuyama H, Krishnamachary B, Kimura H, Zhang L, Huso DL, Semenza GL. Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. *Cancer Res* 2006; **66:** 10849-10854 [PMID: 17108121 DOI: 10.1158/0008-5472.CAN-06-2146]

147 **Miura M**, Miura Y, Padilla-Nash HM, Molinolo AA, Fu B, Patel V, Seo BM, Sonoyama W, Zheng JJ, Baker CC, Chen W, Ried T, Shi S. Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. *Stem Cells* 2006; **24:** 1095-1103 [PMID: 16282438 DOI: 10.1634/stemcells.2005-0403]

148 **Jeong JO**, Han JW, Kim JM, Cho HJ, Park C, Lee N, Kim DW, Yoon YS. Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. *Circ Res* 2011; **108:** 1340-1347 [PMID: 21493893 DOI: 10.1161/CIRCRESAHA.110.239848]

149 **Casiraghi F**, Remuzzi G, Abbate M, Perico N. Multipotent mesenchymal stromal cell therapy and risk of malignancies. *Stem Cell Rev* 2013; **9:** 65-79 [PMID: 22237468 DOI: 10.1007/s12015-011-9345-4]

150 **Bernardo ME**, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 2007; **67:** 9142-9149 [PMID: 17909019 DOI: 10.1158/0008-5472.CAN-06-4690]

151 **Tarte K**, Gaillard J, Lataillade JJ, Fouillard L, Becker M, Mossafa H, Tchirkov A, Rouard H, Henry C, Splingard M, Dulong J, Monnier D, Gourmelon P, Gorin NC, Sensebé L. Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. *Blood* 2010; **115:** 1549-1553 [PMID: 20032501 DOI: 10.1182/blood-2009-05-219907]

152 **Røsland GV**, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lønning PE, Bjerkvig R, Schichor C. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 2009; **69:** 5331-5339 [PMID: 19509230 DOI: 10.1158/0008-5472.CAN-08-4630]

153 **Torsvik A**, Røsland GV, Svendsen A, Molven A, Immervoll H, McCormack E, Lønning PE, Primon M, Sobala E, Tonn JC, Goldbrunner R, Schichor C, Mysliwietz J, Lah TT, Mo-

taln H, Knappskog S, Bjerkvig R. Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track - letter. *Cancer Res* 2010; **70:** 6393-6396 [PMID: 20631079 DOI: 10.1158/0008-5472.CAN-10-1305]

154 **Prockop DJ**, Brenner M, Fibbe WE, Horwitz E, Le Blanc K, Phinney DG, Simmons PJ, Sensebe L, Keating A. Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 2010; **12:** 576-578 [PMID: 20735162 DOI: 10.3109/14653249.2010.507330]

155 **Dillmann J**, Popp FC, Fillenberg B, Zeman F, Eggenhofer E, Farkas S, Scherer MN, Koller M, Geissler EK, Deans R, Ladenheim D, Loss M, Schlitt HJ, Dahlke MH. Treatment-emergent adverse events after infusion of adherent stem cells: the MiSOT-I score for solid organ transplantation. *Trials* 2012; **13:** 211 [PMID: 23151227 DOI: 10.1186/1745-6215-13-211]

P- Reviewer: Amarapurkar DN, Orlando G, Ozden I
S- Editor: Ma YJ **L- Editor:** A **E- Editor:** Zhang DN

LWW	25/01/2020	9:13	4 Color Fig(s): F2-4	Art: TPA-2019-1115

Review

Mesenchymal Stromal Cells in Solid Organ Transplantation

Morgan Vandermeulen, MD,[1,2] Pauline Erpicum, MD, PhD,[1,3] Laurent Weekers, MD,[3] Alexandra Briquet, PhD,[4] Chantal Lechanteur, PhD,[4] Olivier Detry, MD, PhD,[1,2] Yves Beguin, MD, PhD,[4,5,6] and François Jouret, MD, PhD[1,3]

Abstract: Over the past decade, the clinical application of mesenchymal stromal cells (MSCs) has generated growing enthusiasm as an innovative cell-based approach in solid organ transplantation (SOT). These expectations arise from a significant number of both transplant- and non–transplant-related experimental studies investigating the complex anti-inflammatory, immunomodulatory, and tissue-repair properties of MSCs. Promising preclinical results have prompted clinical trials using MSC-based therapy in SOT. In the present review, the general properties of MSCs are summarized, with a particular emphasis on MSC-mediated impact on the immune system and in the ischemic conditioning strategy. Next, we chronologically detail all clinical trials using MSCs in the field of SOT. Finally, we envision the challenges and perspectives of MSC-based cell therapy in SOT.

(*Transplantation* 2019;00: 00–00)

INTRODUCTION

Over the past decade, the clinical application of mesenchymal stromal cells (MSCs) has generated growing enthusiasm as an innovative cell-based approach in solid organ transplantation (SOT). In addition to the attenuation of the inevitable ischemia/reperfusion (I/R) injury associated with SOT, the objectives of MSC administration in SOT mostly concern (1) the prevention or treatment of acute rejection (AR) or interstitial fibrosis and tubular atrophy (IF/TA), (2) the induction of long-term graft tolerance, and (3) the minimization of the adverse effects of immunosuppressive drugs (ISDs).[1-3] These expectations arise from a significant number of both transplant- and non–transplant-related experimental studies investigating the complex anti-inflammatory, immunomodulatory, and tissue-repair properties of MSCs. Promising preclinical results, especially those including the demonstration of the ability of MSCs to inhibit T-cell proliferation and dendritic cell (DC) maturation and to induce regulatory T-cell expansion, have prompted clinical trials using MSC-based therapy in SOT.

The present review includes (1) a summary of the general properties of MSCs, with a particular emphasis on the MSC-mediated impact on the immune system and the ischemic conditioning strategy, and (2) a detailed description of clinical trials using autologous and allogeneic MSCs in SOT. From these observations, we envision the challenges and perspectives of MSC-based therapy in SOT.

GENERAL FEATURES AND PROPERTIES OF MESENCHYMAL STROMAL CELLS

Definition and Sources

MSCs represent a heterogeneous population of fibroblast-like cells whose definition relies on the combination of the following criteria, according to the International Society for Cellular Therapy[4]: (1) adherence to plastic, (2) specific surface antigen expression of CD105, CD73, and CD90 and lack of expression of CD45, CD34, CD14 and/

Received 2 September 2019. Revision received 4 November 2019.

Accepted 19 November 2019.

[1] *Unit of Cardiovascular Sciences, Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA), Cardiovascular Sciences, University of Liège (ULiège), Liège, Belgium.*

[2] *Division of Abdominal Surgery and Transplantation, University of Liège Hospital (CHU ULiège), Liège, Belgium.*

[3] *Division of Nephrology, University of Liège Hospital (CHU ULiège), Liège, Belgium.*

[4] *Laboratory of Cell and Gene Therapy (LTCG), University of Liège Hospital (CHU ULiège), Liège, Belgium.*

[5] *GIGA, I3-Hematology, University of Liège (ULiège), Belgium.*

[6] *Division of Hematology, University of Liège Hospital (CHU ULiège), Liège, Belgium.*

This work was supported by grants with no role in the conduct of the study. M.V., P.E., and F.J. are Fellows of the Fonds National de la Recherche Scientifique (FNRS), and received support from the FNRS (Research Credit 2013/2015) and from the University of Liège (Fonds Spéciaux à la Recherche, Fonds Léon Fredericq [AciRT]). This study was supported by the Fonds d'investissement de recherche scientifique (FIRS) of Liège CHU. The LTCG is supported by the FNRS, the Foundation against Cancer and the "Federal Cancer Plan, Belgium."

The authors declare no conflicts of interest.

M.V., P.E., and L.W. participated in the writing of the article and in the performance of the research. A.B. and C.L. participated in the performance of the research. O.D., Y.B., and F.J. participated in the writing of the article and in research design. M.V. and P.E. contributed equally to this work.

Correspondence: François Jouret, MD, PhD, Division of Nephrology, University of Liège Hospital (CHU ULiège), Ave Hippocrate, 13, B-4000 Liège, Belgium. (francois.jouret@chuliege.be).

ISSN: 0041-1337/19/0000-00

DOI: 10.1097/TP.0000000000003077

<2doi:10.1097/TP.0000000000003077>

or CD11b, CD79α and/or CD19, and HLA-DR, and (3) the potential to differentiate into osteocytes, adipocytes, and chondrocytes.[5] MSCs were initially isolated from the bone marrow (BM-MSC).[6] Since then, cells exhibiting similar in vitro characteristics as BM-MSCs have been alternatively obtained from both adult tissue (especially adipose tissue[7]) and umbilical cord.[8,9] Although sharing analogous features, these cells are not strictly identical, as intersource heterogeneity has been reported.[10,11] This results in different functional properties according to MSC origins.[12,13] BM-MSCs currently represent the predominant source of MSCs used in SOT.

Mechanisms of Action and Properties

MSCs exert anti-inflammatory, immunomodulatory, and tissue repair/regeneration properties that are mediated by (1) the secretion of soluble factors, (2) direct cell-cell contacts, and (3) the production of extracellular vesicles (EV).[14,15] A considerable number of chemokines/adhesion molecules, cytokines, proangiogenic, and/or growth factors constitute the secretome of MSCs.[16] Among immunomodulatory factors, indoleamine 2,3-dioxygenase is likely to be one of the key molecules involved in the immune-related effects of human MSCs.[17] By catalyzing the rate-limiting step for the conversion of tryptophan—an essential amino acid necessary for T-cell proliferation—to kynurenine, indoleamine 2,3-dioxygenase activity leads to a reduction in local tryptophan concentration, as well as in the production of immunomodulatory tryptophan metabolites that will alter proliferation and function of the immune cells.[16,18,19] HLA-G5, a soluble isoform of nonclassic HLA-G class I molecule, is also critical in mediating the immune response in human MSCs, especially for the expansion of functional regulatory T-cells (Treg).[20,21] MSC-secreted prostaglandin E2 (PGE2) induces an increase of the anti-inflammatory/proinflammatory ratio of cytokines secretion in DCs leading to a shift from a T helper (Th)-1 to a Th-2 cell subtype.[22-24] Numerous factors have also been shown to play a direct or indirect role in MSC-mediated properties, including transforming growth factor beta 1(TGF-β),[23] heme-oxygenase-1,[25] hepatocyte growth factor, IL-1 receptor Antagonist, Peptide LL-37, Matrix Metalloproteinase 3 and 9, angiopoietin-1, tumor necrosis factor-α, and insulin-like growth factor-binding proteins.[24,26]

Additionally, the MSC secretome includes EVs, which are categorized into apoptotic bodies, microvesicles, and exosomes according to their size and mechanism of cellular release.[27] MSC-derived EVs may represent a cell-free therapeutic approach.[28,29] A wide variety of bioactive molecules are included in EVs, such as proteins, lipids, RNA subtypes (ie, at least mRNA and miRNA), and DNA subtypes.[30] Beside the release of their content into target cells, MSC-derived EVs may help transfer cytoplasmic materials and organelles,[30] particularly in a potential bidirectional exchange with T-cells.[31]

The strong immunomodulatory properties of MSCs throughout their highly complex interactions with immune cells hold great promise in SOT.[15] Regarding the innate immune system, MSCs influence macrophage differentiation, with a preferential shift towards an anti-inflammatory immunosuppressive M2 phenotype.[14,32] M2 macrophages are rather involved in the repair and maintenance of tissue integrity and are characterized by efficient phagocytic and immunoregulatory activities.[33] The relationship between MSCs and natural killer (NK) cells is complex. MSCs have been reported to inhibit NK cell proliferation and cytotoxicity.[34] Conversely, MSCs might be susceptible to lysis mediated by NK cells[34,35] since they secrete ligands activating NK cell receptors and they express low levels of class I major histocompatibility complex (MHC) molecules.[36] Interestingly, MSCs can affect major stages of the DC cycle,[37] that is, activation, differentiation, maturation, and antigen presentation. MSCs may thus favor the reprogramming of mature stimulatory DC into a more protolerogenic DC phenotype, characterized by (1) a lower immunogenicity, (2) a higher secretion of IL-10 but lower production of IL-12, and (3) an ability to inhibit the proliferation and function of allo-reactive T-cells and to generate allo-antigen specific Treg.[37-40] The interaction between MSC and T-cells has also been broadly investigated. MSCs suppress T-cell proliferation triggered either by allogeneic,[41] mitogenic, or antigen-specific stimuli[42]; impair the activation and differentiation of T-cells[43]; decrease T-cell cytotoxicity[44,45]; regulate the Th1/Th2 balance[22,46]; and favor the differentiation of $CD4^+$ T-cell subsets with a Treg phenotype.[45] The enhancement and/or preservation of Treg function is particularly relevant and attractive in the field of SOT[47] since Treg are crucial mediators of the immune allogeneic response. Moreover, their impact on the T-cell population indirectly results from MSC-mediated modulation of antigen-presenting cells.[48] However, the relative impact of MSCs on memory and preactivated T-cell effectors compared with naive T-cells, in addition to the duration of MSC-induced immunomodulation, are incompletely understood.[46]

Finally, MSCs have been reported to inhibit B-cell proliferation, especially through an arrest in the G0/G1 phases.[49] Of important note, the immunomodulatory properties of MSCs are thought to depend upon the microenvironment to which they are exposed.[50] In addition, critical MSC- and immune cell–related parameters could impact MSC capacities, including cell culture and expansion conditions, tissue origin, cryopreservation, activation signals, and the MSC-to-immune cell ratio.[43] Variability in these parameters may result in heterogeneous outcomes in MSC-based therapy.

Besides their immunomodulatory actions, MSCs are likely to contribute to tissue repair through several mechanisms,[30,51,52] such as (1) promoting angiogenesis, (2) reducing apoptosis, and (3) enhancing the survival and proliferation of endogenous cells. The ability of MSCs to "home" into the primarily damaged tissue remains controversial since most MSCs are actually trapped within the lung capillaries following intravenous administration.[53-58] Furthermore, accumulating evidence has also highlighted the predominant paracrine role of MSCs in establishing a regenerative microenvironment through their interactions with many cells, including fibroblasts, endothelial cells, epithelial cells, and macrophages. The original theory of a transdifferentiation or cell fusion phenomenon has been refuted.[59,60] Angiogenesis is a crucial step in the process of tissue repair, which can be modulated by MSC paracrine factors, especially vascular endothelial growth factor,[61,62] angopoietin-1, hepatocyte growth factors, tumor growth

factor-β, and stromal cell–derived factor 1-α. MSCs regulate several functions of endothelial cells including their proliferation and migration.[61,63] Anti-apoptotic effect is also noted in many studies, via the ability of MSCs to prevent oxidative stress and via the activation of the protein kinase B pathway.[52] MSC anti-inflammatory effects result from a reduction of proinflammatory molecules, such as interferon-γ and IL-1α, and the production of anti-inflammatory cytokines, such as IL-10 and tumor necrosis factor-α–induced protein-6.[30,52] In addition, MSC-derived EVs may help rapidly restore ATP supply following I/R by transferring mitochondria into the damaged cells.[64] Finally, the attenuation of acute kidney injury (AKI) could result from the MSC-mediated modulation of renal metabolism[65] and prevention of lipotoxicity.[66]

Immunogenicity Concerns

The broad impact of MSCs on the immune system is widely admitted. By contrast, the potential of MSCs to elicit allogeneic response remains uncertain. The evidence for an intrinsic immune privileged status of MSCs has been evoked on the basis of (1) their inhibitory functions on various immune cells, (2) the creation of a suppressive microenvironment, and (3) their low immunogenicity.[67] Indeed, culture-expanded MSCs usually express low levels of MHC class I, and no MHC class II or costimulatory molecules such as CD40, B7-1, or B7-2.[68-70] MSCs do not activate allogeneic lymphocytes.[69,71] However, following exposure to interferon-γ, MSCs act as antigen-presenting cells with upregulation of both MHC-I and MHC-II antigens.[72-74] After the preclinical documentation of an immune response against MSCs,[75,76] the paradigm of the absolute immune privileged status of MSCs was questioned. MSC immunogenicity needs to be considered as one of their characteristics since these cells are "immune evasive" but not "immune privileged."[68] These considerations are particularly relevant in SOT. Indeed, MSCs in the settings of SOT can be isolated from the patient who is the recipient of the graft (autologous MSCs), from the graft donor (allogeneic donor-derived MSCs) or from an unrelated healthy donor not matched either to the recipient or to the graft donor (allogeneic third-party MSCs) (Figure 1).[77] The main concerns with the use of allogeneic MSCs in SOT include[67] (1) the rejection of MSCs by the recipient's immune system,[78] (2) establishing the equivalence of efficacy between autologous and allogeneic MSCs, and (3) the induction of an immune response that could be deleterious to the host, including the production of additional graft donor-specific antibodies (DSAs) in cases of shared mismatches between MSCs and graft donors with a potential risk of recipient sensitization.[67] Whether allogeneic MSCs hamper immunological benefits compared with autologous MSCs needs to be further studied, especially over longer periods of time.[78]

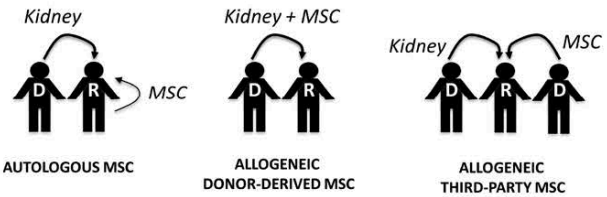


FIGURE 1. Sources of MSC used in SOT. D, donor; MSC, mesenchymal stromal cells; R, recipient; SOT, solid-organ transplantation. Adapted from Pileggi et al.[77]

CLINICAL TRIALS USING MSCS IN KIDNEY TRANSPLANTATION

Convincing preclinical data from animal models have opened new perspectives for the use of MSC-based therapy in kidney transplantation (KTx).[79-81] Interestingly, MSCs significantly inhibit the in vitro proliferation of allo-activated recipient T-cells in mixed lymphocyte reactions of living kidney donor-recipients pairs, either before or 1 month after KTx. Of note, donor-derived and third-party MSCs showed similar potency, suggesting that their inhibitory effect on lymphocyte proliferation occurs independently of HLA.[82] Clinical trials using MSC-based cellular therapy[1,2] aimed at using MSC as (1) induction therapy,[83] (2) follow-up therapy to lower/replace ISDs,[84-87] and (3) treatment of subclinical AR and IF/TA[88] (Table 1).

The administration of autologous MSCs was first performed in 2 living donor kidney recipients in a pilot clinical trial in 2011 in Italy[87] (Figure 2). BM-MSCs were administered intravenously at day 7 post-KTx in addition to an induction regimen by low-dose rabbit anti-thymocyte globulins (rATGs), basiliximab, and steroids. This design relies on previous in vitro data showing that MSC exposition to serum from rATG-treated patients was associated with minimal rATG binding to MSCs, with no repercussion of the MSC's capacity to inhibit T-cell proliferation in mixed lymphocyte reactions. The maintenance regimen consisted of cyclosporine, mycophenolate mofetil (MMF), and steroids. Surprisingly, both patients presented with a deterioration of renal function within 2 weeks following cell infusion. This was not associated with histology-proven AR. Moreover, the levels of intragraft $CD4^+$ T-cells, $CD8^+$ T-cells, $CD14^+$ monocytes, $CD20^+$ cells, and $CD68^+$ macrophages were lower than from patients who had experienced AR. There was also a high number of granulocytes and intragraft staining of C3d. The authors hypothesized that intragraft MSCs may have produced proinflammatory mediators that could have contributed to the recruitment of granulocytes and, in turn, to the deterioration of renal graft function in a so-called process of "engraftment syndrome." Concerning the immune-phenotyping of peripheral blood T-cells, the investigators described an MSC-induced protolerogenic environment. The percentage of Treg markedly decreased during the first 30 days in each patient but considerably increased from day 30 till the end of the study in MSC-treated patients, resulting in a higher ratio of Treg/memory $CD8^+$ T-cells in comparison to controls that had received the same induction therapy. Finally, the $CD8^+$ T-cell cytolytic responses at day 180 post-KTx towards donor and third-party antigens were reduced in both patients in comparison to controls.

In a second clinical attempt by the same group in 2013, and on the basis of a preclinical study showing an improved graft outcome with MSC pretransplant administration,[81] 2 living-related kidney transplant recipients (KTRs) were infused with autologous MSCs 24 hours before KTx (Table 1). In addition, the anti-interleukin (IL)-2-receptor monoclonal antibody, basiliximab, was removed from the induction regimen since it had been associated with a transient decrease in circulating Treg in

TABLE 1.

Clinical trials in KTx

Trial patients	Dose/source; timing; route	IS regimen	Endpoints	Outcomes	Immune modulation	Reference y
Autologous MSC						
NCT00752479 (n = 2) LD	1 inj. of 1.7–2 × 10^6/kg BM-MSC at D7 (IV)	*Induction:* basiliximab, low dose rATG + steroids *Maintenance:* MMF, CsA, and steroids	Safety; feasibility	Early transient deterioration of graft function 7–14 d after MSC infusion	↑ Treg/memory $CD8^+$ T-cells ↓ $CD8^+$ T-cell cytolytic activity in response to donor/third-party Ag No DSA	Perico et al[87] 2011
NCT00658073 (n = 156) LD	2 inj. of 1–2 × 10^6/kg BM-MSC at reperfusion and at W2 (IV)	3 groups: 1. MSC + std-dose CNI (n = 53) 2. MSC + low-dose CNI (n = 52) 3. Anti-IL2R + std-dose CNI (n = 51) + *maintenance:* steroids, MMF	Primary: 1-y BPAR; 1-y eGFR Secondary: safety; survivals	Similar survival: ↓ incidence of BPAR in MSC groups in the first 6 mo ↑ recovery of renal function in MSC groups ↓ opportunistic infections in low-dose CNI + MSC	Not evaluated	Tan et al[83] 2012
NCT02012153 (n = 2) LD	1 inj. of 1–2 × 10^6/kg BM-MSC at D-1 (IV)	*Induction:* low dose rATG + steroids *Maintenance:* MMF, CsA, and steroids	Safety; feasibility	No engraftment syndrome BPAR in 1 patient at D14 after KTx	↑cell number of Treg /memory-effector $CD8^+$ T-cells ↓ $CD8^+$ T-cell cytolytic activity in response to donor/third party Ag	Perico et al[86] 2013
NCT00734396 (n = 6) LD	2 inj. of 1–2 × 10^6/kg; BM-MSC from 6 to 10 mo after KTx (IV)	*Induction:* basiliximab *Maintenance:* CNI, MMF, steroids	Safety; feasibility; treatment of BPAR	Opportunistic infections in 3 patients Resolution of tubulitis without IF/TA in 2 patients	↓ proliferation PBMC upon stimulation with donor PBMC in 5/6 patients	Reinders et al[88] 2013
NCT02409940 (n = 4) LD	2 injections of 0.2–2.8 × 10^6/kg BM-MSC at D-1 and at D30 (IV)	*Induction:* Low-dose ATG *Maintenance:* CNI, MMF, steroids	Safety; feasibility	No graft dysfunction; no BPAR	↓ $CD4^+$ T-cell proliferation	Mudrabettu et al[84] 2015
Allogeneic donor-derived (Dd) or third-party (3p) MSC						
(n = 12) LD: 6 MSC-treated; 6 controls	2 inj. 5 × 10^6/kg Dd BM-MSC at reperfusion (IA) 2 × 10^6/kg Dd BM-MSC and at M1 (IV)	*Induction*: CTX and steroids *Maintenance*: low-dose TAC steroids MMF	Pilot study	No difference in renal function No difference in BPAR	No difference in Treg, NK, CD4, or CD8 cells ↑ B-cells at M3 no detectable chimerism	Peng et al[85] 2013
(n = +20) LD: 10 MSC-treated; 10 controls	2 inj. of 5 × 10^6/kg Dd BM-MSC at reperfusion (IA) and 2 × 10^6/kg Dd BM-MSC at M1 (IV)	*Induction*: CTX and steroids *Maintenance*: low-dose TAC steroids MMF		No difference in renal function No difference in BPAR	↓ NK and CD38 cells No detectable chimerism	Pan et al[90] 2016
n = 7 LD	1 × 10^6/kg Dd BM-MSC at KTx (iliac bone)	*Induction:* ATG *Maintenance*: CNI, MMF and steroids	Efficacy; safety	BPAR in 3 patients	Expansion of Treg from baseline in 2 patients No detectable chimerism	Lee et al[91] 2013
NCT02490020 (n = 42) DD: 21 MSC-treated; 21 controls	2 inj. of 1.2 × 10^6/kg 3p UC-MSC 30 min prior KTx (IV) and 2. 5 × 10^6/kg 3p UC-MSC at reperfusion (IA)	*Induction:* ATG and steroids *Maintenance*: CNI, MMF and steroids	Primary: DGF and 1-y BPAR Secondary: infections	No difference in - BPAR - DGF - renal function M12 - infection rate	Not evaluated	Sun et al[92] 2018
NCT01429038 (n = 20) DD: 10 MSC-treated; 10 controls	2 × 10^6/kg 3p BM-MSC at D3 (IV)	*Induction:* basiliximab and steroids *Maintenance*: CNI, MMF, and steroids	Primary: safety Secondary: survival graft function; BPAR; immunity MSC-DSA	Better eGFR at D7 No difference in - eGFR at 1 y - BPAR - opportunistic infections	↑ % of Treg at D30 ↑ NK cells at D30 DSA against MSC or shared kidney-MSC HLA in 4 patients (lower MFI)	Erpicum, Weekers et al[93] 2019

3p, third party; Ag, antigen; ATG, anti-thymocyte globulin; BM, bone marrow; BPAR, biopsy-proven acute rejection; CNI, calcineurin inhibitor; CTX, cyclophosphamide; Dd, donor derived; DD, deceased donor; DGF, delayed graft function; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; IA, intra-arterial; IF/TA, interstitial fibrosis and tubular atrophy; IL, interleukin; inj., injection; IS, immunosuppression; IV, intravenous; KTx, kidney transplantation; LD, living donor; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MSC, mesenchymal stromal cell; NK, natural killer; rATG, rabbit anti-thymocyte globulin; TAC, tacrolimus; Treg, regulatory T-cells; UC, umbilical cord.

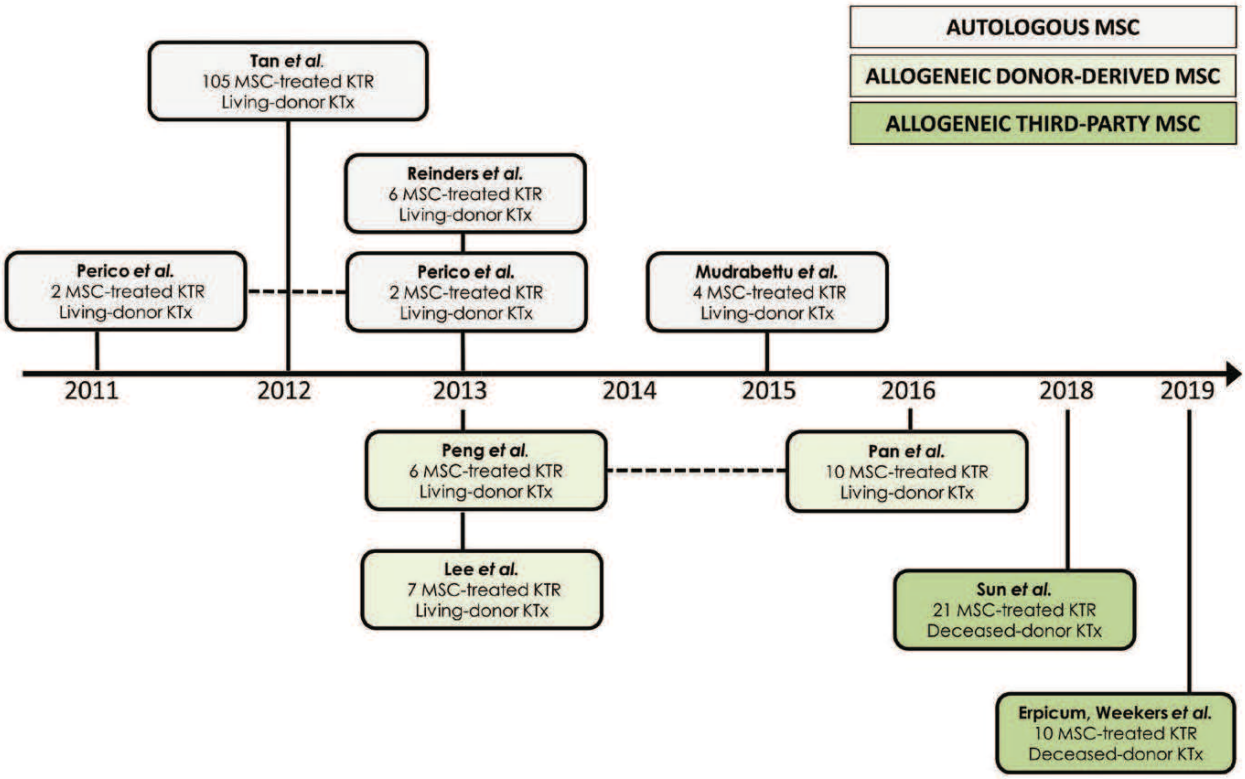


FIGURE 2. Timeline of clinical trials in KTx. KTR, kidney transplant recipients; KTx, kidney transplantation; MSC, mesenchymal stromal cells.

previous studies. No engraftment syndrome was observed in the early posttransplant phase. However, 1 patient developed AR 2 weeks post-KTx (potentially because of the absence of basiliximab in the induction regimen). The expansion of Treg was similar in patients with or without basiliximab induction.[86]

This Italian group has recently published the 5/7-year follow-up of the clinical and immunological profile of these 4 MSC-treated patients.[89] Graft function remained stable in all patients, with no major side-effects. Moreover, the Treg/memory $CD8^+$ T-cell ratio remained increased in 2 patients, in parallel with an expansion in the pool of circulating naïve and transitional B-cells. Of particular note, 1 patient achieved a protolerogenic environment, allowing the withdrawal of cyclosporine and a progressive tapering of the low-dose MMF maintenance.[1,89]

The largest study to date in the field of MSCs in KTx is a prospective randomized clinical trial performed in 2012 in China, which included 156 living-donor KTRs (Figure 3). Tan et al investigated the impact of autologous MSCs in combination with low-dose of calcineurin inhibitor (CNI) (n = 52) or standard-dose CNI (n = 53) in comparison to anti-IL-2 receptor antibody plus standard-dose CNI (n = 51) as an induction therapy (Table 1). BM-MSCs at a dose of 1–2 × 10^6/kg were administered intravenously at the time of reperfusion and 2 weeks after KTx. Maintenance therapy consisted of the adjunction of MMF and steroids. No difference in graft or patient survival was reported at the 12-month follow-up. However, the authors reported a faster recovery of renal function within the first month after KTx and a lower rate of biopsy-proven AR in the first 6 months in both MSC groups. Interestingly, the rate of opportunistic infections was significantly reduced in the MSC low-dose CNI group, but not in the MSC standard-dose CNI, compared with the control group.[83]

In 2015, Mudrabettu et al evaluated the safety of 2 intravenous injections of MSCs, 1 the day before KTx and 1 month later, as an immunosuppressive therapy for 4 recipients of living-donor related KTx (Figure 2). MSC administration was safe and well tolerated. Notably, no graft dysfunction or AR was reported. In addition, MSC-treated patients exhibited a nonsignificant increase in Treg and a reduction in CD4 T-cell proliferation.[84]

In the Netherlands, Reinders et al studied the use of autologous BM-MSCs for the treatment of AR after KTx (Figure 3). Six fully DR-mismatched living-donor KTRs received autologous BM-MSCs because of AR and/or increased IF/TA in the 6-month protocol biopsy compared with the renal biopsy performed 4 weeks after KTx. MSC administration was feasible and well tolerated. Moreover, in 2 patients with AR with a clinical indication of a third biopsy, there was no persistent sign of tubulitis on renal biopsies after MSC treatment. However, 3 MSC-treated patients developed opportunistic infections.[88]

Allogeneic MSCs have also been tested in the field of KTx (Figure 2). In the study of Peng et al in 2013, 6 de novo living-related KTRs received 2 injections of donor-derived BM-MSCs: the first one during the intervention through the renal allograft artery and the second one intravenously 1 month later. Similar to the study of Tan et al, these patients were given a low dose of tacrolimus (±50% of the standard dose) to minimize CNI toxicity, whereas

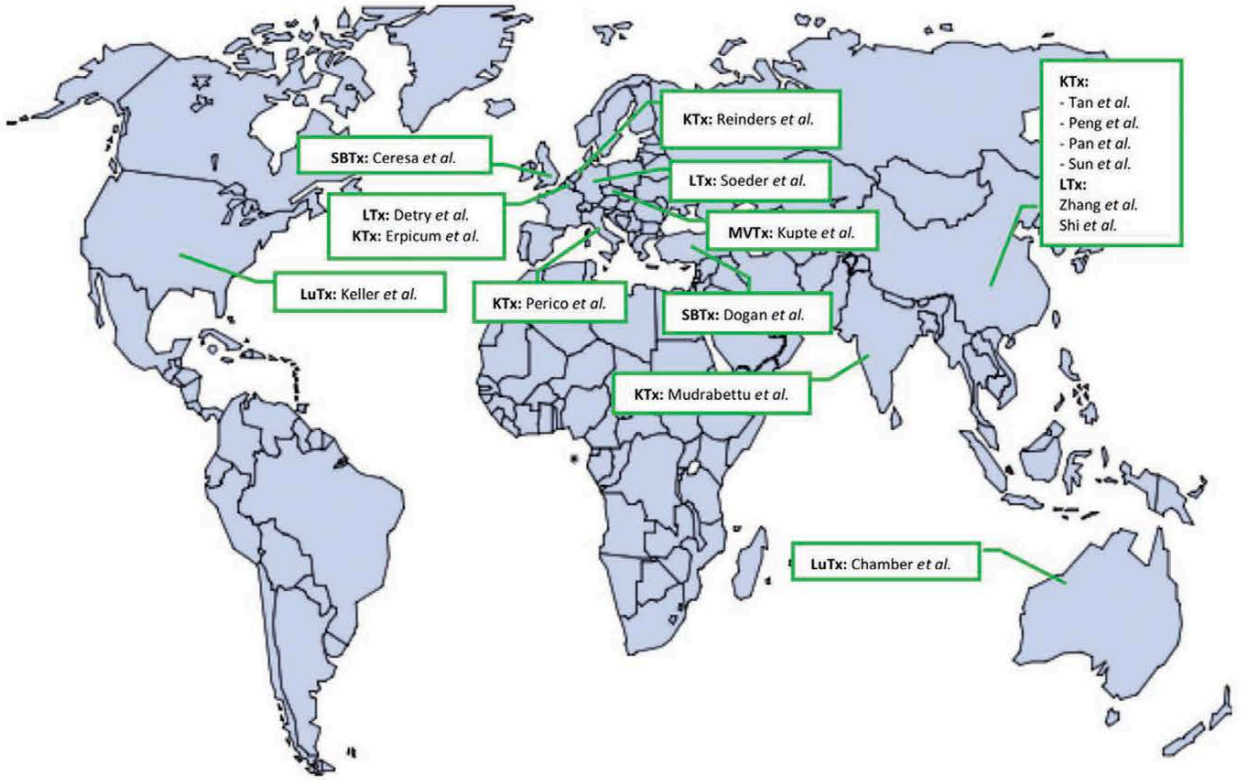


FIGURE 3. Locations where MSC-based cell therapy in SOT has been reported. KTx, kidney transplantation; LTx, liver transplantation; LuTx, lung transplantation; MSC, mesenchymal stromal cell; MVTx, multi-visceral transplantation; SBTx, small bowel transplantation; SOT, solid-organ transplantation. Adapted from http://www.free-world-maps.com/source-political-world-map-a.

the control group (n = 6) received the standard dose of tacrolimus. MSC infusion in combination with low-dose tacrolimus was demonstrated to be safe and feasible with no statistical difference in AR rate, patient and graft survivals, and renal function over the 1-year follow-up.[85] In the continuation of this pilot study, 20 additional patients were enrolled in a second trial, which confirmed, in 2016, that the association of MSCs with low-dose tacrolimus was as efficient as standard doses of tacrolimus for graft survival (at least at 2 y after KTx). In addition, a lower percentage of NK cells were found in the MSC group compared with controls (Table 1).[90]

In 2013, Lee et al studied the safety and feasibility of an intraosseous injection of donor-derived BM-MSCs into the iliac bone of HLA-mismatched living donor KTRs at the time of KTx (Table 1). They did not note any adverse event during or after the injection. No graft failure was reported. However, 3 KTRs experienced biopsy-proven AR during the follow-up. There was no detection of mixed chimerism in the peripheral blood of the recipients at 1 and 8 weeks post-KTx.[91]

Sun et al have recently evaluated the administration of third-party umbilical cord-MSCs (UC-MSCs) in 21 KTRs from deceased donor, compared with 21 paired KTRs in a multicenter randomized trial. MSCs were injected twice: intravenously 30 minutes before KTx and through the renal artery during surgery. Primary endpoints were the rate of biopsy-proven AR and delayed graft function. No difference was found between the groups. In addition, the rate of infections and the estimated glomerular filtration rate (eGFR) were similar in both groups after a 1-year follow-up. Of note, no UC-MSCs were detected at Day 7 post-KTx on the renal graft biopsy of 1 patient, using a multiprobe fluorescence in situ hybridization assay (Table 1).[92]

Finally, our group has recently reported on the results of a prospective phase I–II controlled clinical trial evaluating the feasibility, safety, and tolerability of third-party MSC injection in KTRs from deceased donors, in addition to standard immunosuppression (Table 1).[93,94] No attempt was made to match HLA between kidney recipient and the MSC- or kidney graft donors. No side effect was noted at the time of MSC injection, except 1 patient with a history of ischemic heart disease who had a non-ST-elevation myocardial infarction ~3 hours after MSC infusion. The incidence of opportunistic infections was comparable among groups. The secondary endpoints included the impact of MSCs on graft outcomes and immunity, as well as the occurrence of MSC-DSAs. We observed that MSC therapy was associated with an increased frequency of Treg among $CD4^+$ cells at day 30 post-KTx and with an immediate improvement of allograft function (at d 7 and 14 post-KTx), compared with the whole cohort of KTRs who were transplanted in our center during the study period; with a similar nonsignificant trend versus the 10 control KTRs specifically enrolled in our study. No difference in eGFR was found at 1 year. However, it is important to note that our study was not designed to assess MSC efficacy. Finally, despite numerous HLA mismatches, only 1 patient developed significant antibodies (with mean fluorescence intensity >1500) against MSCs.

TABLE 2.

Clinical trials in LTx

Trial patients	Dose, source/origin, timing, route	IS regimen	Endpoints	Outcomes	Immune modulation	Ref, y
Liver transplantation						
NCT01841632 (n = 3) LD	2 inj. of 1.5×10^8 3p BM-MAPC during LTx (PV) and at D2 (IV)	*Initial:*Basiliximab (D0+D4); steroids; MMF, if needed: +CNI	Primary: acute toxicity	No injection-related adverse event Major AE in n = 3	Transient increase of $CD4^+FoxP3^+CD127^{low}$	Soeder et al (n = 1)[97] 2015 + data from clinicaltrials.gov (n = 2) 2018
			Secondary: acute rejection, middle/long-term safety	Need for CNI No malignancy		
NCT01429038 n = 10 DD	1 inj. of $1.5–3 \times 10^6$/kg 3p BM-MSC at D3 (IV)	TAC; MMF; CS	Primary: toxicity; incidence of infections Secondary: patient and graft survivals	No AE IS withdrawal successful only in 1 patient	None observed	Detry et al[98] 2017
NCT01690247 n = 14 DCD LTR with AR	1 inj. of 1×10^6/kg of 3p UC-MSCs 4 wk after recruitment (IV)	TAC; MMF; CS	Safety and efficacy	No AE ↓ ALT, AST, and TBIL Histological improvement	↑ Treg and Treg/Th17 ratio ↓ $CD4^+$ T-cell activation ↑ TGF-β1 and PGE2	Shi et al[99] 2017
NCT02706132 n = 18 ABO-I	6 inj. of 1×10^6/kg MSC timing/ origin N/A (IV)	*Induction:* - Plasma exchange - Rituximab - IVIG *Maintenance:* - Basiliximab - TAC - MMF - Steroids	Safety and efficacy	No AE No AMR MOF and death in n = 3 Infection in n = 10 Biliary complications in n = 5	N/A	Zhang et al[100] 2017
NCT02223897 N = 12 LTR with ITBL	6 inj. of 1×10^6/kg 3p UC-MSC at 1,2,4,8,12, and 16 wk (IV)	*Conventional IS*	Safety and efficacy	No AE ↓ need for therapy for ITBL ↑ 1-y survival	N/A	Zhang et al[101] 2017

3p, third party; ABO-I, ABO incompatible; AE, adverse event; ALT, alanine aminotransferase; AMR, antibody-mediated rejection; AR, acute rejection; AST, aspartate aminotransferase; BM, bone marrow; CNI, calcineurin inhibitor; CS, corticosteroids; DCD, donation after circulatory death; DD, deceased donor; inj., injection; IS, immunosuppression; ITBL, ischemic-type biliary lesion; IV, intravenous; LD, living donor; LTR, liver transplant recipient; LTx, liver transplantation; MAPC, multipotent adult progenitor cells; MMF, mycophenolate mofetil; MOF, multiple organ failure; MSC, mesenchymal stromal cell; N/A, not applicable; PGE2, prostaglandin E2; PV, portal vein; Ref, reference; TAC, tacrolimus; TBIL, total bilirubin; TGF-β1, transforming growth factor beta 1; Treg, regulatory T-cells; UC, umbilical cord.

CLINICAL TRIALS USING MSCS IN LIVER TRANSPLANTATION

Similar to KTx, convincing results from preclinical liver transplantation (LTx) models supported the launch of clinical trials (Table 2). In animal LTx models, MSCs have shown the ability to (1) prevent I/R injury, (2) enhance liver regeneration, and (3) prevent AR.[26]

The *MISOT-I Study* aimed to investigate the safety and feasibility of multiple injections of MultiStem, which is a commercial product (Athersys Inc., Cleveland, OH) of BM-derived multipotent adult progenitor cells (MAPCs), after LTx (Figure 4). This trial also evaluated the impact of MAPC administration on the time to the first biopsy-proven AR within the first 90 days post LTx. MAPCs belong to the family of MSCs and exhibit very similar properties.[95] The patients enrolled in this single-arm study were supposed to receive 2 doses of third-party MAPCs at the time of LTx and 48 hours later. Cell dose escalation was scheduled for every third patient, in association with basiliximab, mycophenolic acid, and steroids.[96] CNIs were used only after biopsy-proven AR. In 2015, Soeder et al published the first-in-man case of this phase I study.[97] The patient did not present any acute complications in connection with MAPC injections, but he presented major adverse events within the first week, as well as an AR requiring CNI introduction. The leukocyte profile showed an increased level of $CD4^+FoxP3^+CD127^{low}$ Treg from postoperative day 3, with a subsequent normalization at day 29. Two more cases were registered on ClinicalTrials.gov. These patients also developed major adverse events, but none of them were directly linked to MAPC injection. MAPCs associated with CNI-free IS could not replace the classical immunosuppressive regimen. The *MISOT-I Study* was discontinued by the investigators (Table 2).

In 2017, our group published the first monocentric, prospective, phase I–II clinical study evaluating the feasibility, safety, and tolerability of a single infusion of third-party MSCs in 10 liver transplant recipients (LTRs) (Figure 4).[98] The MSC patients received classical ISDs and were infused with a single injection of third-party MSCs ($1.5–3 \times 10^6$/kg) on postoperative day 3 ± 2. They were prospectively compared with 10 LTRs receiving classical immunosuppression (control group). Moreover, immunosuppression weaning was attempted in patients who had a normal liver function and no sign of graft rejection on the protocol 6-month graft biopsy. Our study did not show any infusional toxicity during the week following MSC administration, with no effect on vital parameters, no sign of allergy, and no inflammatory response. During the 1-year follow-up, no de novo cancer or major opportunistic infections were reported in either group. In terms of efficacy, no immunologic effect following this single MSC injection could be found. IS could be completely weaned in only 1 patient of the MSC group with no signs of rejection, and she remained without any ISDs for 12 months. This phase I-II study did not demonstrate any sign or suspicion of toxicity from a single injection of third-party MSCs after 1-year follow-up. However, such a single MSC injection combined with classical immunosuppression was not associated with operational tolerance (Table 2).

In 2017, Shi et al published the results of their trial studying the effectiveness of UC-MSCs to treat liver allograft AR (Table 2).[99] Twenty-seven LTRs under conventional ISDs who presented biopsy-proven AR were enrolled in this study. These patients were randomly assigned into either (1) the MSC-group receiving conventional immunosuppressive treatment associated with a single (n = 13) or multiple (n = 1) injection(s) of UC-MSCs or to (2) the control group treated with conventional IS drugs (n = 13). In terms

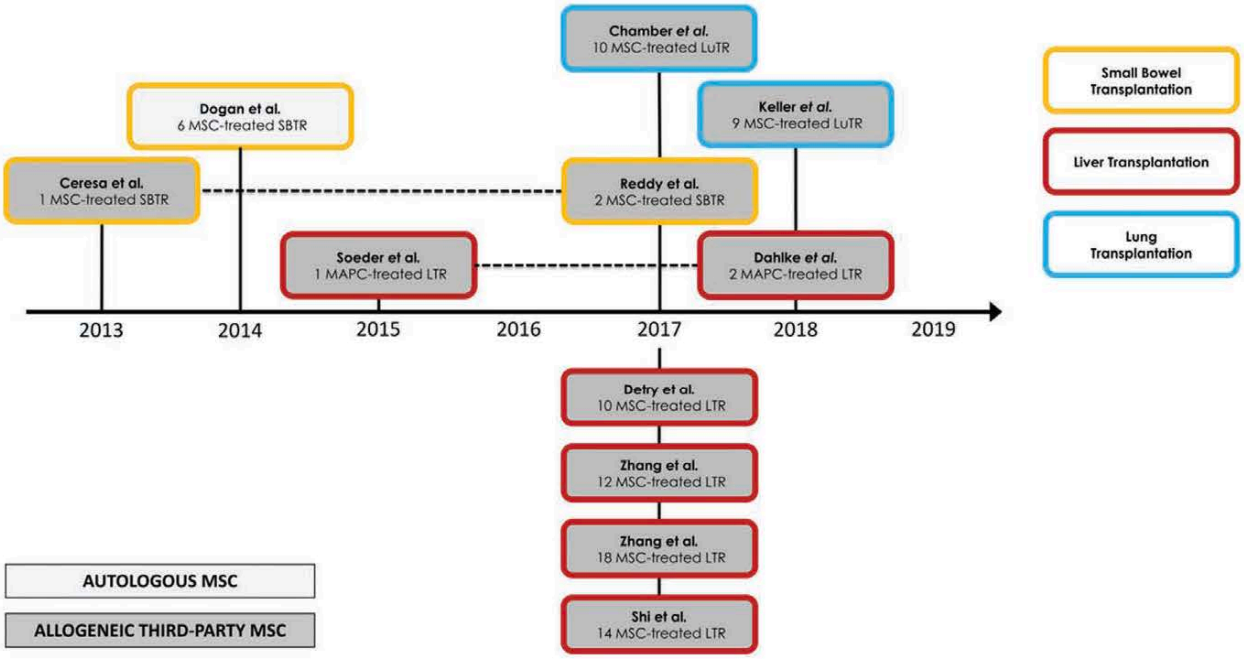


FIGURE 4. Timeline of clinical trials in liver, small bowel, and lung transplantations. LTR, liver transplant recipients; LTx, liver transplantation; LuTR, lung transplant recipients; MAPC, multipotent adult progenitor cells; MSC, mesenchymal stromal cells; SBTR, small bowel transplant recipient.

of safety, no complications were associated with the use of UC-MSCs in the first group at the 24-week follow-up. Furthermore, a significantly higher decrease of the levels of aspartate aminotransferase, alanine aminotransferase, and total bilirubin was observed in MSC-treated versus control patients. Nearly half the MSC-treated patients presented with histological improvements of liver allograft at 4 weeks after infusion, while none of the controls showed histological improvement. MSC-treated patients showed a significant increase of circulating Treg population and Treg/T-helper 17 ratio, while HLA-DR expression on $CD4^+$ T-cell was significantly lower (which suggests an inhibition of $CD4^+$ T-cell activation) 4 weeks after MSC infusion. The soluble factors, that is, PGE2 and TGF-β1, were significantly increased in 86% of the MSC-treated patients after 4 weeks, compared with the controls. Hence, a single MSC injection is safe and could possibly be effective in controlling liver AR. Repetition of MSC injections is feasible (n = 1) in the case of unresponsiveness to the first MSC administration. Still, there is no conclusive information about the efficacy of repeated administrations of MSCs.

The results of a Chinese study initiated in 2014 were presented at the American Transplant Congress in 2018 (Table 2). They evaluated the safety and efficacy of MSC treatment to prevent antibody-mediated rejection (AMR) in a simplified protocol for ABO incompatible LTx in 18 patients with severe hepatic failure (phase I).[100] To prevent AMR, plasma exchange and rituximab were used and associated with injections of intravenous immunoglobulins and MSCs (without splenectomy or local infusion into the graft). Patients received a standard IS protocol. No adverse event in association with MSC injection was reported and no patient developed AMR. During the mean 15-month follow-up, 3 patients died from severe complications (infection or hemorrhage). More than half of the patients developed an infection during the follow-up, and 5 patients developed biliary complications. None of them developed malignancies. At first glance, this study seems to show that MSC injection in this context would be safe and could play a role in preventing AMR for ABO incompatible LTR. Nevertheless, given an insufficiently detailed protocol (eg, no information about the origin of MSCs, the number of cells and injections, the immunological profile of the recipients, etc), it is hard to draw any robust conclusions. The publication of this study is pending.

Besides immunomodulation and induction of tolerance, MSCs have also been tested for the treatment of ischemic-type biliary lesions (ITBLs).[101] Hence, 12 patients with ITBLs post-LTx were treated with repeated injections (n = 6) of UC-MSCs and were retrospectively matched to 70 "comparable" patients with ITBLs who had been "conventionally" treated. The need for interventional therapies was lower in the MSC-treated group (33.3 versus 64.3%, $P < 0.05$) when compared with the historical controls. The investigators also showed that 1-year graft survival was significantly higher in the MSC-treated group (72 versus 100%, $P < 0.05$).[101] This study showed that multiple injections of UC-MSCs were safe and well tolerated and could potentially be effective to treat (or to prevent the worsening of) ITBLs in the context of LTx (Figure 4).

Finally, a prospective pilot trial aiming to investigate the safety and feasibility, and those effects on immunomodulation, of donor-derived MSCs in pediatric living-donor LTx is in the pipeline (MYSTEP1, NCT02957552).[102] Remuzzi et al have also started a study evaluating the safety and tolerance post induction of a single injection of third-party MSCs in the context of LTx (NCT02260375).

MSCS IN OTHER SOT

MSCs in Lung Transplantation

Chronic lung transplant rejection (or chronic lung allograft dysfunction, CLAD) is the major hurdle to long-term survival in lung transplant recipients. In 2017, Chamber et al reported on a single-arm phase I study exploring the feasibility and safety of third-party BM-MSCs for patients with advanced CLAD.[103] Ten eligible patients were infused with 2×10^6/kg body weight/infusion twice weekly for 2 weeks. The authors observed some minor adverse events possibly linked to MSC therapy: halitosis, lower respiratory tract infection symptoms, liver test alteration, and dizziness. The authors also noticed a trend towards a slower decline of the forced expiratory volume in 1 second after MSC infusions, as well as a transient fall in mean arterial pressure and O_2 saturation rapidly after MSC infusion. Two patients died a few months after MSC infusions from CLAD evolution a priori unrelated to cell injection. The authors concluded that MSC infusion for patients with advanced CLAD is safe and feasible (Table 3).

The Mayo Clinic has published the results of a single-arm trial evaluating the feasibility and safety of the use of BM-MSCs for obstructive CLAD, with dose escalation.[104] The 9 treated patients received a single IV infusion of MSC with increasing doses at every third patient from 1×10^6 MSC/kg to 4×10^6/kg. MSC infusions were well tolerated even with higher doses, and no significant adverse events were observed during the first month postinfusion.

MSCs in Small Bowel Transplantation

In 2013, Ceresa et al reported a case in which they treated refractory bowel dysfunction secondary to infection with third-party BM-MSCs in a patient who underwent small bowel transplantation (SBTx).[105] After failure of multiple therapeutic attempts, this patient received an IV single dose of 1×10^6 MSC/kg. Interestingly, a rapid clinical, biological, endoscopic, and histologic improvement was observed, with no relevant side effects. The authors suggested that MSC therapy could, in this case of SBTx-associated bowel dysfunction, have an anti-inflammatory action and play a trigger role in the regenerative process (Table 3). The same team published an abstract reporting on 2 additional SBTx recipients treated with MSCs in the same context. Those patients received 2 doses of MSCs of 2 or 3×10^6 cells/kg 1 week apart. One of those 3 patient developed de novo MSC-DSA (A34 and DR18) which persisted >2 years after MSC infusion.[106]

In 2014, Dogan et al published their experience about the prevention of AR and graft-versus-host disease after autologous BM-MSC therapy in SBTx.[107] Each patient (n = 6) received 3 doses of 1×10^6 MSC/kg: first intraoperatively directly into the transplanted intestinal artery, then on day 15 and day 30 through the mesenteric artery using angiography catheter. They did not encounter any problems related to MSC infusion. However, AR occurred in 4 out of 6 SBTx recipients. Two patients died of sepsis

TABLE 3.
Clinical trials in other SOT

Trial patients	Dose; source/origin; timing; route	IS regimen	Endpoints	Outcomes	Immune modulation	Ref; country; y
Lung transplantation						
NCT01175655 n = 10 LuTR with advanced CLAD	4 inj. of 2×10^6/kg 3p BM-MSC twice weekly for 2 wk (IV)	TAC, MMF, steroids	Primary: safety	No major AE Minor AE: halitosis (n = 12), lower respiratory tract infection symptoms (n = 3), liver test alteration (n = 3), and dizziness (n = 2)	N/A	Chamber et al[103] 2017
			Secondary: effect on lung function, survival	Slow ↓ FEV_1 after infusion Transient small ↓ in mAP and SaO_2 rapidly after infusion		
NCT02181712 n = 9 LuTR with moderate to severe transplant-related BOS	1 inj. of 1×10^6/kg (n = 3), 2×10^6/kg (n = 3), 4×10^6/kg (n = 3) 3p BM-MSC twice weekly for 2 wk (IV)	CNI; MMF or AZA; steroids	Safety and feasibility	No AE No significant clinical, functional, and laboratory change	N/A	Keller et al[104] 2018
Small bowel transplantation						
n = 1 small bowel transplant recipient	1 inj. of 1×10^6/kg 3p BM-MSC at M12 (IV)	*Conventional IS*	Treatment of infective (Candida and Norovirus) bowel dysfunction resistant to conventional therapy	No AE Rapid improvement	N/A	Ceresa et al[105] 2013
n = 6 intestinal transplant recipient	3 inj. 1×10^6 MSC/kg/inj. Auto BM-MSC per-op and then POD 15 and 30 via SMA	*Induction:* ATG, steroid *Maintenance:* mTOR inhibitor, TAC low-dose	Prevention of AR and GVHD	No AE AR in 4 patients 2 patients died after 2 and 3 mo (sepsis)	N/A	Dogan et al[107] 2014

3p, third party; AE, adverse event; AR, acute rejection; ATG, anti-thymocyte globulin; AZA, azathioprine; BM, bone marrow; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; CNI, calcineurin inhibitor; FEV1, forced expiration volume in 1 second; GVHD, graft-vs-host disease; inj., injection; IS, immunosuppression; IV, intravenous; LuTR, lung transplant recipient; mAP, mean arterial pressure; MMF, mycophendate mofetil; MSC, mesenchymal stromal cell; N/A, not applicable; POD, postoperative day; Ref, reference; SaO2, peripheral oxygen saturation; SMA, superior mesenteric artery; SOT, solid-organ transplantation; TAC, tacrolimus.

(which is recognized as a major cause of death after SBTx). This uncontrolled and nonrandomized cohort is too small to draw strong conclusions. Still, MSC infusion was apparently safe, but obviously not efficient in preventing AR in this cohort (Table 3).

DISCUSSION AND PERSPECTIVES

On the basis of a reasonable number of clinical trials in different fields of SOT, one may claim that MSC administration in transplant recipients is feasible and safe, at least on the short term. This is in line with a meta-analysis evaluating the safety of MSCs in general, including nontransplant applications.[108] Nevertheless, side-effects have been potentially linked to MSC infusion, including engraftment syndrome in 2 KTRs exposed to MSCs after KTx.[87] Although such an AKI post–MSC injection has not been observed in larger cohorts, one may remain cautious concerning the timing of MSC infusion in KTx. Similarly, a transient decrease of peripheral oxygen saturation directly after MSC IV infusion has been reported in lung transplant recipient, which putatively suggests infusion-related lung toxicity.[103] One of the main theoretical safety concerns of MSC therapy is an increased risk of opportunistic infections and neoplasms secondary to "over-immunosuppression." To date, the majority of published studies has not found any difference in terms of opportunistic infections in SOT recipients exposed to MSCs. Furthermore, MSCs associated with low-dose CNI significantly reduced the rate of opportunistic infections in China.[83] In the Italian experience, no increased susceptibility to infections was observed in 4 KTRs at 5/7 years post–MSC infusion.[89] By contrast, the Dutch team reported that 3 among 6 MSC-treated KTRs developed opportunistic infections.[88] Concerning the potential increased risk for malignancies in transplanted patients, the currently available literature is limited in both the duration of the follow-up and the number of cases exposed to MSCs.

Concerning the efficacy of MSCs in SOT, the current data are obviously not powerful enough to draw firm conclusions. However, several perspectives may be inferred to support further research in the field. In our opinion, there are 2 main issues that should be considered with regard to the definite purposes of MSC-based cell therapy in SOT: (1) the prevention/attenuation of the I/R injury inherently associated with organ transplantation and (2) the immune modulation for AR treatment or tolerance induction and minimization of ISDs.

The outcomes of SOT are highly influenced by the quality of the graft, including the severity of the I/R injury. Therefore, the development of tools and maneuvers preventing ischemic damage and/or accelerating tissue recovery after reperfusion is an urgent need in SOT. This is even more relevant nowadays since the inadequacy between the increasing number of patients on the waiting list and the limited availability of grafts has prompted the expansion of eligibility criteria for organ procurement, especially for kidneys[109] and livers.[110] These organs from expanded criteria donors are more vulnerable to I/R injury. Preclinical studies support the use of MSCs as an innovative strategy in organ conditioning against I/R.[111-114] In the nontransplant field of ischemic AKI, 2 clinical trials using allogeneic MSCs have been reported. If the preliminary results of the first study (NCT00733876) appear promising in showing a reduction of postoperative deterioration in renal function in the MSC-treated patients compared with historical matched controls,[115,116] no MSC-associated nephroprotection was observed in a randomized, multicenter, double blind, placebo-controlled phase II study (NCT01602328) performed on 156 patients in 2018.[117] Nevertheless, the highly heterogeneous experimental conditions of MSC administration in rodent models of I/R, with various sources, doses, timings, routes of delivery, and vehicles of administration, may partly explain the nontranslation of MSC-induced organ protection from animal models to clinical applications. Therefore, one of the main challenges in both preclinical and clinical research using MSCs remains the standardization of MSC collection, storage, handling, and infusion. In the particular settings of KTx, most of the clinical trials have only included living-donors related to KTRs. Still, from a pragmatic point of view, deceased donors (1) face longer ischemia time and (2) do have significantly worse short- and long-term outcomes compared with living donors.[118] Thus, innovative approaches in ischemic conditioning are especially needed in deceased organ donors. The pilot study of Sun et al was the first one to be designed to assess the prevention of delayed graft function in deceased-donor KTx by MSC infusion. The authors did not observe significant differences between MSC-treated and control patients, possibly because of the limited sample size and the parameters of MSC injections.[92] In our study, MSC-treated KTRs from deceased-donors display a significantly better eGFR on day 7 when compared with the whole cohort.[93] One may speculate that MSC administration has beneficially post-conditioned the ischemic kidney graft, although our study was not statistically powered to assess MSC efficacy. In nonrenal SOT, no clinical trial has thus far evaluated the potential of MSC therapy to reduce I/R injury. In our study comparing 10 MSC-treated LTRs with 10 control LTRs, there was no difference in postoperative immediate peak of liver enzymes.[98] Of note, the feasibility of MSC therapy during organ preservation has been recently successfully tested in a porcine model.[119] Moreover, Brasile et al have demonstrated that MSC exposure during a 24-hour ex vivo "exsanguinous metabolic support" perfusion of human kidneys reduces the renal production of inflammatory cytokines and significantly stimulates kidney regeneration (on the basis of an increased mitosis rate in renal cells).[120] The use of MSC could thus be useful to mitigate I/R injury during organ preservation.

The second major clinical application of MSCs in SOT concerns the minimization of conventional immunosuppression, the treatment of AR, and eventually the induction of tolerance. It "works" in animal models of SOT.[79,81,121-123] In clinical trials, it remains controversial. In our group, only 1 MSC-treated LTR could be weaned of any ISDs at 1-year follow-up,[98] while Soeder et al using MAPC in 3 LTR could not dispense CNI.[97] The Italian group reported 1 KTR with no CNI 6 years after KTx and MSC infusion,[89] despite a CNI trough level during the 1-year follow-up significantly lower in the MSC low-dose CNI group when compared with the 2 others standard-dose CNI group. In China, MSC-treated KTRs showed a lower rate of biopsy-proven AR in the first 6 months.[83] In the Netherlands, 2 KTRs with biopsy-proven AR showed no persistent signs

of tubulitis after MSC treatment.[88] On the basis of these encouraging but variable findings based on a very limited number of subjects, it would be hazardous to claim that MSC-based therapy prevents or treats AR. Concerning the long-term follow-up, no team has convincingly shown operational tolerance after MSC administration in SOT.

In addition to these 2 main applications, MSCs have been tested in the treatment of SOT-associated complications, like CLAD,[103,104] ITBLs,[101] and refractory infective bowel dysfunction.[105]

Finally, no definitive conclusions can be drawn concerning the immunogenicity of MSCs and the clinical relevance of MSC-DSA. Third-party MSCs may offer various advantages, including the selection of healthy donors[124] and the ease of urgent logistical issues at the time of SOT from deceased donors. By contrast, third-party MSCs may expose transplant recipients to additional antigens, thereby risking allo-immunization. The long-term follow-up of patients exposed to third-party MSCs, as well as the information expected from ongoing clinical trials in the field, will help assess the actual immune risk of third-party MSCs compared with autologous cells.

In conclusion, MSC-based cell therapy represents a fascinating opportunity in 2 crucial challenges of SOT: the prevention of I/R injury and the induction of tolerance. The administration of MSCs in SOT recipients is reasonably safe. It is now time to focus on their efficiency. One of the first steps will most probably be the standardization of MSC sources, storage, and culture conditions, as well as the comparative investigations of different timings of infusion and various ISD regimens. Multicenter randomized placebo-controlled double-blinded clinical trials sufficiently powered are basically required.

ACKNOWLEDGMENTS

The authors cordially thank the surgeons (M. Meurisse, C. Coimbra Marques, E. Decker, E. Hamoir, P. Honoré, L. Kohnen, A. Haumann, N. Meurisse, J-P Squifflet), the physicians (J-M Krzesinski, A. Bouquegneau, C. Bonvoisin, C. Bovy, S. Grosch, P. Vanderweckene, L. Vanovermeire and P. Xhignesse), and the members of the local transplant coordination center (M-H Delbouille, M-F Hans and J Monard) for their commitment to kidney transplantation at the University of Liege Academic Hospital in Liege, Belgium, the LTRN (Laurence Poma), and the LTCG for the production of MSCs (Etienne Baudoux, Virginie Bettonville, Alexandre Carpentier, Roland Marini, Sylviane Simar, Pascale Dufour, Amélie Lubke, Amélie Halleux, Tiffany Crepin).

REFERENCES

1. Perico N, Casiraghi F, Remuzzi G. Clinical transplantation of mesenchymal stromal cell therapies in nephrology. *J Am Soc Nephrol.* 2018;29:362–372.
2. Reinders MEJ, van Kooten C, Rabelink TJ, et al. Mesenchymal stromal cell therapy for solid organ transplantation. *Transplantation.* 2018;102:35–43.
3. Rowart P, Erpicum P, Detry O, et al. Mesenchymal stromal cell therapy in ischemia/reperfusion injury. *J Immunol Res.* 2015;2015:602597.
4. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006;8:315–317.
5. Friedenstein AJ, Deriglasova UF, Kulagina NN, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. *Exp Hematol.* 1974;2:83–92.
6. Caplan AI. Mesenchymal stem cells. *J Orthop Res.* 1991;9:641–650.
7. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* 2002;13:4279–4295.
8. Majore I, Moretti P, Stahl F, et al. Growth and differentiation properties of mesenchymal stromal cell populations derived from whole human umbilical cord. *Stem Cell Rev Rep.* 2011;7:17–31.
9. Wang HS, Hung SC, Peng ST, et al. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells.* 2004;22:1330–1337.
10. Strioga M, Viswanathan S, Darinskas A, et al. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev.* 2012;21:2724–2752.
11. Hass R, Kasper C, Böhm S, et al. Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal.* 2011;9:12.
12. Phinney DG, Sensebé L. Mesenchymal stromal cells: misconceptions and evolving concepts. *Cytotherapy.* 2013;15:140–145.
13. Grégoire C, Ritacco C, Hannon M, et al. Comparison of mesenchymal stromal cells from different origins for the treatment of graft-vs.-host-disease in a humanized mouse model. *Front Immunol.* 2019;10:619.
14. Li N, Hua J. Interactions between mesenchymal stem cells and the immune system. *Cell Mol Life Sci.* 2017;74:2345–2360.
15. Gao F, Chiu SM, Motan DA, et al. Mesenchymal stem cells and immunomodulation: current status and future prospects. *Cell Death Dis.* 2016;7:e2062.
16. Shi Y, Su J, Roberts AI, et al. How mesenchymal stem cells interact with tissue immune responses. *Trends Immunol.* 2012;33:136–143.
17. Ren G, Su J, Zhang L, et al. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells.* 2009;27:1954–1962.
18. Meisel R, Zibert A, Laryea M, et al. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood.* 2004;103:4619–4621.
19. Ge W, Jiang J, Arp J, et al. Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation.* 2010;90:1312–1320.
20. Selmani Z, Naji A, Zidi I, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25HIGHFOXP3+ regulatory T cells. *Stem Cells.* 2008;26:212–222.
21. Nasef A, Mathieu N, Chapel A, et al. Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation.* 2007;84:231–237.
22. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood.* 2005;105:1815–1822.
23. Sensebé L, Bourin P. Mesenchymal stem cells for therapeutic purposes. *Transplantation.* 2009;87(9 Suppl):S49–S53.
24. Spees JL, Lee RH, Gregory CA. Mechanisms of mesenchymal stem/stromal cell function. *Stem Cell Res Ther.* 2016;7:125.
25. Chabannes D, Hill M, Merieau E, et al. A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood.* 2007;110:3691–3694.
26. Vandermeulen M, Grégoire C, Briquet A, et al. Rationale for the potential use of mesenchymal stromal cells in liver transplantation. *World J Gastroenterol.* 2014;20:16418–16432.
27. Harrell CR, Fellabaum C, Jovicic N, et al. Molecular mechanisms responsible for therapeutic potential of mesenchymal stem cell-derived secretome. *Cells.* 2019;8:E467.
28. Farzamfar S, Hasanpour A, Nazeri N, et al. Extracellular micro/nanovesicles rescue kidney from ischemia-reperfusion injury. 2019;234:12290–12300.
29. Di Trapani M, Bassi G, Midolo M, et al. Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions. *Sci Rep.* 2016;6:24120.
30. Kusuma GD, Carthew J, Lim R, et al. Effect of the microenvironment on mesenchymal stem cell paracrine signaling: opportunities to engineer the therapeutic effect. *Stem Cells Dev.* 2017;26:617–631.
31. Matula Z, Németh A, Lőrincz P, et al. The role of extracellular vesicle and tunneling nanotube-mediated intercellular cross-talk between mesenchymal stem cells and human peripheral T cells. *Stem Cells Dev.* 2016;25:1818–1832.
32. Cho DI, Kim MR, Jeong HY, et al. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp Mol Med.* 2014;46:e70.

33. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest.* 2012;122:787–795.
34. Spaggiari GM, Capobianco A, Abdelrazik H, et al. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood.* 2008;111:1327–1333.
35. Crop MJ, Korevaar SS, de Kuiper R, et al. Human mesenchymal stem cells are susceptible to lysis by CD8(+) T cells and NK cells. *Cell Transplant.* 2011;20:1547–1559.
36. Reinders ME, Hoogduijn MJ. NK cells and MSCs: possible implications for MSC therapy in renal transplantation. *J Stem Cell Res Ther.* 2014;4:1000166.
37. Zhao ZG, Xu W, Sun L, et al. Immunomodulatory function of regulatory dendritic cells induced by mesenchymal stem cells. *Immunol Invest.* 2012;41:183–198.
38. Liu X, Qu X, Chen Y, et al. Mesenchymal stem/stromal cells induce the generation of novel IL-10-dependent regulatory dendritic cells by SOCS3 activation. *J Immunol.* 2012;189:1182–1192.
39. Li YP, Paczesny S, Lauret E, et al. Human mesenchymal stem cells license adult CD34+ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway. *J Immunol.* 2008;180:1598–1608.
40. Zhang B, Liu R, Shi D, et al. Mesenchymal stem cells induce mature dendritic cells into a novel jagged-2-dependent regulatory dendritic cell population. *Blood.* 2009;113:46–57.
41. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol.* 2002;30:42–48.
42. Plumas J, Chaperot L, Richard MJ, et al. Mesenchymal stem cells induce apoptosis of activated T cells. *Leukemia.* 2005;19:1597–1604.
43. Ménard C, Tarte K. Immunoregulatory properties of clinical grade mesenchymal stromal cells: evidence, uncertainties, and clinical application. *Stem Cell Res Ther.* 2013;4:64.
44. Rasmusson I, Ringdén O, Sundberg B, et al. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation.* 2003;76:1208–1213.
45. Maccario R, Podestà M, Moretta A, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica.* 2005;90:516–525.
46. Duffy MM, Ritter T, Ceredig R, et al. Mesenchymal stem cell effects on T-cell effector pathways. *Stem Cell Res Ther.* 2011;2:34.
47. Braza F, Durand M, Degauque N, et al. Regulatory T cells in kidney transplantation: new directions? *Am J Transplant.* 2015;15:2288–2300.
48. Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood.* 2005;105:2214–2219.
49. Corcione A, Benvenuto F, Ferretti E, et al. Human mesenchymal stem cells modulate B-cell functions. *Blood.* 2006;107:367–372.
50. Krampera M. Mesenchymal stromal cell 'licensing': a multistep process. *Leukemia.* 2011;25:1408–1414.
51. Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair–current views. *Stem Cells.* 2007;25:2896–2902.
52. Khubutiya MS, Vagabov AV, Temnov AA, et al. Paracrine mechanisms of proliferative, anti-apoptotic and anti-inflammatory effects of mesenchymal stromal cells in models of acute organ injury. *Cytotherapy.* 2014;16:579–585.
53. Sordi V. Mesenchymal stem cell homing capacity. *Transplantation.* 2009;87(9 Suppl):S42–S45.
54. Mundra V, Gerling IC, Mahato RI. Mesenchymal stem cell-based therapy. *Mol Pharm.* 2013;10:77–89.
55. Bi B, Schmitt R, Israilova M, et al. Stromal cells protect against acute tubular injury via an endocrine effect. *J Am Soc Nephrol.* 2007;18:2486–2496.
56. Geng Y, Zhang L, Fu B, et al. Mesenchymal stem cells ameliorate rhabdomyolysis-induced acute kidney injury via the activation of M2 macrophages. *Stem Cell Res Ther.* 2014;5:80.
57. Schrepfer S, Deuse T, Reichenspurner H, et al. Stem cell transplantation: the lung barrier. *Transplant Proc.* 2007;39:573–576.
58. Eggenhofer E, Benseler V, Kroemer A, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol.* 2012;3:297.
59. Duffield JS, Park KM, Hsiao LL, et al. Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells. *J Clin Invest.* 2005;115:1743–1755.
60. Prockop DJ. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). *Clin Pharmacol Ther.* 2007;82:241–243.
61. Potapova IA, Gaudette GR, Brink PR, et al. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells in vitro. *Stem Cells.* 2007;25:1761–1768.
62. Tang YL, Zhao Q, Zhang YC, et al. Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. *Regul Pept.* 2004;117:3–10.
63. Tögel F, Weiss K, Yang Y, et al. Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol.* 2007;292:F1626–F1635.
64. Islam MN, Das SR, Emin MT, et al. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med.* 2012;18:759–765.
65. Perico L, Morigi M, Rota C, et al. Human mesenchymal stromal cells transplanted into mice stimulate renal tubular cells and enhance mitochondrial function. *Nat Commun.* 2017;8:983.
66. Erpicum P, Rowart P, Poma L, et al. Administration of mesenchymal stromal cells before renal ischemia/reperfusion attenuates kidney injury and may modulate renal lipid metabolism in rats. *Sci Rep.* 2017;7:8687.
67. Alagesan S, Griffin MD. Autologous and allogeneic mesenchymal stem cells in organ transplantation: what do we know about their safety and efficacy? *Curr Opin Organ Transplant.* 2014;19:65–72.
68. Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: immune evasive, not immune privileged. *Nat Biotechnol.* 2014;32:252–260.
69. Le Blanc K, Tammik C, Rosendahl K, et al. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol.* 2003;31:890–896.
70. Le Blanc K, Tammik L, Sundberg B, et al. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol.* 2003;57:11–20.
71. Tse WT, Pendleton JD, Beyer WM, et al. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation.* 2003;75:389–397.
72. Stagg J, Pommey S, Eliopoulos N, et al. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood.* 2006;107:2570–2577.
73. Schu S, Nosov M, O'Flynn L, et al. Immunogenicity of allogeneic mesenchymal stem cells. *J Cell Mol Med.* 2012;16:2094–2103.
74. Chan WK, Lau AS, Li JC, et al. MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge. *Exp Hematol.* 2008;36:1545–1555.
75. Zangi L, Margalit R, Reich-Zeliger S, et al. Direct imaging of immune rejection and memory induction by allogeneic mesenchymal stromal cells. *Stem Cells.* 2009;27:2865–2874.
76. Eliopoulos N, Stagg J, Lejeune L, et al. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood.* 2005;106:4057–4065.
77. Pileggi A, Xu X, Tan J, et al. Mesenchymal stromal (stem) cells to improve solid organ transplant outcome: lessons from the initial clinical trials. *Curr Opin Organ Transplant.* 2013;18:672–681.
78. Lohan P, Treacy O, Griffin MD, et al. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells and their extracellular vesicles: are we still learning? *Front Immunol.* 2017;8:1626.
79. De Martino M, Zonta S, Rampino T, et al. Mesenchymal stem cells infusion prevents acute cellular rejection in rat kidney transplantation. *Transplant Proc.* 2010;42:1331–1335.
80. Hara Y, Stolk M, Ringe J, et al. In vivo effect of bone marrow-derived mesenchymal stem cells in a rat kidney transplantation model with prolonged cold ischemia. *Transpl Int.* 2011;24:1112–1123.
81. Casiraghi F, Azzollini N, Todeschini M, et al. Localization of mesenchymal stromal cells dictates their immune or proinflammatory effects in kidney transplantation. *Am J Transplant.* 2012;12:2373–2383.
82. Crop MJ, Baan CC, Korevaar SS, et al. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. *Transplantation.* 2009;87:896–906.
83. Tan J, Wu W, Xu X, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA.* 2012;307:1169–1177.
84. Mudrabettu C, Kumar V, Rakha A, et al. Safety and efficacy of autologous mesenchymal stromal cells transplantation in patients undergoing living donor kidney transplantation: a pilot study. *Nephrology (Carlton).* 2015;20:25–33.

85. Peng Y, Ke M, Xu L, et al. Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: a clinical pilot study. *Transplantation.* 2013;95:161–168.
86. Perico N, Casiraghi F, Gotti E, et al. Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation. *Transpl Int.* 2013;26:867–878.
87. Perico N, Casiraghi F, Introna M, et al. Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol.* 2011;6:412–422.
88. Reinders ME, de Fijter JW, Roelofs H, et al. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl Med.* 2013;2:107–111.
89. Perico N, Casiraghi F, Todeschini M, et al. Long-term clinical and immunological profile of kidney transplant patients given mesenchymal stromal cell immunotherapy. *Front Immunol.* 2018;9:1359.
90. Pan GH, Chen Z, Xu L, et al. Low-dose tacrolimus combined with donor-derived mesenchymal stem cells after renal transplantation: a prospective, non-randomized study. *Oncotarget.* 2016;7:12089–12101.
91. Lee H, Park JB, Lee S, et al. Intra-osseous injection of donor mesenchymal stem cell (MSC) into the bone marrow in living donor kidney transplantation; a pilot study. *J Transl Med.* 2013;11:96.
92. Sun Q, Huang Z, Han F, et al. Allogeneic mesenchymal stem cells as induction therapy are safe and feasible in renal allografts: pilot results of a multicenter randomized controlled trial. *J Transl Med.* 2018;16:52.
93. Erpicum P, Weekers L, Detry O, et al. Infusion of third-party mesenchymal stromal cells after kidney transplantation: a phase I-II, open-label, clinical study. *Kidney Int.* 2019;95:693–707.
94. Lechanteur C, Briquet A, Giet O, et al. Clinical-scale expansion of mesenchymal stromal cells: a large banking experience. *J Transl Med.* 2016;14:145.
95. Reyes M, Verfaillie CM. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. *Ann N Y Acad Sci.* 2001;938:231–3; discussion 233.
96. Popp FC, Fillenberg B, Eggenhofer E, et al. Safety and feasibility of third-party multipotent adult progenitor cells for immunomodulation therapy after liver transplantation–a phase I study (MISOT-I). *J Transl Med.* 2011;9:124.
97. Soeder Y, Loss M, Johnson CL, et al. First-in-human case study: multipotent adult progenitor cells for immunomodulation after liver transplantation. *Stem Cells Transl Med.* 2015;4:899–904.
98. Detry O, Vandermeulen M, Delbouille MH, et al. Infusion of mesenchymal stromal cells after deceased liver transplantation: a phase I-II, open-label, clinical study. *J Hepatol.* 2017;67:47–55.
99. Shi M, Liu Z, Wang Y, et al. A pilot study of mesenchymal stem cell therapy for acute liver allograft rejection. *Stem Cells Transl Med.* 2017;6:2053–2061.
100. Zhang Y, Zheng J, Cai J, et al. Clinical application of mesenchymal stromal cells with a simplified protocol for ABO incompatible liver transplantation in severe hepatic failure patients. *Am J Transpl.* 2017;17(Suppl 3). Available at https://atcmeetingabstracts.com/abstract/clinical-application-of-mesenchymal-stromal-cells-with-a-simplified-protocol-for-abo-incompatible-liver-transplantation-in-severe-hepatic-failure-patients/. Accessed May 1, 2019.
101. Zhang YC, Liu W, Fu BS, et al. Therapeutic potentials of umbilical cord-derived mesenchymal stromal cells for ischemic-type biliary lesions following liver transplantation. *Cytotherapy.* 2017;19:194–199.
102. Hartleif S, Schumm M, Döring M, et al. Safety and tolerance of donor-derived mesenchymal stem cells in pediatric living-donor liver transplantation: the MYSTEP1 study. *Stem Cells Int.* 2017;2017:2352954.
103. Chambers DC, Enever D, Lawrence S, et al. Mesenchymal stromal cell therapy for chronic lung allograft dysfunction: results of a first-in-man study. *Stem Cells Transl Med.* 2017;6:1152–1157.
104. Keller CA, Gonwa TA, Hodge DO, et al. Feasibility, safety, and tolerance of mesenchymal stem cell therapy for obstructive chronic lung allograft dysfunction. *Stem Cells Transl Med.* 2018;7:161–167.
105. Ceresa CD, Ramcharan RN, Friend PJ, et al. Mesenchymal stromal cells promote bowel regeneration after intestinal transplantation: myth to mucosa. *Transpl Int.* 2013;26:e91–e93.
106. Reddy S, Vrakas G, Chen M, et al. Third-party mesenchymal stromal cell therapy and risk of allosensitisation in transplant patients. *Transplantation.* 2017;101:S64.
107. Doğan SM, Kılınç S, Kebapçı E, et al. Mesenchymal stem cell therapy in patients with small bowel transplantation: single center experience. *World J Gastroenterol.* 2014;20:8215–8220.
108. Lalu MM, McIntyre L, Pugliese C, et al; Canadian Critical Care Trials Group. Safety of cell therapy with mesenchymal stromal cells (safecell): a systematic review and meta-analysis of clinical trials. *Plos One.* 2012;7:e47559.
109. Metzger RA, Delmonico FL, Feng S, et al. Expanded criteria donors for kidney transplantation. *Am J Transplant.* 2003;3 (Suppl 4):114–125.
110. Gastaca M. Extended criteria donors in liver transplantation: adapting donor quality and recipient. *Transplant Proc.* 2009;41:975–979.
111. Souidi N, Stolk M, Seifert M. Ischemia-reperfusion injury: beneficial effects of mesenchymal stromal cells. *Curr Opin Organ Transplant.* 2013;18:34–43.
112. Jin G, Qiu G, Wu D, et al. Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia-reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats. *Int J Mol Med.* 2013;31:1395–1401.
113. Kanazawa H, Fujimoto Y, Teratani T, et al. Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. *Plos One.* 2011;6:e19195.
114. Erpicum P, Detry O, Weekers L, et al. Mesenchymal stromal cell therapy in conditions of renal ischaemia/reperfusion. *Nephrol Dial Transplant.* 2014;29:1487–1493.
115. Westenfelder C, Togel FE. Protective actions of administered mesenchymal stem cells in acute kidney injury: relevance to clinical trials. *Kidney Int Suppl (2011).* 2011;1:103–106.
116. Gooch A, Doty J, Flores J, et al. Initial report on a phase I clinical trial: prevention and treatment of post-operative acute kidney injury with allogeneic mesenchymal stem cells in patients who require on-pump cardiac surgery. *Cell Ther Transplantation.* 2008;1:31–35.
117. Swaminathan M, Stafford-Smith M, Chertow GM, et al; ACT-AKI investigators. Allogeneic mesenchymal stem cells for treatment of AKI after cardiac surgery. *J Am Soc Nephrol.* 2018;29:260–267.
118. Kramer A, Pippias M, Noordzij M, et al. The European Renal Association – European Dialysis and Transplant Association (ERA-EDTA) registry annual report 2015: a summary. *Clin Kidney J.* 2018;11:108–122.
119. Pool M, Eertman T, Sierra Parraga J, et al. Infusing mesenchymal stromal cells into porcine kidneys during normothermic machine perfusion: intact MSCs can be traced and localised to glomeruli. *Int J Mol Sci.* 2019;20:E3607.
120. Brasile L, Henry N, Orlando G, et al. Potentiating renal regeneration using mesenchymal stem cells. *Transplantation.* 2019;103:307–313.
121. Zhang W, Qin C, Zhou ZM. Mesenchymal stem cells modulate immune responses combined with cyclosporine in a rat renal transplantation model. *Transplant Proc.* 2007;39:3404–3408.
122. Wang Y, Zhang A, Ye Z, et al. Bone marrow-derived mesenchymal stem cells inhibit acute rejection of rat liver allografts in association with regulatory T-cell expansion. *Transplant Proc.* 2009;41:4352–4356.
123. Eggenhofer E, Renner P, Soeder Y, et al. Features of synergism between mesenchymal stem cells and immunosuppressive drugs in a murine heart transplantation model. *Transpl Immunol.* 2011;25:141–147.
124. Reinders ME, Dreyer GJ, Bank JR, et al. Safety of allogeneic bone marrow derived mesenchymal stromal cell therapy in renal transplant recipients: the neptune study. *J Transl Med.* 2015;13:344.