Research Article

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

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**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.



## 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

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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<br>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<br>EBV negative (recipient) |
| Miscellaneous | Auto-immune liver disease (recipient)<br>Endotracheal intubation (recipient)<br>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.

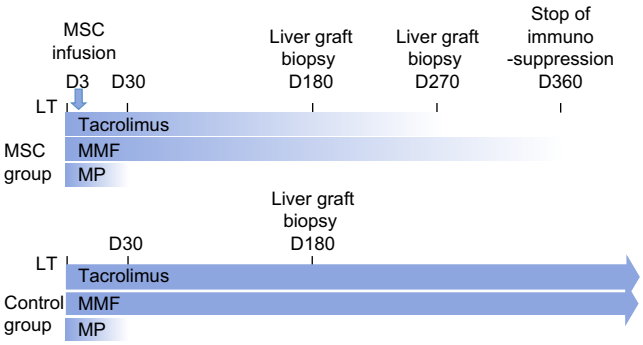


**Fig. 1. Scheme of the study.** MSC: Mesenchymal stromal cell; MMF: mycophenolate mofetil; MP: Methylprednisolone.

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

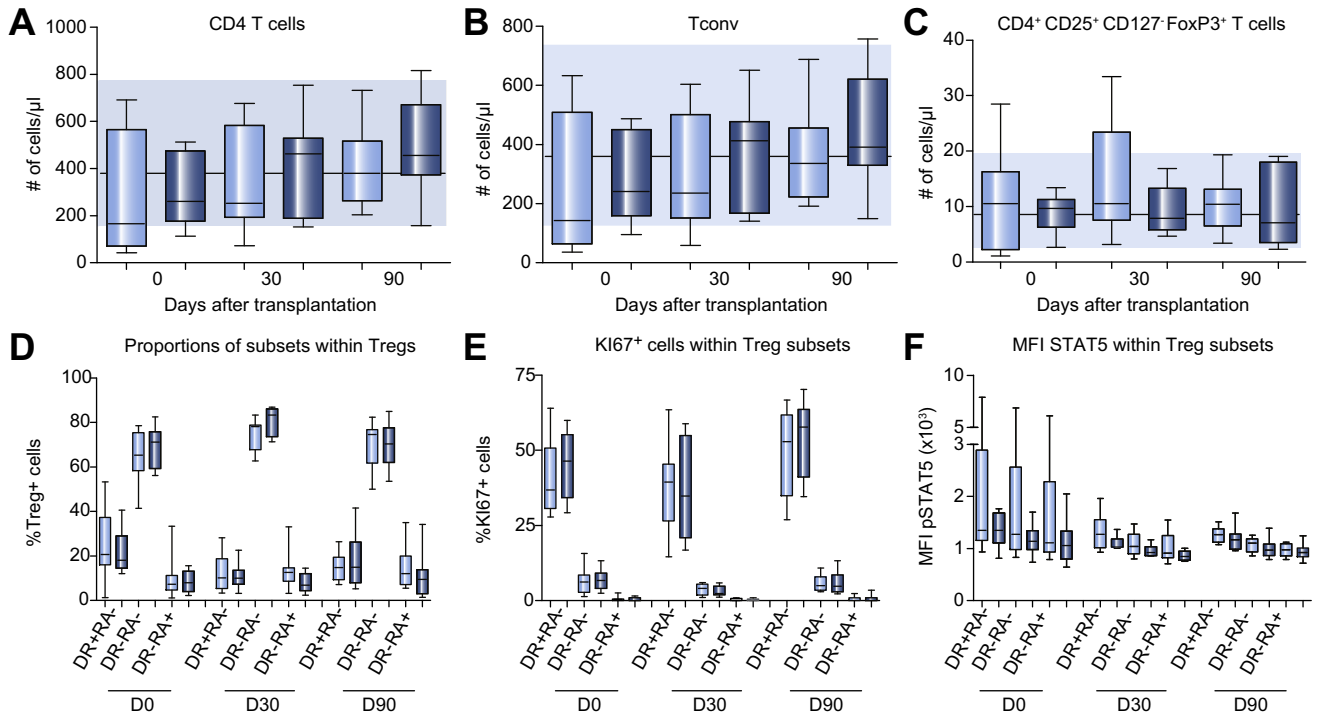


**Fig. 2. Evolution of on peripheral blood CD4⁺ T cells (including Tregs) on days 0, 30 and 90 in the control (light blue boxes) or MSC (dark blue rectangles) groups.** (A) Total CD4⁺ T cells; (B) Conventional CD4⁺ T cells (Tconv); (C) Regulatory T cells (Tregs); (D) Treg subsets; (E) Treg proliferation (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.


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## 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.


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## 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.

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*Author names in bold designate shared co-first authorship*


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