No Evidence for Circulating Mesenchymal Stem Cells in Patients with Organ Injury

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Running title

No Circulating Mesenchymal Stem Cells in Man

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Key words

Circulation, Injury, Migration, Mesenchymal stem cells, Organ

Abbreviations

ISCT: international society for cellular therapy

MSC: mesenchymal stem cells

PBMC: peripheral blood mononuclear cells

SVF: stromal vascular fraction

Abstract

Mesenchymal stem cells (MSC) are present in the bone marrow, from where they are thought to migrate via the blood stream to sites of injury. However, virtually all tissues contain resident MSC that may contribute to local regenerative and immunomodulatory processes, thereby hypothetically pre-empting the need for recruiting MSC via the bloodstream. Although there is some indication for circulating MSC in animal models, there is little solid evidence for the mobilization and migration of MSC in the human circulation. In the present study we were unable to detect MSC in the blood of healthy individuals. We then searched for MSC in the blood of ten patients with end-stage renal disease, ten patients with end-stage liver disease and in eight heart transplant patients with biopsy proven rejection by culturing of mononuclear cells under MSC-supporting culture conditions. In none of these patient categories MSC were identified in the blood. MSC were however found in the blood of a severe trauma patient with multiple fractures, suggesting that disruption of bone marrow leads to the release of MSC into the blood stream. The conclusion of this study is that MSC are not recruited into the circulation in patients with injured solid organs and during aggressive immune responses after transplantation.

Introduction

Mesenchymal stem cells (MSC) are plastic adherent cells with fibroblastic morphology in culture and have regenerative and immunomodulatory properties. They can be induced to undergo multilineage differentiation *in vitro* [1] and are able to modulate immune cell responses [2]. MSC are present in essentially all tissues, where they may have a perivascular localization. MSC express chemokine receptors and integrins, which would allow them to migrate in response to chemokines and home to specific sites. It is commonly suggested that MSC migrate to sites of injury and inflammation, where they assist in dampening immune responses and aiding tissue repair [3-5].

There is, however, controversy about whether MSC can be detected in the circulation in human. While some studies failed to detect MSC in peripheral blood [6], it has been demonstrated that cells with a stromal appearance and osteogenic differentiation potential can be isolated from buffy coats of healthy individuals [7]. Yet, it has been reported that cells with a fibroblast morphology that derive from peripheral blood mononuclear cells (PBMC) in culture actually express a hematopoietic immunophenotype [8] and do therefore not fulfill the criteria for MSC set by the International Society for Cellular Therapy (ISCT) [9]. Others have indicated that MSC can be detected in the peripheral blood in rats after housing them for 3 weeks under hypoxic conditions [10], or in mice after the induction of liver injury by two injections with CCL_4 [11]. These studies demonstrate that MSC can circulate at least under extreme conditions, but do not evidence that this occurs under more physiological conditions. Furthermore, the results may possibly be different in man and murine. In man, there is a report that shows that plastic adherent cells with an MSC immunophenotype are present in the blood of hip fracture patients [12]. It is, however, questionable whether these cells actively enter the circulation via migratory processes or whether they end up in the blood stream via mechanical disruption of the bone tissue. So far, there is no convincing evidence that cells

that adhere to the ISCT criteria for MSC enter the circulation via biological processes in human, while this has been demonstrated for endothelial progenitor cells [13] and hematopoietic progenitor cells [14] and for MSC that are mobilized from graft tissue during organ transplantation [15].

If MSC are capable of migrating via the blood stream to areas of injury, then the highest chance of detecting them would be in diseased individuals. Therefore, in the present study we set out to investigate whether MSC can be isolated from the blood of patients with severe inflammatory and degenerative disease. For this purpose blood samples were collected from end-stage liver failure patients and end-stage kidney failure patients just prior to organ transplantation and from heart transplant patients that went through a rejection episode early after transplantation and examined for the presence of MSC. As a positive control, blood was collected from severe trauma patients with multiple bone fractures.

Materials and Methods

Blood sample collection

Blood was collected from healthy individuals and from 10 end-stage liver disease and 10 end-stage kidney disease patients on the day prior to organ transplantation. Blood from 8 heart transplant patients was collected at the time of a biopsy proven rejection within the first weeks after transplantation. Blood from 2 trauma patients that fell from 10 meter heights thereby acquiring multiple bone fractures and other injuries was collected respectively 8 and 9 days after the accidents. Blood was collected in heparin tubes and PBMC were separated by Ficoll-Paque PLUS (density 1.077g/mL; GE Healthcare, Uppsala, Sweden). PBMC were stored at -150 ℃ until usage. Blood sample collections were approved by the institutional Medical Ethical Committee (MEC-2010-022).

Flow cytometric analysis of PBMC

PBMC were stained for CD45, CD73, CD90 and CD105 (all BD Biosciences, San Diego, CA, USA) and analyzed on a FACSCanto II flow cytometer with BD FACSDiva software (BD Biosciences). At least 500,000 $CD45^-$ events were gated and analyzed for the expression of the MSC cell surface markers CD73, CD105 and CD90. To detect the specificity of the staining, cells stained only for CD45 were used.

Culture of PBMC

PBMC were thawed, washed and seeded at approximately 200,000 cells per cm^2 in minimum essential medium Eagle alpha modification (MEM-α) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin (p/s; 100IU/ml penicillin, 100 µg/ml streptomycin; Lonza) and 15% fetal bovine serum (FBS) (Lonza). The cultures were kept at 37 ℃ in a humidified atmosphere

with 5% CO_2. After three days the culture medium was refreshed to remove non-adherent cells. Subsequently, medium was refreshed twice weekly.

Culture of MSC from adipose tissue

The stromal vascular fraction (SVF) of adipose tissue is a rich source of MSC. To determine whether ficoll separation would affect the culture of MSC, the SVF was isolated as described before [16] and subjected to Ficoll-Paque PLUS. In brief, subcutaneous adipose tissue was collected from healthy individuals that underwent surgery for kidney donation after written informed consent as approved by the institutional Medical Ethical Committee. Adipose tissue was mechanically disrupted and enzymatically digested with collagenase type IV (Life Technologies, Paisley, UK). The obtained SVF was put on Ficoll-Paque PLUS and the mononuclear fraction cultured in culture medium as described above. Culture medium was refreshed twice weekly.

Culture of MSC liver perfusates

We have previously demonstrated that perfusion fluid obtained from liver grafts contains liver-derived MSC, which were found to be highly similar to bone marrow MSC with regard to differentiation potential, gene expression, immunosuppressive and regenerative capacities [15,17]. To test whether ficoll separation and freezing of cells affected the efficiency of MSC culture expansion, liver perfusate cells were separated by Ficoll-Paque PLUS and mononuclear fractions were stored frozen at -150 ℃ until usage. Upon thawing, the cells were treated as described above to serve as positive controls. The use of liver perfusate cells was approved by the institutional medical ethical committee.

Microscopy

Cultures were inspected for the presence of cells with MSC morphology using an Axiovert 40 microscope (Zeiss, Goettingen, Germany). Images were made using a Canon EOS 1000D digital camera and Axiovision software (Zeiss).

Flow cytometric analysis of cultured cells

Cells were removed from culture flasks using 0.05% trypsin-EDTA (Life Technologies, Paisley, Great Britain) and stained for CD13, CD31, CD45, CD73, CD90 and CD105 (all BD Bioscience). Unstained cells were used as negative controls. Cells were analyzed on a FACSCanto II flow cytometer with BD FACSDiva software.

Results

Circulating MSC in healthy individuals

To examine whether cells with an MSC immunophenotype ($CD45^-CD73^+CD90^+CD105^+$), were detectable in the peripheral blood of healthy individuals, PBMC of six healthy volunteers were ficoll-separated and analyzed by flow cytometry. $CD45^+$ cells were out gated and at least 500,000 $CD45^-$ cells, comprising hematopoietic and endothelial progenitor cells, thrombocytes and potentially MSC, were analyzed for expression of the MSC markers CD73, CD90 and CD105. No $CD45^-$ cells that co-expressed CD73, CD90 and CD105 were detected (Figure 1), suggesting cells with a MSC immunophenotype are not present in the blood of healthy individuals. To confirm the lack of MSC in blood by a more sensitive method and to circumvent the possibility that circulating MSC have a different immunophenotype making them undetectable by flow cytometry, PBMC were cultured in MSC supporting medium to detect the outgrowth of MSC colonies from potentially single cells. No MSC colonies were found in cultures of PBMC from healthy individuals (n=5, data not shown).

Circulating MSC in organ failure patients

The lack of MSC in blood of healthy individuals may be explained by the absence of injury or inflammation, which may act as a trigger for the mobilization and attraction of MSC. To examine whether MSC are present in the blood of patients with injury and/or inflammation, blood was collected from three groups of organ failure patients with abundant inflammation and tissue damage. The first group comprised end-stage kidney disease patients on dialysis of whom blood was collected on the day prior to kidney transplantation (n=10). The second group were end-stage liver failure patients of whom blood was taken on the day prior to liver transplantation (n=10). The third group were heart transplant patients up to two months after

transplantation. Blood was taken at the time of biopsy-proven rejection (n=8). Patient characteristics are shown in table 1.

PBMC were put in culture in MSC-supporting culture medium. In all cultures plastic adherent cells were visible within days, but no cells with an MSC/fibroblastic morphology were observed. Instead, the cells showed a macrophage-like morphology (Figure 2A). Flow cytometric analysis of the cultures after 2-3 weeks demonstrated that all cells were $CD45^+$ and a subset was $CD13^+$ and weakly $CD31^+$ and $CD105^+$ (Figure 3A). The cells thereby did not meet the MSC immunophenotype ($CD45^-$, $CD31^-$, $CD13^+$, $CD73^+$, $CD90^+$, $CD105^+$).

MSC from adipose tissue, liver perfusate, and blood from trauma patients

To rule out that the ficoll separation procedure was toxic for MSC and to confirm that the culture conditions used were supportive for MSC, cells of the stromal vascular fraction (SVF) of adipose tissue, known to be rich of MSC [18] and routinely isolated and cultured in our laboratory [16,19], were put on ficoll and brought into culture (n=1). In addition, liver transplant perfusates, previously demonstrated to contain MSC [15], were put on ficoll and the obtained cells were stored frozen. Subsequently, cells were thawed and brought into culture (n=2). Adherent cells with a fibroblastic morphology appeared within days after seeding of the SVF and liver perfusate cell suspensions (Figure 2B,C). Finally, as MSC were previously reported in the blood of hip fracture patients [12], PBMC were collected from the blood of trauma patients with multiple bone fractures (see table 1 for details) and brought into culture. In one of the patients, cells with an MSC morphology appeared in the cultures (Figure 2D). This indicates that viable MSC can be present in the blood and can be brought into culture, although the cells most likely originated from a single colony.

Flow cytometric analysis of the cells cultured from adipose tissue and liver perfusates demonstrated that all cells expressed the MSC cell surface markers CD13, CD73, CD90 and

CD105 and were negative for CD31 and CD45 (Figure 3B,C). In a culture of PBMC from one of the trauma patients a subset of cells was found that was $CD45^-$ and $CD31^-$ (Figure 3D). Analysis of the $CD45^-$ cells demonstrated that the majority of these cells were $CD13^+$, $CD73^+$, $CD90^+$ and $CD105^+$, confirming their MSC phenotype (Figure 3E).

These results indicate that the isolation and culture procedures used in the present study were supportive for the culture of MSC, but that MSC are not present in blood of organ failure patients.

Discussion

The role of MSC in mediating tissue repair and modulating immune responses has been considered in many research and review papers. The mechanisms via which these processes may occur are however largely unclear and therefore widely speculated upon. One of the key questions on MSC biology is whether these cells can migrate towards sites of injury and inflammation. There is some evidence that infused MSC home to such sites [3-5], but other studies indicated that although MSC have a beneficial effect, they act from a distance and are short lived after infusion [20,21].

In the present study we investigated whether MSC could be detected in the circulation of patients with severe organ failure. While MSC could not be found in the blood of healthy individuals by us and others [8,22], it was hypothesized that they would be recruited into the bloodstream by signals coming from injured organs. Although several studies demonstrated the mobilization of hematopoietic stem cells in organ injury [23,24], we could not detect MSC in the blood of patients with end-stage kidney failure, end-stage liver failure, or in patients that underwent a rejection episode shortly after heart transplantation. We could only detect MSC in blood of a trauma patient with multiple bone fractures, which is in line with a previous study that detected MSC in blood of hip fracture patients [12]. In bone fracture patients, MSC may be released into the blood stream by mechanical disruption of the bone marrow rather than by active migration.

The results of this study imply that MSC are not recruited to sites of injury via the bloodstream. It is possible that the medication taken by the large majority of the patients is harmful to circulating MSC. However, medication was very variable between all patients. Furthermore, MSC can be isolated from bone marrow and adipose tissue of patients with end-stage renal disease that are on similar medication [25,26]. Therefore it is reasonable to assume that the blood stream is not the route of migration for MSC. It was recently demonstrated that

adipose tissue-derived MSC can be found in lymph fluid, suggesting that the lymph system is used by MSC as a route for migration [27]. A possible limitation of the present study is that the organ injuries of the studied patients were not acute enough for recruitment of MSC from distant sites. In particular the kidney disease patients suffered from chronic disease, whereas the liver disease patients contained both patients with acute and chronic disease. The heart transplant patients suffered from acute rejection, but the time of diagnosis of rejection may be some time after the onset of the rejection. We cannot totally exclude the possibility that MSC are recruited into the bloodstream shortly after severe organ injury.

The discrepancy between the results of the present study and other, in particular animal, studies may in the first place depend on the differences between the biology of experimental animals and human. Rodents show better regenerative responses than human and this might rely on the recruitment of distant MSC in rodents and the lack of this regenerative mechanism in human. Furthermore, circulating rodent MSC may show a different phenotype than human MSC, making them easier detectable by flow cytometry. Possibly rodent circulatory MSC are better adapted to cell culture conditions than human circulatory MSC, making them better available after seeding in a culture dish. Finally, experimental animals are usually exposed to more extreme conditions than humans and can be studied in a more controlled manner. Trauma can be timed and varied in severity and blood can be taken on chosen time points in animal studies. This is not possible and not desirable in human studies.

Our data from organ transplant patients are in support of the idea that MSC do not migrate via the blood stream towards injured organs. Transplantation is a unique model to pursuit migrating MSC as recipient cells migrating to the donor organ can be detected by HLA typing. Furthermore, transplanted organs are initially not connected to the lymph vasculature, leaving the circulation as the only route towards the organ. We have previously demonstrated that recipient MSC are not present in heart transplants up to six years after transplantation

[28]. These heart transplants suffered from brain death and ischemia-reperfusion related injury and were exposed to alloreactivity for years but even so were found to contain only donor MSC. A study in lung transplants found very similar data [29]. These data are supportive for the lack of evidence for circulating MSC.

Whereas MSC and hematopoietic cells are considered to belong to diverged lineages with specialized functions, there is some evidence that the distinction between the cells may not be as sharp as commonly thought. Circulating $CD14^+$ monocytes can be transformed in multipotent cells *in vitro* that have endothelial and mesenchymal differentiation potential [30]. Adipose tissue-derived hematopoietic cells contain a population of macrophages that, like MSC, can differentiate into osteogenic, adipogenic and chondrogenic lineages [31]. It is therefore not unlikely that under particular conditions hematopoietic cells can be mistaken for MSC. Hypothetically, hematopoietic cells with differentiation capacity migrate to injured organs to contribute to regeneration and immune modulation.

In conclusion, MSC, with the phenotype and characteristics as described by the ISCT, are not present in the circulation. All organs contain resident MSC that can contribute to organ repair and immunomodulation, making the recruitment of MSC from distant sites obsolete.

Disclosures

No competing financial interests exist.

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Legends

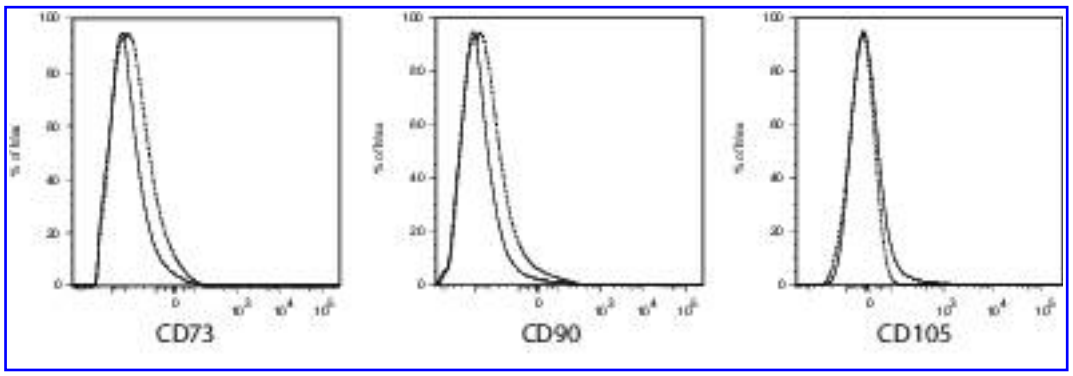


Figure 1. Flow cytometric analysis of PBMC of healthy individuals for the presence of MSC. At least 500,000 $CD45^-$ events were gated and analyzed for expression of the MSC markers CD73, CD90 and CD105. Solid line histograms represent stained cells, dotted histograms represent unstained cells. A representative experiment is shown.

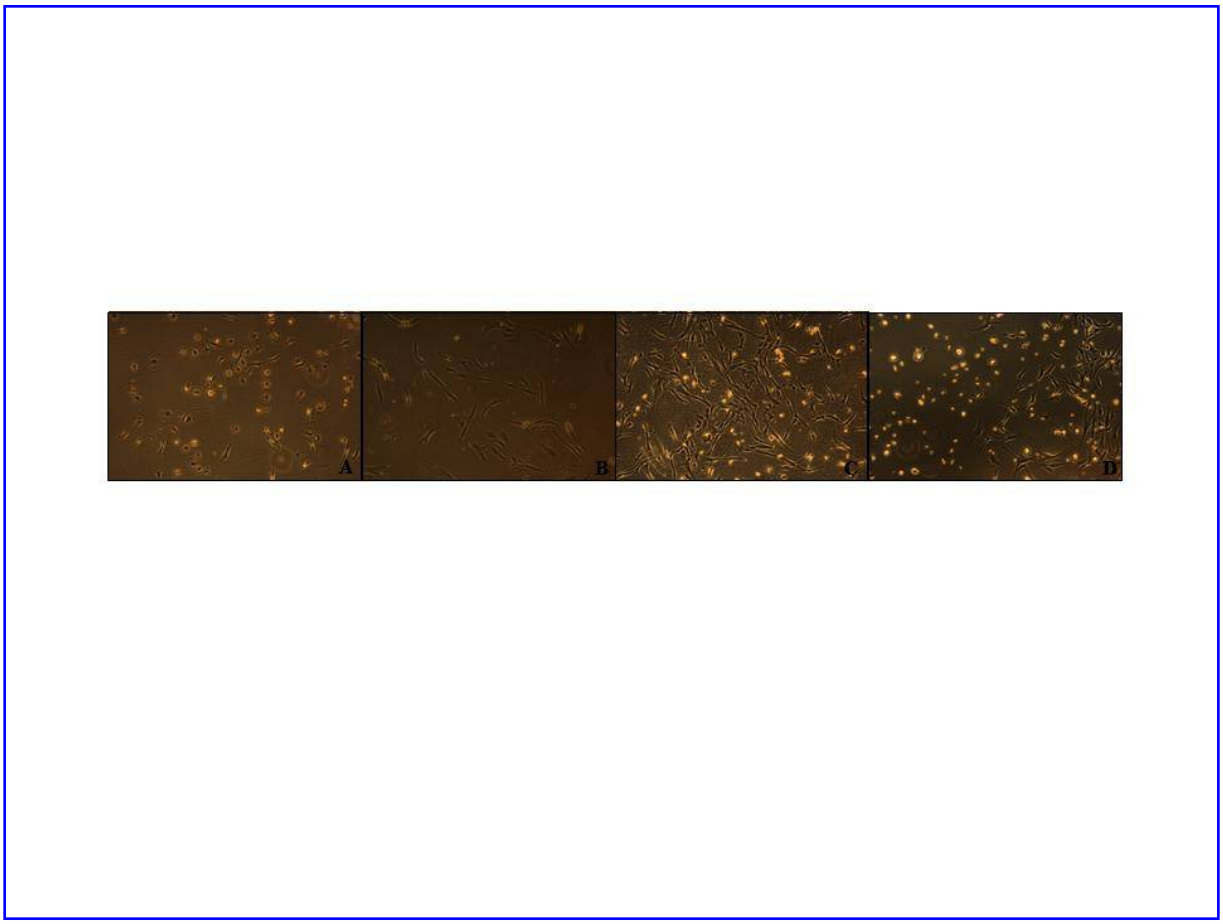
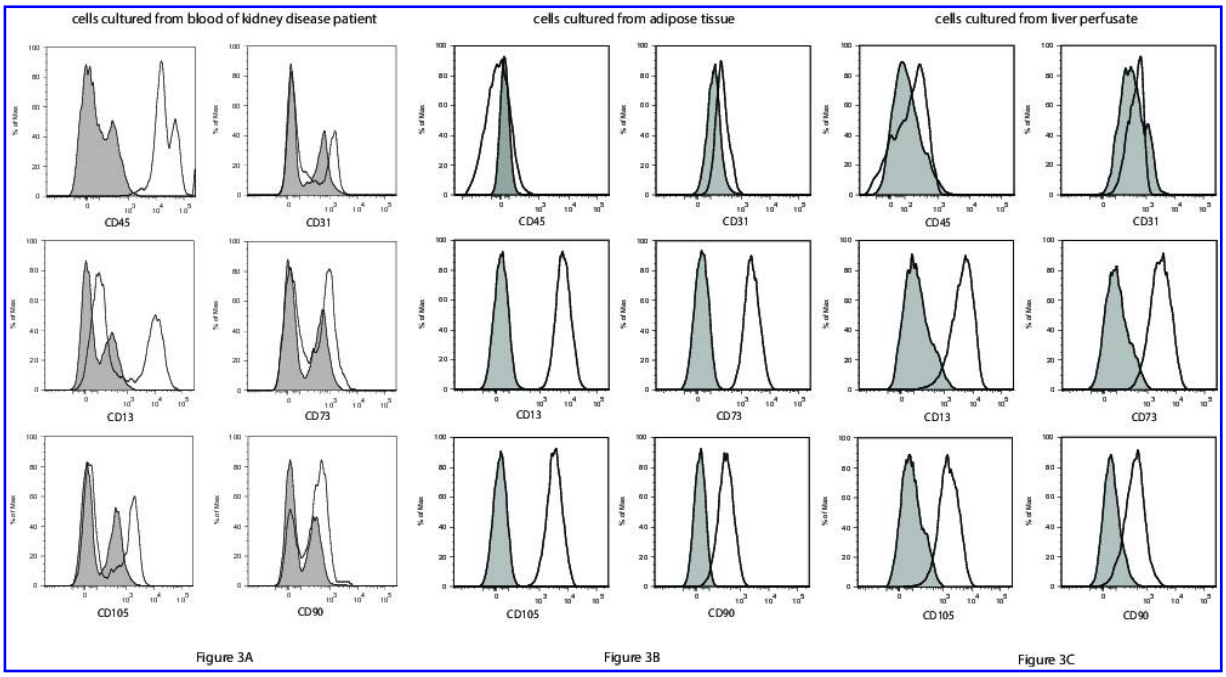


Figure 2. Cultures of PBMC from blood of organ failure patients do not contain cells with an MSC morphology, while adipose tissue, liver perfusates and PBMC from blood from a trauma patient do. A-D: Representative microscopic images of adherent cells cultured for 14 days from blood of a liver disease patient (A), a culture of adipose tissue cells (B), cells from a liver perfusate (C) and adherent cells from blood of a trauma patient.



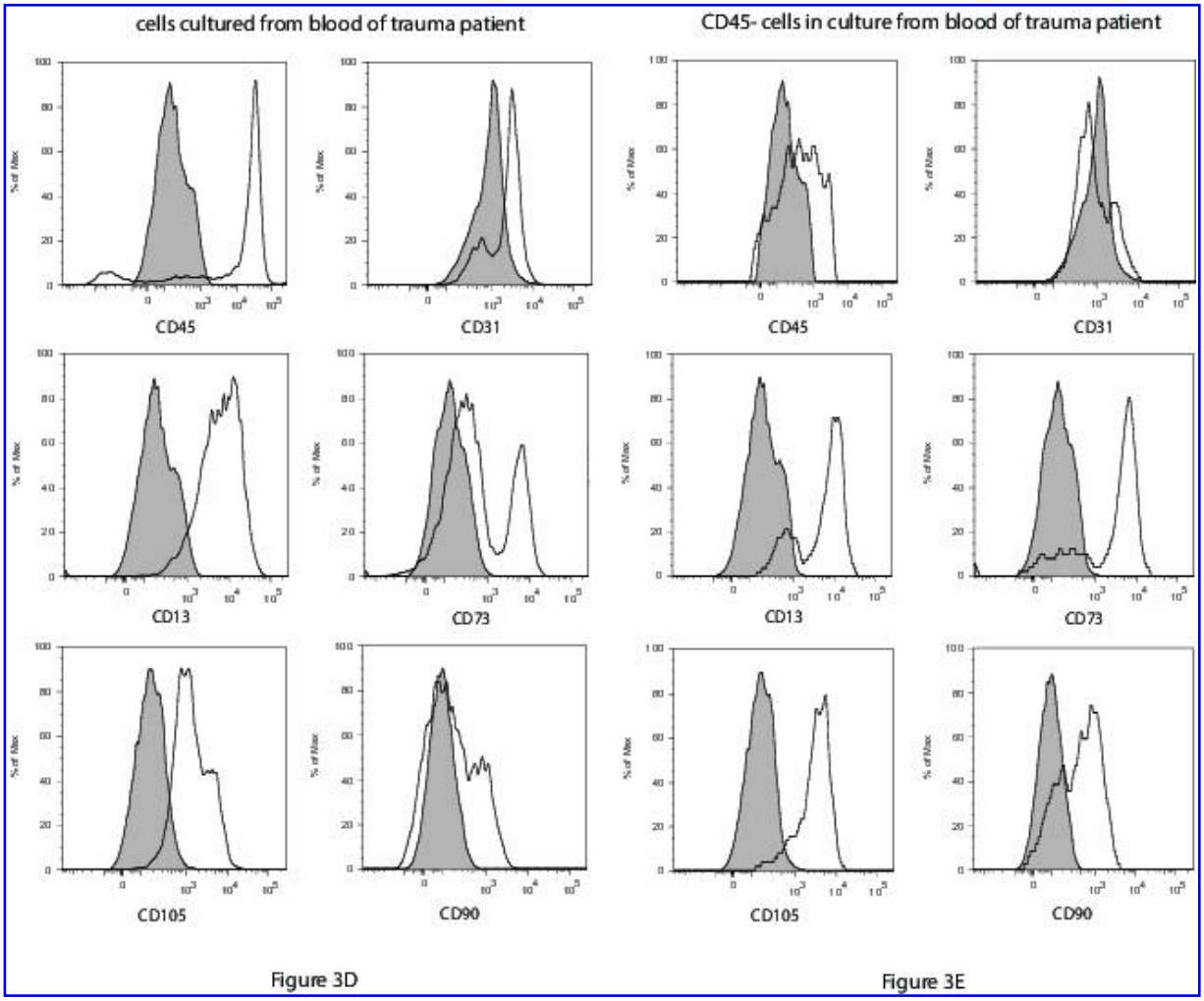


Figure 3. Flow cytometric analysis of adherent cells in cultures of blood from a kidney disease patient (A), adipose tissue (B), liver perfusate (C) and blood of a trauma patient (D-E). A: Only cells with a hematopoietic immunophenotype ($CD45^+$) are present (gated on all cells). B-C: All cells have a MSC immunophenotype ($CD45^-CD31^-CD13^+CD73^+$ $CD90^+CD105^+$) (gated on all cells). D: A population of $CD45^-$ and $CD31^-$ cells is present in the culture (gated on all cells). E: The majority of $CD45^-$ cells express the MSC markers CD13, CD73, CD90 and CD105 (gated on $CD45^-$ cells). Filled histograms represent unstained cells, open histograms represent stained cells.

Patient no.	Age [y] (sex)	Disease	Medication	MSC
Kidney disease patients				
1	75 (M)	Diabetic nephropathy	Amlodipine, Bisoprolol, Alfacalcidol, Sevelamer, Aprovel, Calci-Chew, Insulin	No
2	52 (M)	Chronic kidney failure	Resonium , Telmisartan, Lercanidipine, Colchicine, Carvedilol, Natriumbicarbonaat, Allopurinol, Sevelamer, Ascal, Dianet	No
3	59 (M)	Lithium nephrotoxicity	Loramet, Clopixol, Perindopril, Metoprolol, Ascal, Depakine Chrono, Lamictal, Seroquel, Dianet	No
4	46 (F)	Hypertensive nephropathy	Aranesp, Etalpha, Fraxiparine, Colecalciferol, Calci-Chew, Cinecalcet, Nifedipine, Renagel, Dianet	No
5	68 (M)	Hypertensive and diabetic nephropathy	Rosuvastatine, Metoprolol, Renagel, Colecalciferol, Diamet, Pantozol, Losartan, Perindopril, Barnidipine, Bumetanide, NovoRapid, Lantus, Etalpha, Fraxiparine, Venofer, Aranesp	No
6	65 (F)	Diabetic nephropathy	Alfacalcidol, NovoMix, Sevelamer, Importal, Losartan, Ascal, Metoprolol, Rabeprazol, Pravastatine , Dianet, Calci-Chew, Venofer, Aranesp, Hydroxocobalamine	No
7	52 (M)	Focal segmental glomerulosclerosis	Amlodipine, Atenolol, Furosemide, Crestor, Aprovel, Calci-Chew, Lisinopril, Renvela, Folic acid	No
8	65 (M)	Acute tubular necrosis	Sintrom, Bisoprolol, Dianet, Etalpha, Renvela, Simvastatine	No
9	43 (M)	Hypertensive and diabetic nephropathy	Lyrica, Isoniazide, NovoRapid, Fosrenol, Selokeen, Renvela, Levemir, Ascal, Calci-Chew, Pantozol, Temazepam	No
10	42 (M)	IgA nephropathy	Atrovel, Amlodipine, Metoprolol, Alfacalcidol,	No

			Sevelamer, Fosrenol, Omeprazol, Resonium	
Liver disease patients				
11	58 (F)	End-stage liver disease due to autoimmune hepatitis	Asacol, Furosemide, Sprironolacton, Omeprazol	No
12	48 (M)	Ecstasy induced acute liver failure	-	No
13	49 (M)	End-stage liver disease due to alcoholic liver cirrhosis	Lactulose, Alendronic acid, Folic acid, Pantoprazol, Propranolol, Thiamine, Calcium/vit D	No
14	47 (M)	End-stage liver disease due to primary sclerosing cholangitis	Ursodeoxycholic acid, Spironolacton, Lactulose, Furosemide, Propranolol, Esomeprazol	No
15	39 (F)	End-stage liver disease due to polycystic liver	Lisinopril	No
16	53 (M)	Hepatocellular carcinoma based on alcoholic liver cirrhosis	Metformine, Glicazide, Propranolol, Spironolacton, Furosemide, Lactulose	No
17	52 (F)	Congenital liver fibrosis	Ascal, Metoprolol, Cholestagel, Spironolacton, Ursofalk, Esomeprazol, Pravastatine, Etalpha, Vit A, Vit E	No
18	54 (F)	End-stage liver disease due to alfa-1-antitrypsin deficient liver cirrhosis	Epoetin, Furosemide, Fytomenadion, Lactulose, Thyrax, Metronidazol, Midodrine hydrochloride, Simvastatine, Octreotide	No
19	63 (M)	Hepatocellulair carcinoma based on liver cirrhosis due to hepatitis B	Glycaside, Metformine, Lantus, Metaprolol, Losartan, Lisinopril, Hydrochloorthiazide, Simvastatine, Valaciclovir, Tenofovir	No
20	39 (M)	End-stage liver disease due to primary sclerosing cholangitis	Ursochol, Calci-Chew, Paracetamol, Propranolol, Furosemide, Spironolacton	No
Heart transplant patients				
21	52 (F)	HTx, hypertrophic cardiomyopathy	Tacrolimus, Everolimus, Prednison, Aspirine, Pravastatine, Pantazol, Potassium chloride, Ezetimibe	No
22	35 (M)	HTx, chemotherapy induced cardiomyopathy	Tacrolimus, Prednison, MMF, Pyrimethazine, Clindamycine, Folic acid, Levothyrax, Furosemide, Ferrosulphate, Theolair, Norvasc, Nexium, Magnesium oxide	No
23	63 (F)	HTx, dilated cardiomyopathy	Tacrolimus, MMF, Prednison, Aspirine, Pravastatine, Clindamycine, Pyrimethamine, Folic acid, Pantozol, Amlodipine, Valganciclovir, Cetirizine, Paracetamol	No
24	54 (M)	HTx, dilated cardiomyopathy	Ciclosporine, MMF, Prednison, Furosemide, Aspirine, Pravastatine, Valganciclovir, Pyrimethamine, Sulfadiazine, Folic acid, Pantozol, Potassium chloride, Calcium/vit D	No
25	36 (F)	HTx, cardiac infarct	Isoprenaline, Furosemide, Paracetamol, Ceftazidim, Fungizone, Primperan, Bromazepam, Bromazepam, Antagel, Esomeprazol, Prednisolon, Danaparoid, Mianserine, Movicolon, Tacrolimus, MMF	No
26	46 (F)	HTx, dilated cardiomyopathy	Tacrolimus, MMF, Prednisolon, Acetylsalic acid, Pravastatine, Calcium/vit D, Esomeprazol, Insuline, Temazepam, Pyrimethamine, Clindamycine, Folic acid	No

27	51 (M)	HTx, ischaemic cardiomyopathy	Ciclosporine, MMF, Prednison, Insuline, Valganciclovir, Losartan, Amlodipine, Pantozol, Burinex, Triamtereen, Ketokonazol, Ascal, Pravastatine, Calcium/vit D, Theolair	No
28	59 (M)	HTx, hypertrophic cardiomyopathy	Lasix, Actrapid, Fraxiparine, Prednison, Fungizone, Antagel, Haldol, Tacrolimus, MMF, Movicolon	No
Positive culture controls				
29	37 (F)	Living kidney donor (fat tissue)	-	Yes
30	41 (F)	Non heart beating liver donor (liver perfusate)	Potassium, Cefotaxim, Nitroprusside	Yes
31	59 (F)	Heart beating liver donor (liver perfusate)	Potassium, Phenylefrin, Morphine, Propofol	Yes
32	30 (M)	Trauma patient; fractures in rib, sacrum, pelvis, multiple vertebra, arms and legs	Movicolon, Paracetamol, Metamizol, Ceftriaxon, Fentanyl, Nadroparine	No
33	26 (M)	Trauma patient; fractures in 8 ribs, pelvis, multiple vertebra and skull, hematomas in lungs, brain and retroperitoneal	Movicolon, Paracetamol, Quetiapine, Oxycodon, Oxycontin, Duratears, Haloperidol	Yes

Table 1. Patient characteristics. M = Male; F = Female; HTx = Heart transplantation; MMF = Mycofenolate mofetil