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RESEARCH ARTICLE

Clinical-scale expansion of adipose-derived stromal cells starting from stromal vascular fraction in a single-use bioreactor: proof of concept for autologous applications

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Abstract

Adipose-derived stromal cells (ASCs) are adult multipotent cells increasingly used for cell therapy due to their differentiation potential, their paracrine effect and their convenience. ASCs are currently selected from stromal vascular fractions (SVFs) of adipose tissue and expanded in 2D flasks following good manufacturing practices. This process is limited in surface area, labour-intensive and expensive, especially for autologous applications requiring selection and expansion steps for every patient. Closed and automated bioreactors offer an alternative for scalable and cost-effective production of ASCs. This study investigated a single-use stirred-tank bioreactor that can expand ASCs from SVFs on microcarriers. A preliminary microcarrier screening in static and spinner flask conditions was performed to evaluate the best candidate for adhesion, amplification and harvest. The selected microcarrier was used for process development in the bioreactor. The first experiments showed poor selectivity and growth of the ASCs from the SVF ($n = 2$). The process was then adjusted by two means: (1) decreasing the platelet lysate in the medium for enhancing cell adherence; and (2) adding a shear protectant (Pluronic F68). Following these modifications, we demonstrated that the number of population doublings of ASCs from SVFs was not significantly different between the bioreactor and the 2D controls ($n = 3$). In addition, the ASC characterization after culture showed that cells maintained their clonogenic potential, phenotype, differentiation potential and immunosuppressive capacities. This study provides the proof of concept that isolation and amplification of functional ASCs from SVFs can be performed in a stirred-tank bioreactor combined with microcarriers.

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1. Introduction

Mesenchymal stromal cells (MSCs) are adult multipotent cells increasingly used in clinical trials for cell therapy (Squillaro *et al.*, 2015). They have the capacity to differentiate towards cell types of mesodermal origin, i.e. osteoblasts, adipocytes and chondrocytes (Gimble and Guilak, 2003; Pittenger *et al.*, 1999), making them great tools for tissue engineering approaches (cell replacement therapy). They also have immunomodulatory properties (Le Blanc *et al.*, 2003; Puissant *et al.*, 2005), and a broad paracrine effect, widely used for regenerative medicine approaches (Meirelles Lda *et al.*, 2009). MSCs are located in several tissues and are well-characterized in bone marrow (BM) and adipose tissue (AT). AT is a suitable reservoir of MSCs, commonly called adipose-derived stem cells (ASCs), because this tissue is abundant, easy to harvest and has a high abundance of ASCs in comparison with the number of MSCs in BM (100–500-fold more concentrated than BM; Bourin *et al.*, 2008; Gimble and Guilak, 2003). These properties and convenience lead to the wide use of ASCs for clinical applications (Bura *et al.*, 2014; Hur *et al.*, 2016; Tzouvelekis *et al.*, 2013). Clinical trials using ASCs often require several hundred million cells, for allogeneic and autologous applications. Despite their high frequency, producing such quantities of ASCs requires an *in vitro* amplification. As they are adherent cells, ASCs are principally expanded in static 2D culture flasks, which can be labour-intensive and may be surface area limited. Moreover, production processes have to comply with good manufacturing practices (GMP), including standards of safety and efficacy similar to those established in the pharmaceutical industry (Sensebe *et al.*, 2011, 2013). Consequently, current partially open processes have to be performed in highly classified rooms, with strong environment and staff constraints, leading to expensive production campaigns (Bourin *et al.*, 2011).

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In this context, bioreactor-based systems are an attractive alternative to implement cost-effective production following the guidelines of the pharmaceutical industry. Bioreactors are functionally closed and allow automated regulation of process parameters [agitation, temperature, pH, dissolved oxygen (DO) and other gases] and potentially making scale-up of ASCs affordable. There are a variety of ways to maintain nutrient and metabolite levels and process parameter set-points in a bioreactor setting. Stirred tank bioreactors can be run in batch, fed-batch, media exchange, perfusion mode and other novel paradigms. Thus, they have the flexibility to support the needs of a variety of cells while pH, DO, temperature and agitation set-points are maintained. However, due to a non-static environment, culture of anchorage-dependent cells requires the use of scaffolds or microcarriers. Unlike static culture where nutrients diffuse to cells and where gas exchange occurs at the media–gas interface, stirred conditions reduce the stagnant layer surrounding cells and create a homogeneous environment. Additionally, it allows sampling of representative cell culture material. However, dynamic conditions can generate shear and the transfer of a static culture to a non-static culture is not trivial, especially for poorly adherent cells as native ASCs within AT (internal observations).

In this study, we investigated the ability to expand ASCs using a stirred tank bioreactor and microcarriers. A key feature of anchorage-dependent cell culture on microcarriers is the spontaneous cell transfer to empty or nearly empty microcarriers ('bead to bead transfer'; Ohlson *et al.*, 1994; Wang and Ouyang, 1999). The ability to increase the available surface area through the addition of microcarriers without the need for detachment provides flexibility for developing the expansion process to accommodate low starting numbers of cells while supporting a high yield of cells.

Most studies based on the microcarrier technology focus on allogeneic applications with the main objective of scaling-up ASC productions starting from *in vitro* pre-amplified ASCs (Carmelo *et al.*, 2015; Dos Santos *et al.*, 2014). Our study focused on autologous applications corresponding to moderate scale-up starting from stromal vascular fraction (SVF) directly isolated from fresh AT. Our experience showed that the native ASCs located in the SVF are less adherent than pre-expanded ASCs. Moreover, the SVF is a mixture of several cell populations composed of ASCs, haematopoietic cells, immune cells and endothelial cells (Bourin *et al.*, 2013; Tallone *et al.*, 2011). Consequently, our main goal was to bring the proof of concept that a homogeneous and functional population of ASCs can be obtained in a non-static environment, starting from SVF, using functionally closed, automated and scaled-up conditions. With such a process, cost-efficient autologous ASC production can be achieved due to decreased labour intensity and decreased clean-room requirements. It could allow ASC production in lower classified manufacturing space.

Moreover, although the use of a chemically defined medium is the ultimate achievement for standardized production, internal observations and other works (Tan *et al.*, 2015) showed that serum-free formulations are cell-line specific and that successful culture of cells in serum-free conditions in planar culture does not predict success in microcarrier culture environments. Moreover, other studies showed that medium supplemented with platelet lysate (PL) compared with fetal bovine serum (FBS)-containing medium, delivers increased growth and comparability between monolayer and microcarrier culture (Heathman *et al.*, 2016). Pending the developments of new serum-free media formulations, this study was conducted using a medium supplemented with human PL.

This study required preliminary investigations to identify the best microcarrier for ASC culture and especially for culture of the SVF. The first step of screening was performed in static conditions and a second step in suspension in spinner flasks. At the end of these screening steps, the selected microcarrier was used for scale-up in the 3-l bioreactor. The transfer from small-scale (100 ml in spinners flasks) to medium-scale (0.8–2 l in the bioreactor) required optimization to allow the start-up of the culture. Unlike single cell suspensions, which can be scaled across bioreactors using power per unit volume, solid–liquid suspensions such as those of stem cells grown on a microcarrier can utilize a principle of 'just suspended'. This is a state where no microcarriers remain on the bottom of the vessel although there is a visible gradient of microcarriers in the vessel. The impeller speed that maintains this state of suspension is called the just suspended speed (N_{js}) and can be calculated using the Zwietering correlation (Zwietering, 1958). ASCs isolated and expanded in the bioreactor were compared with ASCs isolated and expanded in 2D standard conditions by assessing population doublings, phenotype, clonogenic potential, differentiation capacities and immunosuppression potential.

2. Materials and methods

This study was approved by the Institutional Review Board, Rangueil University Toulouse Hospital, and was performed in accordance with the principles of the Declaration of Helsinki (1964) and in agreement with French bioethics laws of July 7th, 2011. After informed consent obtained from the patient, AT was harvested during surgery from healthy human adult patients ($n = 13$) at Rangueil University Toulouse Hospital. The mean age was 36 ± 11 years.

2.1. Culture medium

For culture on microcarriers in static conditions and in spinner flasks, and for their respective controls in 2D flasks, culture medium consisted of α-modified Eagle's medium (α-MEM; Thermofisher Scientific, Waltham, MA, USA) supplemented with 2 mg/ml ciflox (Bayer,

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Leverkusen, Germany), 1 U/ml heparin Choay (Sanofi-Aventis, Franckfurt, Germany) and 2% PL. This medium corresponded to standard medium (SM).

After the first two experiments with SM in the bioreactor and their respective controls, the following three experiments were performed in the bioreactor and their respective 2D controls with medium consisting of α-modified Eagle's medium supplemented with 2 mg/ml ciflox, 1 U/ml heparin Choay, 5% PL and 0.1% Pluronic® F68 (Thermofisher Scientific, Waltham, MA, USA).

2.2. PL preparation

Platelet lysate was prepared from outdated platelet concentrates provided by the French Blood Institute. Platelet concentrates were frozen (−80 °C)/thawed (+37 °C) twice, then frozen (−80 °C)/thawed (+4 °C) once to release growth factors. PL was centrifuged and filtered through 0.8-μm and 0.2-μm filters (Thermofisher Scientific, Waltham, MA, USA). PL was stored at −80 °C until use.

2.3. SVF isolation

Subcutaneous ATs were washed with phosphate-buffered saline (PBS). Tissues were then digested by collagenase NB4 (Coger, Paris, France) with agitation, at 37 °C, for 45 min. Enzymatic action was stopped and digestate was filtered through a 100-μm cell strainer (Becton Dickinson, Franklin Lakes, New Jersey, USA). The digestate was centrifuged for 10 min, at 600 ***g***. The pellet (= SVF) was suspended in SM.

2.4. 2D control culture

The SVF was seeded at 4000 cells/cm^2 in plastic flasks (TPP, Trasadingen, Switzerland and EMD Millipore, MA, USA). The medium used was the same in the plastic flask and the corresponding experiment with microcarriers. Cells were washed at day 1 with PBS to remove non-adherent cells. Medium was changed twice a week. Cells were harvested at the same time and using the same enzymatic solution for both the control and the microcarrier cultures. For experiments longer than 8 days, an intermediate harvesting and re-seeding was performed at day 8.

2.5. Culture on microcarriers

2.5.1. In static conditions: screening

These experiments (n = 3) were performed in six-well Ultra Low Attachment plates (total volume = 14 ml/well; Corning, New York, USA). The medium used was SM and the working volume was 2 ml (depth of the medium = 0.5 cm). The Corning® microcarriers (Corning, New York, USA): Enhanced Attachment and Synthemax II (Table 1); and the Solohill® microcarriers (Solohill, MI, USA): Collagen, Fact III, Plastic, Plastic plus, Glass and Pronectin F (Table 1) were added at a final concentration of 15 g/l. The Solohill® microcarriers: Hillex II (Table 1), were plated at a final concentration of 10 g/l. For all, the available surface on microcarriers was 9.3 cm^2/well.

The SVF was seeded with a density of 4000 cells/cm^2; 50% of SM was changed at day 1, and then twice a week. Cells were detached using TrypLE Select 1X (Thermofisher Scientific, Waltham, MA, USA), separated from microcarriers with a 100-μm cell strainer (Becton Dickinson, Franklin Lakes, New Jersey, USA) and counted at day 8 using trypan blue exclusion (Thermofisher Scientific, Waltham, MA, USA).

2.5.2. In stirred and small-scale conditions

Screening experiments (n = 3) were performed with three microcarriers previously selected in static conditions: the Corning® Enhanced Attachment and Synthemax II microcarriers; and the Solohill® Plastic Plus microcarriers (Table 1). After screening, all experiments (n = 7) were performed with the selected Corning® Enhanced Attachment microcarriers (Table 1). For all experiments, cultures were performed in spinner flasks (Corning, New York, USA) with a final microcarrier concentration of 15 g/l, and an available surface between 240 and 540 cm^2 (1 g microcarriers = 360 cm^2). The medium used was SM and the cell seeding density was 4000 cells/cm^2. The agitation speed was 20 rpm until day 4, and 35 or 45 rpm until day 7. A washing step with PBS was performed at day 1, and complete medium exchanges were performed twice a week after cell settling. Cells were harvested and counted at day 7, with the same protocol as the static conditions.

2.5.3. In the Mobius® 3-l bioreactor

These experiments (n = 5) were performed in the Mobius® 3-l bioreactor (Merck KGaA, Darmstadt, Germany) in collaboration with Millipore S.A.S. (Molsheim, France) and EMD Millipore (Bedford, MA, USA). This system is a single-use stirred-tank bioreactor that comes pre-assembled and gamma irradiated. It is fitted with a marine impeller, a vent filter, three options for gassing (overlay, open-pipe and sintered

Table 1. Characteristics and nomenclature of microcarriers

Manufacturer	Name	Treatment/ Coating	Code
Corning	Enhanced attachment	Physical surface treatment	A
	Synthemax II	Extracellular matrix peptides	B
Solohill	Collagen	Type A porcin gelatin	C
	Fact III	Type A porcin gelatin, cationic	D
	Plastic	Styrene copolymer	E
	Plastic Plus	Styrene copolymer, cationic	F
	Glass	Glass	G
	Pronectin F	Recombinant protein	H
	Hillex II	Trimethylammonium, cationic, semi-porous	I

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microsparger), three probes ports, one thermowell, four fluid addition lines, one sub-surface inlet/outlet, one bottom harvest and one sampling valve. All tubing (except gas lines) are made of Cflex®. The maximum working volume is 2.4 l. The bioreactor was combined with an Applikon EZ Controller (Applikon Biotechnology B.V., Delft, the Netherlands) provided with three probes: pH (AppliSens); temperature (PT100); and DO (AppliSens), used for monitoring and control. A Terumo SCD IIB tube welder (Terumo, Tokyo, Japan) was used to add fresh media and wash solution as well as to withdraw spent media in a sterile manner (Figure 1). For all experiments, sterile bags containing the mixture medium/microcarriers, cells, PL, wash buffer and enzyme for detachment were previously prepared using a peristaltic pump under biosafety cabinet. After these preparations, experiments were performed outside the biosafety cabinet. Cell growth was daily evaluated by sampling (using the sampling valve), and metabolites were measured (glucose and lactate) using the Contour TS Blood Glucose Monitoring System (Bayer, Leverkusen, Germany) and the Lactate Pro Blood Lactate Test Meter (Arkray, Kyoto, Japan).

The first two runs were performed in a volume of 800 ml of SM, with 12 g of Corning® Enhanced Attachment microcarriers [4320 cm^2 and an initial seeding of 17.3 million cells (4000 cells/cm^2) using a constant speed of 35 rpm]. Air and CO_2 were added to the bioreactor in the overlay at a constant flow rate; respectively, 27 ml/min and 1.6 ml/min. DO and pH were monitored only. Temperature was controlled at 37 °C. A washing step was performed at day 1. The culture was stopped at day 8 and cells were harvested with the same protocol as the static conditions.

The following two runs were performed in an initial volume of 764 ml of the same medium as previously, but with 0.5% PL instead of 2% and supplemented with 0.1% Pluronic® F68 (Thermofisher Scientific, Waltham, MA, USA), with 12 g of Corning® Enhanced Attachment microcarriers (4320 cm^2) and an initial seeding of 17.3 million cells (4000 cells/cm^2) using a constant speed of 35 rpm. Air was added at 27 ml/min in the overlay and DO only was monitored. The pH was controlled at 7.5 with CO_2 (headspace) only and temperature was controlled at 37 °C. After 24 h, 36 ml of PL was added in the tank to obtain a final concentration of 5% PL and a final volume of 800 ml. A single washing step was performed at day 1 or day 5. The culture was stopped at day 8, and cells were harvested with the same protocol as the static conditions.

The last run was performed as the previous two runs until day 8. After sampling and cell numeration at day 8, 1.2 l of medium containing 5% PL and 18 g of Corning® Enhanced Attachment microcarriers were added in the tank to feed the cells and increase the surface area (4320–10 800 cm^2). Cells were harvested at day 12 and counted using Trypan blue exclusion.

2.5.4. Fluorescent cell labelling on microcarriers

Cells attached to microcarriers were washed with PBS and fixed with paraformaldehyde (3.7%). Cells were stained with phalloidin (1/40), which is a high-affinity filamentous actin (F-actin) probe conjugated to green-fluorescent Alexa Fluor® 488 dye. Nuclei were stained with DAPI (1/10 000). Cell staining on microcarriers was observed with a fluorescence microscope (Olympus IX71) combined to a DSD system (ANDOR) and analysed with the image analyser software NIS-AR (Nikon).

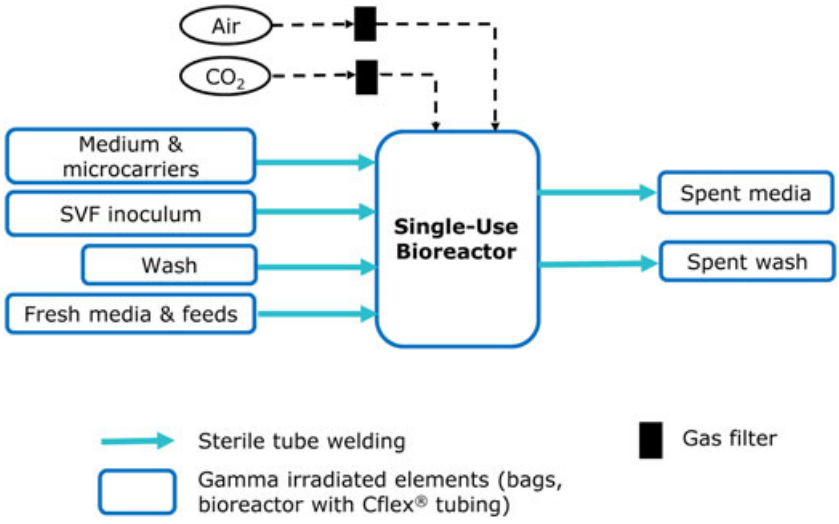

Figure 1. Culture process flow diagram of the bioreactor. The bioreactor is a single-use stirred-tank pre-equipped with weldable C-flex® tubing. The gases are filtered prior to entering the bioreactor with 0.2-µ filters. Seeding: the bag containing the mixture medium/microcarriers was welded to the bioreactor via a fluid addition line, and the mixture was transferred into the tank by gravity or using a peristaltic pump. The stromal vascular fraction (SVF) inoculum pre-conditioned in bag was then welded to the bioreactor via a fluid addition line and transferred into the bioreactor in the same manner as previously. Washing: the agitation was stopped so that the microcarriers settled and then the spent medium was removed by gravity through a sub-surface inlet/outlet line. A buffer bag was then connected to a fluid addition line and the buffer was transferred into the tank as previously described. Stirring is stopped again to remove the spent buffer via the sub-surface inlet/outlet line. Feeding: fresh media (± microcarriers) or platelet lysate (PL) were added in the bioreactor in the same way as for seeding after pre-conditioning of reagents in bags. [Colour figure can be viewed at wileyonlinelibrary.com]

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2.6. Clonogenicity (CFU-F) and flow cytometry assays

For colony-forming unit fibroblast (CFU-F) assays ($n = 7$ for spinner flasks and $n = 3$ for the 3-l bioreactor), cells after culture were plated at 16 cells/cm^2 in SM. After 11 days of culture, cells were fixed and stained with the MCDh Ral stainer kit (RAL diagnostics, Martillac, France) according to manufacturer's instructions. Stained colonies were counted.

For flow cytometry assays ($n = 6$ for spinner flasks and $n = 3$ for the bioreactor), cells after culture were incubated with fluorochrome coupled-antibodies against CD90, CD105, CD29, CD34, CD14, CD45, HLA-DR, HLA-ABC (all Becton Dickinson, Franklin Lakes, New Jersey, USA), CD73 (Miltenyi Biotec Bergisch Gladbach, Germany) and CD31 (eBioscience, San Diego, CA, USA). After 30 min of incubation at +4 °C, cells were washed and suspended in MACS buffer (Miltenyi Biotec Bergisch Gladbach, Germany). The labelled cells were analysed by flow cytometry with the ADP CYAN flow cytometer and the Kaluza software (both Beckman-Coulter, Pasadena, California, USA).

2.7. Multi-lineage differentiation assays

2.7.1. Osteogenesis assays (n = 2)

Cells were seeded at 20 000 cells/cm^2 using osteogenic induction medium consisting of α-MEM (Thermofisher Scientific, Waltham, MA, USA), 1 U/ml heparin Choay (Sanofi-Aventis, Frankfurt, Germany), 0.8% PL, 2 mM L-glutamine (Thermofisher Scientific, Waltham, MA, USA), 2 mg/ml ciflox (Bayer, Leverkusen, Allemagne), 50 μM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate (both Sigma-Aldrich, St Louis, Missouri, USA) and 50 ng/ml rhBMP7 (R&D Systems, Minneapolis, MN, USA). Medium was changed twice a week and induction was stopped at day 7.

2.7.2. Adipogenesis assays (n = 2)

Cells were seeded at 20 000 cells/cm^2 using adipogenic induction medium consisting of α-MEM, 10% FBS (both Thermofisher Scientific, Waltham, MA, USA), 2 mg/ml ciflox, 1 μM dexamethasone, 0,45 mM isobuthyl methylxantine, 60 μM indomethacin (all Sigma-Aldrich, St Louis, Missouri, USA). Medium was changed twice a week and induction was stopped at day 14.

2.7.3. Chondrogenesis assays (n = 2)

Cells were plated in 24-well plates; 2.5×10^5 cells concentrated in a droplet of 12.5 μl medium were seeded and incubated for 2 h at +37 °C, 5% CO_2. After incubation, chondrogenic induction medium, consisting of Dulbecco's modified Eagle's medium-high-glucose (Thermofisher Scientific, Waltham, MA, USA), 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM L-proline, 62.5 ng/ml bovine insulin, 62.5 ng/ml transferrin, 53.3 ng/ml selenous acid, 53.3 ng/ml linoleic acid, 12.5 μg/ml bovine serum albumin (all Sigma-Aldrich, St Louis, Missouri, USA) and 0.01 μg/ml TGF-β3 (R&D Systems, Minneapolis, MN, USA) was added. Medium was changed three times a week and induction was stopped at day 7.

The capacity to differentiate into the three cell lineages was evaluated through the expression quantification of specific genes.

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR)

For the study of the multi-lineage differentiations, total RNA was extracted after inductions. The extractions were performed using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Reverse transcription was performed from total RNA for cDNA synthesis using the High-Capacity cDNA Reverse Transcription kit (Thermofisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. For all studied genes, specific cDNA was amplified by real-time PCR using the SsoFast™ EvaGreen® Supermix (Biorad, Hercules, CA, USA). The concentrations of forward and reverse primers (Eurogentec, Liège, Belgium) were 500 nM. All reactions were performed using the CFX96 detection system instrument (Biorad, Hercules, California, USA) under the following conditions: 98 °C, 2 min, followed by 40 cycles of 98 °C, 2 s and 60 °C, 10 s. For each gene of interest, the relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method using normalization of the differentiated samples with their undifferentiated controls. The studied genes for multi-lineage differentiations were: osteogenesis: runt-related transcription factor 2 (Runx2), distal-less homeobox 5 (*DLX5*), *Osterix*, integrin-binding sialoprotein (*iBSP*) and NOP2/Sun domain family, member 5 (*NSUN5)* as housekeeping gene; adipogenesis: CCAAT/enhancer binding protein α (*CEBPα*), peroxisome proliferator-activated receptor γ (*PPARγ*), lipoprotein lipase (*LPL*), adipocyte protein 2 (*AP2*), adiponectin (*ADIPOQ*) and peptidyl-prolyl cis-trans isomerase A (*PPIA*) as housekeeping gene; chondrogenesis: SRY (sex-determining region Y)-box 6 (*Sox-6*), SRY (sex-determining region Y)-box 9 (*Sox-9*), *Aggrecan*, Collagen 2A1 (*Col 2A1*), Collagen 10A1 (*Col 10A1*) and Translocase of Inner Mitochondrial Membrane 17 Homolog B (*TIMM17B*) as housekeeping gene. The primer sequences used in these assays are shown in Table 2.

2.9. Immunosuppression assay

2.9.1. T-lymphocyte immunosuppression (n = 2)

Adipose-derived stromal cells were seeded at 10^5 cells/well in 48-well plates. Human T-cells were purified after PBMC isolation from buffy coat (French Blood Institute) using 'Pan T-cell isolation kit' (Miltenyi Biotec Bergisch

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Table 2. Primers sequences used for real-time PCR

GENE	FORWARD	REVERSE
NSUN5	AGA CCA GTC ACT TGG CTG CTC T	AGC CAG TTC ACA GCA AGA GAC G
Runx2	GGC CCA CAA ATC TCA GAT CGT T	CAC TGG CGC TGC AAC AAG AC
DLX5	GCC ATT CAC CAT TCT CAC CTC	TAC CCA GCC AAA GCT TAT GCC
Osterix	CTC CTG CGA CTG CCC TAA T	GCC TTG CCA TAC ACC TTG C
iBSP	GGG CAG TAG TGA CTC ATC CGA AG	CTC CAT AGC CCA GTG TTG TAG CAG
PPIA	GCC GAG GAA AAC CGT GTA CTA T	TCT TTG GGA CCT TGT CTG CAA
CEBPα	GCC GGG AGA ACT CTA ACT CC	CTG CAG GTG GCT GCT CAT
PPARγ	GAT ACA CTG TCT GCA AAC ATA TCA C	CCA CGG AGC TGA TCC AA
LPL	GGT CGA AGC ATT GGA ATC CAG	TAG GGC ATC TGA GAA CGA GTC
AP2	AGT GAA AAC TTT GAT GAT TAT A	CCA TGC CAG CCA CTT TCC T
AdipoQ	TGC CCC AGC AAG TGT AAC C	TCA GAA ACA GGC ACA CAA CTC A
Timm17B	GGA ACT CTA TCA CCA GTG GAG C	AGG ATG CCA ACG CCC TCA ATG A
Sox-9	AGC GAA CGC ACA TCA AGA C	GTC GTA GTG TGG GAG GTT GAA
Aggrecan	TGC ATT CCA CGA AGC TAA CCT T	GAC GCC TCG CCT TCT TGA A
Col 2A1	GGC AAT AGC AGG TTC ACG TAC A	CGA TAA CAG TCT TGC CCC ACT T
Col 10A1	GGG CAG AGG AAG CTT CAG AAA G	CAG ATG GAT TCT GCG TGC TG

Gladbach, Germany) and were labelled with CFSE (2.5 μM, 10 min at 37 °C; Thermofisher Scientific, Waltham, MA, USA); 10^5 labelled T-cells were added to ASCs at a ratio of 1:1 and stimulated with anti-CD3/CD28 beads (Thermofisher Scientific, Waltham, MA, USA) at a ratio of T-cells:beads of 2:1. After 5 days, the co-cultures were stopped and cells were stained with APC-Vio770™ coupled anti-CD3 and VioBlue®-conjugated anti-CD45 antibodies (Miltenyi Biotec Bergisch Gladbach, Germany). Data were collected on the ADP CYAN flow cytometer and analysed with Kaluza software (both Beckman-Coulter, Pasadena, California, USA). The percentage of T-cell proliferation, especially the percentage of T-cells able to achieve five cycles, was evaluated.

2.10. Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, California, USA). Differences were assessed using Wilcoxon tests and considered statistically significant with P-values < 0.05.

3. Results

3.1. Microcarriers screening

3.1.1. In static conditions

The first step consisted of testing nine different microcarriers. They were all composed of a polystyrene core with different coatings or surface treatments (Table 1). The purpose was to determine the best three microcarriers for adhesion, proliferation and recovery of ASCs when starting from SVF. This extensive screening was performed in static conditions with SM. The static screening paradigm was designed to quickly eliminate poor performing conditions, and internal observations (EMD Millipore) showed that it recapitulates the ranking performed in spinner flasks. Microscopic observations after cell fluorescence labelling using phalloidin-Alexa488 coupled to DAPI demonstrated that the 'native' ASCs could adhere to all tested microcarriers in static conditions (Figure 2a). The number of harvested cells/cm^2 after 8 days of culture starting from SVF was not significantly different between tested microcarriers and 2D controls in flask (Figure 2b). Nevertheless, the microcarriers C and D were eliminated due to the composition of their coating with animal proteins, which is not preferred for clinical use. Even with a lack of significant difference, three microcarriers were chosen for the next screening step. The microcarrier A: Enhanced attachment (Corning®) was chosen for its manufacturing simplicity consisting of a physical surface treatment (less expensive), for its sterile packaging and for its GMP grade, suitable for clinical use. The microcarrier B: Synthemax II (Corning®) coated with extracellular matrix peptides was selected due to the best screening results and the same advantages as microcarrier A: sterility and GMP grade. The observed variability could potentially be explained by a partial detachment of cells, which tended to aggregate on microcarriers B in static conditions. Indeed, microscopic observations of these microcarriers labelled after cell recovery showed more remaining cells on their surface than the other microcarriers (data not shown). Finally, the microcarrier F: Plastic Plus (Solohill®) was selected due to the reproducibility of the results and to its manufacturing simplicity, consisting of a positively charged surface.

3.1.2. In stirred conditions

After their pre-selection in static conditions, the microcarriers A (Enhanced attachment, Corning®), B (Synthemax II, Corning®) and F (Plastic Plus, Solohill®) were tested in stirred conditions using spinner flasks, to control adhesion and proliferation of native ASCs in a dynamic environment. Cells were grown in SM for 7 days, with a low speed during the first 4 days to allow adhesion of cells, and with the optimal speed allowing the suspension of all microcarriers for the remaining days. After 7 days of culture, the number of cells harvested (Figure 2c) and the number of population doublings (Figure 2d) were calculated. No significant difference was observed between the three tested microcarriers and the 2D standard flask. Nevertheless, the microcarrier F (Plastic Plus, Solohill®) was eliminated because of aggregates that were not observed in static conditions. The microcarrier B (Synthemax II, Corning®) was

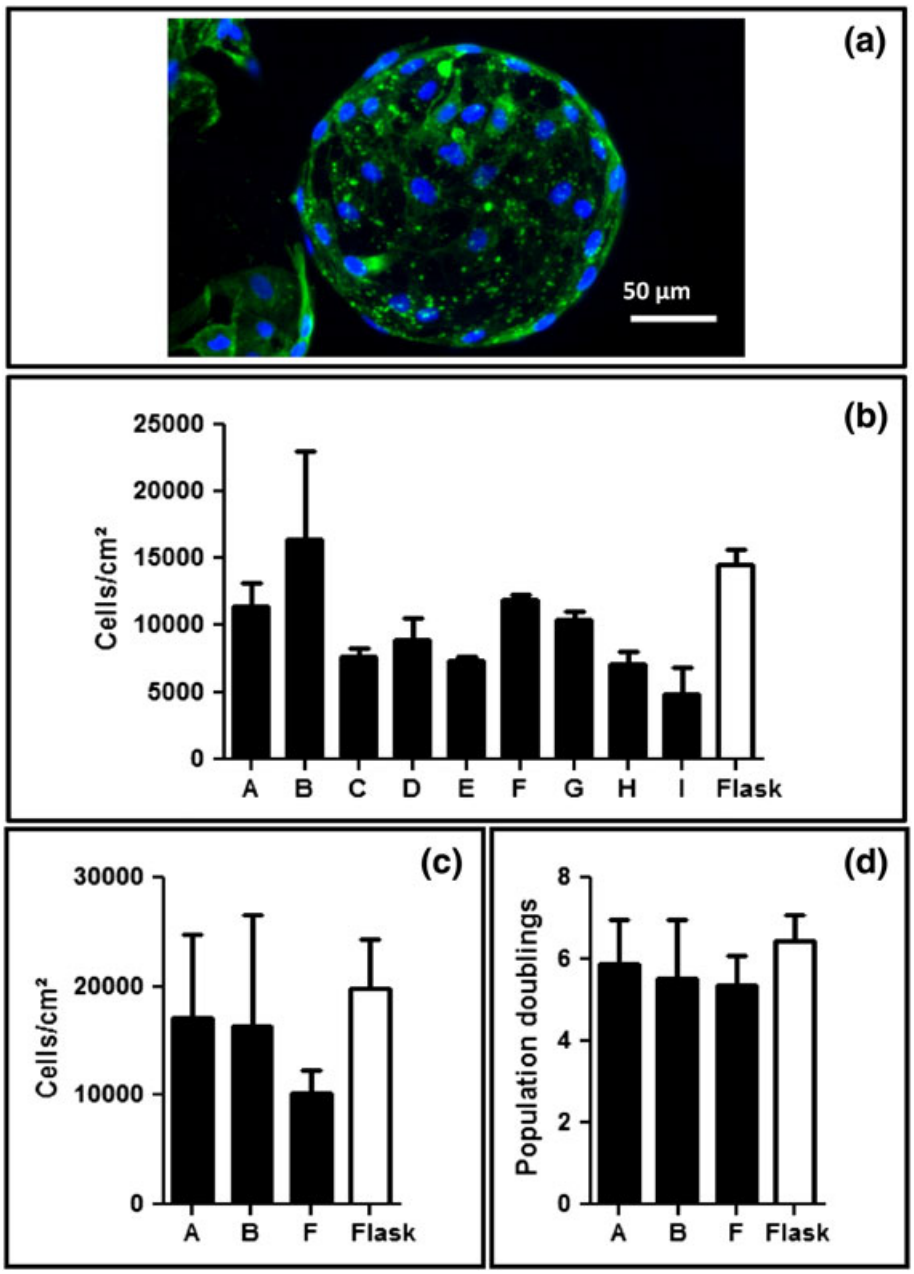

Figure 2. Microcarrier screening. Representative pictures of sdipose-derived stromal cells (ASCs) on microcarriers following double staining phalloidin-Alexa 488 (green)/DAPI (blue) (a). Microcarrier screening in static conditions: number of cells/cm^2 recovered after 8 days of culture on microcarriers (A–F refer to the nomenclature of Table 1) in ultra-low-attachment plates vs. standard 2D flasks, using stromal vascular fraction (SVF) from three donors (b). Microcarrier screening in stirred and small-scale conditions after selection of the best three microcarriers in static conditions: number of recovered cells/cm^2 (c) and number of population doublings (d) after 7 days of culture on microcarriers (A, B, F refer to the nomenclature of Table 1) in spinner flasks vs. standard 2D flasks using SVF from three donors. Values are represented as mean ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

eliminated because of the strong variability observed in static conditions and confirmed in stirred conditions. The selected microcarrier was consequently the microcarrier A (Enhanced Attachment, Corning®) due to the good results obtained in static and stirred conditions starting with SVF, in addition to its sterile packaging and to its GMP grade.

3.2. Culture of ASCs on the selected microcarrier in stirred small-scale conditions

Following the results of these two screening steps, supplementary experiments were performed in spinner flasks to confirm the choice of the Enhanced Attachment microcarriers. The results in Figure 3a demonstrated that the number of population doublings was similar to the 2D controls and highly reproducible. At the end of the culture, cells obtained from both culture conditions were characterized. The results showed that they maintained the same clonogenic potential (Figure 3b) and that they had a similar phenotype for studied markers (Figure 3c), confirming the recovery of a homogeneous population of ASCs after selection and amplification on microcarrier A, in stirred conditions. These results confirmed that the selected microcarrier was a good candidate for scale-up experiments in bioreactors.

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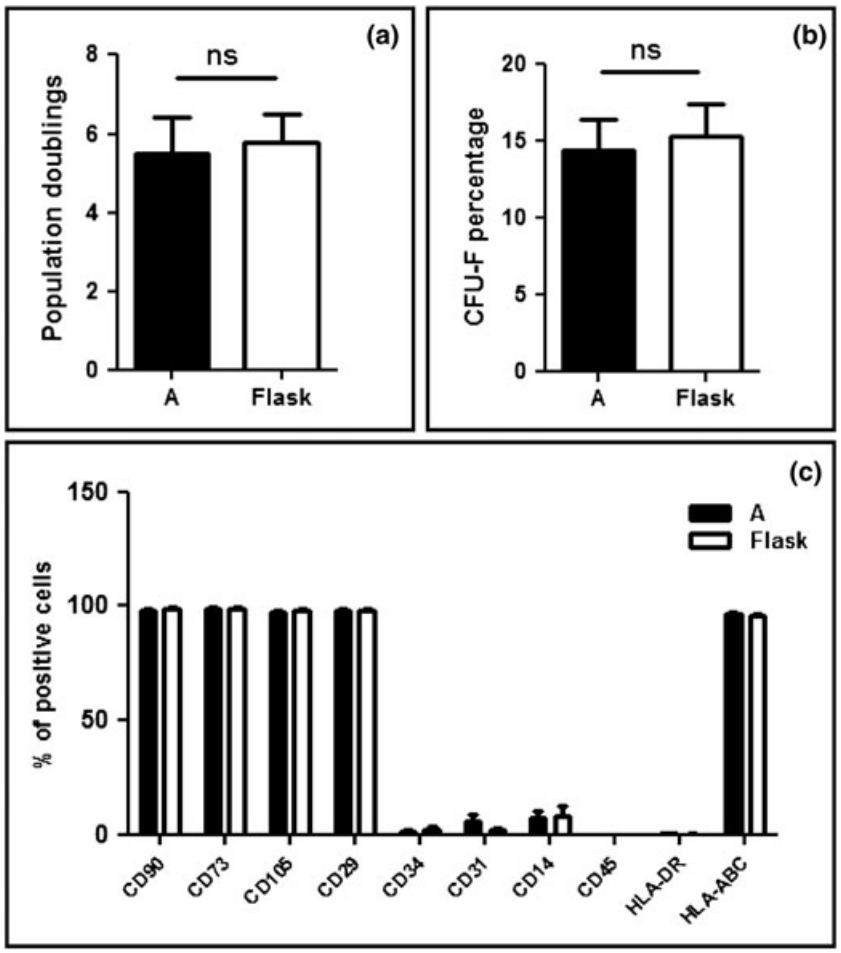

Figure 3. Culture of adipose-derived stromal cells (ASCs) on Enhanced Attachment microcarriers (Corning®) in stirred and small-scale conditions. Number of population doublings after 7 days of culture on the Enhanced Attachment microcarrier (Corning®): A, in spinner flasks vs. standard 2D flasks using stromal vascular fraction (SVF) from seven donors (a). Quantification of the colony-forming unit fibroblast (CFU-F) percentage in the cell suspension after culture, $n = 7$ (b). Phenotypic analysis of ASCs after culture, showing the percentage of positive cells for studied markers, $n = 6$ (c). Values are represented as mean ± SEM

3.3. Scale-up and automation in the 3-l bioreactor

The scale-up experiments were performed in the 3-l bioreactor (Figure 4a). The first two experiments were performed with the same culture conditions as the spinner flasks (4000 cells/cm^2, 2% PL) with an increased volume (800 ml) and automated temperature control and gassing control (pH monitored only). However, the stirring speed was constant throughout the culture and was not increased during the process as in spinner flasks. Aggregate formation was observed as soon as the stirring speed was below the optimal speed for the suspension of all microcarriers. No amplification of ASCs was observed in the 3-l bioreactor in contrast to 2D controls, using these conditions (Figure 4b). It is possible that the higher speed used at the beginning of the culture prevented good adhesion of native ASCs to the microcarriers. Several parameters were modified for the following three runs to promote cell adhesion, to reduce shear forces on cells and to create a more consistent environment. The first one was the modulation of PL concentration. The PL percentage was reduced to 0.5% during the first 24 h of culture to increase cell adhesion (Himes and Hu, 1987; Mukhopadhyay *et al.*, 1993), and increased to 5% for the remaining culture to boost the ASC proliferation after adhesion. In addition to this modification, Pluronic® F-68, which is a non-ionic surfactant, was added to the culture medium as a shear protectant (Gigout *et al.*, 2008; Hu *et al.*, 2011; Murhammer and Goochee, 1990; Wu, 1995; Wu *et al.*, 1995). The final change was the control of the pH at 7.5 in order to promote a more consistent environment to the cells. With these modifications, the attachment and amplification of ASCs were quite similar to those obtained in the static 2D controls (Figure 4b). The last run was extended through a feeding strategy by the addition of medium and microcarriers at day 8, and then stopped after 12 days of culture, allowing the recovery of 178 million cells. These results showed that with suitable adhesion conditions, the selection and the amplification of ASCs when starting from SVF in the 3-l bioreactor was possible and as good as the 2D control flasks. Moreover, monitoring and control of process parameters were supported throughout the run, ensuring a successful expansion.

3.4. ASC characterization after culture in the 3-l bioreactor

After culture in the 3-l bioreactor, the characteristics and properties of cells were evaluated and compared with

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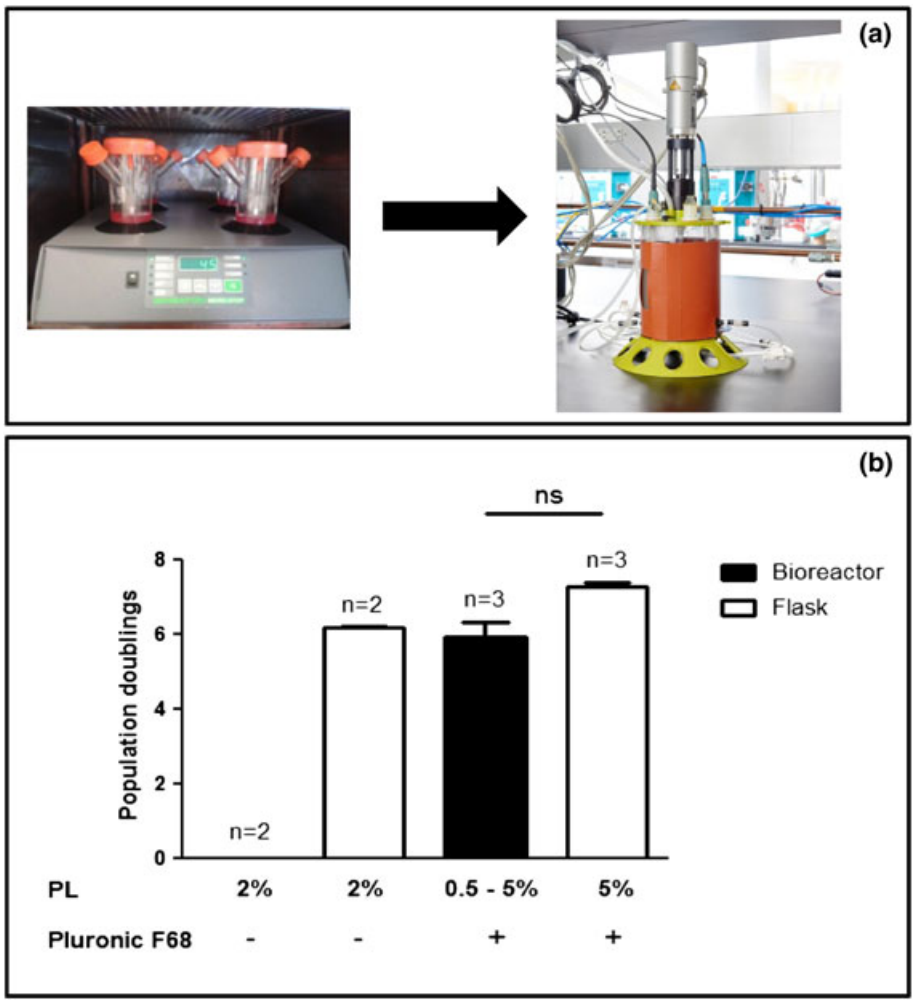

Figure 4. Transfer to large-scale and automated conditions. Spinner flasks (a, left) and the Mobius® 3-l bioreactor (a, right). Number of population doublings after culture of adipose-derived stromal cells (ASCs) on Enhanced Attachment microcarriers (Corning®) in the bioreactor vs. standard 2D flasks, with or without the non-ionic surfactant Pluronic F68 and using different platelet lysate (PL) percentages, first 2% and then 5%. For the bioreactor, the condition '0.5–5% PL' corresponds to a culture with 0.5% PL during the first 24 h and 5% PL during the remaining culture (b). Values are represented as mean ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

the same cells grown in 2D control flasks. The clonogenic potential of cells was evaluated by determining the CFU-F frequency in the cell suspension. The results showed that there was no significant difference between both conditions (Figure 5a). The cell phenotype was also studied for the following markers: CD90, CD73, CD105, CD29 (mesenchymal markers), CD34 (expressed by native ASCs and decreased during culture), CD31, CD14 and CD45, respective markers of endothelial, immune and haematopoietic cells, which are all present in the starting SVF. The expression of human leucocyte antigens (HLAs), class 1 and 2, was also evaluated. No significant difference was observed for all studied markers between both culture conditions (Figure 5b). These results showed that the cell population obtained after selection and amplification in the 3-l bioreactor was a homogeneous population of ASCs. The cell functionality was then evaluated. The differentiation potential of cells after culture was assessed by inducing the osteogenic, adipogenic and chondrogenic lineages, and by quantifying the expression of specific genes. The expression of genes involved in the osteogenic commitment (Figure 5c) and those involved in the adipogenic commitment (Figure 5d) were increased with induction for both compared conditions (3-l bioreactor and 2D controls). Concerning chondrocyte differentiation, the expression of specific genes (Figure 5e) was slightly or not increased with induction after culture in the bioreactor. While additional experiments are required to confirm their ability to develop chondrocytes, our results confirm that ASCs produced in the bioreactor remain undifferentiated and multipotent stem cells. Taken together, ASCs produced in the bioreactor maintained their differentiation potential for osteogenic and adipogenic lineages.

3.5. Immunosuppression capacities of ASCs after culture in large-scale and automated conditions

The immunosuppressive potential was assessed by studying the capacity of ASCs to inhibit T-cell proliferation. As shown in Figure 6a, the ASCs slowed proliferation of stimulated T-cells and reduced the number of division cycles. The proliferation percentage of stimulated T-cells and particularly of T-cells that performed five division cycles were similarly decreased

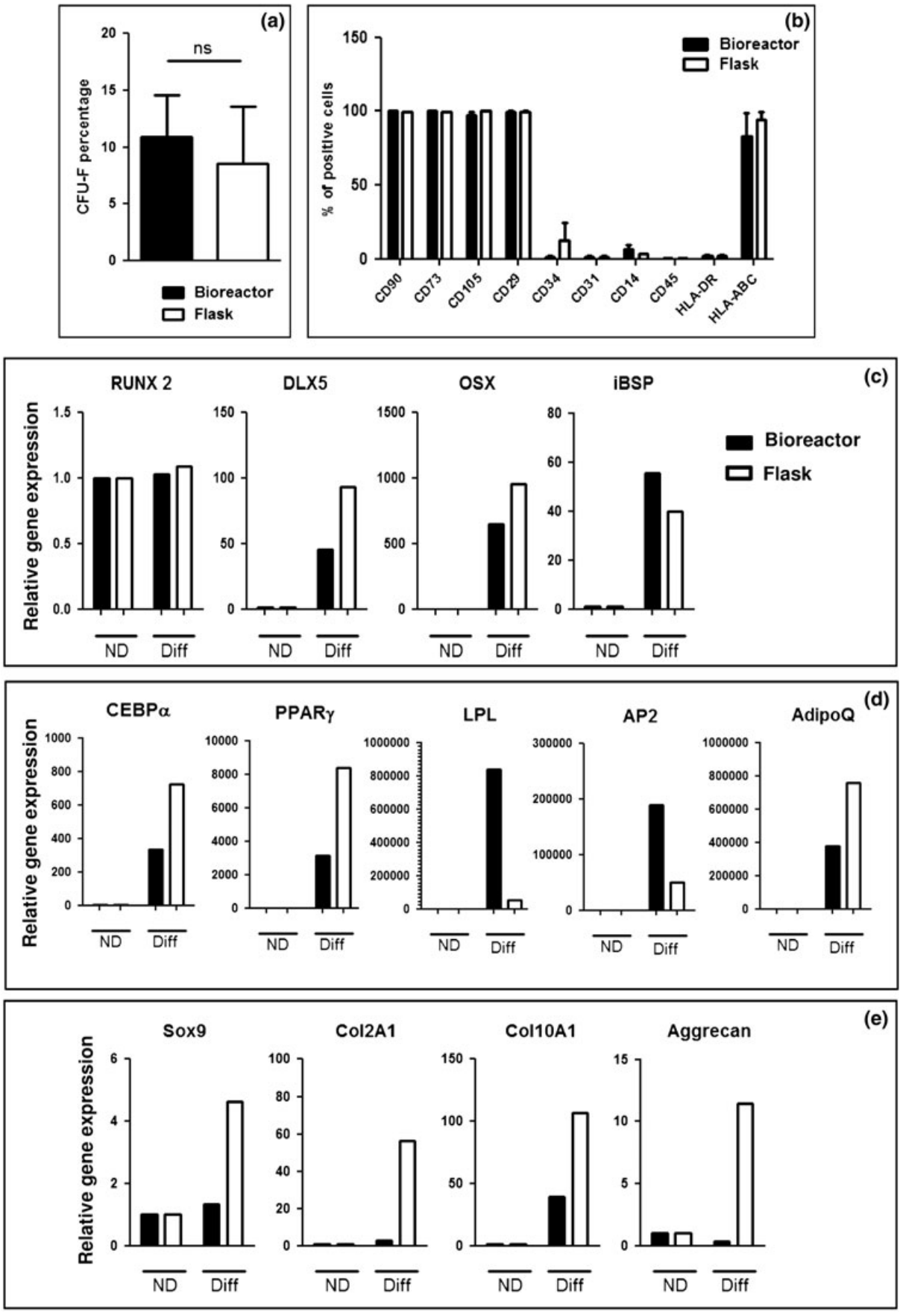

Figure 5. Adipose-derived stromal cells (ASCs) characterization after culture in large-scale and automated conditions. Quantification of the colony-forming unit fibroblast (CFU-F) percentage in the cell suspension after culture, $n = 3$ (a). Phenotypic analysis of ASCs after culture, showing the percentage of positive cells for studied markers, $n = 3$ (b). Values are represented as mean ± SEM. Relative expression of genes involved in the osteogenic (c), adipogenic (d) and chondrogenic (e) lineages commitment, quantified by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and calculated after normalization of differentiated samples (Diff) with their undifferentiated control (ND), using the $2^{-\Delta\Delta CT}$ method, $n = 2$. Values are represented as mean

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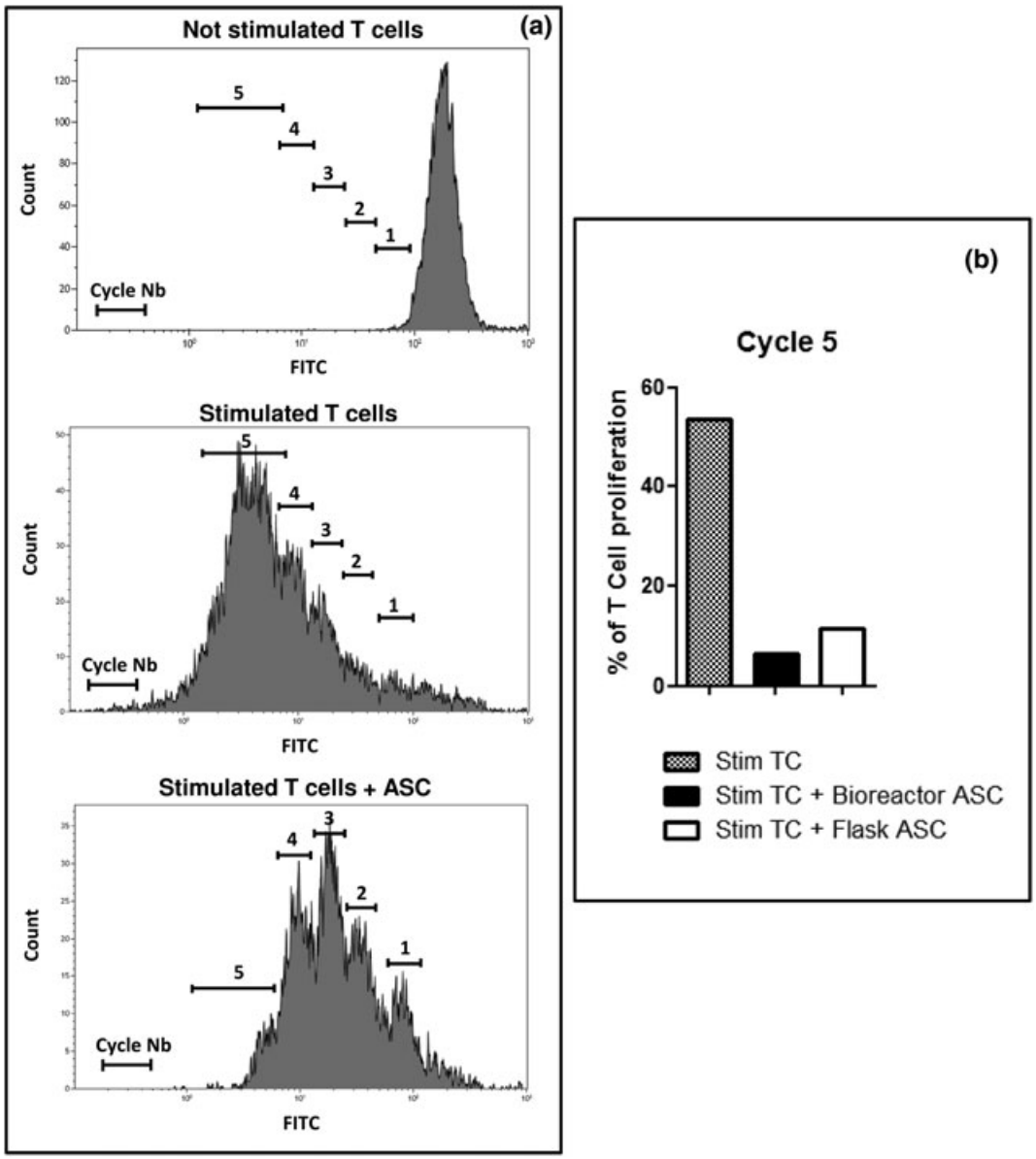

Figure 6. Immunosuppression capacities of adipose-derived stromal cells (ASCs) after culture in large-scale and automated conditions. Histograms display the fluorescence intensity of T-cells stained with CFSE without stimulation (a, top), after stimulation causing T-cell proliferation and CFSE dilution after each cell cycle without (a, middle) or with ASCs (a, bottom). Percentage of T-cells reaching five cycles after stimulation, without ASCs, with ASCs from the bioreactor and with ASCs from 2D flasks, $n = 2$ (b). Values are represented as mean

in the presence of ASCs from either the 3-l bioreactor or from their 2D controls (Figure 6b). These results showed that cells selected and amplified in the 3-l bioreactor retained their immunosuppressive properties.

4. Discussion

The increasing use of MSCs for cell therapy clinical trials requires closed, scalable and cost-efficient production processes in compliance with the principles of GMP. Bioreactor-based strategies were identified to meet these requirements by providing automated and closed scale-up of MSCs production. Bioreactors are often presented as useful tools for significant scale-up for applications requiring large cell quantities for allogeneic applications (Hanley *et al.*, 2014). However, bioreactor strategies are less considered for applications requiring a moderate number of cells, such as autologous applications. However, these productions must also comply with the principles of GMP (Migliaccio and Pintus, 2013), which results in expensive productions due to space requirements (batch productions have to be spatially separated), specific and expensive environments (B classrooms), and staffing requirements (two operators for each batch; Heathman *et al.*, 2015). Moreover, most studies concerning culture of adherent cells using bioreactors were performed by inoculation of cells previously amplified in 2D flasks using an open process (Carmelo *et al.*, 2015; Dos Santos *et al.*, 2014). For clinical trials focusing on autologous applications, this initial phase of cell isolation and selection from tissue is repeated for every patient. The development of processes in bioreactors, including the initial selection step, could strongly increase the cost-efficiency of autologous productions, despite a moderate cell quantity requirement.

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In this context, our study was performed from AT and specifically from SVF obtained after fat tissue digestion. However, our previous experience with SVF inoculation in bioreactors based on perfused technologies (hollow fibres and multi-layered vessels) showed disappointing results because of poor adhesion of native ASCs in those dynamic conditions. The current study was conducted using a stirred technology and microcarriers in suspension, offering a new alternative for the culture of SVF in bioreactor. Moreover, this technology allows an accurate modulation of the available surface for cell growth.

Two initial rounds of microcarriers screening, in static and small-scale stirred conditions, were performed, and the sterile and GMP-grade Enhanced Attachment microcarrier (Corning®) was selected. The number of population doublings on this microcarrier was similar to 2D control flasks. The cell phenotype studied after culture in stirred conditions (spinner flasks) showed that a homogeneous population of ASCs was obtained, despite the inoculation of SVF containing several cell types. We also showed that cells retained their clonogenic potential despite dynamic conditions.

The culture conditions used in spinner flasks were then transferred to a larger scale in the Mobius® 3-l single-use bioreactor. This system, pre-installed with weldable tubes, allowed the culture in a functionally closed environment after pre-packaging of ancillary reagents in sterile bags. This transfer from spinner to the 3-l bioreactor required some optimization in terms of agitation as the mixing technologies were different (magnetic bar vs. marine impeller). At the larger scale, microcarriers aggregation was observed at 20 rpm but not at 35 rpm at the starting volume of 800 ml. Indeed, 35 rpm was identified as 'the just suspended speed', meaning that it was the minimal speed required to suspend all the microcarriers. At larger volumes, the speed was maintained at 35 rpm to maintain a balance between the suspension of the microcarriers and the limitation of shear forces that could detach the cells from the microcarriers.

In addition, because other studies demonstrated that cell adhesion to microcarriers was improved using low serum quantity (Himes and Hu, 1987; Mukhopadhyay *et al.*, 1993), we decreased the PL percentage to 0.5% at the beginning of the culture. A non-ionic surfactant, the Pluronic® F68, was also added to the culture medium as a shear protectant (Gigout *et al.*, 2008; Hu *et al.*, 2011; Murhammer and Goochee, 1990; Wu, 1995; Wu *et al.*, 1995). Both modifications and the control of the pH supported the culture of ASCs in the 3-l bioreactor and population doublings close to control flasks. The study of cell phenotype after culture showed that a homogeneous population of ASCs was obtained. Moreover, their clonogenic potential, their immunosuppressive capacities and their differentiation capacity into osteoblasts and adipocytes were maintained, whereas the cell commitment toward the chondrogenic lineage was decreased or delayed.

Our results showed that after optimization, we obtained results similar to control flasks despite a non-static environment and the poor adherence of native ASCs. Nevertheless, there is still room to optimize the cell amplification and cell recovery. Different strategies could be considered to increase the cell proliferation. The first one could be the feeding strategy: medium exchange, batch or fed-batch can result in a complete renewal of medium or medium stagnation. For stirred tanks, the feeding strategies mostly used are batch or fed-batch, where medium is not exchanged (Carmelo *et al.*, 2015; Hupfeld *et al.*, 2014; Schop *et al.*, 2010). Batch and fed-batch strategies could decrease the required medium quantity for cell production, but the persistence of cells, such as immune cells or endothelial cells present in SVF and not removed by medium exchange, could affect the ASC potential, including their immunomodulatory properties and their secretory profile. However, studies showed that the therapeutic effect of ASCs was mainly related to their paracrine action (Meirelles Lda *et al.*, 2009). The proliferation step optimization could also be conducted by modulation of other parameters, such as microcarrier concentration, cell seeding density, stirring speed and gassing option. The last critical step is the cell recovery requiring cell detachment and separation of cells from microcarriers. For the cell detachment by enzymatic action, two parameters could be optimized: the duration and the stirring speed during incubation. The separation of cells from microcarriers is also a critical step; it must be reliable enough to prevent residual microcarriers in the final cell suspension. The most straightforward strategy may be the use of a membrane separating cells from microcarriers by size exclusion, without clogging and loss of cells. The choice of microcarriers is also important for cell recovery because size homogeneity is required for a reliable separation by size exclusion. Moreover, potential collisions between carriers should not generate particles remaining in the final cell suspension. Finally, the whole process of cell recovery including enzymatic action, stirring and separation must maintain the cell integrity.

In conclusion, we provide for the first time the proof of concept that isolation and amplification of functional ASCs after inoculation of SVF could be performed with microcarriers in a stirred-tank bioreactor. We showed that the results were similar to those obtained in control 2D flasks, demonstrating that a stirred-tank bioreactor is an attractive alternative to planar culture for the expansion of ASCs from the initial collection of SVF.

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Authorship

Contribution: MG, MB, NE and CB performed the research; MG and JM designed the research study; SR, BC and LSa contributed reagents and tools; MG wrote the paper; and LSe, LSa, NE, MB and JM revised it.

Conflict of interest statement

The authors confirm that there are no conflicts of interest.

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