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Good manufacturing practice-compliant animal-free expansion of human bone marrow derived mesenchymal stroma cells in a closed hollow-fiber-based bioreactor

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ABSTRACT

Mesenchymal stroma cells (MSC) are increasingly recognized for various applications of cell-based therapies such as regenerative medicine or immunomodulatory treatment strategies. Standardized large-scale expansions of MSC under good manufacturing practice (GMP)-compliant conditions avoiding animal derived components are mandatory for further evaluation of these novel therapeutic approaches in clinical trials.

We applied a novel automated hollow fiber cell expansion system (CES) for in vitro expansion of human bone marrow derived MSC employing a GMP-compliant culture medium with human platelet lysate (HPL). Between 8 and 32 ml primary bone marrow aspirate were loaded into the hollow fiber CES and cultured for 15–27 days. 2–58 million MSC were harvested after primary culture. Further GMP-compliant cultivation of second passage MSC for 13 days led to further 10–20-fold enrichment. Viability, surface antigen expression, differentiation capacity and immunosuppressive function of MSC cultured in the hollow fiber CES were in line with standard criteria for MSC definition. We conclude that MSC can be enriched from primary bone marrow aspirate in a GMP-conform manner within a closed hollow fiber bioreactor and maintain their T lymphocyte inhibitory capacity. Standardized and reliable conditions for large scale MSC expansion pave the way for safe applications in humans in different therapeutic approaches.

1. Introduction

Mesenchymal stroma cells (MSC) are immature fibroblasts possessing an enormous growth capacity in vitro and the inherent capability to differentiate into a variety of tissues [1]. MSC harbor a distinct antigen expression profile [1,2] and exhibit immunomodulatory function [3–5]. Their ability to migrate to sites of tissue injury [6,7] make MSC useful in tissue repair [8–10] or treatment of autoimmune disorders [11,12], steroid refractory graft versus host disease (GvHD) [13,14] or chronic allograft rejection [15]. MSC can be derived from various human tissues like fat [16], umbilical cord [17] or cord blood [18]. The use of bone marrow as a source of human MSC has been first described by Friedenstein et al. [19,20] and represents still the most reliable source for adult MSC until today. Recently, a hollow fiber bioreactor system (Quantum® Cell Expansion System, Terumo BCT) has been developed providing an expansion surface for adherent cells [21]. In order to move MSC transplantation into properly controlled clinical studies, safe and standardized ex vivo expansion protocols in a GMP-compliant manner are required. Efforts have been made to avoid fetal calf serum for MSC cultivation [22–24] because of the risk of transmission of pathogens as well as xenoimmunization against bovine antigens [25–28]. In addition, growth of MSC in conventional cell culture monolayer requires large surfaces for sufficient cell expansion in a sterile GMP-compliant microenvironment. Therefore, cell-stack chambers placed into incubators [29] or rotating bioreactor devices [30,31] have been used for clinical-grade MSC expansion. We have applied a GMP-compliant culture medium based on human platelet lysate (HPL) for MSC expansion from primary adult human bone marrow in a novel closed hollow fiber cell expansion system (CES) with a fibronectin-coated surface of 2.1 m in order to establish a safe, efficacious and standardized protocol according to the guidelines of the European Medicines Agency (EMA).

Abbreviation: CES, cell expansion system.

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2. Materials and methods

2.1. Collection of bone marrow and pre-enrichment of MSC

Bone marrow was aspirated from the iliac crest of volunteer healthy donors. For pre-enrichment of MSC, spongiform bone fragments were obtained from hip replacement surgery as described previously [32]. The procedures had been approved by the local ethics committee at the Philipps-University Marburg (study no. 64/01 and 25/10) and patients had been given written informed consent. 10 ml syringes prefilled with 160 I.U. of heparin anticoagulant (Sarstedt, Nuembrecht, Germany) were used for bone marrow collection.

In brief bone marrow fragments were subjected to density gradient centrifugation, washed with PBS (PAA, Linz, Austria) and resuspended in Dulbecco's modified medium (DMEM) with low glucose (Biochrom, Berlin, Germany) containing 1% N(2)-L-alanyl-L-glutamine (Dipeptamin, Fresenius Kabi, Germany), and 10% HPL, manufactured at the Institute for Clinical Immunology and Transfusion Medicine, Giessen, Germany in a GMP-compliant manner as described [23,33]. Preparation of pooled HPL is an efficient replacement for animal serum-free human stem cell cultures. For expansion of MSC in plastic flasks, cells were plated at a concentration of 5000 cell/cm^2 (Greiner Bio-One, Frickenhausen, Germany). Initially medium was replaced after the first 24 h, afterward medium was exchanged every 3–4 days until the cells reached ~80% confluence. With every splitting procedure the passage number was increased. The pre-enriched MSC of passages 2–3 were loaded into the Quantum cell expansion system (TerumoBCT). For primary MSC expansion, unprocessed BM aspirates were directly transferred into a Quantum system cell inlet bag (TerumoBCT, Lakewood, USA) under clean room conditions.

2.2. Expansion of MSC within the hollow fiber cell expansion system

One day prior loading of cells the expansion tubing set was placed into the Quantum system (provided by TerumoBCT) and coated with 10 mg fibronectin (BD Biosciences, Heidelberg, Germany) according to manufacturers instructions. The Cell Inlet Bag (CIB) containing bone marrow aspirate or pre-enriched MSC was then filled with medium (DMEM + 10% HPL + 1% N(2)-L-alanyl-L-glutamine) up to a total volume of 100 ml, connected to the cell expansion system via tubing welder (TSCDII, Terumo, Tokyo, Japan) and loaded into the cell expansion system using the automated functionality of the system. After 24 h, a complete medium exchange was performed in order to remove unattached cells (e.g. red blood cells or non-viable MSC). Cells were continuously fed with medium during the expansion. Loading, washing and feeding tasks were performed by the Quantum system following a program sequence according to manufacturer's instructions. Medium samples were collected regularly for determination of glucose and lactate levels. The inlet rate was started with 0.1 ml/min and was increased 2-fold when either glucose levels dropped below 70 mg/dl or lactate levels rose above 4 mmol/l. Cell harvest was initiated when glucose levels dropped below 70 mg/dl at a flow rate of 48 ml/h. For MSC harvest a CIB pre-filled with 180 ml of 0.25% trypsin/EDTA solution (PAA) was sterilely welded to the CES tubing and introduced into the hollow-fiber-system via automated tasks. After an incubation time of 4–6 min the cells were released into the cell harvest bag (TerumoBCT) by flushing with 500 ml medium. Cell count and viability were assessed using trypan blue staining and a Neubauer counter chamber.

Table 1
MSC yield after primary expansion of unprocessed bone marrow from 5 healthy volunteers in the hollow fiber cell expansion system (A). Subsequent cultivation of primary culture MSC from donor no. 5 and early passage MSC pre-cultured in plastic flasks led to further propagation of cells (B).

Donor	1	2	3	4	5
(*A*) *MSC expansion from primary bone marrow aspirate*					
Bone marrow aspirate (ml)	30	8	30	26	32
Expansion time (days)	15	19	15	18	27
Harvest (million cells)	2	6	7	9	58
(*B*) *Expansion from preselected MSC*					
	A	B	C	5	
No. of seeded cells (million)	2	3.5	5	7.6	
Expansion time (days)	6	11	13	13	
Harvest (million cells)	18.7	50	98	17.2	
Fold expansion	9.4	14.2	20	2.3	

2.3. Immunophenotyping of bioreactor-derived MSC by flow cytometry

The surface marker expression of MSC expanded in HPL-supplemented medium was analyzed with a four-color flow cytometer (FACSCalibur, BD Biosciences). In brief, MSC were stained for 15 min at 4 °C with fluorochrome-labeled monoclonal antibodies CD14, CD45, CD34, CD73, CD105, CD90, CD44 (all obtained from BD Biosciences) and HLA-DR (Beckman Coulter GmbH, Krefeld, Germany), washed with PBS and resuspended in FACSFlow™ (BD Biosciences) with 3% formaldehyde (Merck, Darmstadt, Germany). The samples were measured with a four-color flow cytometer

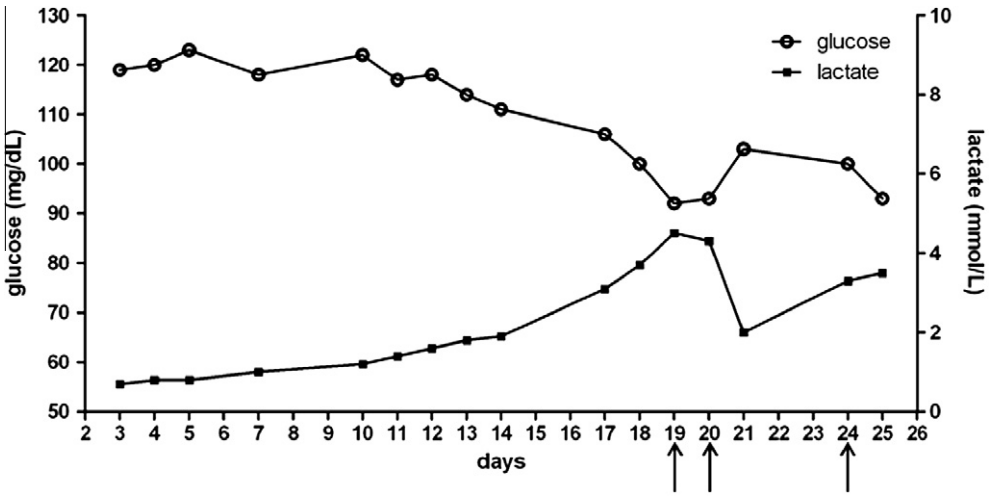

Fig. 1. Determination of glucose and lactate levels during MSC expansion within the bioreactor as indicators of cell growth. The medium flow rate was increased (→) at latest when lactate values rose above 4 mmol/l.

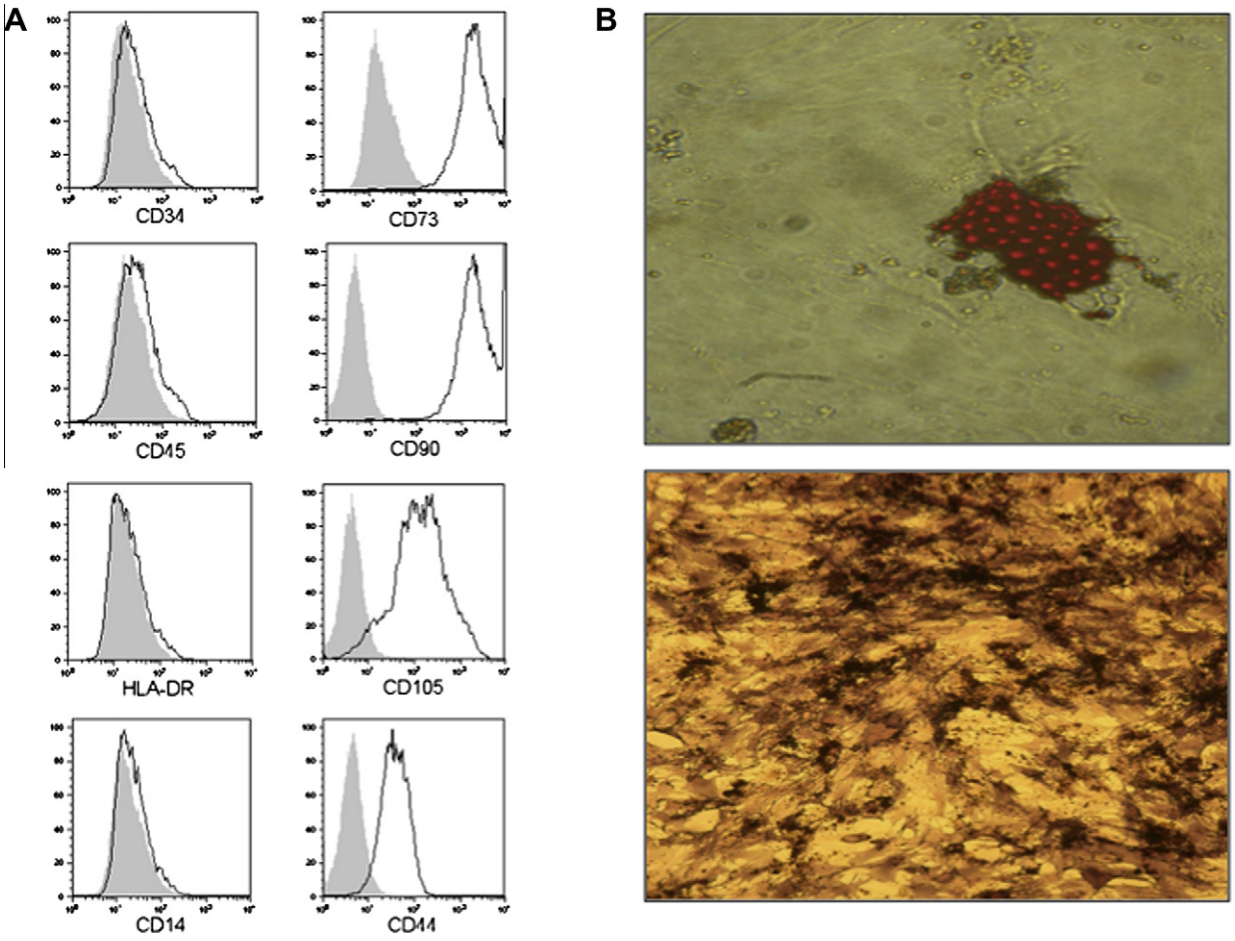

Fig. 2. (A) Flow cytometric analysis of bioreactor-expanded MSC in HPL-supplemented cultures. MSC are positive for CD90, CD105, CD44 and CD73 but lack expression of CD14, CD34, CD45 and HLA-DR. Results are representative for 3 independent experiments. (B) Bioreactor-derived MSC were differentiated into adipogenic and osteogenic cell lineages in vitro as indicated by red fat vacuoles upon Oil-red O staining (upper picture) and calcium deposits appearing black after von Kossa stain (lower picture) with $AgNO_3$.

(FACSCalibur™) with CellQuest Pro™ Software (both BD Biosciences). Isotype-matched antibodies were used as negative controls (BD Biosciences). FCS data were analyzed with FlowJo™ software version 9.5.3 (TreeStar Inc., Ashland, USA).

2.4. Induction of differentiation into osteocytes and adipocytes

Adipogenic differentiation of MSC was carried out with the NH-AdipoDiff Media (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following manufacturer's instructions. Cells were seeded in 35 mm cell culture dishes (8500 cells/cm^2) with NH-AdipoDiff Media. Afterward dishes were incubated at 37 °C in a humidified air chamber at 5% CO_2. Medium was replaced every 3–4 days. After 18–21 days cells were stained with a 0.5% Oil-Red O solution (Sigma–Aldrich, St. Louis) for 20 min.

Osteogenic differentiation was started immediately after harvest. Cells were plated out in 35 mm cell culture (5000 cells/cm^2) with NH-OsteoDiff Media (Miltenyi Biotec) and incubated for 10–14 days at 37 °C (5% CO_2) by replacing the medium every 2–3 days. Cells were subsequently fixed with acetone/methanol (1:1) at −20 °C for 5 min in order to perform von Kossa staining of the extracellular calcium matrix as described previously [32].

2.5. T-cell proliferation assay

Immunosupressive capacity of MSC was assessed using a co-culture of PBMC and CES-grown MSC. Briefly, one million human peripheral blood mononuclear cells (PBMC) and CES-grown MSC in different concentrations were co-cultured for 5 days. First, MSC were seeded in 24 well plates (Greiner Bio-One) in order to let them attach and equilibrate. After 24 h PBMC were added to the MSC. Prior to co-culture PBMC had been labeled with 1 μM 5,6-carboxyfluorescein di-acetate N-succinimidyl ester (CFSE) (Molecular Probes, Oregon, USA) for 10 min in the dark at 37 °C. Proliferation of PBMC was induced by addition of CD3 and CD28 antibodies (both from Biolegend, San Diego) at a concentration of 1 μg/ml. After incubation in a humidified air chamber at 37 °C with 5% CO_2 for 5 days in DMEM (+10% HPL + 1% N(2)-L-alanyl-L-glutamine + 1% Penicillin/Streptomycin), PBMC were collected and labeled with monoclonal antibodies against CD4 and CD8 (Biolegend) as described above. Dead cell exclusion was performed using Sytox Dye (Life Technologies, Carlsbad). CFSE intensity on $CD4^+$ and $CD8^+$ T-cells was measured using a BD FACS Canto II™ (BD Biosciences) with BD FACS DIVA™ software and further analyses were performed with FlowJo™ and GraphPad Prism™ software. ANOVA testing was employed for calculation of statistical significance.

3. Results

3.1. GMP-compliant expansion of MSC in a closed hollow fiber bioreactor

Given the potential variability in the biology of primary cells from different individuals and the long duration of cell expansion,

high standardization is mandatory for MSC manufacturing for therapeutic purposes. In order to comply with the EMA requirements for advanced therapy medicinal products (ATMPs) ex vivo expansion of MSC must follow a reliable and reproducible protocol to ensure quality and safety of the generated cell products.

To evaluate two different clinical scenarios, both primary MSC expansion in the hollow fiber bioreactor, as well as secondary expansion of pre-selected MSC were analyzed.

Primary bone marrow from 5 healthy volunteers was aspirated into heparin-coated syringes and transferred to a Cell Inlet Bag under grade A clean room conditions. Loading of cells into the fibronectin-coated hollow fiber bioreactor and all further steps of feeding, washing and harvest were performed in a closed tubing system with standardized control programs of the Quantum system, thus avoiding bacterial contamination during the expansion process [34]. Employing DMEM medium with low glucose levels and 10% HPL we were able to grow 2–58 million MSC from 8–32 ml primary human bone marrow. The duration of expansion lasted between 15 and 27 days. Expansion of MSC in the hollow fiber bioreactor was monitored by regular measurement of glucose and lactate levels. Increase of lactate and decrease of glucose prompted us to increase the flow rate in order to deliver sufficient amount of medium to the cells as depicted in Fig. 1. Glucose and lactate levels were also used as indicators for an appropriate harvest time. Moreover, we analyzed MSC expansion of primary bioreactor-expanded MSC versus secondary culture of preselected MSC. By loading 2–7.6 million preselected MSC into the bioreactor and providing continuous medium supply for 6–13 days, we achieved up to 20-fold cell expansions (Table 1).

MSC harvested from the bioreactor after culture of primary bone marrow proved to have a very good viability and proliferation capacity when subsequent flask-cultures were performed. Trypan blue stains and FACS analyses revealed a relatively high amount of fragments from dead blood cells in the harvest solution, which is consistent with the large population of red blood cells loaded into the system with the whole human bone marrow. However, the debris obviously did not influence MSC biology or further growths. Viability of MSC after the second passage cultivation or

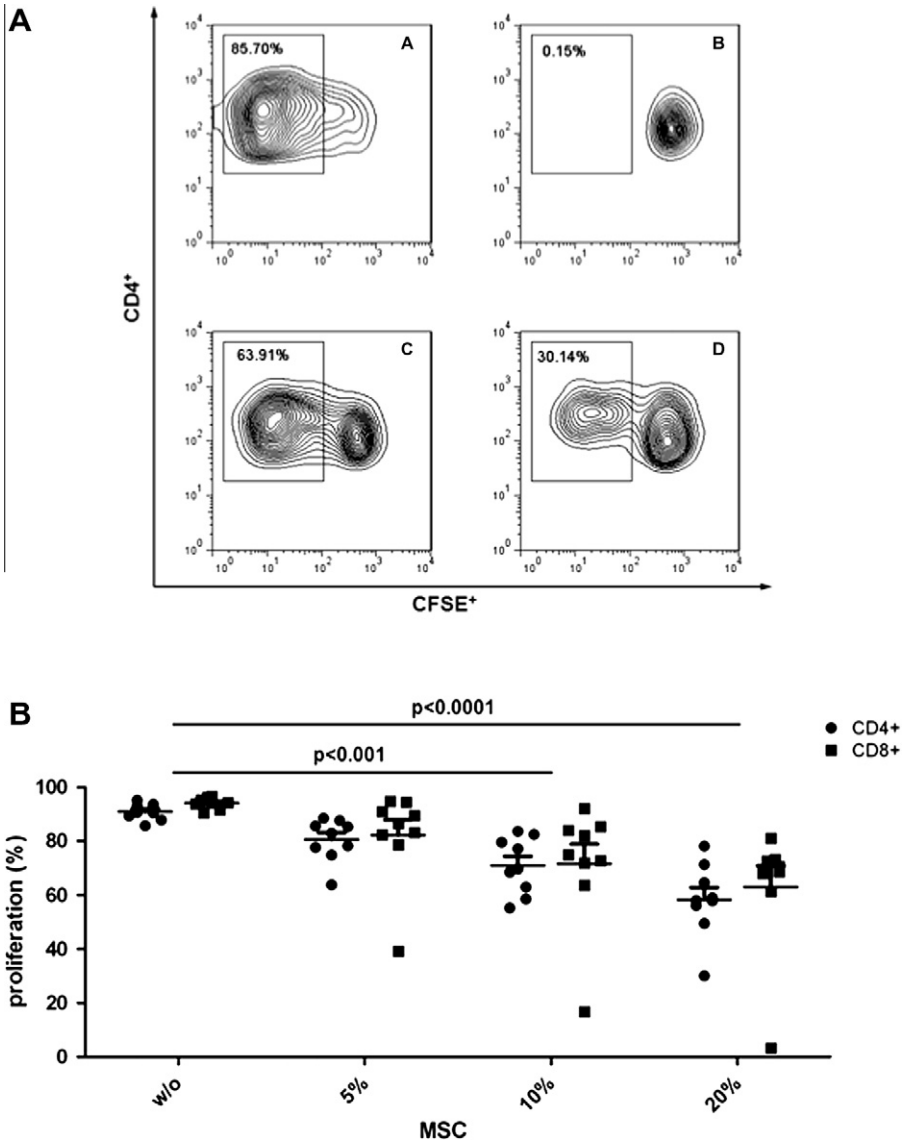

Fig. 3. Bioreactor-derived MSC inhibit T-cell proliferation in a dose dependant manner. (A) T-cell proliferation was determined in CFSE-labeled cells by flow cytometry after CD4 and CD8 surface staining. Proliferation of T-cells was high without addition of MSC (A) and no proliferation occurred without T-cell stimulating antibodies CD3/CD28 (B). MSC inhibited T-cell growth in a dose dependant manner at 10% (C) and 20% (D). (B) T-cell proliferation was significantly inhibited by MSC co-culture. Results are representative for $n = 9$ independent experiments.

after expansion of pre-enriched MSC proved to be 90%–99%. Thus MSC can be expanded efficiently under standardized GMP-compliant conditions in a hollow fiber bioreactor starting from 8 to 30 ml unprocessed primary bone marrow aspirate and clinical-scale cell amounts can be achieved after only one or two following passages of MSC culture.

3.2. Surface antigens and differentiation capacity of bioreactor-derived MSC

In order to address the criteria for defining cells as MSC according to the position statement of the *International Society for Cellular Therapy* [2], immunophenotyping and in vitro differentiation is required. Expression of surface antibodies was determined by flow cytometric analysis. Bioreactor-derived MSC proved to express the characteristic antigens CD90, CD73, CD44 and CD105, but lacked expression of CD34, CD45, CD14 and HLA-DR as depicted in Fig. 2A. MSC grown in the closed hollow fiber bioreactor with a GMP-compliant animal-free culture medium exhibited the typical antigen expression profile in accordance with the consensus criteria for MSC.

In order to test the bioreactor-derived MSC for their in vitro differentiation capacity cells were seeded into cell culture dishes immediately after harvesting and subjected to osteogenic and adipogenic differentiation media. After 2–3 weeks Oil-red O and von Kossa staining was performed as described [32]. Bioreactor-expanded MSC depicted the typical morphology of fat vacuoles and granules. The MSC exhibited the characteristic staining pattern with red vesicles proving adipogenic differentiation, as well as calcium deposits in response to induction of osteogenic differentiation visualized by $AgNO_3$ (Fig. 2B). Thus, bioreactor-expanded MSC exhibited the same differentiation capacity as flask-expanded MSC.

3.3. Inhibition of $CD4^+$ and $CD8^+$ T-cell proliferation by bioreactor-derived MSC

Suppression of T-cell proliferation is another hallmark of MSC biology and important to determine because MSC have been used for immunosuppressive treatment of autoimmune disorders and graft-versus host disease that do not respond to other therapies [13,35–37]. Therefore bioreactor-derived MSC were co-cultured with allogeneic PBMC and co-stimulatory antibodies at different ratios. T-cell proliferation was measured in $CD4^+$ and $CD8^+$ T-cells after carboxyfluorescein succinimidyl ester (CFSE) staining by subsequent surface staining and flow cytometric analysis. Bioreactor-derived MSC significantly inhibited both $CD4^+$ and $CD8^+$ T-cell proliferation in a dose dependant manner, as shown in Fig. 3. These results indicate that growth of MSC in a closed hollow fiber system with HPL supplemented medium does preserve the reported immunosuppressive properties of MSC.

4. Discussion

Adult pluripotent mesenchymal stem cells can be rapidly expanded in vitro. Their regenerative capacity, plasticity and immunomodulatory functions have been extensively characterized [38]. In addition many phase 1/2 clinical trials have suggested therapeutic potential of systemic or local application of autologous and allogeneic MSC [8–10,12–14]. Large-scale expansion in a standardized and GMP-compliant manner is a prerequisite for safe clinical implementation of MSC. We show that MSC can be expanded from un-processed bone marrow aspirate within a closed hollow fiber bioreactor employing animal-free medium components such as HPL instead of fetal calf serum and preserve their immunosuppressive capacity. By employing conventional tube-sealing and welding devices for connecting media bags to the expansion system we did not observe bacterial culture contamination even when the expansion duration was extended up to 27 days. The required tasks for cell manufacturing were supported by a control software providing continuous reports during all processing steps, thus making careful monitoring and standardized cultivation conditions possible. Surface antigen expression and differentiation capacity of bioreactor-manufactured MSC did meet minimal criteria for MSC definition as recommended by the ISCT [2]. Moreover, analysis of the modulation of T-cell proliferation through bioreactor-expanded MSC demonstrated significant $CD4^+$ and $CD8^+$ T-cell inhibitory capacity known from conventionally grown MSC. Cell numbers obtained after 2–4 weeks of primary culture of total bone marrow were always sufficient to initiate a secondary expansion. Hence the hollow fiber bioreactor system is suitable to generate about 100 million MSC within two or three passages, sufficient for clinical applications. In our hands, doubling times were similar or better in the Quantum system than flask-based expansion. Higher MSC numbers may be possible when a larger number of primary cells is seeded into the bioreactor. Using low passage MSC is always desirable in order to avoid alterations of biologic functions due to senescence [39] or the risk of genetic or epigenetic instability [40].

Taken together we demonstrate a robust, reproducible and efficient culture process for MSC in a closed hollow-fiber bioreactor system. This novel GMP-compliant approach may facilitate the clinical application of MSC.

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