REGULAR ARTICLE

Isolation, expansion and characterization of bone marrow-derived mesenchymal stromal cells in serum-free conditions

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Abstract Bone marrow-derived mesenchymal stromal cells (BM-MSCs) heralded a new beginning for regenerative medicine and generated tremendous interest as the most promising source for therapeutic application. Most cell therapies require stringent regulatory compliance and prefer the use of serum-free media (SFM) or xeno-free media (XFM) for the MSC production process, starting from the isolation onwards. Here, we report on serum-free isolation and expansion of MSCs and compare them with cells grown in conventional fetal bovine serum (FBS)-containing media as a control. The isolation, proliferation and morphology analysis demonstrated significant differences between MSCs cultured in various SFM/XFM in addition to their difference with FBS controls. BD Mosaic™ Mesenchymal Stem Cell Serum-Free media (BD-SFM) and Mesencult-XF (MSX) supported the isolation, sequential passaging, tri-lineage differentiation potential and acceptable surface marker expression profile of BM-MSCs. Further, MSCs cultured in SFM showed higher immune suppression and hypo-immunogenicity properties, making them an ideal candidate for allogeneic cell therapy. Although cells cultured in control media have a significantly higher proliferation rate, BM-MSCs cultured in BD-SFM or MSX media are the preferred choice to meet regulatory requirements as they do not contain bovine serum. While BM-MSCs cultured in BD-SFM and MSX media adhered to all MSC characteristics, in the case of few parameters, the performance of cells cultured in BD-SFM was superior to that of MSX media. Pre-clinical safety and efficiency studies are required before qualifying SFM or XFM media-derived MSCs for therapeutic applications.

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Introduction

Bone marrow-derived mesenchymal stromal cells (BM-MSCs) have great potential to repair or regenerate damaged tissue and thus hold promise to treat various human diseases (Dominici et al. 2006; Pittenger et al. 1999). MSCs can be conveniently isolated and expanded to a great extent from various sources (Spitkovsky and Hescheler 2008; Zuk et al. 2002) and have been extensively studied with respect to tissue engineering and cell therapy. BM-MSCs have to meet the specifications set by the International Society of Cellular Therapy (ISCT), including surface marker profile (Dominici et al. 2006; Horwitz et al. 2005) in order to be used for therapeutic applications. In addition, MSCs exhibit powerful immunomodulatory properties and suppress the proliferation of activated allogeneic lymphocytes in vitro and in vivo (Di Nicola et al. 2002; Le Blanc 2003) allowing MSCs to cross histocompatibility barriers. In addition, MSCs possess homing ability by means of which they can reach the site of injury and perform regeneration/repair (Papayannopoulou 2003) by

mobilization through several growth factors, cytokines and chemokines, resulting in increased therapeutic efficiency or repair mechanisms (Ding et al. 2011). Furthermore, MSCs have proven safe in most international clinical trials (Caimi et al. 2010; Fouillard et al. 2007; Marmont et al. 2006). An abundance of human safety data for therapeutic application makes MSC therapy a new clinical paradigm (Ankrum and Karp 2010). BM-MSCs are a heterogeneous population of cells and are characterized by surface marker expression, proliferation rate and differentiation potential. Their functional properties are known to differ with passage number, cell-seeding density and the media used for cell propagation (Prockop 2009).

While various new sources of MSCs have been uncovered in recent times, these may not be the ideal source for clinical applications due to the lack of in-depth studies of them. BM-MSCs are increasingly being used in clinical applications due to the availability of a large amount of data on their cell characteristics and safety in clinical studies. But the therapeutic MSCs developed for a particular disease indication should demonstrate enhanced disease-specific efficacy. This paradigm shift in the development of therapeutic MSCs is based on their transplantability, differentiation ability, secretion of trophic factors and immunosuppressive capacity (Ankrum and Karp 2010).

Currently, successful clinical translation of MSCs largely depends on the regulatory requirements to meet the safety, purity and potency of them as a biological product (21 CFR 601.2, 21 CFR part 210 and part 211). Current standard practice of culturing BM-MSCs is based on supplementing the fetal bovine serum (FBS) in basal medium of Dulbecco's modified Eagle Medium (DMEM) of low glucose (LG) or knock out (KO) or alpha-DMEM (Gottipamula et al. 2012; Gupta, et al. 2013; Nimura et al. 2008; Pal et al. 2009). But the use of FBS raises concerns with respect to lot-to-lot variability and immunological reactions by bovine sialic acid and protein derivatives in MSCs (Martin et al. 2005; Spees et al. 2004). Thus, xeno-free serums like autologous serum or human platelet lysate (HPL) can be used for culturing cells (Gottipamula et al. 2012; Kobayashi et al. 2005; Schallmoser et al. 2007; Shahdadfar et al. 2005). But it would be difficult to obtain large amounts of autologous serum and HPL for large-scale expansion of MSCs.

Serum-free media (SFM) development for BM-MSCs not only overcomes the regulatory requirements but also increases the performance of production systems by minimizing the variability of production batches and thus helps in understanding cell metabolism and nutritional requirements for biomedical research. At present, there are several SFM formulations published allowing cultivation of MSCs in defined media and several groups have developed the donor MSC-specific in-house SFM for higher cell growth (Chieregato et al. 2011; Jung et al. 2010, 2012; Mimura, et al. 2011). However, these disclosed formulations require safety of the materials used, as there is an increasing concern for transmission of infectious agents from animals to humans. Thus, media used in MSC culture for therapy require the development of GMP-compliant media, which can take care of all regulatory requirements in manufacturing of media for cell therapy products.

Many commercial SFM or xeno-free media (XFM) address the challenges posed by in-house SFM/XFM that have been developed as promising alternatives for the manufacturing of clinical-grade MSCs. These media selectively isolate the MSCs of homogenous cell populations and enrich MSC characteristics. Moreover, the spent media of SFM- or XFM-cultured cells can be used effectively as cell-free therapeutic products more efficiently than FBS-based spent media (Baglio et al. 2012; Cho et al. 2012; Jayaraman et al. 2013). It is necessary to identify the most efficient SFM/XFM for isolation and clinical-scale expansion of MSCs. Due to donor-to-donor variability in MSC proliferation kinetics, it may be necessary to adjust the media formulation for selected donor MSCs. The basic criteria for selecting donor MSCs are proliferation kinetics and functional properties.

Morphology, growth profile characteristics and functional and immunological characteristics of MSCs are essential for successful commercial translational research to become a viable option for cell therapy. A few authors have studied serum-free MSC characteristics in StemPro-SFM-XF (SP-XF), where they compared it with MSCs grown in FBS-supplemented media (Chase et al. 2010, 2012). Comparison of more than one SFM really provides the innate potential of MSC proliferation capacity as well as the robustness of SFM to support the growth of MSCs. Additionally, studying the immunomodulatory function of MSCs derived from different culture media will help to select suitable MSCs for clinical applications. These are the crucial steps towards characterization of cells as a drug and it is important to know the properties of MSCs grown in at least a few commercially available SFM as well as traditional standard (FBS) serum-containing media (SCM) as controls. Therefore, current studies focus on comparative characterization of MSCs in BD Mosaic™ Mesenchymal Stem Cell Serum-Free media (BD-SFM), Mesencult-XF (MSX) and KO-FBS media from isolation onwards. Further characterization includes induction of MSCs with interferon-gamma (IFN-γ) to elicit HLA-DR expression patterns as well as Indoleamine 2,3-dioxygenase (IDO) activity in different culture conditions. To date, IFN-γ induction studies have mostly been done on SCM to compare the differences of immune-modulation between bone marrow-derived MSCs and WJ-MSCs (Prasanna, et al. 2010). The behavior and characteristics of MSCs are highly variable in SCM and SFM and, from a regulatory point of view, there is a need for alternative vendors for the critical raw materials in process.

Based on our previous studies (Gottipamula et al. 2013), we selected Mesencult-XF (MSX) and BD Mosaic™ (BD-SFM) for culturing BM-MSCs and compared them with DMEM-KO supplemented with 10 % FBS as control. We investigated morphological characteristics, growth kinetics, cell surface markers, differentiation potential, immunosuppression and immunogenicity of BM-MSCs grown in BD-SFM-, MSX- and KO-FBS-containing media.

Materials and methods

Human bone marrow mononuclear cells isolation

BM aspirates were obtained from healthy volunteers aged between 19 and 23 years, after signed informed consent and under the ethics guidelines of the Kasturba Hospital Institutional Ethics Committee. The bone marrow mononuclear cells (BM-MNCs) were isolated by the Lymphoprep (Axis-Shield PoC)-based density gradient centrifugation method as previously reported (Pal et al. 2009).

Isolation of MSCs

Flasks (T-25) were coated with CELLstart™ (Invitrogen) for StemPro MSC SFM XenoFree™ (SP-XF) cultures (Invitrogen) before using SP-XF media prepared as per the manufacturer's instructions. Mesencult attachment substrate was used for Mesencult-XF™ (Stemcell Technologies, Canada) cultures before using MSX media prepared as per the manufacturer's instructions. And BD surface coating for BD Mosaic™ Mesenchymal Stem Cell Serum-Free media (BD-SFM) was used before using BD-SFM prepared as per the manufacturer's instructions. Frozen MNCs were quickly thawed in a 37 °C water-bath, diluted with pre-warmed respective media and centrifuged at 1,200 rpm for 10 min. The resulting pellet was resuspended and the mono-dispersed MNCs were plated in pre-coated T-25 flask at passage 0 (P0). For SP-XF, MSCs were isolated using SP-XF supplements with 2.5 % pooled human AB serum at a seeding density of 50 K (50, 000) per cm^2 and the same seeding density was used for KO-FBS, BD-SFM and MSX during P0 isolation of MSCs from MNCs. At later stages of passage (from P1), the SP-XF media devoid of human serum components were used. BM-MNCs cultured in Dulbecco's modified Eagles medium-knock Out (DMEM-KO) supplemented with 10 % FBS (Cat.no. SH30084.03, Lot no. GRH0054; Hyclone, Waltham, MA, USA), 2 mM L-glutamax (Invitrogen, Carlsbad, CA, USA), 1X penstrep (Invitrogen) served as control (KO-FBS.c, where c denotes control-without bFGF). The MSCs cultured in KO-FBS,c media supplemented with 2 ng/mL bFGF (Sigma) were also evaluated for isolation of MSCs and media designated as KO-FBS.b (where b denotes

control with bFGF). During the culture process, media were exchanged with respective freshly prepared SFM for every alternate day until five media changes had been completed or the colonies of MSCs reached 80–90 % confluence. The cells were harvested by digestion for 1–2 min at 37 °C with trypsin (0.25 %)/ethyelenediamine tetra acetic acid (EDTA; 1 mM) (Gibco, USA) for control cultures and TrypLE Select 10× (Invitrogen) for SP-XF cultures as well as BD-SFM cultures. However, Mesencult enzymatic dissociation solution was used for the dissociation of MSX cultures. During cultivation, morphological changes were photographed by phase contrast microscopy. Experiments in each condition were performed in triplicates.

Sub-culturing of MSCs

The P0-harvested MSCs were re-suspended in respective culture media and the plated (P1) at a density of 1,000/3,000 per cm^2 (1 K/3 K). Cells were screened every alternate day and media change was done at around 50 % confluency. At 80–90 % confluency, cells were further passaged while a portion was removed for further analysis.

Isolation and proliferation kinetics

Isolation kinetics of MSCs in KO-FBS media with and without bFGF supplementation, BD-SFM and MSX were analyzed on culture days 3, 7 and 11 at P0. BM-MSCs grown in various SFM from each donor of P0 were used to study the growth kinetics. MSCs isolated without bFGF supplements in KO-FBS cultures were used for sequential sub-culturing at 1 K seeding density from P1 to P4 and served as control whereas, for BD-SFM and MSX cultures, 3 K seeding density was used. Population doubling time (PDT) of BD-SFM, MSX and KO-FBS was calculated. Cumulative population doublings (CPD) were calculated by sequential sub-culturing from P1 to P4 and harvested at 80–90 % confluency in each passage. However, we did not calculate CPD for SP-XF cultures as this media did not support the growth of cells beyond the P0 stage.

Cell size distribution analysis

The MSCs cultured in KO-FBS, BD-SFM and MSX were analyzed for cell size and distribution profile through a Vi-cell XR-cell analyzer as per the manufacturer's instructions. One million cells from KO-FBS, BD-SFM and MSX at P4 were used to run the Vi-cell analyzer and its video imaging system was used to analyze various parameters like cell size, size distributions and circularity. This analyzer automates the trypan blue exclusion protocol and data were analyzed by the built-in software. Further, cell size distribution profiles were analyzed by forward scatter (FSC) signals of FSC height,

fluorescence intensity plots and cell distribution by side scatter (SSC) and FSC for BM-MSCs grown in KO-FBS, BD-SFM and MSX at P4.

Immunophenotyping

Immunophenotyping of the cultured BM-MSCs in SFM and control media were performed using flow cytometry to identify the presence of specific cell surface antigens. Briefly, BM-MSCs were harvested and re-suspended in wash buffer at a concentration of 1×10^6 cells/mL. The wash buffer consisted of phosphate buffered saline (PBS) supplemented with 1 % (v/v) FBS and 1 % (w/v) sodium azide. Cell viability was measured by flow cytometry using 7-amino actinomycin D (7AAD, which can penetrate through cell membranes of dead cells). About 200 μl of cell suspension were incubated in the dark for 30 min at 4 °C with saturating concentrations of fluorescein iso-thiocyanate (FITC)- or phycoerythrin-(PE)-conjugated antibodies. Appropriate isotype matched controls were used to set the instrument parameters. After incubation, cells were washed thrice with wash buffer and re-suspended in 0.5 mL of wash buffer for analysis. Analysis was performed in flow cytometry (EasyCyte; Guava Technologies). Cells were identified by light scatter for 10,000 gated events and analyzed using Guava Express Pro software (Guava Technologies). An antigenic event was observed when the fluorescence was greater than 25 % or above its isotype control. The following markers were analyzed: CD34-PE, CD133-PE, CD90-PE, CD14-FITC, CD19-FITC, CD105-PE (chemicon), CD45-FITC and CD73-PE, CD166-PE and HLA-DR-FITC (BD Pharmingen, San Diego, USA).

Differentiation potential

Osteoblast differentiation was induced by culturing P4 BM-MSCs cultured in respective media of KO-FBS, BD-SFM and MSX supplemented with 10^{-8} M dexamethasone, 30 μg/mL ascorbic acid and 10 mM β - glycerophosphate (all Sigma-Aldrich) for 3 weeks. Calcium accumulation was assessed by Von Kossa Staining. The differentiated cells were washed with PBS and fixed with 10 % formalin for 30 min. The fixed cells were incubated with 5 % $AgNO_3$ for 60 min under UV light and then treated with 2.5 % sodium thiosulphate for 5 min, and the images were captured.

To induce adipogenic differentiation, at P4, BM-MSCs were cultured in respective media of KO-FBS, BD-SFM and MSX, supplemented with 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 1 μg/mL insulin and 100 μM indomethacin (all Sigma-Aldrich) for 21 days. The supernatant was then aspirated and cells were fixed in 10 % formalin for 20 min. Further, 200 μl of oil red O staining solution was added and incubated for 10 min at room temperature. The cells were rinsed five times with distilled water and images were captured.

To induce chondrogenic differentiation, at P4, BM-MSCs from KO-FBS, BD-SFM and MSX were cultured in STEMPRO (Invitrogen) chondrogenesis differentiation medium (Chondrocyte differentiation basal medium with chondrogenesis supplement). Chondrogenesis differentiation medium was replenished every 3 days. For staining, cells were fixed with 4 % formalin for 30 min. Safranine O staining solution was added and incubated for 5 min. The cells were rinsed with distilled water and images were captured. Images of BM-MSCs differentiated towards osteogenic, chondrogenic and adipogenic lineages were captured using an Nikon Eclipse 90i microscope (Nikon, Japan, www.nikon.com) and Image-Pro Express software (Media Cybernetics, Silver Spring, MD, USA, www.mediacy.com).

MTT assay

Cell proliferation was determined using a 3-(4, 5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MSCs were plated at a density of 5×10^4 to 1.25×10^4 cells/well in 96-well plates. Proliferation was assessed after 24 h post-seeding by Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich), according to the manufacturer's instructions. The absorbance of the samples was measured at 570 nm using a microplate reader. Experiments were performed in triplicate and the results were expressed relative to the untreated control.

CFU-F assay

For CFU-F assay, 100 MSCs were plated in KO-FBS, BD-SFM and MSX ($n=5$ of each condition) on a 100-mm^2 cell culture dish. The plates were stained with crystal violet after 14 days of incubation and visible colonies were counted.

Post-thaw viability

One million BM-MSCs cultured in three different media (KO-FBS, BD-SFM and MSX) at P4 were cryopreserved in standard serum-free cryopreservation medium containing 10 % Dexamethasone (DMSO), human serum albumin and PlasmaLyte A and post-thaw viability was assessed by 7AAD after 3 months storage. Each sample was thawed after removing the vials from the liquid nitrogen dewar (cryo-freezer) and immediately placed in a 37 °C water bath until ice crystals just disappeared. The contents of each sample vial were transferred to a 15-mL centrifuge tube and diluted at a ratio of 1:9 within 3–5 min in complete media to a final volume of 10 mL. These were then centrifuged to remove the DMSO and suspended in complete media, then viability was assessed. 7AAD viability along with phenotypic marker expression were done immediately after thawing and washing

of MSCs. 7AAD and phenotypic marker expression were analyzed by using flow cytometry as described previously (Gottipamula et al. 2012).

Immunogenicity assay

Mixed lymphocyte reaction (MLR) mediated assay

Mitomycin C (10 μg/mL for 2.5 h) treated MSCs were seeded in 96-well plates (Corning) at cell numbers ranging from 8×10^4 to 1×10^4 cells /well and allowed to attach overnight. Two $\times 10^5$ responder peripheral blood mononuclear cells (PBMC) were then added to each well and the cultures were maintained for 5 days during which they were pulsed with 5-bromo-2-deoxyuridine (BrdU) for the final 24 h. Cell proliferation was measured using a fluorimetric immunoassay kit (Calbiochem) for the quantification of BrdU incorporation, according to the manufacturer's instructions. One-way MLRs at the corresponding stimulator to responder ratios were used as positive controls for PBMC proliferation in response to alloantigen.

MSC priming based assay

BM-MSCs cultured in KO-FBS, BD-SFM and MSX at P4 were primed by IFN-ϒ induction. The exogenous and endogenous expression of IDO, an anti-inflammatory marker and endogenous expression of interleukin-1 beta (IL-1β), a pro-inflammatory marker were analyzed. Briefly, BM-MSCs at P4 of KO-FBS, BD-SFM and MSX cultures were treated with IFN-ϒ at 0, 50 and 100 ng per million cells concentration for 24 h to induce IDO expression. The spent media were collected for exogenous determination of IDO activity and harvested cells were tested for inducible HLA-DR expression. The biological activity of IDO was evaluated by measuring the level of tryptophan degradation product, L-kynurenine, present in the spent media by using Ehrlich's reagent as described (Prasanna et al. 2010).

Quantification of endogenous IDO and IL-1β

Endogenous IDO and IL-1β were measured through qRT-PCR. For IDO, CAAAGGTCATGGAGATGTCC was used as forward primer and CCACCAATAGAGAGACCAGG as reverse primer. The total length of the primer was 233 bp. For IL-1β, GCTGAGGAAGATGCTGGTTC was used as forward primer and GTGATCGTACAGGTGCATCG as reverse primer. The total length of the primer was 146 bp. Briefly, total RNA isolation was carried out using a RNeasy minikit (Qiagen). cDNA was prepared with superscript III reverse transcriptase enzyme. PCR conditions were initial denaturation at 94 °C for 10 min, followed by denaturation at 94 °C for 15 s, annealing for 1 min, extension at 72 °C for 1 min for

35 cycles and final extension was carried out at 72 °C for 5 min. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as a control for normalization of RNAs. PCR products were analyzed using ethidium bromide-stained 2 % agarose gels. Analysis of the gel images was carried out using the Biorad imaging system. qRT-PCRs were carried out with SYBR Green master mix and AB real-time thermocycler (AB StepOne Plus).

Immunosuppression assay

For immunosuppression assays, mitomycin C-treated MSCs were seeded in 96-well plates at cell numbers ranging from 8×10^4 to 1×10^4 cells /well and allowed to attach overnight. A one-way MLR at a stimulator: responder ratio of 1:2.5 was then added to each well. To set up the MLR, 2×10^5 responder PBMC were mixed with 8×10^4 allogeneic stimulator PBMC, which had been previously treated with 25 μg/mL mitomycin C for 0.5 h. Lymphocyte proliferation was measured at the end of 5 days using a fluorimetric immunoassay kit as per the manufacturer's instructions. A set of MLR wells cultured in the absence of MSCs was considered as the proliferation control. For all assays, treatments were performed in triplicate in RPMI 1640 medium (Invitrogen) supplemented with 10 % FBS (Hyclone), 2 mM glutamine (Invitrogen) and 0.05 μM β-mercaptoethanol (Sigma-Aldrich).

Statistical analysis

All values were expressed as mean ± SEM (standard error of mean). Data were analyzed by paired Student's *t* test using Graphpad Prism (v.5; Graphpad Software, La Jolla, CA, USA). Student's *t* test (2-tailed) was performed in order to compare means between groups. A *p* value <0.05 was considered significant.

Results

Isolation kinetics of mesenchymal stromal cells

BM-MNCs were isolated and incubated in five different cell culture media: KO-FBS supplemented with bFGF (KO-FBS-b), KO-FBS as control (KO-FBS-c), BD-SFM, MSX and SP-XF media. It was not possible to isolate the MSCs in SP-XF culture media even with 2 % AB serum at P0. P0 cultures showed significantly higher proliferation rates in KO-FBS-b medium compared to the rest of the media. As the flow cytometric analysis of harvested MSCs at P0 cultures of KO-FBS-b revealed higher HLA-DR expression (>40 %), they were excluded from further multi-passage proliferation and characterization studies. BD-SFM and MSX have supported the attachment and expansion of MSCs. Adherent cells

could be observed as early as 48–72 h post-seeding of MNCs in all cultures but the forming of spindle-shaped MSCs appeared early in BD-SFM and MSX compared to KO-FBS media. Further, the growth pattern of MSCs attachment and proliferation at P0 was studied by harvesting across different days (days 3, 7 and 11) in various SFM. Distinctly different isolation patterns and proliferation abilities were found across the five different culture media (Fig. 1a). The isolation efficiency of MSCs cultured in BD-SFM was more compared to other culture media tested at days 3 and 7 of P0. Isolation efficiency was significantly enriched by supplementing with bFGF compared to bFGF-free media (Fig. 1b). There was a significantly higher number of MSCs in BD-SFM and MSX cultures compared to KO-FBS.c.

Proliferation kinetics

The results showed that BM-MSCs grown in KO-FBS cultures had a higher proliferation ability than BD-SFM and MSX grown MSCs. Cumulative growth profiles of BM-MSCs cultured in BD-SFM were similar to those of MSX cultures as shown in Fig. 2a. It was observed that the average CPD was higher in KO-FBS (5.27/passage) than in BD-SFM (3.97/passage) or in MSX (3.92/passage). The PDT increased as the passage number increased in SFM, whereas KO-FBS cultures had relatively consistent PDT. The average PDT of BM-MSCs cultured in KO-FBS, BD-SFM and MSX were found to be 43.94, 37.84 and 38.12 h, respectively (Fig. 2b). The MSCs cultured in KO-FBS, BD-SFM and MSX retained all morphological features, cell yields and surface markers until P4. The signs of senescence were prominent in cells cultured in all three media at P5 onwards, as they were unable to reach even 50 % confluence at P5. Hence, P5 cultures were not considered for further culturing or characterization. BM-MSCs cultured in all these three media were only able to grow up to P4 without losing basic MSCs characteristics.

Cell morphology and cell analysis

The BM-MSCs expanded in KO-FBS, BD-SFM and MSX showed distinct morphological features at various confluency (10–30, 50–60 and 80–90 %) levels in the same passage. But BM-MSCs expanded in all tested SFM along with KO-FBS showed the basic features as fibroblastic-like morphology (Fig. 3). BD-SFM-cultured cells have shown two distinct morphological features, they were broad in the middle and were short and spindle shaped, whereas MSX-cultured cells are thin, long, slender spindles with long tapering ends. MSCs grown in KO-FBS showed size compression when they reached 80–90 % confluency but the BM-MSCs grown in other SFM did not showed such characteristics. Interestingly, BD-SFM cultures at P4 and above showed bundles of bright, elongated, slender MSCs in a group with vacuoles as a distinct morphological feature as compared to the previous passages. Further, these distinct morphological characteristics were tested by performing size distribution analysis. Supplementary Fig. S1a shows flow cytometry-based size distribution of BM-MSCs grown in KO-FBS, BD-SFM and MSX at P4 by FSC signals. The size (FSC height) of all the media samples population was normally distributed, thus the peak of each distribution was chosen to be representative of mean cell size. A comparison of average cell size showed that cells cultured in BD-SFM and MSX were similar in size when compared to KO-FBS. (Electronic Supplementary Material, Fig. S1a, b).

Another parameter used to check the cell size was FSC and SSC by flow cytometery. BM-MSCs expanded in KO-FBS had a FSC cell cluster near to debris and had heterogeneous populations (Electronic Supplementary Material, Fig. S1c). BM-MSCs expanded in BD-SFM showed homogeneous, large and dense clusters compared to KO-FBS and MSX (Electronic Supplementary Material, Fig. S1d). MSCs grown in MSX showed a similar FSC scatter to that of BD-SFM but showed a slightly lower SSC (Electronic Supplementary Material, Fig. S1e). FSC and SSC of BD-SFM revealed that
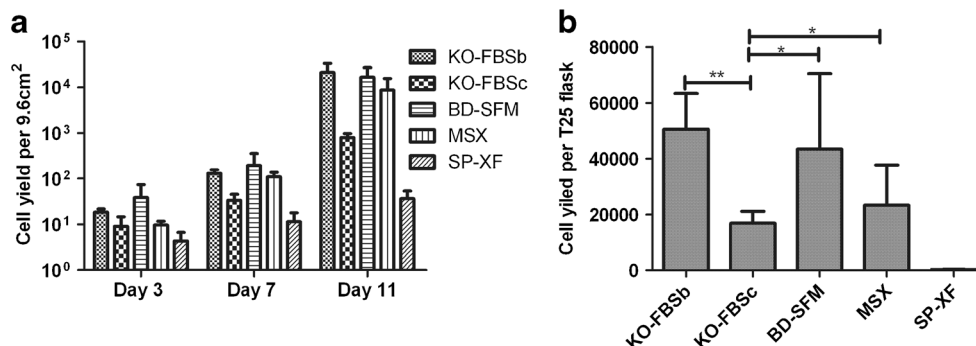
Fig. 1 BM-MSCs isolation efficiency of culture media. a Isolation kinetics of SFM at days 3, 7 and 11. b Comparison of yield of MSCs in three different serum-free media along with KO-FBS media with and without bFGF as control. (KO-FBS$_b$ control serum containing media with bFGF, KO-FBS$_c$ control serum containing media without bFGF). KO-FBS$_b$, BD-SFM and MSX show a statistically significant higher cell yield than KO-FBS$_c$ and SP-XF. The values are the mean ± SD (n=3); *p<0.05; **p≤0.02

Fig. 2 Proliferation kinetics of BM-MSCs. **a** Comparative cumulative population doublings (CPDs) of KO-FBS, BD-SFM and MSX. KO-FBS has higher CPDs than BD-SFM or MSX. **b** Comparison population doubling time (PDT) of BM-MSCs in the KO-FBS, BD-SFM and MSX cultures
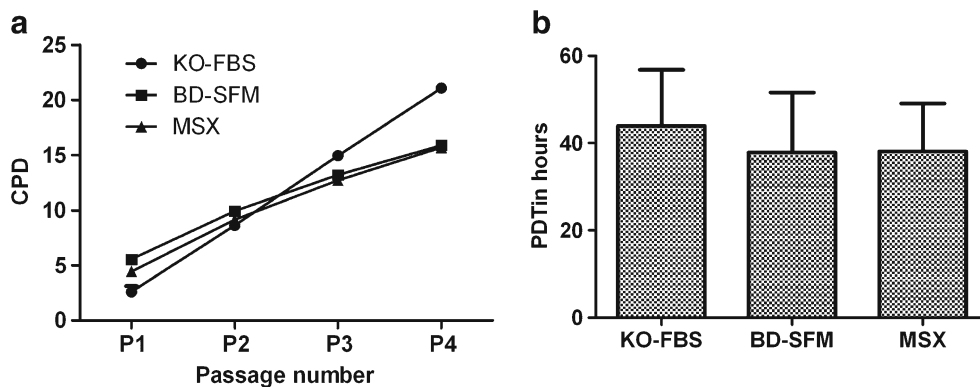
the cells were more homogeneous in nature when compared to the cells cultured in the other two media. BM-MSCs cultured in BD-SFM showed higher granularity as compared to the cells cultured in the other two media, as shown by the FSC and SSC plots (Electronic Supplementary Material, Fig. S1d).

BM-MSCs at P4 of KO-FBS, BD-SFM and MSX were further analyzed for cell size and distribution using the Vi-cell analyzer and the results were expressed in terms of average cell diameter. Approximate mean cell sizes of BM-MSCs cultured in KO-FBS, BD-SFM and MSX were 13.95, 17.49 and 16.12 μm, respectively but BD-SFM and MSX cultures showed similar cell size (Electronic Supplementary Material, Fig. S1f–h).

Altogether, with the morphological observations, FSC height, FSC-SSC plots and Vi-cell image analysis, the data suggest that growth media influenced the morphological as well as cell size characteristics to some extent. As a result, BD-SFM MSCs were more homogeneous and slightly larger than the smaller and heterogeneous sub-populations found in KO-FBS cultures.

Immunophenotyping

The immunophenotype was evaluated and analyzed by using flow cytometry for BM-MSCs cultured in serum and serum-free media; BM-MSCs showed similar expression of CD markers in serum (KO-FBS) and serum-free (BD-SFM and MSX) MSCs. BM-MSCs cultured in all three media were analyzed for their phenotypic expression at passage 4 ($n=3$). The CD markers expression in all culture media tested showed low levels (<3 %) of hematopoietic markers such as $CD45^+$,

Fig. 3 Morphology of expanded BM-MSCs at P4. **a**, **d**, **g** BM-MSCs cultured in KO-FBS at a seeding density of 1,000 cells/cm^2; **b**, **e**, **h** BM-MSCs cultured in BD-SFM with 3,000 cells/cm^2 seeding density; **c**, **f**, **i** BM-MSCs cultured in MSX with 3,000 cells/cm^2. **a–c** Microphotographs of 5–10 % confluency, **d–f** 45–55 % confluency and **g–i** 80–90 % confluency. Magnification ×10; *scale bar* ~100 μm
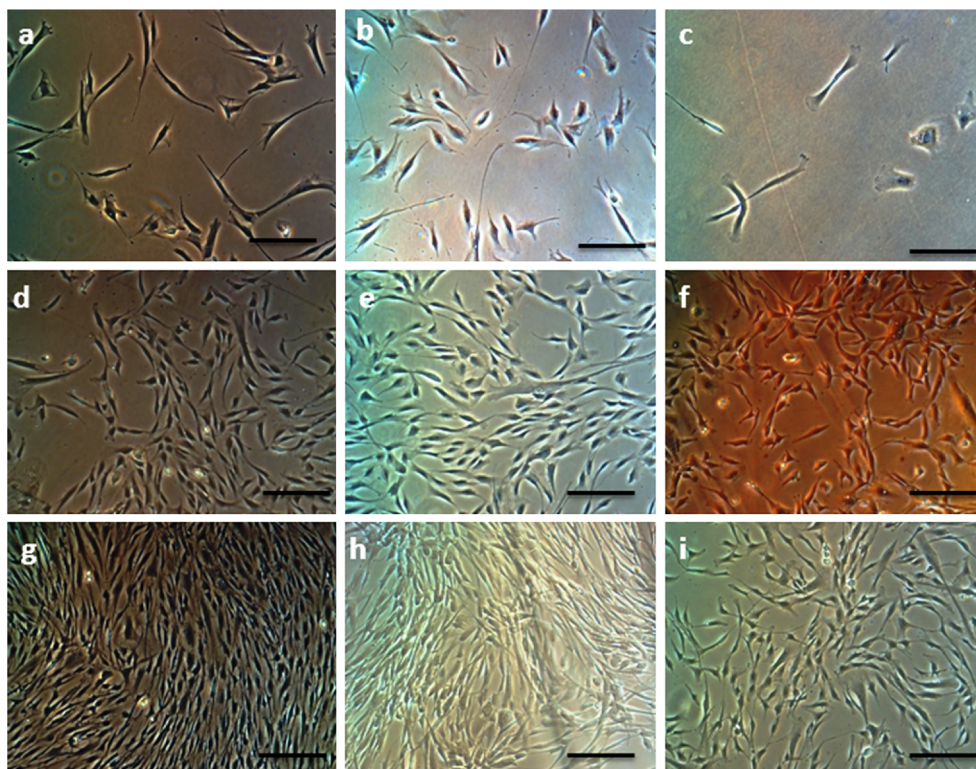
CD14$^+$ and HLA-DR and high levels of typical MSC-positive markers such as CD73, CD90, CD105 and CD166. There were no differences in the expression levels of any of the markers tested but HLA-DR expression was lowest in BD-SFM cultures. The BM-MSCs were positive for CD73, CD90, CD105 and CD166 and negative for CD34, CD133, CD14, CD19, CD45 and HLA-DR (Electronic Supplementary Material, Fig. S2a).

Mean fluorescence intensity (MFI) is a parameter strictly related to the amount of antigen expressed from a cell population, because it depends on the amount of fluorescence antibody binding to the antigen, which in turn depends on the amount of antigens expressed by the cells. Moreover, MFI is a straight-forward value calculated by flow cytometric software. The analysis of MFI revealed that MSCs from serum-free cultures expressed higher amounts of positive markers and lower amounts of negative markers, suggesting that the cell populations were purer than KO-FBS media (Electronic Supplementary Material, Fig. S3a–b). The percentage of positive or negative cells and MFI collectively provides the characteristics and nature of the expressed markers.

Differentiation potential

To determine whether SFM media (BD-SFM and MSX) alter the functional tri-lineage potential of MSCs, we performed in vitro tri-lineage differentiation assay by inducing P4-harvested cells to osteoblasts, adipocytes and chondrocytes. The differentiation towards adipocytes was observed by oil red O staining (Fig. 4a–c). The characteristics of the fatty vacuole deposits were evident after differentiation of KO-FBS, MSX and BD-SFM expanded cells from days 6, 5 and 4 onwards, respectively. The differentiation toward osteoblasts was observed by Von Kossa staining of calcium deposits (Fig. 4d–f). The differentiation toward chondrocytes was observed by safranine O staining after 10 days of induction (Fig. 4g–i). Overall, BM-MSCs cultured in KO-FBS, BD-SFM and MSX showed comparable tri-lineage potential as evidenced by their staining characteristics (Fig. 4). However, interestingly, KO-FBS MSCs showed more propensity towards chondrogenic differentiation, whereas BD-SFM MSCs showed more propensity towards adipogenic differentiation as evidenced by early appearances of differentiated cells containing lipid droplets.

MTT assay

The proliferation activity of BM-MSCs cultured in KO-FBS, BD-SFM and MSX at P4 was analyzed by MTT assay. Electronic Supplementary Material, Fig. S4 shows the proliferation of MSCs in three different densities of all culture media used. BM-MSCs cultured in KO-FBS showed an apparently higher proliferation ability at all three densities tested but significantly higher proliferation was observed at 25 K cell

densities when compared to BD-SFM and MSX (Electronic Supplementary Material, Fig. S4).

CFU assay

Typically, MSCs are characterized by a clonogenic potential as determined by CFU-F assay where more than 50 MSCs are considered as one colony. The mean CFU-F number at P4 in BD-SFM was significantly higher than KO-FBS and MSX cultures, as shown in Fig. 5a. The observed CFU-F in various media cultures indicated that there are considerable differences in colony morphology and proliferation rates among MSC colonies. Morphology and numbers of CFU-F in KO-FBS media were smaller and fewer (Fig. 5b). MSC colonies in BD-SFM were smaller but more colonies were observed compared to KO-FBS (Fig. 5c). MSC colonies in MSX were only a few but they were large and dispersed (Fig. 5d).

Post-thaw viability

No significant decrease in viability was observed in BM-MSCs derived from serum-free cultures (BD-SFM and MSX) before and after cryopreservation. Post-thaw viability of BM-MSCs derived from all three culture media tested were found to be above 90 %. Overall, serum-free cultures (BD-SFM and MSX) maintained significantly high post-thaw viability (95 %) when compared to KO-FBS cultures (92 %) (Fig. 6a). The percentage of dead cells were found as green scatter on the right side in the 7AAD plot and green scatter was higher in KO-FBS (Fig. 6b), compared to BD-SFM (Fig. 6c) and MSX (Fig. 6d).

Immunogenicity assays

MLR mediated assay

To determine the immunogenicity of KO-FBS, BD-SFM and MSX MSCs, the PBMC proliferative response was measured in the assay. BD-SFM MSC elicited a higher proliferative response in allogeneic PBMC as compared to MSC cultured in KO-FBS (Fig. 7a), although the difference was not statistically significant at all ratios tested. However, the BD-SFM MSCs were still found to be hypo-immunogenic as compared to the MLR control, indicating that allogeneic MSCs cultured in BD-SFM will not elicit an immune reaction in an unrelated recipient.

MSC priming assay

BM-MSCs cultured in KO-FBS, BD-SFM and MSX media respond differently to various doses of IFN-γ and are shown as percentage HLA-DR expression (Fig. 7b). A higher level of HLA-DR expression was observed in MSCs derived from KO-FBS and MSX culture media compared to BD-SFM. The higher IDO expression in KO-FBS-cultured cells and

Fig. 4 Comparative in vitro differentiation potential of BM-MSCs cultured in KO-FBS, BD-SFM and MSX. **a–c** BM-MSCs of KO-FBS, BD-SFM and MSX differentiate consistently into the adipogenic lineage, **d–f** osteogenic lineage, **g–i** chondrogenic lineage and **j–l** non-induced as controls. Stains shown are representative for passage 4. **a–c** Adipogenic differentiation potential was detected by oil droplet formation, stained with oil red O. **d–f** Osteogenic differentiation potential indicated by calcium accumulation (Von Kossa Staining). **g–i** Chondrogenic differentiation was stained by safronin O. Representative results of three independent experiments are shown. Magnification ×10, *scale bar* ~100 μm
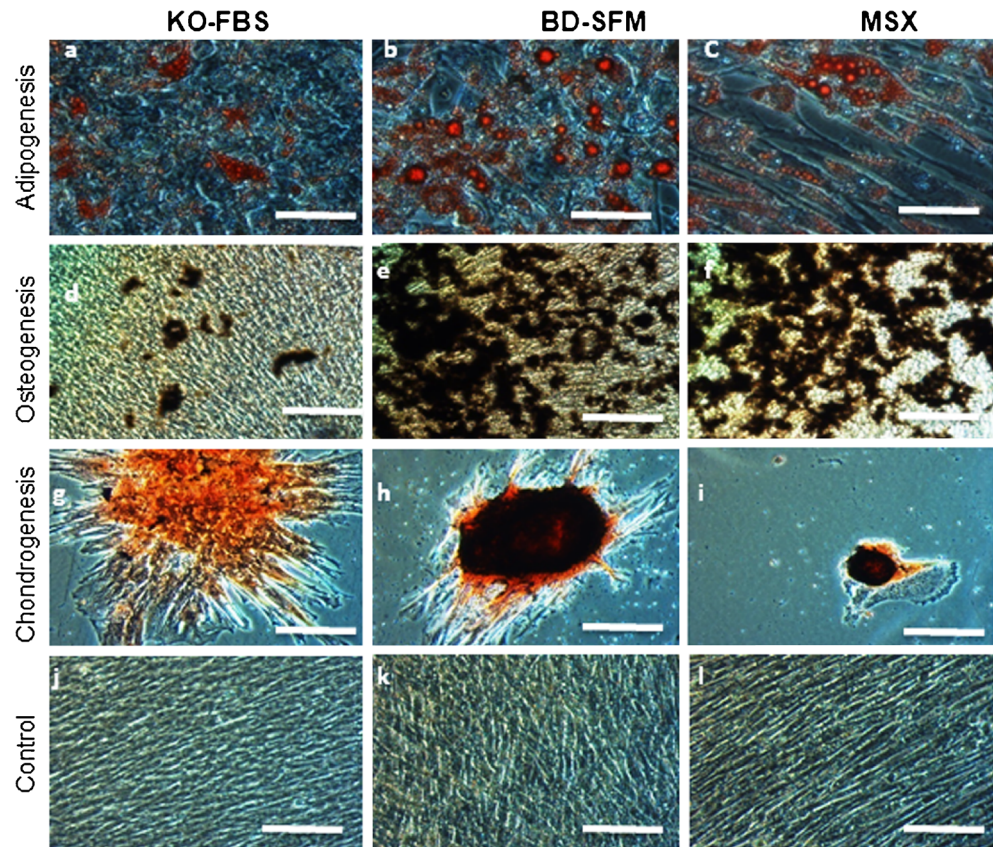
conditioned media indicated a higher immunosuppression property of these cells followed by BD-SFM- and MSX-grown cells in response to IFN-γ induction (Fig. 7c, d). The IFN-γ treatment increased the expression level of endogenous anti-inflammatory cytokine IDO in all culture conditions. The variable endogenous level of the pro-inflammatory marker IL-1β was observed in different cultures. BD-SFM cultures with 100 ng of treatment showed minimum levels of IL-1β, which is found to be much lower than the KO-FBS cultures without IFN-γ treatment (Fig. 7e). This gives a strong indication that IFN-γ-induced BD-SFM cultures had the ability to maintain lower expression of pro-inflammatory cytokines.

Immunosuppression assay

The immunosuppressive properties of MSCs cultured in BD-SFM and KO-FBS were compared by testing their ability to suppress a MLR. Lymphocyte proliferation was highly suppressed in the presence of MSCs cultured in both SFMs and this inhibition was found to be dose-dependent (Fig. 7f). BD-SFM MSCs were found to be equivalent to KO-FBS MSCs at the highest dose tested and the observed differences in all other doses were not significant. Thus, MSCs cultured in BD-SFM were found to exhibit an immunosuppressive capacity inherent to MSCs.
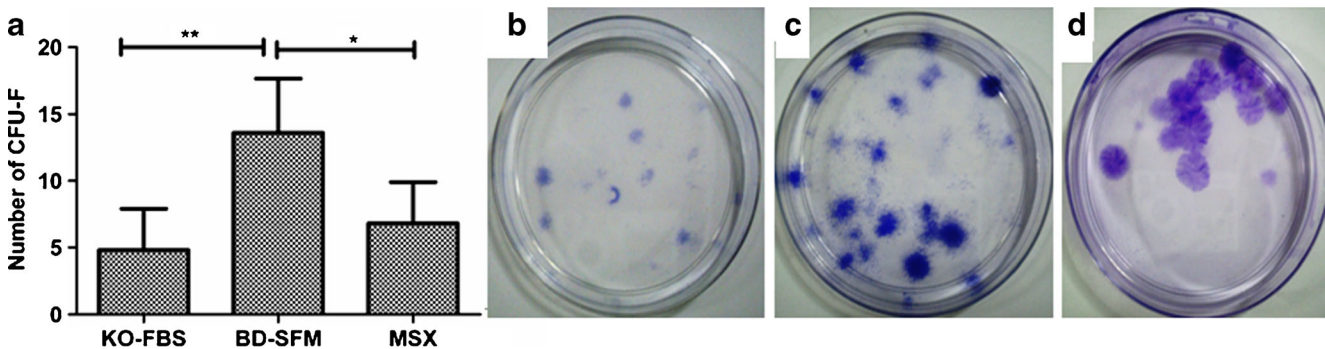
Fig. 5 Colony-forming unit-fibroblast (CFU-F) assay. **a** CFU-F colony formation of BM-MSCs in KO-FBS, BD-SFM and MSX media; Significant differences exist between KO-FBS with BD-SFM ($n=5$; **$p<0.02$) and BD-SFM with MSX ($n=5$; *$p<0.05$). **b–d** Representative photograph of CFU-F of KO-FBS, BD-SFM and MSX
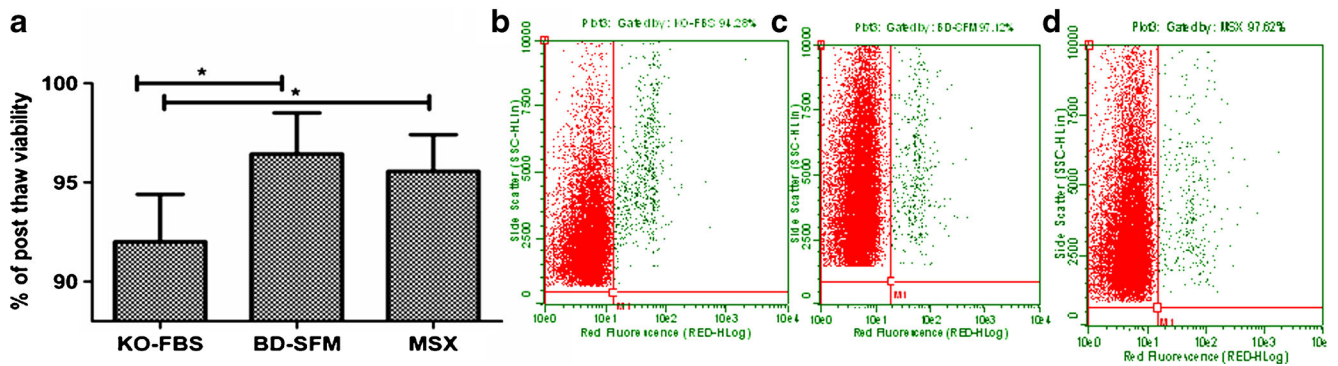
Fig. 6 Post-thaw viability of BM-MSCs in serum and serum-free conditions. **a** Post-thaw viability of BM-MSCs cultured in KO-FBS, BD-SFM and MSX. **b–d** 7AAD plot of KO-FBS BD-SFM and MSX, showing significantly higher post-thaw viability in BD-SFM and MSX compared to KO-FBS ($n=3$; *$p<0.05$). A representative 7AAD plot from three independent samples was drawn from three different culture media tested

Discussion

Over the past few decades, BM-MSCs have gained a tremendous interest in regenerative medicine and several pre-clinical models and clinical trials have demonstrated their safety and efficacy in various clinical indications (Lalu et al. 2012). Large-scale production of these clinically relevant cells can only be achieved by properly standardized cell expansion procedures. We previously reported that pre-isolated BM-MSCs can be expanded in optimized serum-free conditions at low seeding densities, even if a donor bank has already been derived from serum conditions at earlier stages (Gottipamula et al. 2013). As a logical step forward, in the present study, we isolated, expanded and characterized the BM-MSCs in
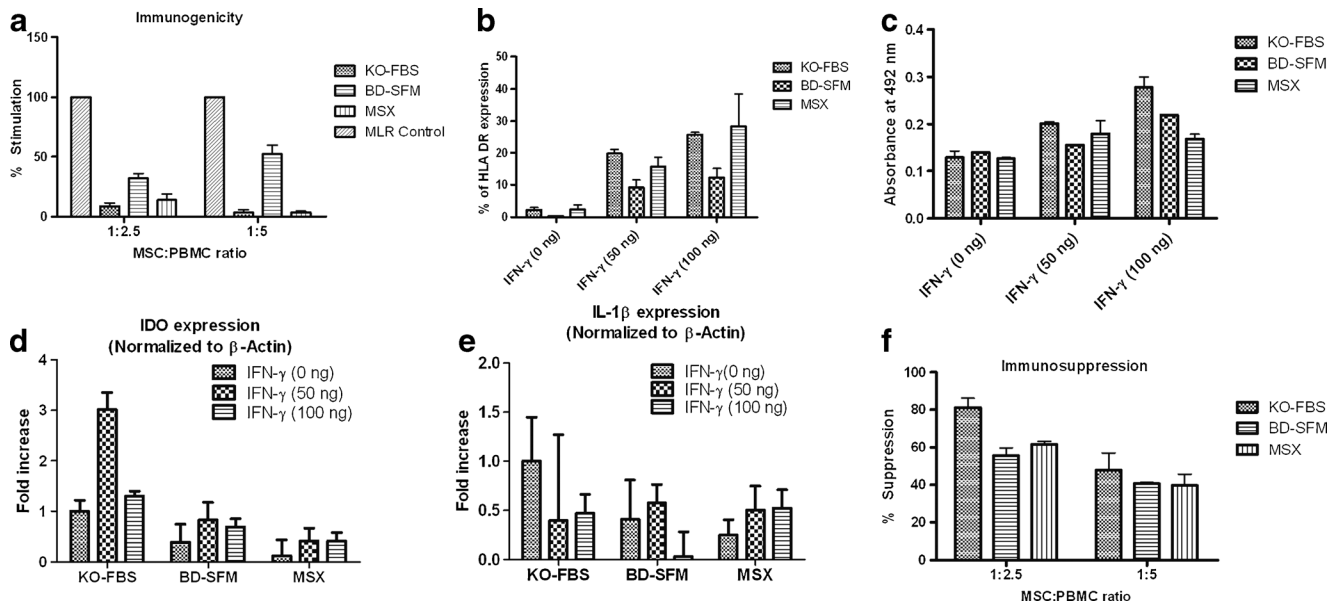
Fig. 7 Comparative in vitro immune-modulatory efficiency of BM-MSCs in KO-FBS, BD-SFM and MSX media. **a** Immunogenicity of MSCs showing dose-dependent kinetics of BD-SFM MSCs. **b** HLA-DR expression after being induced by variable concentrations of IFN-ϒ in serum (KO-FBS) and serum-free cultures (BD-SFM and MSX). **c** Kyneurine secretion was estimated in spent media of KO-FBS, BD-SFM and MSX. Variable secretory levels of Kyneurine exist among KO-FBS, BD-SFM and MSX ($n=2$). The enzyme indolamin-2, 3-dioxygenase (IDO) is induced by IFN-γ at various levels in MSCs derived from three different media. The enzyme catalyzes the degradation of L-tryptophan to N-formyl-kynurenine, which becomes L-kynurenine. The detection of IDO activity by measuring the secretion of kynurenine from hMSC in vitro could be used as a marker for the potency of implanted hMSC. No clear differences in kynurenine levels were detected among the samples, indicating that the IDO can be induced equally well in cells that were expanded in both types of media, BD-SFM and MSX. **d** Endogenous IDO expression in IFN-γ-induced BM-MSCs. **e** Endogenous expression of IL-1β in IFN-γ-induced BM-MSCs. **f** Immunosuppression of MSCs showing dose-dependent kinetics of MSCs derived from three different media. *Bars* mean ± SD of triplicates ($p<0.05$). No significance differences exist among MSCs derived from three different media

defined serum-free conditions using BD-SFM and MSX media and compared them with conventional methods of serum-containing (KO-FBS) media. The BM-MSCs showed similar isolation efficiency under serum-free conditions in BD-SFM and MSX. In addition, they exhibited typical MSCs characteristics such as fibroblastoid morphology, CFU-F efficiency, multipotent differentiation capacity and typical surface markers.

Results from isolation kinetics showed that the isolation efficacy of primary culture was higher in serum-free conditions compared to control (KO-FBS without bFGF). Similarly, Agata et al. (2009) found higher isolation efficiency of MSCs in serum-free conditions in all donors they tested when compared to controls. Further, Chase et al (2012) used SFM-XF supplemented with 2.5 % pooled human AB serum for derivation of MSCs from BM-MNCs and showed higher isolation and proliferation efficiency than controls. In all these controls, there was no added growth factor, which led to lower isolation efficiency than SFM/XFM. Isolation efficiency and proliferation rates were improved by adding compatible cytokines like bFGF to control media during isolation. Hence, we have shown isolation efficacy in controls supplemented "with and without bFGF" (Fig. 1). Jung et al. (2012) used several commercially available SFM like MSX, Therapeak lonza and SP-XF and found that they did not support isolation and expansion of MSCs. But SP-XF did not support MSC isolation even with 2 % of human serum whereas MSX and BD-SFM showed similar isolation and proliferation activity. Similarly, Miwa et al. (2012) reported that xeno-free isolation and proliferation using MSX found a higher proliferation than control.

BM-MSCs derived from serum-free conditions (BD-SFM and MSX) along with KO-FBS cultures maintain and retain their morphology, phenotype and differentiation ability. Average cell size of MSCs grown in KO-FBS was smaller than MSCs cultured in BD-SFM and MSX. However, the culturing of MSCs in disclosed in-house formulation media showed that the size of hMSCs in SFM is smaller and the proliferation rate is higher than controls (Hudson et al. 2011; Jung et al. 2011). This discrepancy has arisen because the controls did not contain bFGF and thus cannot be directly compared with our study. Although BM-MSCs cultured in serum-free conditions (BD-SFM and MSX) express the phenotypic characteristics similar to that of KO-FBS (Electronic Supplementary Material, Fig. S2), the intensity of the positive markers CD90 and CD73 was higher as compared to KO-FBS. This might be because of the purest population being selected in P0 cultures and being further expanded. Expression of CD90 is one of the positive markers for more immature cell progeny (Baum et al. 1992; Grisendi et al. 2010) and might be selected during isolation and subsequently those populations were expanded and maintained in further passages in BD-SFM. The HLA-DR expression in SFM-

cultured MSCs was low compared to that of KO-FBS cultures, a critical phenotypic marker for defining hMSCs (Dominici et al. 2006). A few studies have reported that BM-MSCs cultured in FBS-supplemented control media with bFGF have a risk of expressing HLA-DR (Bocelli-Tyndall et al. 2010; Sotiropoulou et al. 2006). Hence, BD-SFM would be the safer culture media for large-scale production leading to low HLA-DR at any passage.

IFN-γ has been shown to induce expression of MHC-I and, to a lesser extent, also MHC-II, increasing the antigen-presenting capacity and hence altering the immunogenicity of MSCs. High doses of IFN-γ-treated MSCs (100 ng/million cells) could activate T cells and initiate proliferation of allogeneic T cells. Thus, after activation, MSCs can lose their immunoprivileged status, which depends on their resistivity towards IFN-γ in the three different media, as shown by percentage HLA-DR expression. On the other hand, IFN-γ-inducible secretion of IDO in human MSCs has become one of the identified characteristics for T cell immunosuppression (Ren et al. 2009). Thus, we measured the endogenous and exogenous IDO expression levels of IFN-γ-primed and unprimed MSCs of all three different media. IFN-γ responsiveness for MSCs immunosuppressive function varied with the culture media (Fig. 7b–e). Therefore, the type of culture media also has an influence on MSCs in responding to and secreting immunosuppression agents. In addition, there was a minimum level of expression of the endogenous level of the pro-inflammatory molecule IL-1β in MSCs of BD-SFM cultures with 100 ng of IFN-γ treatment when compared to KO-FBS cultures without IFN-γ treatment. This suggests that MSCs derived from SFM are showing higher immune suppression and hypo-immunogenicity properties.

The proliferative ability of MSCs through MTT assay in all three cell culture media showed that KO-FBS has a higher proliferative ability than serum-free cultures and the difference is significant at 25 K per well in MTT assay (Electronic Supplementary Material, Fig. S4). The probable reason might be because of the complex and rich nutrient formulation of FBS media compared to SFM, which helped them to increase proliferation of MSCs. However, the clonogenic ability of MSCs cultured in KO-FBS was less than that of cells cultured in serum-free media. In contrast, many other groups showed both higher clonogenic ability and increased proliferation of MSCs in SCM (Doucet et al. 2005; Jung et al. 2011).

BM-MSCs of SFM media (BD-SFM and MSX) have shown higher post-thaw viability (95 %) than KO-FBS (92 %) (Fig. 6). Higher post-thaw viability in serum-free MSCs might be because of the usage of a mild cell detachment reagent, TrypLE Select, whereas trypsin was used in serum-supplemented cultures. Carvalho et al. (2011) reported cell viability of higher than 95 % at a time point of 3–20 min by treating the adipose-derived stem cells with TrypLE and

TrypZean, supporting the mild nature of these detachment agents. The other reasons for comparatively low post-thaw viability in KO-FBS media might be because of very smaller cell (RS-1/2) size or because of higher CPD (5.27/passage) compared to the 3.9/passage of SFM (BD-SFM and MSX). This indicates that the in vitro aging or replicative senescence of MSCs in KO-FBS cultures may be faster compared to serum-free cultures (Wagner et al. 2010).

The major challenge of the MSCs cultured in SFM compared to KO-FBS is the low cell yield per passage. Serum-supplemented KO-FBS supports higher PDs compared to BD-SFM and MSX. As the newer generations of serum-free media are being developed by various companies, the low yield issue has to be addressed appropriately to facilitate SFM to replace SCM for large-scale culturing of MSCs.

Conclusion

This study clearly shows that BM-MSCs can be isolated and expanded in SFM without losing their basic morphology, growth kinetics and functional properties. This is the first comprehensive study to provide the characterization and derivation of MSCs from BM-MNCs in two commercially available SFM/XFM (BD-SFM and MSX) along with KO-FBS medium. Isolation of MSCs from BM-MNCs is generally considered as a limiting step because the MSCs yield depends on the choice of SFM and the coating substrate along with other standard culture conditions. The initial isolation efficiency was higher in BD-SFM than KO-FBS and MSX. In contrast, at later passages, BD-SFM and MSX produced a lower cell yield than KO-FBS. These commercially available media have certain challenges, like the extra step of coating the culture flask, low yield during expansion, etc., which have to be addressed before considering them for large-scale settings. But, certainly, these BD-SFM and MSX media fulfill the primary requirements in terms of their isolation and multi-passage expansion of MSCs, while complying with regulatory concerns on transmittable disease agents. As different media produce MSCs with different properties, the most suitable protocol for MSC expansion should be chosen accordingly. Although, in recent times, the cell yield in many in-house serum-free formulations was similar to that of FBS (Jung et al. 2010, 2011, 2012), these disclosed formulations are required to ensure the bio-safety of the media ingredients. The SFM to be used in the production of MSCs for therapeutic applications requires to fulfill cGMP and regulatory compliance.

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