Cell and Tissue Transplantation and Therapy

ORIGINAL RESEARCH

Immune Modulatory Responses of Mesenchymal Stem Cells from Different Sources in Cultures and In Vivo

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Abstract: We present a comparison of immune-properties of mesenchymal stem cells (MSC) isolated from human placenta, umbilical cord matrix, adipose, and bone marrow. MSC collected from all these sources possess low levels of MHC-I and lacked MHC-II. These cells inhibited the mixed lymphocytic reaction in culture conditions when incubated with human allogeneic peripheral blood mononuclear cell (PBMNC) population. Even after differentiation into different cell lineages, these MSC are able to suppress the mixed lymphocytic reaction when cultured with stimulated allogeneic PBMNC. The cytokine profiles were determined for all types of MSC when co-cultured with PBMNC. IFNγ and IL-1β increased in supernatants of all co-cultures and whereas TNF-α, IL-1α, IL2 levels diminished. The low immunogenicity in vitro suggests that these cells can be used for allogeneic transplantation. A single infusion of MSC into immunocompetent BALB/c mouse resulted in immune suppressive changes in lymph nodes and spleen. Bone marrow and thymus remained largely unchanged. These findings suggest that MSC from AD, PL, and WJ could be substituted for BMSC. One can look for these sources particularly in cases where it is difficult to get the required number of autologous mesenchymal stem cells of bone marrow.

Keywords: immune-response, cytokines, lymphoid organs, cell transplantation

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Introduction

Mesenchymal stem cells (MSC) from bone marrow are proven to be an important contributor in haematopoietic niche and adult multipotent stem cells. With a capacity to differentiate into any of the three embryonic germ cell lineages, these cells are sought after for cell replacement therapy. The scope of cell replacement therapy has increased recently due to advances in directed differentiation of human embryonic stem cells. These cells however face risks of tumorigenesis and immune rejection. Bone marrow derived MSC (BMSC) stand a better chance as they have been attributed with immunosuppressive and immunomodulatory properties. These cells inhibit the immune response elicited by the receiver and prevent graft vs. host disease (GvHD).[1–4] MSC have been shown to be an effective suppressor of allogeneic lymphocyte proliferation in vitro.[3,5–8] The immunomodulatory properties of BMSC are attributed to the low levels of MHC-I and absence of MHC-II expression on these cells.

MSC are shown to exert influence on functions of T, B, dendritic, and NK cells through various pathways mediated by cytokines or direct cell interactions. It has been shown that MSC can suppress proliferation and cytokine production by T cells stimulated with mitogens or allogeneic cells.[6,9,10] Reports on immunomodulatory properties of MSC state that a number of cytokines or soluble factors are involved in MSC functions, both in inflammatory or non inflammatory situations.[6] All these properties make MSC a candidate for universal cell therapy.

The drawback with BMSC is the source, yield, and reduction in the number of cells with ageing. MSC have also been derived from other sources including placenta (PL), Wharton's jelly (WJ), cord blood, fat, cartilage as well as medical wastes. These cells have been extensively explored for their capacity to expand and show pluripotency.[11–13] Unlike bone marrow, cells derived from these sources display good potential to replace BMSC as these can be harvested in larger number and exhibit equally good potential for expansion and differentiation.

MSC have been envisaged to play a major role in cell based therapies, where an infusion of MSC near the injury site or administered intravenously may bring recovery and regeneration of the affected organ. The chances of using allogeneic MSC is high in cases of aged patients where the MSC may not be retrievable or cannot be expanded in large numbers. With their propensity to migrate to all organs and home in especially to the injured site, what effect these cells could have on the lymphoid organs or immunity in general is not clear. In a parallel study, we had observed that when MSC derived from placenta was implanted at the injury site by injection in a bone drill injury model, there was no immune rejection of the cells or inflammation at the implantation site.[14] The present study has been designed to evaluate the immune modulation by MSC derived from four different sources: human placental derived MSC (PMSC), human Wharton jelly derived MSC (WMSC), and human adipose tissue derived MSC (AMSC) with respect to human bone marrow derived MSC (BMSC) both in vivo and in vitro. This study deals with immune-responsiveness of MSC cultured with allogeneic hPBMNC. Cytokines released in response to allogeneic cell have been estimated to shed light on the immunomodulatory properties of different MSC. MSC injected into BALB/c mice with or without challenge with hPBMNC show immune suppressive changes in lymphoid organs.

Materials and Methods

Isolation and culturing of MSC

Placenta and Wharton's jelly were collected from the hospitals following Caesarean section, while lipoaspirates were collected from patients undergoing liposuction. All samples were transported to the laboratory under sterile conditions. Bone marrow was collected by scraping the femur or sternum of the medically terminated fetus samples. The necessary consent was taken wherever required before collection of samples.

The placenta, lipoaspirate, and cord (without blood and blood vessels) were washed with Hank's Balanced Salt Solution (HBSS). In the case of WMSC, umbilical cords were washed in HBSS to remove blood components, and vessels were removed to avoid endothelial cell contamination before digestion. Solid tissues were diced into smaller pieces and digested with cocktail of Dispase (0.1 mg/mL), Trypsin (0.1%) and DNase (0.025%) for 30 minutes at 37 °C. After digestion the tissue was filtered through a 100 μm mesh, and cells were enriched by Ficoll gradient (Histopaque from Sigma Aldrich). The lipoaspirate was incubated

with collagenase for 60 minutes at 37 °C and centrifuged at 500 g for 5 minutes. The supernatants were discarded and cell pellets were plated after washing once with HBSS. Because of difficulty to obtain human adult BM cells, we relied on bone marrow harvested from fetal femur or sternum. Cells were counted in Neubauer's haemocytometer chamber, and 1×10^6 cells or spicular clumps were plated in IMDM (Iscove's modified Dulbecco's Medium) with 15% FCS containing 0.1 mM β-mercaptoethanol and 0.1 mM hydrocortisone. Cells were passaged upon 80% confluence and a doubling time of roughly 2.5 was maintained at each passage.

Immunophenotype studies of cultured MSC

For immunophenotype analysis, culture expanded MSC (1×10^6 cells) from four different sources were harvested after trypsinization and washed with PBS containing 0.5% Bovine Serum Albumin (BSA) (Sigma). Cells were incubated with primary antibodies (5 µg/mL) of CD29 (ab19525, Chemicon), CD44 (ab4065, Chemicon), CD73 (LS14111, lifespan), CD90 (LS 73837, Lifespan), CD105 (LS-c40929, Lifespan), MHC I (B421, Abcam) and MHC II (CVS20, Abcam) overnight at 4 °C. Cells were then washed with PBS containing 0.5% BSA and incubated with fluorophore-conjugated secondary antibody (1 µg/mL) (Sigma) for 40 minutes at room temperature. Cells without any antibody staining were used as negative control. Cell analysis was performed with FACSCalibur system from BD Biosciences using Cell Quest software.

Differentiation studies

Cultured MSC derived from adipose were allowed to differentiate into osteoblasts by using growth medium supplemented with a cocktail of dexamethasone (0.1 µM), β-glycerol phosphate (10 mM) and ascorbic acid (50 µM). Cells were treated for three weeks with weekly assessment of the osteogenic phenotype.[15] Cells were stained with alizarin Red S and osteocalcin antibody for phenotypic analysis. Endothelial phenotype was induced with growth medium supplemented with VEGF (50 ng/mL) containing 2% FCS, after 7 days, the cells were immunostained for VEGF and CD31 to assess differentiation.[12] MSC were induced to differentiate into neuron like cells using standard protocol as used by Woodbury et al.[16] Cells were initially induced with 10 ng/ml beta FGF overnight followed by induction with 2% DMSO and 200 µM butylated hydroxyanisole in the growth medium containing 2% FCS. After 5 hours, the medium was modified for long term induction by adding 25 mM KCl, 2 mM valproic acid, 10 µM forskolin, 1 µM hydrocortisone and 5 µg insulin. Cells were stained for tyrosine hydroxylase and β tubulin type III after 15–20 days.

Mixed lymphocyte reaction (MLR)

MSC from bone marrow, placenta, wharton's jelly, and adipose tissues were treated with 5 µg/mL mitomycin C for 2 hours and used as stimulators in one way mixed lymphocyte reaction (MLR). Cells were trypsinized and plated in 6 well plates at a density of about 50,000 cells per well. Peripheral blood was collected from a healthy individual and hPBMNC were enriched by Ficoll gradient. For each aliquot of 1×10^6 cells/mL, 2 × CFSE [Carboxyfluorescein succinimidyl Ester-10 µM] was added. Cells were gently mixed and incubated for 7 minutes at room temperature. The reaction was stopped with the addition of FCS and cells were washed with 5% FCS and centrifuged at 400 g for 10 minutes. The cells were washed with two changes of PBS recounted and added as responders to MSC cultures.

After 24 hours, each MSC containing well received CFSE labelled lymphocytes (1×10^6 cells) in RPMI media containing 10% FCS, and were incubated for 3 days in a CO_2 incubator at 37 °C. Cells were harvested and analysed by MoFlo flow-cytometer for the different fluorescent intensities of CFSE stain.

ELISA for quantification of human cytokines

Cytokines such as interferon-γ (IFNγ), tumour necrosis factor-α (TNF α), interleukin-1α, IL-1β and IL-2 from the culture supernatants were quantified using enzyme linked immunosorbent assay (bender med systems GMBH, http://www.bendermedsystems.com). Quantification was done according to manufacturer's protocol.

In vivo studies

BALB/c J mice from animal house were housed in accordance with the guidelines for care and use of

animals in scientific research (Indian National Science Academy, New Delhi, India) in a CPCSEA (Committee for the purpose of control and supervision of experiment on animals) registered animal facility. The animals were maintained in at standard environmental condition (Temperature 22 °C–25 °C, Humidity 40%–70%) with 12:12 dark/light photoperiod. The protocols were approved by Institutional Animal Ethical Committee (IAEC). Animals were randomly allocated to six groups. The Group 1–4 animals were injected with PMSC, WMSC, AMSC and BMSC alone or along with hPBMNC respectively. 1×10^6 cells each were injected subcutaneously near the region of pre scapular and pre-crural lymph nodes. Positive control (Group 5) received hPBMNC and negative controls (Group 6) received no injection of cells. In another set of experiments, the animals were injected with cells through intravenous route. After 25–30 days of transplantation, animals were sacrificed as per standard protocols for euthanasia. Spleen, Thymus and pre scapular, precrural and mesenteric lymph nodes were collected and fixed in 10% formalin. Fixed and paraffin embedded tissues were cut at 5 µm thickness, stained with haematoxylin and eosin following standard procedure and examined under light microscope.

Results

Immunoprofiling of MSCs

MSC cultures obtained from placenta, adipose, bone marrow, and wharton's jelly were adherent and fibroblastic in shape. The cultures of expanded cells up to passage 3 were analysed using flow cytometry for the presentation of MSC markers. These cells were positive for MSC specific surface markers such as CD29, CD44, CD73, CD90, CD105 and negative for CD34, CD45 (Fig. 1). Besides MSC markers, cells stained for MHC antigens showed absence of MHC-II on MSC derived from all the sources and were positive for MHC I (Figs. 1 and S1). All types of MSC exhibited their pluripotent nature by undergoing differentiation into osteoblasts, neuron like cells, and vascular endothelial cells Fig S4.

Lymphocyte proliferative assay by flow cytometry

MSC were analysed for their ability to induce lymphocytic proliferation in culture conditions.

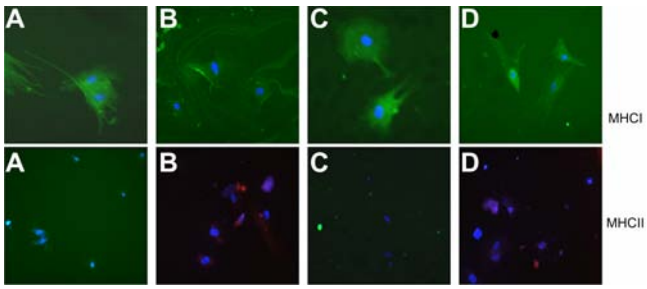

Figure 1. Immunocytochemistry of MSC derived from adipose (**A**) bone marrow (**B**), Placenta (**C**) and Wharton's jelly (**D**) stained with antibodies for MHCI and MHCII.
Note: Cells show presence of MHCI marker but absence of MHCII.

PBMNC (1×10^6) labelled with CFSE, were co-cultured with mitotically inactive MSC (5×10^4) of different origins for 3–7 days and harvested for analysis in MoFlo using Laser 488 nm. Only PI negative cells were used for analysis. CFSE labelled PBMNC with or without induction with 0.8% PHA, were used as positive and negative controls respectively. As positive control divides rapidly upon induction with PHA, cells shows 3–4 peaks of different fluorescent intensities. PHA stimulated allogeneic lymphocytes cultured with MSC show up as a single peak (Fig. 2). The percentage of cells divided in co-cultures with PMSC, AMSC, BMSC, and WMSC were significantly lower with respect to mitogen induced MNC ($P < 0.05$), as shown in the Figure 3, demonstrating that MSC suppressed lymphocytic proliferation in the cultures. PBMNC cultured with MSC differentiated in vitro into osteoblasts (78% showing osteogenic markers) did show a small peak suggesting some lymphocytic proliferation. MSC differentiated to neuron like cells (65% showing neurogenic markers) and endothelial cells (73% endothelial markers) co-cultured with CFSE labelled PBMNC do not support the lymphocyte proliferation ($P < 0.05$) (Figs. 2 and 3).

Analysis of cytokines released by the MSC

Cytokines released during MLR were quantified using culture supernatants. PHA stimulated PBMNC cells incubated with or without MSC were used for investigation. MSC and lymphocytes by themselves (negative controls) produced negligible amounts of the cytokines IFN-γ, IL 1α and β, IL2 and TNF α. Stimulated lymphocytes (positive controls) showed significant changes in the levels of these cytokines. MLR supernatants

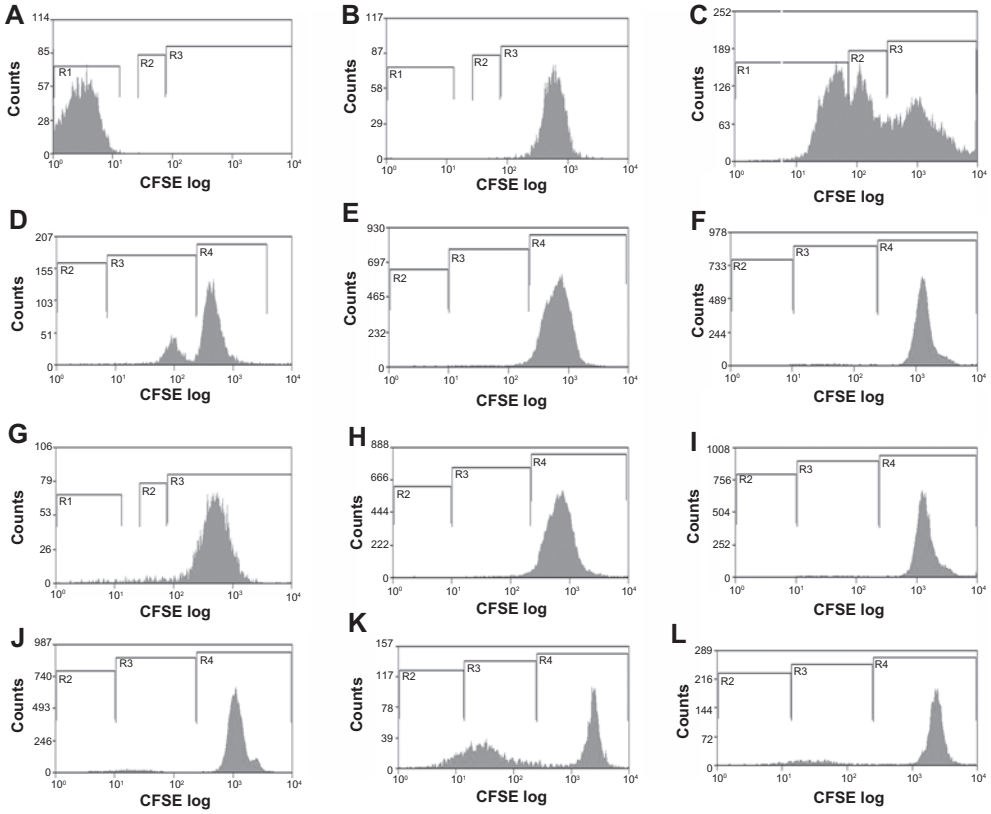

Figure 2. Mixed lymphocyte reaction using human peripheral blood monocytes loaded with CFSE co-cultured with mitotically inactive MSC. Unloaded controls (**A**), Loaded negative Control (**B**) and positive control ie, cells treated with PHA (**C**) and LPS (**D**) profiles are shown. Human PBMNC were grown with MSC derived from Adipose (**E**), Bone marrow (**F**) Placenta (**G**) Wharton's Jelly (**H**) and mouse MSC (**J**). Cells were also exposed to MSC populations that have been differentiated in cultures to Endothelial (**J**) Osteo (**K**) and Neurogenic lineages (**L**).
Note: None of these interactions induced the MLR response.

contained higher levels of all the cytokines tested, IFN-γ and IL1 (α and β) levels increased by 2.5–3 fold. The levels of TNF-α and IL 2 increased 5 and 8 times respectively in stimulated PBMNC (Fig. 4).

When the MLR was performed in the presence of different MSC, production of cytokines altered in all cases (Figs. 4 and 5). IFN-γ levels increased significantly (*P* value 0.0001) with all the MSC. AMSC showed highest levels; about 8 fold increase (48.34 ± 18.86 pg/mL) compared to positive control (6.1 ± 3.6 pg/mL). IL-1β levels also showed a slight increase in production, in MLR supernatants for all the MSC (*P* value 0.02). BMSC showed maximum production of IL-1β, 261.67 ± 127.1 pg/mL compared to 152.67 ± 24.21 pg/mL in positive control. TNF-α, IL-1α and IL-2 levels declined in the MLR supernatant with all the MSC. IL-1α declined to almost background levels as seen in negative controls (*P* value 0.5). IL-2 and TNF-α levels decreased but remained higher compared to background levels (*P* value 0.01 and 0.05 respectively).

MLR was also performed in presence of MSC induced to differentiate into neurogenic, osteogenic or endothelial cell types, the levels of most of the cytokines dropped to background levels. Only TNF-α, IL-1α and 1β showed higher levels in the MLR supernatant performed in presence of osteogenic cells.

In vivo experiments

Gross pathology

Use of MSC for cell therapeutics usually involves injection of MSC at the site of injury or delivery of cells through IV route. We wanted to see the kind of response generated by the MSC in the neighbouring/proximal lymph nodes, spleen, or thymus. We also challenged the animals with PBMNCs to induce an immune response and then inject MSC in order to see the immunosuppression. No mortality was observed in any group of animals in the entire experimental period. There were no tumour formations in any group or any unusual growth in the viscera of any animal. Positive control (Group 5) animals that were

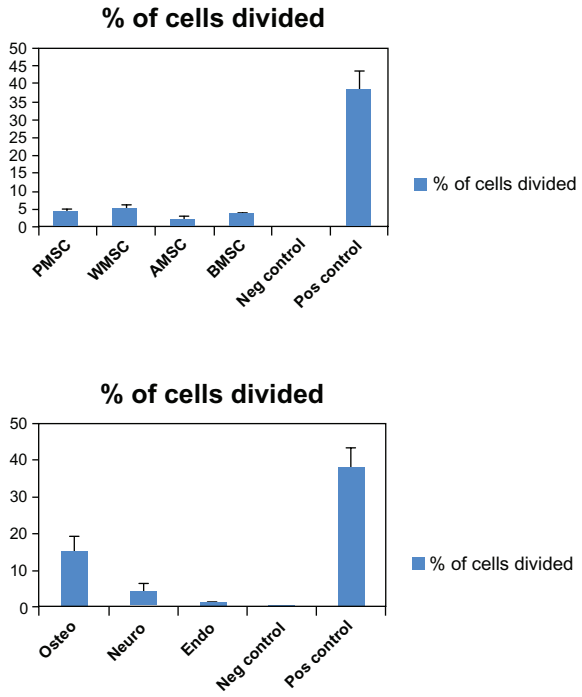

Figure 3. Lymphocytic proliferation during the MLR reaction for MSC derived from different sources.
Notes: About 30% cells divided upon PHA induction, whereas in coculture with MSC the lymphocytic proliferation was significantly suppressed. The lower panel shows the cells that had been induced to differentiate also are able to suppress the lymphocytic proliferation.

injected with PBMNC showed bulging of cortical surface as palpation of all the lymph nodes (precrural, pre-scapular and mesenteric). No changes were observed in spleen of positive control group. All the MSC injected animals showed mild enlargement of cervical and precrural lymph nodes. Angiogenesis and blood filled cyst were observed in the cortical surface of precrural and cervical lymph nodes. Mild enlargement of spleen was observed in Group 1(PMSC) and Group 4 (BMSC) animals.

Histopathology

Animals challenged with hPBMNC showed proliferation of more number of lymphoblast cells inside the lymphatic follicles of spleen and lymph nodes. No changes were observed in the lymphatic follicles of spleen and lymph nodes of negative control (Group 6) animals (Fig. 6A and B).

Lymphoid depletion and follicular atrophy was observed in all the experimental groups. Animals that received AMSC showed fatty cysts filled with fat cells and dead necrotic debris in lymph node also in the medullary area (Fig. 6).

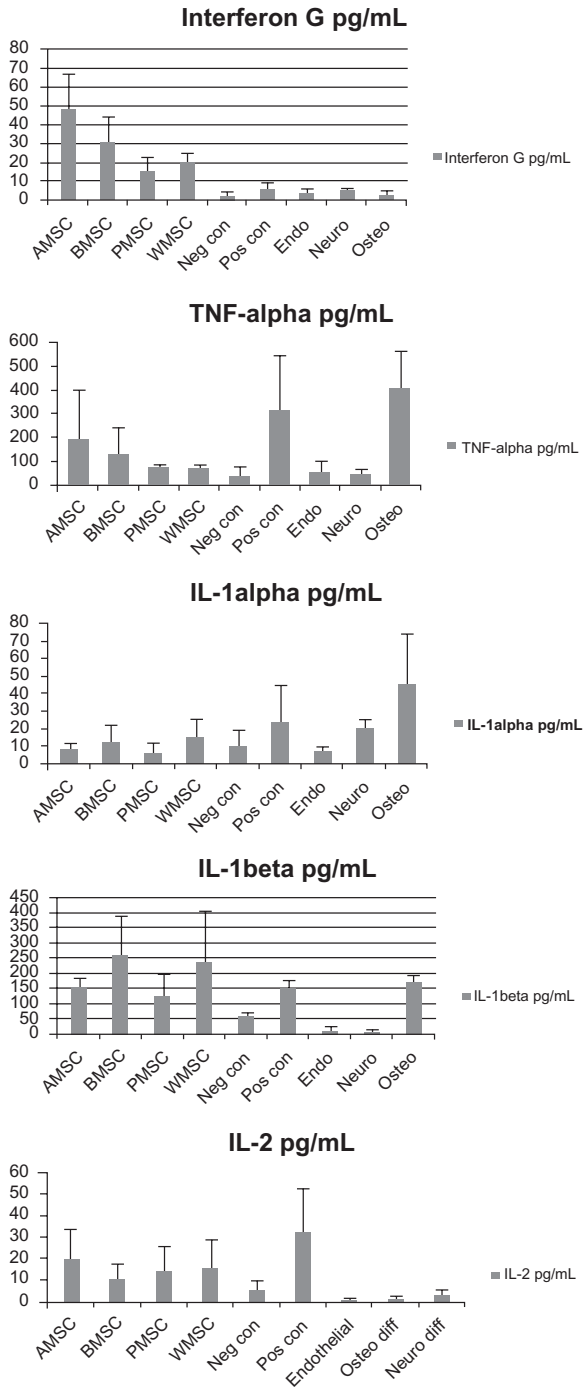

Figure 4. Cytokine production in lymphocyte/MSC co-cultures.
Note: There is a marked change in cytokine production as compared to control in all cases of MSC-Lymphocyte interaction in MLR supernatants.

Moderate angiogenesis were observed in the lymphoid follicles of both spleen and lymph node. The animals that were challenged before MSC injection also showed lymphoid depletion in follicles and fatty cells. Thymus remained largely unchanged with well developed cortex and medullary regions,

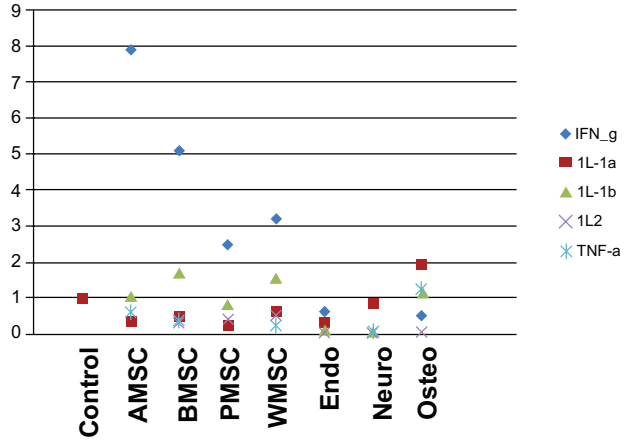

Figure 5. Cytokine production in the culture supernatants of PBMNC with differentiated MSC show altered profile of cytokines production as compared to positive control.
Notes: Scatter plot for cytokine production in MLR supernantants compared to control. All the values are normalized by taking positive control for each cytokine as 1.

though there was little angiectasis and angiogenesis (Fig. 6C and D, Fig. S3).

Bone marrow MSC injected mice showed degeneration of primary follicles. Interfollicular region had macrophages, adipogenic cells, and vacuolar areas in animals injected with BMSC. Vacuolar and fatty degeneration with individual lymphocyte apoptosis and mild necrotic foci was observed in the spleenic and lymphatic follicles (Fig. 6E). Most of the follicles showed decline in cellularity in mice challenged before MSC infusion (Fig. 6F). Thymus only showed increased angiogenesis with well developed cortical and medullary areas (Fig. S3).

The animals treated with PMSC and WMSC showed severe angiogenesis in spleenic and lymphoid follicles. Interfollicular region showed vacuolization due to T cell depletion. Angiogenesis and or blood filled vascular spaces throughout the lymph node along with atrophy of follicles was also noticed. These effects were more severe when MSC were injected after PBMNC challenge. The paracortical area showed severe cell depletion. Blood filled cyst angiectasis with RBC and clotted blood was observed in the red pulp and follicular region of the spleen (Fig. 6G–J, Fig. S3A).

Thymus of most animals injected with MSC showed no changes; ratio of cortical areas to medullary area remained largely unchanged in all the groups. No macrophages or apoptotic activity seen in thymus (Fig. S3A and B).

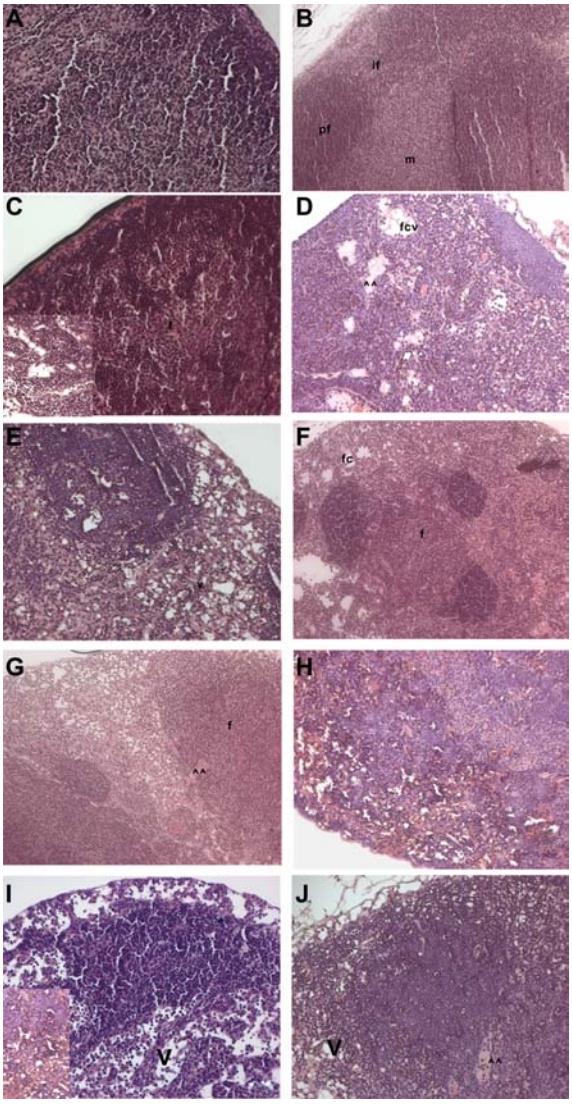

Figure 6. Histopathology studies. Mice were treated with human MSC obtained from different sources. Another set of mice received MSC after challenge with hPBMNC. Positive control mice (**B**) received hPBMNC alone and showed classic lymphoid follicle (pf) formation interspersed with interfollicular area (if) full of cells in the cortical region. Negative control mice (**A**) did not show much change. In the mice injected with MSC lymph node showed general follicular atrophy, angiogenesis (^^) and adipogenesis (fcv). Animals that received only MSC from adipose (**C**), Bone marrow (**E**), Placenta (**G**) and Wharton's Jelly (**I**) showed follicular degeneration to various degrees. Interfollicular regions (if) showed severe cell depletion (**V**) Angiogenesis (^^) was predominantly seen in the lymph nodes of the mice that were challenged with mononuclear cells followed by MSC from adipose (**D**), Bone marrow (**F**) placenta (**H**) and Umbilical cord matrix (**J**).

Just to ensure there is cell migration to these organs, we injected some animals with CFSE labelled cells. Lymph nodes were examined after cryosectioning to look for CFSE positive cells and also counted for CFSE positive cells (Fig. 7). Animals injected with MSC derived from adipose and bone marrow showed maximum infiltration of cells in lymph nodes.

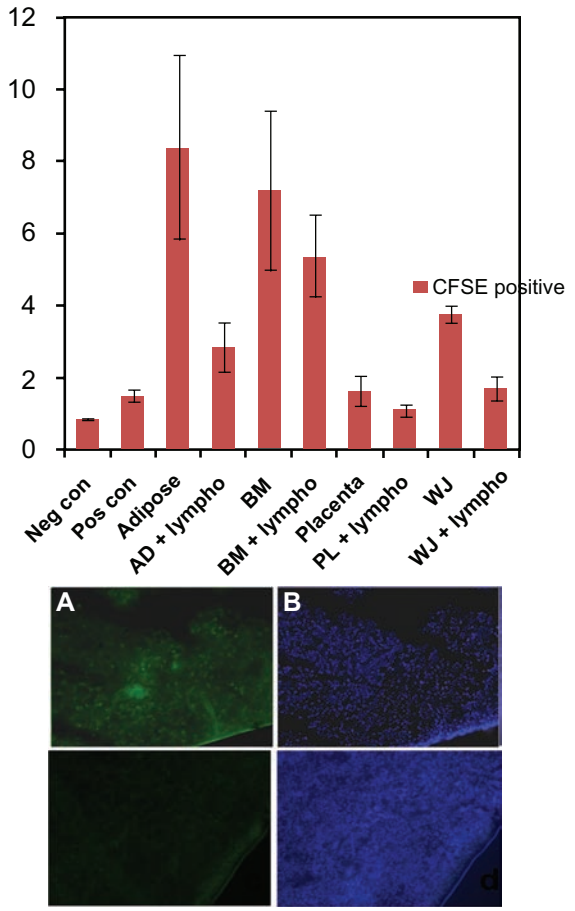

Figure 7. Animals were injected with MSC labelled with CFSE.
Notes: Lymph nodes of these animals were harvested and used for counting the number of positive cells using FACS. The bars represent the percent of labelled cells recovered. The lower panel shows positive staining seen in the lymph node sections.

Discussion

Reports on BM derived MSC showed that these cells are self renewable and capable of differentiation into several lineages under appropriate conditions. Due to their regenerative capacity, these cells have been used in clinical applications and tissue engineering for repairing or regenerating the damaged tissue.[12,14] MSC also have low inherent immunogenicity and can modulate or suppress the immune responses by interacting with the immune cells. This helps in reducing graft rejections or Graft versus Host Disease (GvHD) raised after the transplantations.[1–3,17–21] Several reports have shown the immunosuppressive properties exhibited by MSC in vitro and in vivo.[18,21] These properties make MSC an attractive candidate for cell based therapies. Generally the cells can be derived from the marrow of patients. An allogeneic donor is desirable in cases where the recovery of cells is not good, such as in cases of aging or when the MSC cannot be expanded to the desired number. As MSC have been reported from various extraembryonic sources also, these can be used for harvesting cells in large numbers. In this study we have shown that PMSC, WMSC, AMSC, and BMSC can suppress the lymphocytic proliferation and alter the production of immunostimulatory cytokines in culture conditions. Single injection of MSC in the mice also reveals some immune suppressive changes.

In the present study, MSC isolated from placenta, cord stroma, fat tissue, and bone marrow showed similar phenotype and differential potential (15/15). All these MSC stained positive for Mesenchymal lineage antigens CD29, CD44, CD73, CD90, CD105, and histocompatibility antigen MHC I as per International Society for Cellular Therapy norms. However, all the MSC stained negative for MHC class II antigen, which plays an important role in antigen presenting and allogeneic response. MSC were induced to differentiate into osteogenic, neurogenic, and endothelial lineages under appropriate conditions, which showed similar differentiation potentials.

In a mixed lymphocyte reaction, all the MSC suppress the hPBMNC proliferation significantly ($P < 0.01$) with respect to the controls. When co-cultured, PMSC, WMSC, AMSC and BMSC showed 88%, 85%, 94% and 90% inhibition respectively in lymphocyte proliferation assay. With regards to the percentage of cells divided, AMSC showed least induction in lymphocyte proliferation in comparison to other types of MSC. MSC that were induced to differentiate into osteoblasts, neuron like cells, and vascular endothelial cells also did not elicit significant hPBMNC proliferation ($P < 0.01$). When cultured with the differentiated cells, few PHA stimulated allogeneic lymphocytes showed division. Suppression induced was osteogenic (61%), neurogenic (85%), and endothelial (97%). MSC cultured with allogeneic hPBMNC for longer period (more than 10 days) also did not elicit MLR. Thus, present data supports the previous reports that MSC from various tissues suppress the MLR similar to BMSC[5,18,21–28] and may retain the immunomodulatory properties even after differentiation.

Immunomodulatory properties of MSC are shown to be not innate but induced by proinflammatory cytokines like IFN γ along with TNFα, IL-1α, or IL-1β.[29]

Quantitation of these chemokines was done after co-culturing of MSCs with allogeneic hPBMNC. MSCs by themselves do not show secretion of these chemokines but do influence their production when cultured with allogeneic lymphocytes. IFN γ production by lymphocytes was increased several fold when cultured in presence of PMSC, WMSC, BMSC and AMSC (as $P < 0.05$). Level of IFN γ dropped to basal level when MLR was done in presence of MSC induced to osteoblasts, neuron like cells, and vascular endothelial cells. IFN-γ is very important in increasing cell surface HLA I and II class antigen expression, and reinforces the T-lymphocyte activities.[9,29,30] IFN-γ has been shown to be high in murine MSC-MLR and has been attributed to up-regulation of B7-H1 molecule implying role of cell contact mechanism.[28] TNF α production was suppressed in the MLR cultured with undifferentiated and differentiated MSC. Culture supernatants analyzed for cytokines showed that the production of IL-2 was completely suppressed in MLR in presence of MSC. (I do not know how to correct this sentence). IL-2 is necessary for the growth, differentiation, and survival of antigen selected T-cells and supports long term T-cell proliferation. As addition of IL2 has been shown to abrogate the MSC suppression,[10] negligible levels of IL2 in the MLR supernatants in these experiments supports the fact that MSCs create a suppressive environment for lymphocyte proliferation.

IL-1α level was significantly reduced in cultures grown in presence of MSCs, except for differentiated osteogenic cells that induced IL-1α levels. MSC and osteoblasts did not show any significant induction or suppression in IL-1β expression. Conversely, MLR with vascular endothelial and neuron like cells showed greater reduction. Consistent results were seen throughout the experiments clearly indicating that the levels of expression of IL-1(α and β), TNF α, IFN γ and IL-2 were altered in presence of MSC showing its immunomodulatory properties. These results support that MSC derived from all these sources display antiproliferative effects on lymphocytes possibly by the cytokine mediated pathways.

In case of therapies, it is expected that a patient will receive an injection of cells either at the site of injury or intravenously. It is unknown what will happen to the lymphoid organs or how they will respond to these immunosuppressive cells. What happens when the subjects are already challenged with antigens? The real test of immune-modulation by MSCs is required to be demonstrated in vivo. Therefore, further experiments were conducted in vivo to determine the changes evoked by MSCs using BABL/c mouse. These studies are different from GvHD study in that the host immune cells are not killed by irradiation nor suppress innate immunity by any other means. The consequences of injecting MSC into animals for cellular therapy were observed. All the groups of animals injected with MSC were healthy throughout the experiment with no change in weight and locomotion. This demonstrated that no toxicity was elicited by MSC infusion and that these cells survived in a xenogenic environment. Infusion of MSC by intraperitoneal, subcutaneous, intravenous routes have demonstrated the efficacy of MSC in honing to all organs and differentiating into different cell types as well as restoring organ functions.[11,14,18,25–29]

In this study observations were made at lymphoid organs (primarily the lymph nodes, thymus, and spleen) as MSC in lymphoid organs may directly affect various immune cells and modulate immune response. Histology of lymphoid organs has been proposed to be an effective approach to evaluate immunosuppression or tolerance. Though immune competent cells are found all over the body, spleen, thymus, and lymph nodes are a good indicator of immune modulation. A slight enlargement was seen in all animals injected with MSC at the cervical and precrural lymph nodes and angiogenesis with blood filled cyst on the cortical surface. By studying the histopathology of lymph node and spleen, it was clearer that animals injected with PMSC, WMSC, and BMSC showed severe angiogenesis. Additionally, angiectasis in the red pulp and follicular region of the spleen followed by lymphoid depletion and atrophy in the lymphatic and spleenic follicles was observed. The animals injected with AMSC showed fatty cysts filled with fat cells and dead necrotic debris in spleen with mild angiogenesis in the lymphoid follicles of lymph and spleen. Conversely, animals injected with induced hPBMNC showed proliferation of a higher number of lymphoblast cells inside the lymphatic follicles of spleen and lymph nodes. This may be due to activation of inflammatory responses.

In conclusion MSC are not only able to suppress the lymphocytic proliferation but also deplete the lymphoid

cells leading to atrophy. This could be a reason for the angiogenesis and blood filled cysts in the depleted regions and the formation of fat cells with animals injected with ADMSC. MSC have been shown to accumulate in lymph nodes between the boundary of paracortical areas and germinal centre and influence various immune cells.[30] Christensen et al[31] reported the infusion of MSC affect the cell contents of lymphoid organs and have transient, time, and dosage dependent effect on GvHD in a recent study.

Additionally, our data reveals that MSC from alternate sources are effective in suppression of immune response. AMSC, BMSC, PMSC, and WMSC can suppress mitogen induced T-cell proliferation in that order and suppress the effect of soluble factors produced by activated T-cells in cultures. In vivo studies reveal the suppressive effect of MSC on the lymphoid organs. The immune suppression is effective even when the animals are primed for immune response. These findings suggest that MSC derived from AD, PL and WJ could be good substitute for BMSC as these are reasonably effective in suppressing an allogeneic immune response.

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Author Contributions

TK, AM were responsible for conducting experiments, data collection and analysis. SG has helped with FACS experiments; MJK and TRA have contributed with animal studies and histology. SS was responsible for the study design and preparation of manuscript. All authors have read and approved the manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

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Supplementary Figures

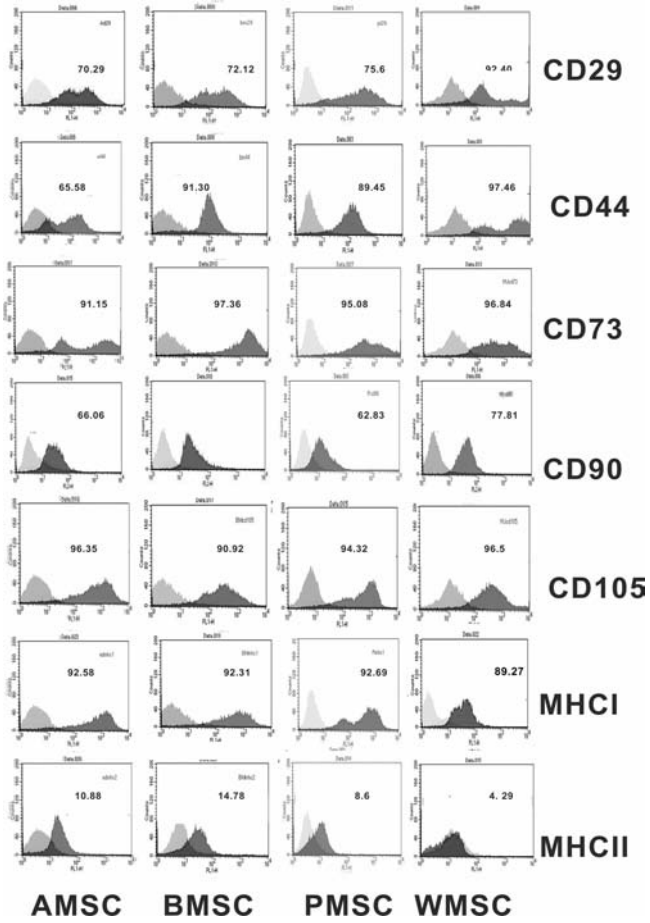

Figure S1. Immunoprofiling of MSC using antibodies against specific stem cell markers.
Notes: Representative data from single coloured FACS analysis of cells isolated and cultured from adipose, bone marrow, placenta and Wharton's Jelly and stained for biomarkers. Cell suspensions were stained for CD29, CD44, CD73, CD90, CD105, MHCI and MHCII analyzed for stained cells using FITC-conjugated second antibody. The numbers indicate percent of positive cells in three experiments. Each histogram represents an overlap of two samples, one unstained (light) and a stained with antibody (dark).

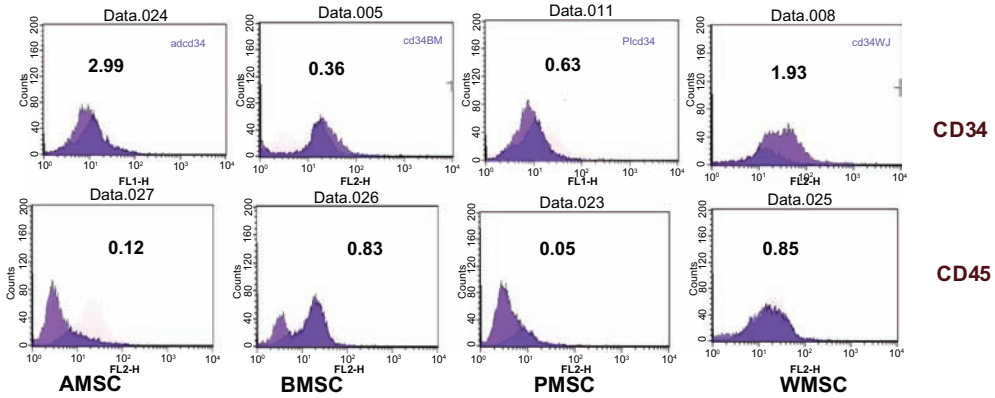

Figure S2. Immunoprofiling of MSC using antibodies against CD34 and CD45.
Notes: Representative data from single coloured FACS analysis of cells isolated and cultured from adipose, bone marrow, placenta and Wharton's Jelly. Cell suspensions were stained for CD34 and CD45 antibody conjugated to FITC. The numbers indicate percent of positive cells in three experiments. Each histogram represents an overlap of two samples, one unstained (light) and a stained with antibody (dark).

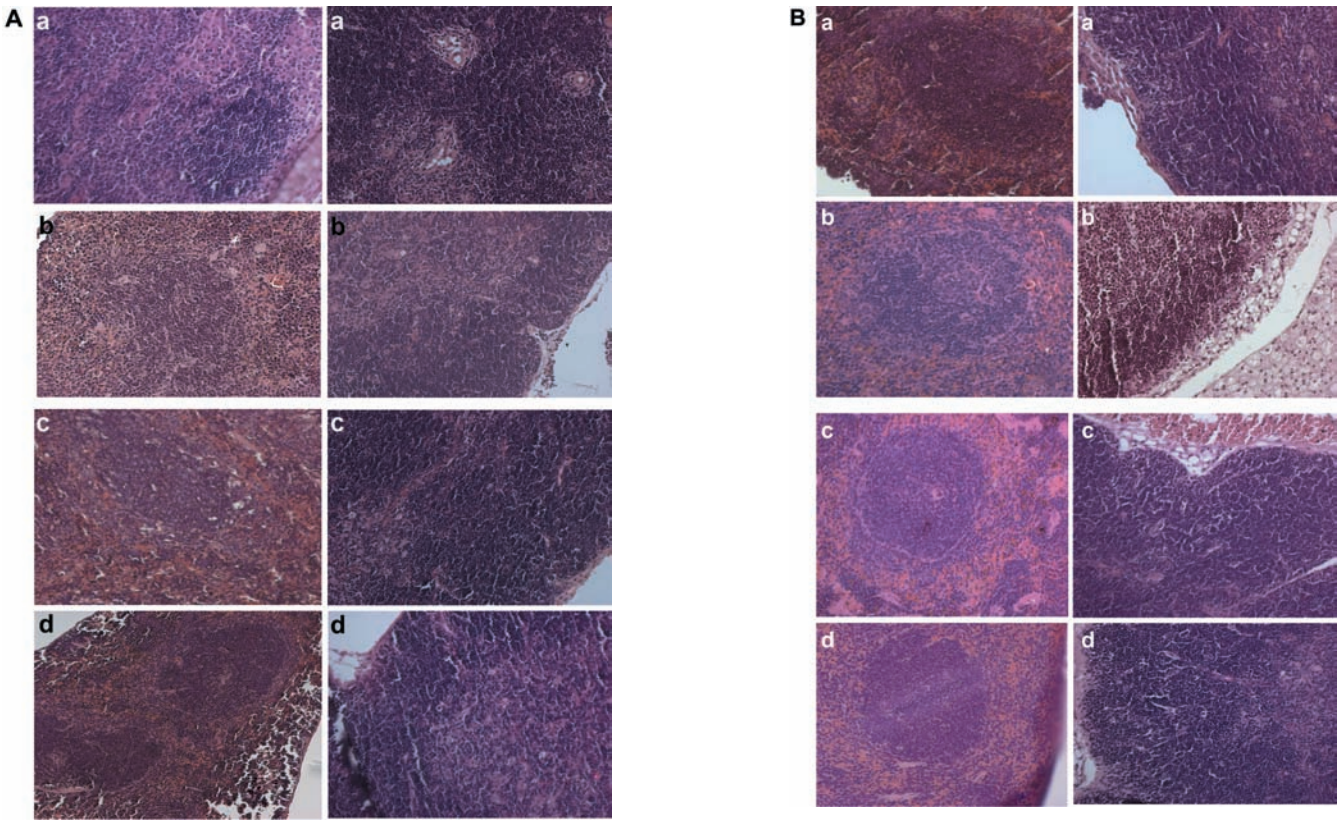

Figure S3. (**A**) Histopathology of spleen(s) and Thymus (t) from mice infused with AMSC (a), AMSC after challenge with PBMNC (b), BMSC (c) and MSC after challenge (d). (**B**) spleen and thymus PMSC (a), PMSC after challenge (b), WMSC (c) and after challenge (d). Spleen sections show angiogenesis and angiectasis in follicles and in red pulp area. Thymus mostly remains unchanged in cellularity.

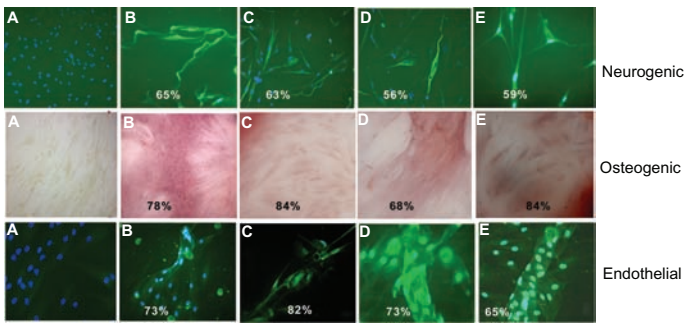

Figure S4. Differentiation of MSC derived from different sources. Induced MSC display the markers associated with the differentiated cell types and alteration in morphology. Induced Neurogenic cells were stained with tyrosine hydroxylase antibodies raised in rabbit and alexa fluor 488-tagged anti-rabbit secondary antibody. Undifferentiated AMSC (**A**); MSC differentiated into neurogenic phenotype-AMSC (**B**); BMSC (**C**); PMSC (**D**) and WMSC (**E**). Osteogenic cells were stained with Alizarin Red S. Uninduced cells MSC (**A**) remain negative. Osteogenic cells derived from AMSC (**B**); BMSC (**C**); PMSC (**D**) and WMSC (**E**). Endothelial cells were stained with VEGF antibodies raised in mouse and alexa fluor 488 tagged to anti-mouse antibody. Undifferentiated MSC (**A**); AMSC (**B**); BMSC (**C**); PMSC (**D**) and WMSC (**E**) differentiated to endothelial cell.
Note: Numbers indicate the percentage of cells positive in Facs Analysis.