Menstrual Blood-Derived Stromal Stem Cells Augment CD4+ T Cells Proliferation

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Abstract

Background: It is more than sixty years that the concept of the fetal allograft and immunological paradox of pregnancy was proposed and in this context, several regulatory networks and mechanisms have been introduced so far. It is now generally recognized that mesenchymal stem cells exert potent immunoregulatory activity. In this study, for the first time, the potential impact of Menstrual blood Stem Cells (MenSCs), as surrogate for endometrial stem cells, on proliferative capacity of CD4+ T cells was tested.

Methods: MenSCs and Bone marrow Mesenchymal Stem Cells (BMSCs) were isolated and assessed for their immunophenotypic features and multi-lineage differentiation capability. BMSCs and MenSCs with or without IFNγ pre-stimulation were co-cultured with purified anti-CD3/CD28-activated CD4+ T cells and the extent of T cell proliferation at different MenSCs: T cell ratios were investigated by CSFE flow cytometry. IDO activity of both cell types was measured after stimulation with IFNγ by a colorimetric assay.

Results: MenSCs exhibited dual mesenchymal and embryonic markers and multi-lineage differentiation capability. BMSCs and MenSCs with or without IFNγ pre-stimulation were co-cultured with purified anti-CD3/CD28-activated CD4+ T cells and the extent of T cell proliferation at different MenSCs: T cell ratios were investigated by CSFE flow cytometry. IDO activity of both cell types was measured after stimulation with IFNγ by a colorimetric assay.

Conclusion: Although augmentation of T cell proliferation by MenSCs can be a basis for maintenance of endometrial homeostasis to cope with ascending infections, this may not fulfill the requirement for immunological tolerance to a semi-allograft fetus. However, more investigation is needed to examine whether or not the immunomodulatory properties of these cells are affected by endometrial microenvironment during pregnancy.

Keywords: Endometrium, Immunological tolerance, Menstrual blood stem cells, Pregnancy, Proliferation, T lymphocytes

Introduction

One of the most controversial issues in reproductive biology is dealing with the fact that a fully functional immune system in women should simultaneously fight off the invading pathogens and tolerate semi-allograft fetus throughout the pregnancy. Indeed, a successful pregnancy is supposed to remain unresponsive to paternal antigens originating from semi-allograft fetus.

Thus far, extensive attempts and studies have been performed to unravel immunosuppressive mechanisms involved in immunological tolerance of gestation. En-
MenSCs Induced CD4+ T Cell Proliferation

MenSCs and BMSCs collections

MenSCs were obtained from 10 apparently healthy women (25-35 years). The women were monitored to exclude those with a history of vaginal infection or consumption of oral contraceptives, corticosteroids and Nonsteroidal Anti-inflammatory Drug (NSAIDs) during the last 3 months, endometriosis, autoimmune diseases and infection with such blood transmittable viruses such as HCV, HBV and HIV. A written consent was obtained from all donors before enrolment to the study. BMSCs were from four healthy donors admitted for bone marrow transplantation and provided by Reproductive Biotechnology Research Center, Avicenna Research Institute, Tehran, Iran. MenSCs were collected on the 2nd day of menstruation phase using menstrual cup. Samples were transferred to the lab in a transfer medium comprising DMEM/F12, 100 μg/ml penicillin, 100 IU/ml streptomycin and 0.25 μg/ml fungizone (Invitrogen, Carlsbad, CA). Clots and tissue derbies were separated using cell strainer with 70 μm pore size. Then, menstrual blood was cultured in DMEM/F12 media supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA) and with the same concentration of antibiotics as mentioned above. Every two or three days, media were replenished, suspended cells were removed and the adherent cells were passaged up to 5 times. These cells were considered as MenSCs and frozen for the following experiments.

Immunophenotyping

MenSCs and BMSCs were harvested after two passages and evaluated for their immunophenotype characteristics using antibodies against MSCs markers: CD9, CD10, CD44, CD73 and CD105, embryonic stem cells markers; Oct-4, Nanog, Stro-1 and SSEA-4 and hematopoietic markers; CD34, CD38, CD45, and CD133. The specification of antibodies is summarized in table 1.

Multi-lineage differentiation

MenSCs and BMSCs were differentiated into adipogenic, chondrogenic and osteogenic lineages using specific polarizing media as per method described previously 24,25. In brief, MenSCs and BMSCs were seeded in 24-well plates at 5×10^4 cell/well. For adipogenic differentiation, MenSCs or BMSCs were cultured in DMEM-F12/FBS 10% supplemented with 1 μM rosiglitazone (St Louis, MO, USA), 10 μg/ml human recombinant insulin, 0.5 mM IBMX (3-Isobutyl-1-me-
Peripheral blood samples were obtained from healthy donors. Then Peripheral Blood Mononuclear Cells (PBMCs) were isolated using density gradient Ficollicapue medium (Amersham, UK). CD4\(^+\) T cells were purified from PBMCs using magnetic beads negative selection kit (Miltenyi Biotec, Germany) with approximate purity of 95\%. CD4\(^+\) T cells were co-cultured (at 4\(\times\)10\(^5\) cells/well) with MenSCs at 1:2 to 1:128 ratios (MenSCs: CD4\(^+\) T cells) in 24-well plates for five days. During culture, CD4\(^+\) T cells were stimulated with anti-CD3 and anti-CD28-loaded activation beads at a ratio of 1:4 (bead:cell) (Miltenyi Biotec, Germany).

**Pre-treatment of MenSCs with IFN\(\gamma\)**

In some settings, MenSCs and BMSCs were stimulated with 25 ng/ml IFN\(\gamma\) in 24-well plates for 48 hr before co-culture with CD4\(^+\) T cells. Thereafter, MenSCs and BMSCs were co-cultured for five days with CD4\(^+\) T cells as above, at ratios of (MSCs: CD4\(^+\) T cells) 1:4-1:8 and 1:5, respectively.

### Table 1. Antibody panel for immunophenotyping

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD9</td>
<td>FITC</td>
<td>M-L13</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD10</td>
<td>PE</td>
<td>H110a</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD29</td>
<td>PE</td>
<td>MAR4</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD34</td>
<td>FITC</td>
<td>581</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD38</td>
<td>FITC</td>
<td>HIT2</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD44</td>
<td>PE</td>
<td>515</td>
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<tr>
<td>Anti-CD45</td>
<td>PE</td>
<td>H1D9</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD73</td>
<td>PE</td>
<td>AD2</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD105</td>
<td>PE</td>
<td>166707</td>
<td>R&amp;D systems</td>
</tr>
<tr>
<td>Anti-CD133</td>
<td>PE</td>
<td>W6B3C1</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-Nanog</td>
<td>-</td>
<td>Polyclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-Oct-4</td>
<td>-</td>
<td>Polyclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-SSEA-4</td>
<td>-</td>
<td>MC813-70</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-Stro-1</td>
<td>-</td>
<td>STRO-1</td>
<td>R&amp;D systems</td>
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<tr>
<td>Anti-rabbit IgG</td>
<td>FITC</td>
<td>Polyclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-mouse IgG</td>
<td>FITC</td>
<td>Polyclonal</td>
<td>Sina Biotech</td>
</tr>
</tbody>
</table>

**IDO activity assay**

IDO activity in MenSCs and BMSCs supernatant was assessed with or without IFN\(\gamma\) pre-stimulation. MenSCs or BMSCs were seeded at 1\(\times\)10\(^5\) cell/well in 650 \(\mu\)L DMEM-F12/FBS 10\% (24-well plate). To evaluate IDO activity, 100 \(\mu\)g/ml tryptophan (Sigma, St Louis, MO, USA) was added to each well in the presence or absence of 100 ng/ml IFN\(\gamma\) (control wells contained only culture media) and incubated in a humidified incubator for 48 hr. Supernatant was harvested and prepared as described elsewhere. IDO activity was then assessed through measurement of tryptophan catabolite (kynurenine) concentration, using a plate reader (Biotec, VT, USA) at 450 nm.

**Statistical analysis**

Flow cytometry data were analyzed using Flowjo 7.6.1 Software (Tree Star Inc., Ashland, USA). All colorimetric experiments were performed in triplicate.

Mann-Whitney was used to evaluate the differences. All graphs are displayed using median and range. P values less than 0.05 were considered statistically significant. The analysis was done using Prism software 6.0 (GraphPad Software Inc., San Diego, USA).

**Results**

**MenSCs exhibited dual mesenchymal and embryonic markers and multi-lineage differentiation capacity**

MenSCs and BMSCs expressed MSCs markers including CD9, CD10, CD29, CD44, CD73 and CD105, they were also negative for hematopoietic markers, CD34, CD38, CD45 and CD133. MenSCs also expressed Oct-4 but failed to express SSEA-4, while the opposite pattern was the case for BMSCs (Figure 1), (Table 2). Both MenSCs and BMSCs were capable of differentiating into adipogenic, chondrogenic and osteogenic lineages confirming their MSCs identity. MenSCs showed less potency to differentiate into osteogenic and adipogenic lineages compared to BMSCs (Figure 2).

**MenSCs augmented CD4+ T cells proliferation**

To assess the immunomodulatory capability of MenSCs, they were co-cultured with CFSE-labeled anti-CD3/anti-CD28-activated CD4+ T cells at different ratios. As shown in figure 3, MenSCs significantly increased proliferation of CD4+ T cells at ratios 1:2, 1:4 and 1:8 (p<0.001). Although the rate of proliferation at higher ratios was higher compared to the CD4+ T cells.
MenSCs Induced CD4+ T Cell Proliferation

MenSCs and BMSCs were induced CD4+ T cell proliferation. BMSCs as the most studied source of MSCs with potent immunomodulatory impact on T cell proliferation, upon stimulation with pro-inflammatory cytokines such as IFNγ, IL-1β and TNF-α were tested as positive control. IFNγ pre-stimulated BMSCs significantly suppressed proliferation of CD4+ T cells compared to the control wells (p<0.05). Although CD4+ T cells proliferation was reduced in the presence of IFNγ pre-stimu-

Table 2. Expression of mesenchymal and embryonic stem cell markers by MenSCs and BMSCs

<table>
<thead>
<tr>
<th>Markers</th>
<th>MenSCs</th>
<th>BMSCs</th>
</tr>
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<tbody>
<tr>
<td>CD34</td>
<td>1.7±0.9%</td>
<td>0.9±0.4%</td>
</tr>
<tr>
<td>CD38</td>
<td>1.4±0.7%</td>
<td>1.9±1.3%</td>
</tr>
<tr>
<td>CD45</td>
<td>1.1±0.7%</td>
<td>1.7±0.8%</td>
</tr>
<tr>
<td>CD133</td>
<td>1.9±0.7%</td>
<td>2.1±1.6%</td>
</tr>
<tr>
<td>CD9</td>
<td>90.8±6.7%</td>
<td>92.1±5.9%</td>
</tr>
<tr>
<td>CD10</td>
<td>89.4±5.7%</td>
<td>40.1±18%</td>
</tr>
<tr>
<td>CD29</td>
<td>98.7±1.26%</td>
<td>94.9±5%</td>
</tr>
<tr>
<td>CD44</td>
<td>99±0.2%</td>
<td>96.97±3%</td>
</tr>
<tr>
<td>CD73</td>
<td>99.7±0.2%</td>
<td>97.6±1.2%</td>
</tr>
<tr>
<td>CD105</td>
<td>89.6±9.6%</td>
<td>99.7±0%</td>
</tr>
<tr>
<td>STRO-1</td>
<td>4.7±1.4%</td>
<td>8.7±3.4%</td>
</tr>
<tr>
<td>Oct-4</td>
<td>99.5±0%</td>
<td>1.4±1.1%</td>
</tr>
<tr>
<td>Nanog</td>
<td>2.5±0.4%</td>
<td>7±2.4%</td>
</tr>
<tr>
<td>SSEA-4</td>
<td>1.8±0.8%</td>
<td>78.3±11.4%</td>
</tr>
</tbody>
</table>

BMSCs as the most studied source of MSCs with potent immunomodulatory impact on T cell proliferation, cultured alone, the differences were not reached to the statistically significant level.

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**Figure 1.** Immunophenotyping of MenSCs and BMSCs. MenSCs and BMSCs were evaluated for the expression of MSCs markers, CD9, CD10, CD29, CD44, CD73 and CD105, hematopoietic makers, CD34, CD38, CD45 and CD133, and pluripotency makers, Nanog, Oct-4, SSEA-4 and STRO-1. The grey and empty histograms represent unstained sample and test samples, respectively. Results are representative of three individual experiments.

**Figure 2.** Multi-lineage differentiation potential of MenSCs and BMSCs. The left and right pictures of each panel represent differentiated (Dif) and undifferentiated (Undif) stem cells, respectively. Differentiation of stem cells toward osteocytes, chondrocytes and adipocytes were assessed by Alizarin red, Alcian blue and Oil red staining, respectively. Results are representative of three individual experiments.
lated MenSCs (p<0.05), it was still significantly higher than the control (p<0.0001) (Figure 4).

**IFNγ induced IDO activity in both MenSCs and BMSCs**

IDO has been widely studied due to its role in tolerance. IFNγ is the most potent stimulator of IDO activity. In this context, MenSCs and BMSCs were stimulated with IFNγ and IDO activity was measured in cell culture supernatant. Our results showed that in MenSCs, IDO activity was induced in both cell types after stimulation with IFNγ compared to the un-treated cells. Both MenSCs and BMSCs exhibited higher IDO activity compared with controls, after stimulation with IFNγ (p<0.0001) (Figure 5).

**Discussion**

Although plenty of mechanisms and regulatory networks for establishment of immune tolerance at the feto-maternal interface have been introduced, the potential immunomodulatory role of endometrial stromal stem cell has been largely ignored. During the past couple of years, the immunomodulatory properties of mesenchymal stem cells have attracted interest of many researchers and to a large extend have foregrounded the principal application of this cell population in regenerative medicine. In this study, the potential immunomodulatory impact of MenSCs, as surrogate cells for endometrial mesenchymal stem cells, on T cell proliferation was addressed. As with previous reports 25, it was shown that MenSCs possessed minimal criteria necessary for defining a cell type as MSCs exemplified by the expression of markers associated with mesenchymal origin and multi-lineage differentiation 25. Expression of the embryonic marker, Oct-4, by MenSCs is a further support to the previous reports on higher proliferation capacity of these cells compared to BMSCs 27.

In the next step, the potential modulatory effect of MenSCs on CD4+ T cells proliferation was examined in reference to BMSCs. It was shown that at MenSCs: T cell ratios of 1:2-1:8, MenSCs supported CD4+ T cells proliferation. This finding seems to have contradiction with our previous results 22, because in that report MenSCs were able to suppress allogenic MLR at 1:1 and 1:2 (MenSCs: PBMCs) ratios. Notably, in allogeneic MLR, a mixture of pro-inflammatory cytokines profiles is produced by responder cells including IL-1β and TNF-α 28 which are able to induce anti-inflammatory phenotype in MSCs 29. Hence, it could be inferred that inflammatory milieu during MLR reaction may help to boost MSCs immunomodulatory capabilities. On the other hand, MSCs use monocyte-dependent mechanism to halt T cell responses 30-32 which was absent in the system reported here. Although by taking our initial concept into consideration, this finding was out of our expectation, the following explanation could be put forth. The upper part of...
female reproductive tract is sterile and in non-pregnant women, immune system needs to be on a stand-by mode to properly respond to any invading pathogen; hence, it seems logical to assume that every potential immunotolerance mechanism remains at low functional level during non-pregnant state. Dual anti-inflammatory or pro-inflammatory phenotype of MSCs depending upon microenvironment milieu has already been reported.

The onset of pregnancy and blastocyst implantation is associated with inflammatory processes initiated by insemination and recruitment of dNK cells. Besides endometrial immune cells such as dNK cells which produce IFNγ, endometrial non-immune cells are also a potential source for establishment of inflammatory milieu. Interestingly, most MSCs acquire anti-inflammatory phenotype upon treatment with such pro-inflammatory cytokines as IFNγ. With this in mind, effect of IFNγ pre-treatment on modulatory activity of MenSCs on the proliferative response of CD4+ T cells was evaluated in the next step.

As expected, IFNγ-treated BMSCs significantly inhibited T cell proliferation, which was in accordance with results reported by other groups. Although IFNγ treatment of MenSCs reduced their capacity to augment T cell proliferation, it was still significantly higher than control. This finding may be due to the lower expression level of IFNγ receptor in MenSCs compared with BMSCs. Almost a similar result was observed in umbilical cord-derived MSCs co-cultured with PHA-activated PBMCs. On the other hand, induction of IDO activity in MenSCs treated with IFNγ implies that suppressive activity of MenSCs IDO on T cell proliferation was not sufficient enough to overcome yet undetermined proliferation supportive mechanisms of this cell population.

Figure 4. Effect of IFNγ stimulation of MenSCs on proliferation of CD4+ T cells: A) MenSCs were co-cultured with CD4+ T cells at 1:4 and 1:8 (MenSCs:CD4+ T cells) ratios with or without IFNγ pre-stimulation for five days and the percent of proliferation was assessed by CFSE flow cytometry. B) IFNγ pre-stimulated BMSCs were used as positive control in CD4+ T cells proliferation assay. Figures on the right in each panel represent histogram plots of corresponding proliferation assays. The empty histograms represent biological controls (BC) (CD4+ T cells cultured alone) and grey histograms represent test samples (co-culture). Results are representative of ten individual experiments *: p<0.05 and ****: p<0.0001.

Figure 5. Assessment of IDO activity in MenSC and BMSC supernatants after stimulation with IFNγ. IDO activity was measured using kynurenine colorimetric assay. The results are median and range of four BMSCs and six MenSCs samples ****: p<0.0001.
It is notable that, IFNγ is not the only pro-inflammatory cytokine in early pregnancy decidua. Expression of other pro-inflammatory cytokines including IL-1, TNF-α, and IL-18 in early pregnancy decidua is upregulated. Interestingly, IL-1β and TNF-α are among the pro-inflammatory cytokines that have been proven to induce anti-inflammatory phenotype in MSCs. Thus, it remains to be investigated whether endometrial microenvironment during pregnancy can affect the immunomodulatory properties of MenSCs.

Conclusion

Our results showed that MenSCs induce proliferation of CD4+ T cells which could be a basis for maintenance of endometrial homeostasis to cope with ascending infections. This feature, however, seems to be contradictory to the requirement for immunological tolerance to semi-allogeneic fetus. Whether or not this immune enhancement capacity of MenSCs is modulated during pregnancy under the influence of immunosuppressive hormones and mediators needs to be determined.

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References


