Original Article
The CD200/CD200R mechanism in mesenchymal stem cells’ regulation of dendritic cells

Yulei Zhao, Guohong Su, Qing Wang, Ruihuan Wang, Minjuan Zhang
The Second Department of Hematology, Cangzhou Central Hospital, 16 West Xinhua Road, Yunhe, Cangzhou, China
Received January 27, 2021; Accepted April 12, 2021; Epub August 15, 2021; Published August 30, 2021

Abstract: Objective: To investigate the CD200/CD200R pathway mechanism in mesenchymal stem cells’ (MSC) regulation of dendritic cells (DC) (MSc). Methods: We collected marrow samples from 40 patients admitted to our hospital from January 2018 to December 2019. The bone marrow MSCs were cultivated, and the peripheral blood mononuclear cells (PBMC) and peripheral blood DC were isolated to establish an in vitro immune response model. The expressions of the CD200 molecule on the surface of MSC were measured. Anti-CD200 blocking antibodies were added to the culture system to observe the effect of the PBMC differentiation and the immature DC (imDC) to mature DC (mDC). Then the impact of the different positive rates of CD200 in the same MSC on imDC maturity was measured. Results: After adding mitogen pHA, the IL-4, IL-10, and TNF-α secretions were increased (all P<0.05), and the OD value of the PBMC+pHA group was higher than it was in the PBMC group. After stimulated by pHA, the CD200 of the MSC group was higher than it was in the MSC+PBMC group (P<0.05). The MSC+PBMC group co-culture inhibited the development of imDC to mDC. Adding anti-CD200 antibodies to the MSC+PBMC co-culture system, MSC could still inhibit the differentiation of PBMC to imDC, and MSC had a significant inhibition effect on imDC to mDC maturation (P=0.006). The addition of MSC reduces the maturation markers on the surface of mDC (P<0.05). The addition of MSC inhibited the ability of mDC to stimulate PBMC (P_{OD}<0.05) and decreased the IL-12 (P_{IL-12}<0.05) levels. The addition of the anti-CD200 antibody increased the proliferation ability of mDC to stimulate PBMC (P_{OD}<0.05), and it also increased the IL-12 levels in mDC (P_{IL-12}<0.05). The expression of the DC mature immune phenotype in the CD200 high expression group was weak (P_{CD83,CD86}<0.05). Conclusion: The mechanism by which MSC inhibits DC may be achieved through the CD200/CD200R pathway, and the CD200/CD200R pathway mainly acts on the process from imDC to mDC.

Keywords: Mesenchymal stem cells, dendritic cells, CD200/CD200R

Introduction
Mesenchymal stem cells (MSC) are a kind of non-hematopoietic adult stem cells with a multidirectional differentiation ability. They are mainly derived from the bone marrow, bones, fat, muscles, and other tissues. They are predominantly used to treat various blood system diseases and for the identification of the immune response in vitro due to their unique immunosuppressive effect [1, 2]. Their immunomodulatory mechanism is mainly achieved by inhibiting the expansion of B cells and affecting the further differentiation and maturation of dendritic cells (DCs). As the most powerful antigen-presenting cells of naive T cells, DCs are crucial in the process of transplantation immu-
The CD200/CD200R mechanism in the regulation of dendritic cells

CD200, it can also transmit inhibitory immune regulatory signals with CD200, thereby inhibiting the DC immune response. Hence, the exploration of the CD200/CD200R pathway mechanism in MSCs’ regulation of DC is of vital significance and can provide a theoretical basis for clinical treatment involving allogeneic transplantation.

Materials and methods

Material collection

We collected bone marrow samples from 40 patients admitted to our hospital from January 2018 to December 2019, and we collected 10 ml of bone marrow from each patient’s posterior or superior iliac spine using heparin anticoagulation tubes. All the samples were examined using bone marrow cytology to determine the disease type. And all patients voluntarily signed an informed consent form, and the Ethics Committee of Cangzhou Central Hospital approved the protocol of the experiments, and the approval No. is 2017-252-01 (Z).

Experimental methods

Cultured bone marrow MSCs and isolated human peripheral blood mononuclear cells (PBMC): After aseptically harvesting the bone marrow, a bone marrow cell suspension was prepared, bone marrow mononuclear cells were separated using density gradient centrifugation, and bone marrow-derived MSCs were obtained through adherent cultures, inoculated in a petri dish (medium: 10% fetal bovine serum+2 mM glutamine low-sugar DMEM) at a density of 5×10^5/cm^2^, and cultured in a 37°C, 5% CO_2_ incubator, and the medium was changed after 48 h. The bone marrow MSCs were digested and passaged when the adherent cells’ confluence reached over 90%.

We collected 20 ml of peripheral blood from healthy volunteers, and we separated the PBMCs using density gradient centrifugation.

Establishing an in vitro immune response model to determine the expressions of the CD200 molecules on the MSCs’ surfaces: The digested and washed MSCs were counted, the cell concentration was adjusted, and then we inoculated 1×10^4_ cells per well in a 96-well plate. 20 µg/ml mitomycin was added to subject the cells to an adherent culture, the culture medium was discarded after being incubated for 3 h, and then we washed the solution three times with a PBS buffer. The PBMCs were suspended in an RPMI 1640 medium (containing 10% FBS) and inoculated into inactivated MSCs.

Phytohemagglutinin was added to accelerate the transformation of the mononuclear cells. The PHA stimulated mononuclear cells without MSC were defined as the positive control group, and those without PHA were defined as the negative control group, and the two groups of cells were incubated. The optical densities (OD) of the negative control group (PBMC), the positive control group (PBMC+PHA), and the experimental group (MSC+PBMC, PBMC+MSC+PHA) were measured at λ=492 nm using ELISA, and the measurement was repeated three times to get the average.

After inoculating the MSCs into a 6-well plate and co-culturing them with the PBMCs, PHA was added. And the MSC+PBMC and the MSC+PHA were set as the control group. After 72 hours of culturing, the adherent cell MSCs were collected and resuspended in PBS, and the CD200 expression changes were measured using FACS.

Culturing and measuring the human peripheral DC: We took 50 ml of peripheral blood from healthy adults and isolated the PBMCs using Ficoll density gradient centrifugation. Then, the cells were washed twice with PBS, inoculated at 2×10^6_/well on a 6-well culture plate (10% fetal calf serum RPMI+1640 medium was used to resuspend the cells), and then the cells were placed in an incubator (37°C, 5% CO_2_) for two hours. Next, the non-adherent cells were discarded, and the solution was washed with a RPMI-1640 medium once more. 2 ml of 10% fetal bovine serum RPMI-1640 medium (containing IL-4+GM-GSF) was added, cultured in an incubator (37°C, 50% CO_2_) until day 3 and day 5 to half medium change, and the immature DCs (imDC) could be observed on day 5. Subsequently, the antigen differentiation cluster was fluorescently labeled and its fluorescence intensity was measured. Lipopolysaccharide (LPS) was added to promote the maturation of some cells, and mature DC (mDC) was obtained on day 7. The fluorescence intensity was measured as above.
The CD200/CD200R mechanism in the regulation of dendritic cells

The effect of anti-CD200 blocking antibodies on PBMC differentiation: MSCs with CD200 positive rate >50% after digestion were obtained, washed, and resuspended, and anti-CD200 blocking antibody was added to the MSC resuspension, incubated in an incubator (37°C) for three hours, then inoculated 2×10^5/well in a 6-well plate, and the adherent was cultured for 8 hours. The PBMCs were extracted and added to the adherent MSC6 well plates and divided into two culture groups: PBMC and MSC+PBMC. We referred to the DC culture procedure to obtain imDC and mDC, and we measured their immunophenotypes.

The effect of the different CD200 positive rates of the same MSC on the maturation of imDC: The PBMCs were isolated to obtain imDC. 8 hours before culturing the imDC, CD200 molecules with different positive rates of the same MSC (positive rate <25% or >80%) were obtained, and we inoculated them into 6-well plates at 2×10^5/well. Next, imDC was added to the adherent MSC6 well plates as a simple imDC group, catalyzed, and then we obtained the mDC. The immunophenotype of the mDC was determined. The effect of DC on the proliferation of PBMC was observed using ELISA, and the IL-12 level in the supernatant was measured.

Statistical analysis

SPSS 23.0 software was used to conduct the data analysis and GraphPad Prism 7.0 to plot the graphics. The measurement data were expressed as (χ±s), and analyzed using t tests. The count data were expressed as n (%), and analyzed using χ² tests. P<0.05 indicated statistical significance.

Results

The expressions of the CD200 molecules on the surface of MSC in the immune response environment

In the in vitro immune response model of the PBMCs, it was found that all groups secreted a small amount of cytokines such as IL-4, IL-10, and TNF-α, and the IL-4, IL-10, and TNF-α secretions in each group after adding the pHA increased (P<0.05, Table 1). The proliferation in each group was measured using the ELISA method, and the measurements showed that the OD value of the PBMC+pHA group was significantly higher compared to the PBMC group [(0.85±0.07) vs (0.38±0.06)] (t=16.12, P<0.05), as shown in Figure 1. At the same time, after the PBMC was stimulated by the PHA, the expressions of the CD200 molecules on the surface of the MSC group increased compared with the MSC+PHB group (51.73±9.68 vs 36.79±8.63, t=3.643, P=0.002).

DC general characteristics

The cell phenotype results demonstrated that PBMC showed a high expression of CD14 and a low expression of CD1a. After we induced the differentiation of the PBMCs, the imDC showed low expressions of CD83 and CD86, and no

Table 1. Changes in the cytokine IL-4, IL-10, and TNF-α levels in PBMC before and after adding mitogen PHA into the culture system (χ±s)

<table>
<thead>
<tr>
<th></th>
<th>IL-4 (ng/L)</th>
<th>IL-10 (ng/L)</th>
<th>TNF-α (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before adding</td>
<td>15.72±5.03</td>
<td>48.79±4.35</td>
<td>21.36±6.28</td>
</tr>
<tr>
<td>After adding</td>
<td>287.3±96.85</td>
<td>226.4±86.30</td>
<td>136.7±36.53</td>
</tr>
<tr>
<td>t</td>
<td>8.857</td>
<td>6.500</td>
<td>9.847</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
The CD200/CD200R mechanism in the regulation of dendritic cells

related CD14 expression, but the mDC showed high expressions of CD83 and CD86. After adding the PBMCs, it can promote its activation and proliferation, showing the effect of the antigen presentation.

The expressions of CD200 and CD200R on PBMC and imDC

A total of five samples of PBMC and imDC were tested, and their CD200 and CD200R expressions are displayed in Figure 2.

The effect of MSC on the immune phenotype of DC

The PBMC group and the MSC+PBMC group were co-cultured for five days. No cell surface molecules in the MSC+PBMC group differentiated into the imDC phenotype. In addition, it could inhibit the development of imDC to mDC. See Table 2.

The effect of the anti-CD200 blocking antibodies on the DC immune phenotypes

After adding the anti-CD200 blocking antibody to the MSC+PBMC co-culture system, MSC can still inhibit the differentiation of PBMC towards imDC, and there was no significant difference in the changes in the surface immune molecules of imDC (P>0.05), but MSC had a significant inhibitory effect on the maturation of imDC to mDC (P=0.006).

MSC was added to treat the maturation process of imDC to mDC, and it was found that the expression levels of the maturation markers on the surface of mDC decreased (P<0.05). After the intervention with the anti-CD200 blocking antibodies, the expressions of the mature immune phenotypes of mDC were significantly higher than the expressions of the MSC suppression group (P<0.05). See Table 3.

The effect of the anti-CD200 blocking antibody on the tendency of imDC to mDC

After adding the MSCs to treat the maturation of mDC, they inhibited the ability of mDC to stimulate the PBMCs (OD value<0.05), and reduced the mDC’s inflammatory factor IL-12 level (IL-12<0.05). After adding the anti-CD200 blocking antibody, compared with the MSC inhibition group, the PBMC proliferation ability stimulated by mDC increased (OD value<0.05), and the mDC’s inflammatory factor IL-12 level also increased (IL-12<0.05). See Table 4.

The effect of the CD200 MSCs on the different positive rates on imDC

In the comparison between the CD200 low expression group and the high expression group, the CD200 high expression group showed a weaker DC mature immunophenotype expression (CD83, CD86<0.05). The proliferation inhibition rate and the secretion ability of IL-12 in the high expression group on PBMCs were weakened by ELISA (OD, IL-12<0.05). See Table 5.

Discussion

In recent years, evidence supporting the role of MSCs in immune regulation has increased.
MSCs can regulate immunity by inhibiting the expression of DC, affecting the activity of T cells, and the immune response of transplantation, and they have achieved certain results in the treatment of graft-versus-host disease [6, 7]. Many scholars have stated that soluble factors play a key role in mediating the inhibition of MSC, and the isolation of MSC and lymphocytes in a co-culture system has no impact on the inhibition of MSC [8, 9]. However, there are studies suggesting that the method of contact between cells may also play an important part in mediating the MSC inhibitory pathway [10]. The CD200/CD200R signaling pathway has a significant inhibitory effect on immune regulation. By the comprehensive analysis of the immune function of MSC and the molecular characteristics of CD200, it is speculated that the molecular expression of CD200 may be related to the immune mechanism of MSC. Ho et al. [11] pointed out that the immunomodulatory effect of the route of exposure of MSC and the effector cells may be related to the CD200/CD200R pathway. Yang et al. [12] revealed that for transplanted bone marrow MSCs in a rat model of ischemic stroke after the injection of CD200R antibodies, the MSCs can inactivate the microglia around the infarcted tissue through ionized calconectin antigens to exert neuroimmunological regulation, suggesting that the transplanted MSC may participate in the inactivation of microglia around the infarction through the CD200/CD200R pathway, exerting an immunosuppressive effect.

In our study, we explored the changes in the CD200 molecules on the MSC surfaces by establishing an immune response environment. The results showed that the PBMCs proliferated significantly after PHA was added to the system in vitro, and the proliferation of PBMC was significantly inhibited after adding MSC to the system. If immune stimulating factors are excluded, the growth of the PBMCs in the in vitro environment will not be affected by the MSCs, suggesting that the PBMCs have produced an immune response under the action of the immune stimulating factors. In addition, in the model of adding PHA to stimulate the immune response of MSC+PBMC co-culture, the PBMCs activated the proliferation, and the expressions of CD200 on the surfaces of the MSCs was up-regulated. MSC alone has no significant immunosuppressive effect on T lymphocytes. But after activating the immune response, it can increase the IL-4, IL-10, and TNF-α levels, plays a certain role in inhibiting the proliferation of immune effector cells, reduces the inflammatory factor levels, and regulates the tendency of T lymphocytes subsets to Th2 [13, 14]. Therefore, the immune regulation mechanism of MSC may guide the immune response to stablish the internal environment [15].

Some foreign scholars have pointed out that MSC can inhibit the differentiation and maturation of PBMC-derived DC in vitro [16, 17]. Zhang et al. [18] found that MSCs can inhibit the differentiation stage of PBMC-derived DC (the expression of CD1a, CD86, human leukocyte DR antigens, etc.), and they also have an inhibitory effect on the later mature stage of DC (the CD83 and CD86 expressions). MSC changes the cytokine secretions and gradually directs DC expressions towards immature immunophenotypes by inhibiting the expression of CD83. MSC inhibits the maturation of DC and induces immune tolerance through intercellular contact [19, 20]. We thus examined its specific regulatory mechanism in this study. In evaluating the effect of CD200 molecules on the maturation and characteristics of DCs in MSCs, anti-CD200 blocking antibodies were added to the PBMC+MSC co-culture system. It was observed that the MSCs had high CD1a expressions and low CD14 expressions, but they had no significant immunosuppressive effect on the immunophenotype of imDC. In addition, the CD200R molecule on the surface of imDC was highly expressed, and the addition of the anti-CD200 blocking antibody to the imDC+MSC co-culture system can improve

<table>
<thead>
<tr>
<th></th>
<th>PBMC</th>
<th>MSC+PBMC</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14 (%)</td>
<td>33.76±9.27</td>
<td>6.43±1.28</td>
<td>9.235</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD1a (%)</td>
<td>12.48±3.13</td>
<td>76.35±8.51</td>
<td>22.270</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD83 (%)</td>
<td>60.83±15.74</td>
<td>91.42±12.16</td>
<td>4.863</td>
<td>0.001</td>
</tr>
<tr>
<td>CD86 (%)</td>
<td>67.28±11.23</td>
<td>85.49±9.53</td>
<td>4.658</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The inhibitory effect of MSC on the DC immunophenotype
DC mature immune phenotype expression, the antigen presentation effect and the inflammatory factor IL-12 level. The above results confirmed that the CD200/CD200R pathway is involved in the process of MSC inhibiting DC maturation, and plays an important role in the stage of imDC tending to mDC. Effective interventions on this signaling pathway are of a certain significance in the clinical treatment of graft-versus-host disease and autoimmune diseases.

Acknowledgements

This study was supported by the Science and Technology Program of Cangzhou, China (Program Number: 162302182).

Disclosure of conflict of interest

None.

Address correspondence to: Yulei Zhao, The Second Department of Hematology, Cangzhou Central Hospital, 16 West Xinhua Road, Yunhe, Cangzhou, China. Tel: +86-15720306668; E-mail: zhaoyulei198411@163.com

References

The CD200/CD200R mechanism in the regulation of dendritic cells


