Journal of Research in Clinical Medicine

J Res Clin Med, 2022, 10: 22
doi: 10.34172/jrcm.2022.022
https://jrcm.tbzmed.ac.ir

TUOMS PRESS

Original Article

Mesenchymal stem cell secretome induced the acquisition of anti-inflammatory phenotype in rat cortical microglia *in vitro*

Babak Davand-Barenji[1,2], Morteza Eskandani[3], Reza Rahbarghazi[1,4*], Mohammad Hossein Geranmayeh[5,6*]

[1]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Biology, Islamic Azad University, Tabriz Branch, Tabriz, Iran
[3]Research Center for Pharmaceutical Nanotechnology (RCPN), Biomedicine Institute, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Department of Applied Cell Sciences, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[5]Stem Cells Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[6]Neurosciences Research Center (NSRC), Tabriz University of Medical Sciences, Tabriz, Iran

Article info

Article History:
Received: 14 May 2021
Accepted: 20 Jan. 2022
e-Published: 2 Nov. 2022

Keywords:
- Rat cortical microglia
- Bone marrow mesenchymal stem cells
- Paracrine activity
- Phenotype acquisition
- M1/M2 phenotype polarization

Abstract

Introduction: Here, we aimed to address the impact of mesenchymal stem cells-conditioned medium (MSCs-CM) on M1/M2 phenotype shifting of rat microglia after 48 hours *in vitro*.

Methods: Rat neonatal cortical microglia were randomly allocated into four different groups as follows: Control; MSC-CM; IL-4; and Anti-IL-4 groups. In the MSC-CM, microglia were treated with MSC condition media. In the interleukin-4 (IL-4), cells received 20 ng/mL IL-4; conceived as a positive M2 control group). In the Anti-IL-4, the combination of IL-4 peptide and anti-IL-4 antibody was used. After 48 hours, protein levels of Iba1, CD86, and CD206 were monitored using immunofluorescence imaging and flow cytometry analysis.

Results: According to immunofluorescence imaging, 48-hour incubation of rat cortical microglia with stem cells condition media increased protein levels of CD206 and decreased CD86, showing the polarization of microglia toward M2 type lineage compared to the non-treated control group. We found a similar trend in the group that received IL-4. By contrast, the incubation of microglia with anti-IL-4 antibody blunted the stimulatory effect of IL-4 to promote M2-type microglia. Similar to the immunofluorescence data, flow cytometry analysis revealed a significant increase of CD206 positive microglia after exposure to MSC-CM and IL-4 ($P<0.05$). Treatment with anti-IL-4 antibody significantly reduced the percent of CD206 positive cells, showing the inhibition of M1-to-M2 phenotype acquisition.

Conclusion: The current study highlighted a notable anti-inflammatory effect of MSC secretome on cortical microglia by promoting M1-to-M2 phenotype acquisition.

Introduction

Microglia have pivotal roles during pathological and physiological conditions.[1] Microglia consist of certain macrophage-like lineage with dynamic activity. These cells are divided into two M1 and M2 phenotypes with certain pro-inflammatory and anti-inflammatory properties, respectively.[2] The conversion of microglia toward the M1 phenotype triggers the release of certain factors such as reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and nitric oxide. By contrast, microglia polarization toward M2 phenotype induces the production of IL-4, IL-10, IL-13 and transforming growth factor-beta (TGF-β).[3] M1 phenotype microglial can respond in hours or days to the entrance of external factors to the brain. This behavior is triggered through various types of receptors of microglia such as toll-like receptors and many other scavenger receptors.[2] M2 and M1 glial cells express surface markers such as CD206, and CD86, respectively.[4,5] Upon initiation of pathological conditions, microglia respond immediately by migrating toward the damaged areas. In these conditions, microglial polarization toward M1 phenotype is enhanced which promotes the inflammatory responses.[6] Currently, the therapeutic effects of mesenchymal stem cells (MSCs) have been indicated in the alleviation of central nervous system injuries.[7] MSCs have the potential to promote healing procedures and exert palliative effects inside brain tissue via using different mechanisms.[7]

Here, we investigated the possible changes in the polarization of rat microglia (M1/M2 shift) upon the treatment with MSCs-conditioned medium (MSCs-CM). It seems that the results of this study can help address

***Corresponding Authors:** Reza Rahbarghazi, Email: rezarahbardvm@gmail.com; Mohammad Hossein Geranmayeh, Email: mhgeranmayeh@gmail.com

© 2022 The Author(s). This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

the palliative effects of MSCs in neurological disease by regulation of microglia polarization.

Methods

Rat MSCs culture

In this study, adult male Wistar rats (8–12 weeks old) were used for the isolation of MSCs. Rats were kept under standard conditions [free access to water and chewing food]. Following euthanization with an overdose of ketamine and xylazine, both right and left femurs were removed aseptically and cells were extracted based on the previously described protocol.[8] In short, the extremities were cut and medullary content was collected by the injection of culture medium. To yield a single suspension cell, the samples were roughly triturated using a 10 mL syringe. After three-time PBS washes, the mononuclear cell fraction was isolated using Ficoll-Hypaque gradient centrifugation. Cells were re-suspended in DMEM/LG culture medium (Gibco) supplemented by 10% FBS (Cat no: 12070106, Gibco) and 1% penicillin-streptomycin (Cat no: 15140148, Gibco) and kept inside a CO_2 incubator. Using 0.25% Trypsin-EDTA solution, rat MSCs were detached and sub-cultured. Cells at passages 3 to 6 were used in this study. For MSC characterization, cells were incubated with anti-CD90 (FITC), CD105 (PE), CD34 (FITC), and CD45 (PE) for 30 minutes at 4˚C and analyzed using FACSCalibur system. Data were analyzed using FlowJo version 7.6.1. CM was collected from MSCs upon reaching 70-80% confluence according to the previously published data.[9]

Isolation of neonatal rat cortical microglia

Five-day-old neonatal rats were used for brain microglia isolation.[10] Briefly, neonatal rats were euthanized and the brain cortex was immediately isolated and washed with PBS. Thereafter, samples were exposed to 0.05% Trypsin-EDTA for 20 minutes. Following digestion, samples were centrifuged at 300 g for 5 minutes. Pellets were cultured in DMEM/F-12 culture medium [FBS 10% (v/v) and penicillin-streptomycin 1% (v/v)]. Microglia displayed in round-shape morphology on the astrocytes monolayer. To isolate microglia from astrocytes, the mixed glial cells were gently agitated at 180 rpm for 3 hours at 37°C. Non-adherent microglia were immediately collected, centrifuged and plated in 6 well-plates at an initial density of 5×10^4 cells per cm^2.

Microglial immunophenotyping

Expanded microglia were confirmed using an antibody against Iba-1 (Cat no: sc-32725; Santa Cruz Biotechnology Inc.).[11] Briefly, microglia were cultured on coverslips. Cells were fixed and permeabilized using pre-cold paraformaldehyde (PFA; 4 v/w %) and 0.1% Triton X-100 solution, respectively. For reduction of non-specific binding, 1% bovine serum albumin (BSA) was used. Cells were then incubated with 4 µg/mL FITC-conjugated Iba1 antibody for 1.5 hours and washed with PBS. DAPI was used for nucleus staining. A similar protocol was performed to detect CD206 and CD86 positive microglia (Santa Cruz Biotechnology, Inc., USA).[12]

Microglia exposure to MSCs-CM

To this end, microglia were randomly allocated into four different groups as follows; Control; MSC-CM; IL-4; and anti-IL-4 groups. Control microglia were cultured in a normal DMEM/F-12 culture medium. In the MSC-CM group, microglia were exposed to MSC-CM. In the IL-4 group, IL-4 (20 ng/mL) was used to induce microglia polarization toward M2 type (Cat no: 776902; BioLegend). In the anti-IL-4 group, the combination of anti-rat IL-4 (Cat no: 511905; BioLegend) and IL-4 peptide was used. In all groups, the culture medium was enriched with 2% FBS and cells were maintained under standard conditions for 48 hours.

Detection of microglia polarization using flow cytometry analysis

To detect microglial polarization toward the M2 phenotype, protein levels of CD206 were monitored in all groups using flow cytometry analysis. After incubation, M2-type microglia were detected based on surface marker CD206. On this basis, cells were collected from different groups, permeabilized using pre-cold PFA, and blocked with 1% BSA. In the next step, cells were incubated with PE-conjugated CD206 (Cat no: sc-58986, Santa Cruz Biotechnology Inc.) according to the manufacturer's instructions. Cells were analyzed using the BD FACSCalibur system and FlowJo software (ver. 7.6.1).

Statistical analysis

Data were analyzed by GraphPad Prism (version 8.0.2) using a one-way analysis of variance (ANOVA) and Tukey post hoc test. Values below 0.05 were considered statistically significant. Three sets of experiments were done for each test otherwise mentioned.

Results

MSCs characterization and immunofluorescence imaging of isolated microglia

Flow cytometry analysis revealed that the isolated MSCs are CD45 and CD34 negative. According to our data, MSCs can express CD105 and CD90 (Figure 1). Immunofluorescence imaging showed that the isolated microglia were highly positive for Iba-1, indicating the efficiency of our protocol in isolation and expansion of rat microglia (Figure 2). In the microscopic field, both amoeboid and branched morphologies were detected which were displayed with elongated cell bodies and lamellipodia. According to our data, the control microglia were CD206 negative (Figure 2). Noteworthy, 48-hour incubation of rat microglia with MSC-CM induced the synthesis of CD206. Interestingly, a similar trend was

found in the group that received IL-4 peptide. By contrast, the incubation of rat microglia with an IL-4 neutralizing antibody reversed the stimulatory effect of IL-4 on CD206 (Figure 2). These data showed that MSC-CM can induce the polarization of rat microglia toward M2 type. It seems that these effects were similar when glial cells were exposed to IL-4.

Data showed that non-treated control microglia can express CD86 after incubation with a normal culture medium (Figure 3). The exposure of these cells to MSC-CM and IL-4 peptide blunted the expression of CD86 in rat microglia after 48 hours. The inhibition of IL-4 peptide using an appropriate antibody (anti-IL-4) diminished the stimulatory effect of IL-4 on M1 to M2 phenotype acquisition (Figure 3). Taken together, these data showed that the incubation of rat microglia with MSC-CM can help them acquire M2 phenotype. These effects were similar when rat microglia were incubated with IL-4 peptide.

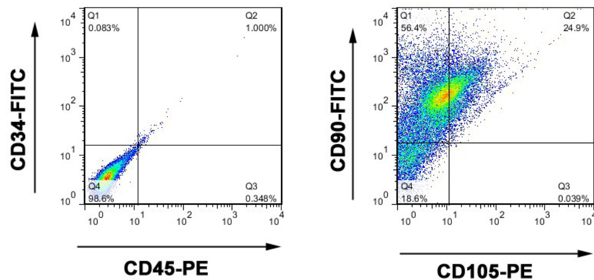

Figure 1. Flow cytometry analysis of isolated MSCs using CD90, CD105, CD34 and CD45

CD206-positive microglia were increased after exposure to MSC-CM

According to our data, 48-hour after IL-4 treatment of rat microglia increased the percent of CD206$^+$cells compared to the non-treated control microglia ($P < 0.001$; Figure 4). Along with these changes, the incubation of rat microglia with MSC-CM resulted in a significant, increase in the number of CD206$^+$cells compared to the control group

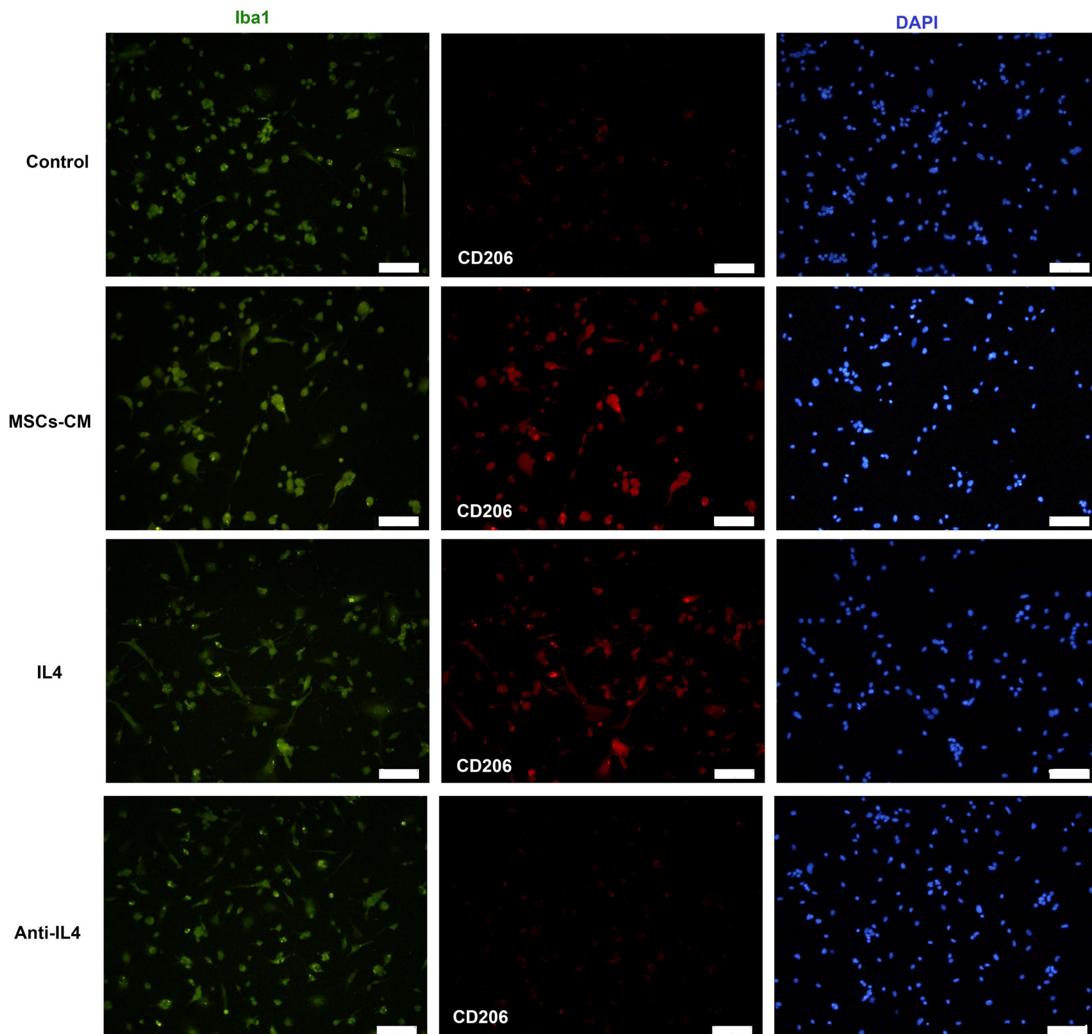

Figure 2. Immunofluorescence imaging revealed isolated rat microglia can express Iba-1, showing the efficiency of the current protocol. Data showed that incubation of microglia with MSC-CM and IL-4 induced the synthesis of M2 type cell-associated marker namely CD206 after 48 hours *in vitro*. Unlike, the application of IL-4 reduced the number of CD206 positive cells. These features were similar to the target protein in non-treated control microglia (Scale bar = 50 μm).

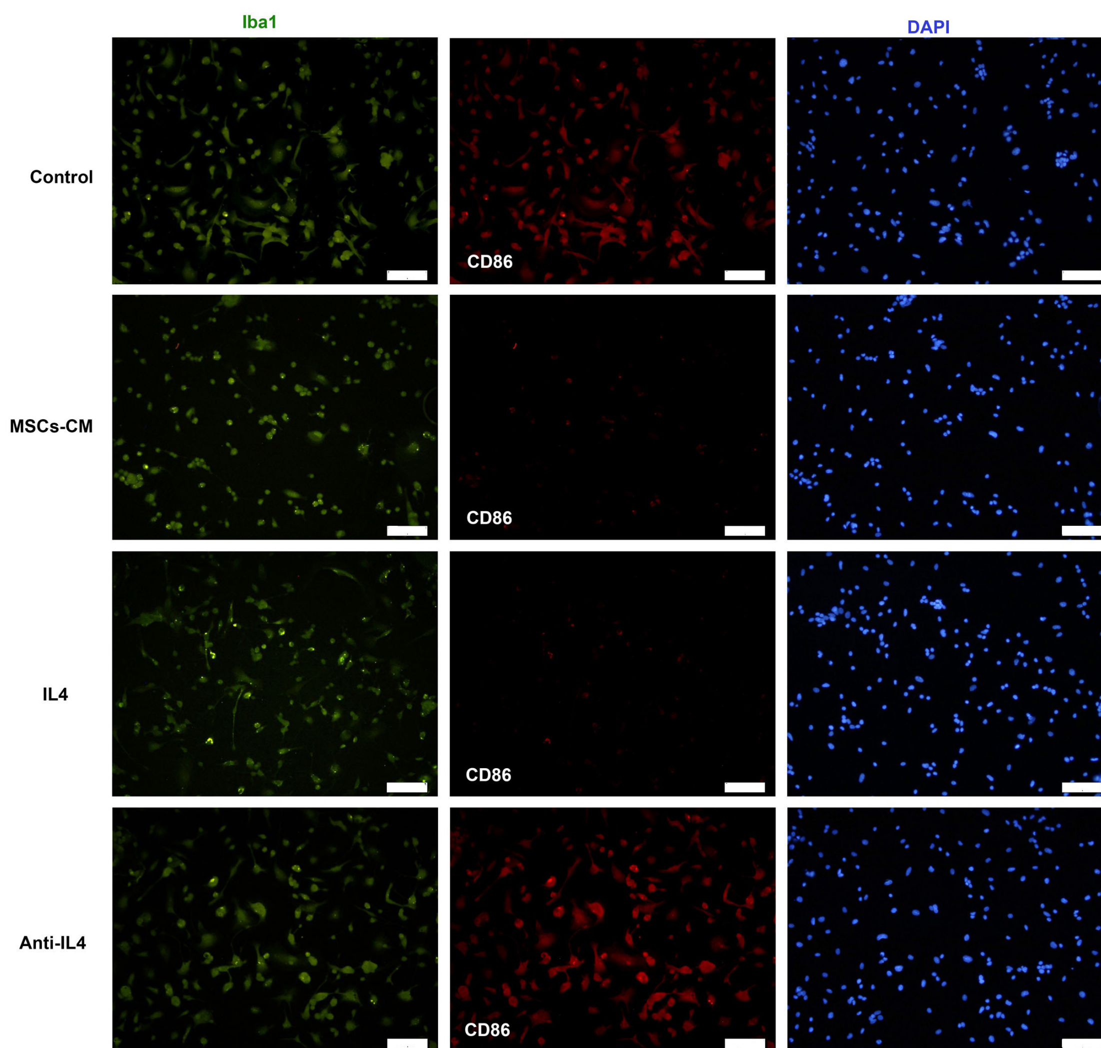

Figure 3. Measuring protein levels of CD86 using immunofluorescence imaging. In control microglia and anti-IL-4 groups, cells are positive for CD86. These effects were prominent in cells that received anti-IL-4 antibody. The incubation of rat microglia with MSC-CM or IL-4 suppressed the production of surface marker CD86, showing the loss of M1 type lineage (Scale bar = 50 μm)

($P > 0.0001$). Similar to immunofluorescence imaging, the incubation of rat microglial with the combination of IL-4 and anti-IL-4 yielded a significant reduction of CD206 positive cells when compared to all groups ($P < 0.0001$; Figure 4). Commensurate with these descriptions, both MSCs-CM and IL-4 peptide can induce the polarization of rat cells toward M2-type microglial. These data showed that MSC-CM harbor certain factors with the potential to promote M1-to-M2 phenotype shifting.

Discussion

We found that MSCs-CM increased rat microglia shifting toward an M2 phenotype *in vitro*. A similar trend was notified for cells that received IL-4 peptide. The inhibition of IL-4 using an appropriate antibody blunted these effects and induced a control group-like appearance. Previously, it was suggested that IL-4 can accelerate the conversion of microglia toward the M2 phenotype.[13,14] Here, we indicated that MSC-CM was more potent to induce M2 phenotype in rat microglia as compared to the IL-4 group. Different experiments have shown that several subsets of stem cells such as C-kit$^+$cells and CD146$^+$MSCs were potent to promote regeneration of injured tissues via paracrine activity and secretion of certain signaling molecules.[15,16] Consistent with our results, previous data confirmed that MSCs could regulate microglia bioactivity in a paracrine manner by releasing TGF-β. The activation of nuclear factor-κB and TLR2/MyD88 signaling axis participated in phenotype polarization toward M2 type.[17,18] Similarly, the incubation of microglia with human exfoliated deciduous teeth stem cell-CM increased the commitment of these cells toward M2 lineage.[19] It seems that the up-regulation of arginase-1 and activation of PI3K/Akt, Rho GTPases Rac1, and Cdc42 are involved in M1/M2 phenotype acquisition.[20] In line with these findings, MSC-CM induced M2 phenotype shifting in BV-2 microglial cell line after 24 hours exposed oxygen-glucose deprivation/re-oxygenation.[21] The inhibition of IL-4Rα can blunt the expression of arginase-1 and reduce M2 phenotype acquisition in rat microglia.[22] Therefore, IL-4 is possibly

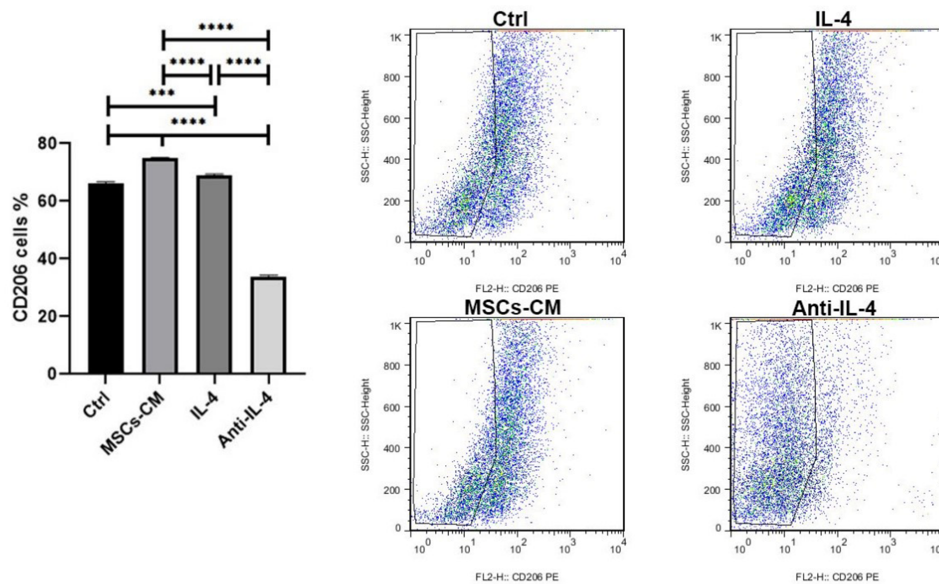

Figure 4. Flow cytometry analysis of CD206 marker for the detection of M2 type microglial 48 hours after treatment with MSC-CM. Like immunofluorescence imaging, flow cytometry analysis revealed the increase of CD206 positive cells in rat microglia treated with MSC-CM and IL-4. By contrast, anti-IL-4 antibody blunted these effects. One-Way ANOVA and Tukey post hoc analysis. ***$P < 0.001$ and **** $P < 0.0001$

a determinant cytokine in M1/M2 phenotype shifting. In support of this notion, the inhibition of IL-4 by an antibody can faint the IL-4 peptide on rat microglia. Previous experiments have shown that MSCs secret notable levels of IL-4 in a paracrine manner.[23] Therefore, one could hypothesize that the stimulatory effect of MSC-CM on M2 type acquisition of microglia can be associated in part with the existence of certain cytokines such as IL-4. Whether IL-4 possesses a dominant role in M1/M2 phenotype shifting or collaborates with other cytokines needs more investigations. Taken together, we showed MSC-CM can induce polarization of rat microglia toward CD206 positive lineage which can control inflammatory responses during brain injuries. Here, we did not measure the levels of IL-4 in MSC-CM. It is suggested that future studies monitor the levels of IL-4 which can result in valuable information about the IL-4 in the paracrine activity of MSCs.

Conclusion

The result of the current study highlights MSCs paracrine activity on the polarization of microglia toward the M2 phenotype, showing the induction of anti-inflammatory status. This strategy can help controlling the intensity of inflammatory responses during neurological diseases.

Acknowledgments

This study was a part of MSc thesis of Babak Davand-Barenji. The authors would like to acknowledge the personnel of the Neurosciences Research Center (NSRC) and Stem Cells Research Center of Tabriz University of Medical Sciences.

Authors' Contribution

BDB and ME performed the experiment, collected data, and prepared the draft. RR and MG designed and supervised the study. All authors reviewed and approved the final version.

Study Highlights

What is current knowledge?

The importance of stem cell paracrine activity on M1/M2 phenotype shifting of microglia

What is new here?

Stem cells can change the phenotype of microglia via paracrine activity.

Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

This study was approved by the Local Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1398.115).

Funding

This study was supported by a grant from the Immunology Research Center of Tabriz University of Medical Sciences (No. 62672).

References

1. Jäkel S, Dimou L. Glial cells and their function in the adult brain: a journey through the history of their ablation. Front Cell Neurosci. 2017;11:24. doi: 10.3389/fncel.2017.00024.
2. Orihuela R, McPherson CA, Harry GJ. Microglial M1/M2 polarization and metabolic states. Br J Pharmacol. 2016;173(4):649-65. doi: 10.1111/bph.13139.
3. Tang Y, Le W. Differential roles of M1 and M2 microglia in neurodegenerative diseases. Mol Neurobiol. 2016;53(2):1181-94. doi: 10.1007/s12035-014-9070-5.
4. Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? Nat Neurosci. 2016;19(8):987-91. doi: 10.1038/nn.4338.
5. Yang X, Xu S, Qian Y, Xiao Q. Resveratrol regulates microglia M1/M2 polarization via PGC-1α in conditions of

neuroinflammatory injury. Brain Behav Immun. 2017;64:162-72. doi: 10.1016/j.bbi.2017.03.003.

6. Zhou S, Zhu W, Zhang Y, Pan S, Bao J. S100B promotes microglia M1 polarization and migration to aggravate cerebral ischemia. Inflamm Res. 2018;67(11-12):937-49. doi: 10.1007/s00011-018-1187-y.
7. Jahed FJ, Rahbarghazi R, Shafaei H, Rezabakhsh A, Karimipour M. Application of neurotrophic factor-secreting cells (astrocyte-like cells) in the in-vitro Alzheimer's disease-like pathology on the human neuroblastoma cells. Brain Res Bull. 2021;172:180-9. doi: 10.1016/j.brainresbull.2021.04.014.
8. Cho DI, Kim MR, Jeong HY, Jeong HC, Jeong MH, Yoon SH, et al. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. Exp Mol Med. 2014;46(1):e70. doi: 10.1038/emm.2013.135.
9. Zhang X, Kong Y, Sun Y, Qian Z, Gao C, Shi X, et al. Bone marrow mesenchymal stem cells conditioned medium protects VSC4.1 cells against 2,5-hexanedione-induced autophagy via NGF-PI3K/Akt/mTOR signaling pathway. Brain Res. 2018;1696:1-9. doi: 10.1016/j.brainres.2018.04.028.
10. Lian H, Roy E, Zheng H. Protocol for primary microglial culture preparation. Bio Protoc. 2016;6(21)e1989. doi: 10.21769/BioProtoc.1989.
11. Hendrickx DAE, van Eden CG, Schuurman KG, Hamann J, Huitinga I. Staining of HLA-DR, Iba1 and CD68 in human microglia reveals partially overlapping expression depending on cellular morphology and pathology. J Neuroimmunol. 2017;309:12-22. doi: 10.1016/j.jneuroim.2017.04.007.
12. Hesp ZC, Yoseph RY, Suzuki R, Jukkola P, Wilson C, Nishiyama A, et al. Proliferating NG2-cell-dependent angiogenesis and scar formation alter axon growth and functional recovery after spinal cord injury in mice. J Neurosci. 2018;38(6):1366-82. doi: 10.1523/jneurosci.3953-16.2017.
13. Rossi C, Cusimano M, Zambito M, Finardi A, Capotondo A, Garcia-Manteiga JM, et al. Interleukin 4 modulates microglia homeostasis and attenuates the early slowly progressive phase of amyotrophic lateral sclerosis. Cell Death Dis. 2018;9(2):250. doi: 10.1038/s41419-018-0288-4.
14. He Y, Gao Y, Zhang Q, Zhou G, Cao F, Yao S. IL-4 switches microglia/macrophage M1/M2 polarization and alleviates neurological damage by modulating the JAK1/STAT6 pathway following ICH. Neuroscience. 2020;437:161-71. doi: 10.1016/j.neuroscience.2020.03.008.
15. Sheshpari S, Shahnazi M, Ahmadian S, Nouri M, Mesgari Abbasi M, Beheshti R, et al. Intra-ovarian injection of bone marrow-derived c-Kit+cells for ovarian rejuvenation in menopausal rats. Bioimpacts. 2022;12(4):325-35. doi: 10.34172/bi.2021.23499.
16. Yamchi NN, Rahbarghazi R, Bedate AM, Mahdipour M, Nouri M, Khanbabaee R. Menstrual blood CD146+mesenchymal stem cells reduced fibrosis rate in the rat model of premature ovarian failure. Cell Biochem Funct. 2021;39(8):998-1008. doi: 10.1002/cbf.3669.
17. Yang H, Wu L, Deng H, Chen Y, Zhou H, Liu M, et al. Anti-inflammatory protein TSG-6 secreted by bone marrow mesenchymal stem cells attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia. J Neuroinflammation. 2020;17(1):154. doi: 10.1186/s12974-020-1731-x.
18. Liu Y, Zeng R, Wang Y, Huang W, Hu B, Zhu G, et al. Mesenchymal stem cells enhance microglia M2 polarization and attenuate neuroinflammation through TSG-6. Brain Res. 2019;1724:146422. doi: 10.1016/j.brainres.2019.146422.
19. Shimojima C, Takeuchi H, Jin S, Parajuli B, Hattori H, Suzumura A, et al. Conditioned medium from the stem cells of human exfoliated deciduous teeth ameliorates experimental autoimmune encephalomyelitis. J Immunol. 2016;196(10):4164-71. doi: 10.4049/jimmunol.1501457.
20. Neubrand VE, Pedreño M, Caro M, Forte-Lago I, Delgado M, Gonzalez-Rey E. Mesenchymal stem cells induce the ramification of microglia via the small RhoGTPases Cdc42 and Rac1. Glia. 2014;62(12):1932-42. doi: 10.1002/glia.22714.
21. Yu H, Xu Z, Qu G, Wang H, Lin L, Li X, et al. Hypoxic preconditioning enhances the efficacy of mesenchymal stem cells-derived conditioned medium in switching microglia toward anti-inflammatory polarization in ischemia/reperfusion. Cell Mol Neurobiol. 2021;41(3):505-24. doi: 10.1007/s10571-020-00868-5.
22. Cherry JD, Olschowka JA, O'Banion MK. Arginase 1+microglia reduce Aβ plaque deposition during IL-1β-dependent neuroinflammation. J Neuroinflammation. 2015;12:203. doi: 10.1186/s12974-015-0411-8.
23. Enam SF, Kader SR, Bodkin N, Lyon JG, Calhoun M, Azrak C, et al. Evaluation of M2-like macrophage enrichment after diffuse traumatic brain injury through transient interleukin-4 expression from engineered mesenchymal stromal cells. J Neuroinflammation. 2020;17(1):197. doi: 10.1186/s12974-020-01860-y.