

Ovarian cancer stem cells induce the M2 polarization of macrophages through the PPAR γ and NF- κ B pathways

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Abstract. Increasing evidence suggests an association between cancer stem cells and the tumor microenvironment. Ovarian cancer stem cell (OCSC) factors can influence the tumor microenvironment and prognosis. However, the effects of OCSCs on macrophage M1/M2 polarization are not yet completely understood. In the present study, we evaluated the effects of OCSCs on macrophage M1/M2 polarization. In addition, we investigated whether the activation of the peroxisome proliferator-activated receptor γ (PPAR γ)/nuclear factor- κ B (NF- κ B) pathway is involved in these effects, thus modulating the M1/M2 differentiation of monocytes into macrophages. The expression levels of markers of the M1 state, such as tumor necrosis factor (TNF)- α , inducible nitric oxide synthase (iNOS) and CD86, as well as those of markers of M2 activation, such as mannose receptor (MR), interleukin (IL)-10 and arginase-1 (Arg-1), were measured by RT-qPCR. We found that the OCSCs promoted the M2 polarization of Raw264.7 macrophages by upregulating the expression of MR, IL-10 and Arg-1, while the expression levels of M1 macrophages markers, including TNF- α , iNOS and CD86 were suppressed. In addition, treatment with OCSCs activated PPAR γ and suppressed NF- κ B in the Raw264.7 cells. Furthermore, the PPAR γ antagonist GW9662, attenuated the promoting effects of OCSCs on the M2 polarization of macrophages. To the best of our knowledge, the findings of the present study, provide the first evidence that OCSCs promote the M2 polarization of macrophages through the PPAR γ /NF- κ B pathway.

Introduction

Ovarian cancer is the leading cause of cancer-related mortality among women worldwide and it predominately affects postmenopausal women, with approximately 204,000 women being diagnosed with the disease each year (1). More than 80% of patients with ovarian cancer eventually have a relapse with chemoresistant disease (2,3); therefore, causes a negative impact on the treatment of ovarian cancer.

Cancer stem cells (CSCs) are a small subset of cells capable of initiating and sustaining tumor growth (4). The CSC hypothesis posits that CSCs are a minority population of self-renewing cancer cells that fuel tumor growth and remain in patients after conventional therapy has been completed (5). Therefore, therapies focusing on CSCs seem to be more effective for the treatment of cancer.

Several studies have identified the existence of CSCs in an increasingly longer list of solid tumors (4), including ovarian cancer (6-10). Recurrent ovarian tumors are known to be enriched with CSC-like cells (11). It has been suggested that ovarian CSCs (OCSCs) have the capacity to survive treatment and they may recreate the original patient tumor in animal models (12,13).

A recent study on cancer biology demonstrated that CSCs and the tumor microenvironment seem to be related (14). Tumors can influence the microenvironment by releasing extracellular signals, promoting tumor angiogenesis and inducing peripheral immune tolerance, while immune cells in the microenvironment can affect the growth and evolution of cancer cells, such as in immunoediting (15-17). A recent study revealed that OCSC factors can influence the tumor microenvironment and prognosis (18). The current study was designed to investigate the role of OCSCs in the M1/M2 polarization of macrophages, as well as to elucidate the underlying molecular mechanisms.

Materials and methods

Cell culture. The mouse monocyte macrophage cell line, Raw264.7, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂. The peroxisome proliferator-activated receptor γ (PPAR γ) antagonist, GW9662,

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was purchased from Cayman Chemical Co. (Ann Arbor, MI, US) and dissolved in 25% DMSO. The Raw264.7 cells were treated with GW9662 at the concentration of 0.1 μ M.

Isolation of OCSCs. The mouse ovarian cancer cell line, OVHM, was obtained from the Key Laboratory of Gynecological Oncology, Qilu Hospital of Shandong University, Jinan, China and cultured in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies) at 37°C with 5% CO₂. A stem-like cell subpopulation from the ovarian cancer cells was obtained and separated through the suspension culture of the OVHM cells in serum-free medium. The OVHM cells were cultured in serum-free DMEM supplemented with epidermal growth factor (20 ng/ml; Invitrogen Life Technologies), basic fibroblast growth factor (20 ng/ml; Invitrogen Life Technologies) and B27 (2%; Gibco-BRL). The cells in suspension formed spheroid structures. The primary spheres were dissociated to generate single cells and serially diluted to plate into 96-well plates at one cell per well. The cells were visualized under a microscope (TS100; Nikon, Tokyo, Japan), and the wells containing one single cell were marked. The secondary spheres were dissociated and plated into serum-free medium at 50 cells/cm². Sphere-forming cells were passaged up to passage (P)7 and then used for the experiments.

Co-culture of Raw264.7 cells with OCSCs. For co-culture experiments, 0.4- μ m pore size Transwell inserts (Corning Inc., Corning, NY, USA) were plated into a 6-well plate. The Raw264.7 cells were seeded at the bottom well, while the OCSCs were seeded onto the inserts.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies). The reverse transcription of 1 μ g of RNA was performed using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania), following the manufacturer's instructions. Quantitative PCR (qPCR) was performed using SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA, USA) in a 7900HT fast Real-Time PCR System (Applied Biosystems). The relative levels of gene expression were calculated by the comparative CT method.

Western blot analysis. The specific primary antibodies were rabbit polyclonal to PPAR γ (1:800; sc-7196; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal to nuclear factor κ B (NF- κ B; 1:1000; sc-372; Santa Cruz Biotechnology) and rabbit polyclonal to GAPDH (1:2000; ab37168; Abcam, Cambridge, MA, USA). The cells were lysed in ice-cold cell lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 1% sodium deoxycholate, 1% SDS and 2 mM phenylmethylsulfonyl fluoride). Total cellular protein (20 mg) was loaded per lane, separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) by electroblotting. After blocking with 5% non-fat milk (Inner Mongolia Yili Industrial Group Co., Ltd., Inner Mongolia, China) at 4°C overnight, the membrane was incubated with primary antibodies for 2 h at room temperature followed by incubation with horseradish

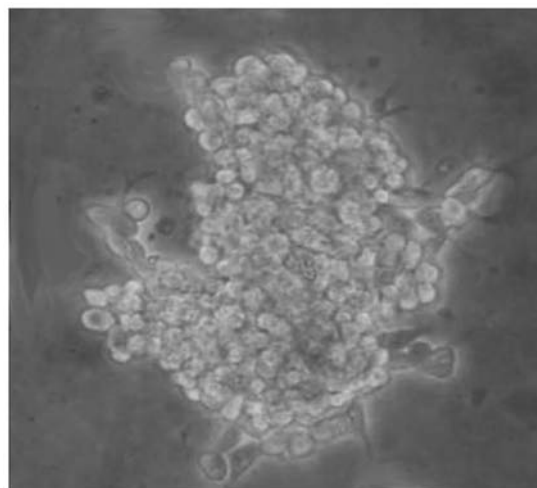


Figure 1. Representative photomicrograph of the OVHM ovarian cancer cells organized in spheroid structure in serum-free medium. Magnification, $\times 400$.

peroxidase-conjugated anti-rabbit secondary antibody (1:2000; sc-2004; Santa Cruz Biotechnology) for 1 h at room temperature. The signals were probed using an ECL Western Blotting kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Immunofluorescence staining. CD117 and CD44 are specific markers of OCSCs (6,19). Immunofluorescence staining was used in the present study to detect the expression of CD117 and CD44 in the OCSCs. After washing with PBS, the cells were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO, USA) at 4°C for 1 h. The cells were then permeabilized by exposure to 0.2% TritonX-100 (Sigma) at 4°C for 1 h, followed by blocking with normal goat serum (Maixin, Fuzhou, Fujian, China) at 4°C for a further 1 h. The cells were then incubated with primary antibodies [rabbit polyclonal to CD117 (1:800; ab5506) and rabbit monoclonal to CD44 (1:400; ab51037); Abcam] for 2 h at room temperature. After washing with PBS, [Alexa Fluor 488 labeled Goat anti-rabbit IgG (1:400; ab150077); Abcam] was added and the cells were incubated at 37°C for 1 h. The nucleus was stained with DAPI (Invitrogen Life Technologies) and the slides were visualized under a fluorescence microscope (80i; Nikon).

Statistical analysis. The results were analyzed using SPSS statistical software version 19.0 (SPSS, Inc., Chicago, IL, USA). All the data are presented as the means \pm SD. Statistically significant differences between 2 groups were compared using the Student's t-test, and P-value <0.05 was considered to indicate a statistically significant difference.

Results

Identification of OCSCs. In the present study, a self-renewing, stem-like cell subpopulation from the ovarian cancer cell line, OVHM, was isolated using non-adherent suspension culture. Fig. 1 shows the spheroid structure of the OCSCs in serum-free medium. Immunofluorescence staining was used to confirm the specific markers expressed in the isolated OCSCs. The images from immunofluorescence staining are presented in Fig. 2. The OCSCs showed positively staining for CD117 and CD44.

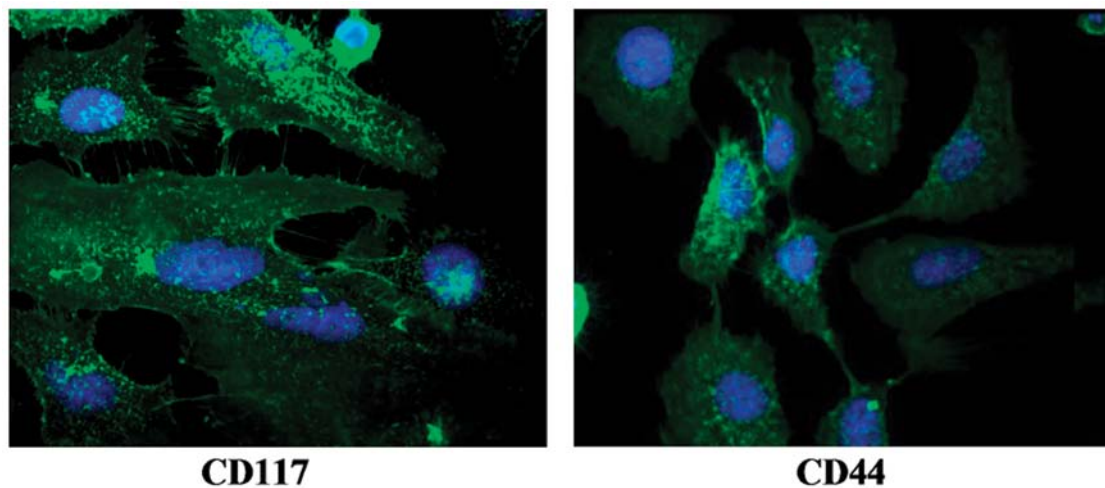


Figure 2. Immunofluorescence staining of CD117 and CD44 in the ovarian cancer stem cells (OCSCs). Cells with a positive signal were stained green. Nuclear DNA was stained with DAPI (blue). Magnification, x400.

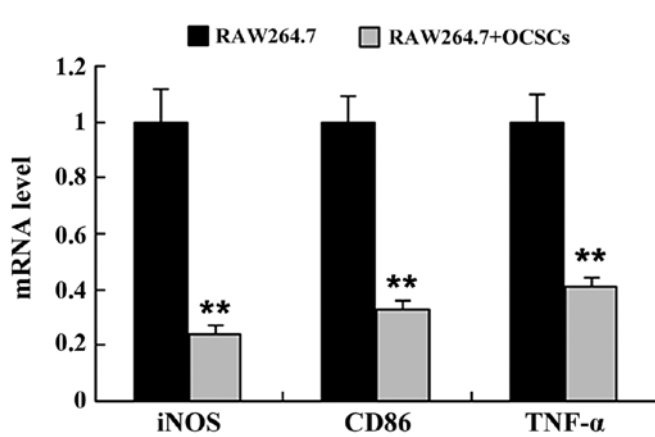


Figure 3. Expression of M1 macrophage markers in the Raw264.7 cells cultured alone or co-cultured with OCSCs determined by RT-qPCR. **P<0.01, compared with the Raw264.7 group. OCSCs, ovarian cancer stem cells; iNOS, inducible nitric oxide synthase; CD86, CD86 molecule; TNF-α, tumor necrosis factor-α.

OCSCs promote the M2 polarization of macrophages. To determine whether OCSCs affect macrophage polarization, the Raw264.7 cells were co-cultured with the OCSCs, and the expression levels of markers of the M1 macrophage phenotype [inducible nitric oxide synthase (iNOS), CD86 and tumor necrosis factor (TNF)-α] and the M2 macrophage phenotype [mannose receptor (MR), interleukin (IL)-10 and arginase-1 (Arg-1)] were measured by RT-qPCR. As shown in Figs. 3 and 4, co-culture of the Raw264.7 cells with OCSCs significantly induced the mRNA expression of markers of the M2 macrophage phenotype, including MR, IL-10 and Arg-1, whereas the mRNA expression of markers of the M1 macrophage phenotype, including iNOS, CD86 and TNF-α was markedly decreased.

OCSCs affects PPARγ/NF-κB expression in Raw264.7 cells. Subsequently, we examined the expression of PPARγ and NF-κB in the Raw264.7 cells co-cultured with OCSCs.

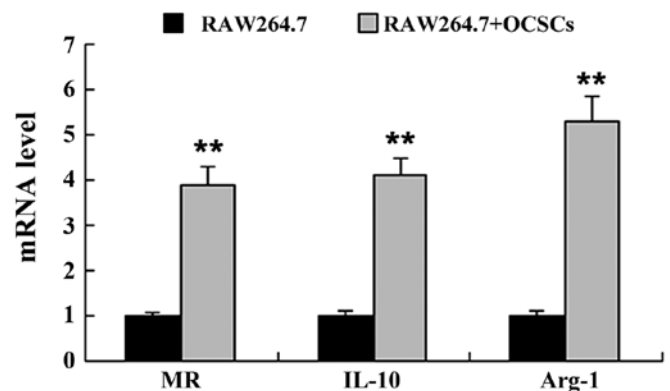


Figure 4. Expression of M2 macrophage markers in the Raw264.7 cells cultured alone or co-cultured with OCSCs determined by RT-qPCR. **P<0.01, compared with the Raw264.7 group. OCSCs, ovarian cancer stem cells; MR, mannose receptor; IL-10, interleukin 10; Arg-1, arginase I.

Western blot analysis revealed that, in comparison with the Raw264.7 cells cultured alone, co-culture with OCSCs significantly increased the relative protein level of PPARγ, while it decreased the relative protein level of NF-κB (Fig. 5).

GW9662 affects PPARγ/NF-κB expression in Raw264.7 cells. GW9662 was used to treat the Raw264.7 cells co-cultured with OCSCs, and the protein expression levels of PPARγ and NF-κB in the Raw264.7 cells were the measured by western blot analysis. As is shown in Fig. 6, following treatment with GW9662, the expression of PPARγ was significantly decreased in the Raw264.7 cells co-cultured with the OCSCs. In addition, NF-κB expression in the Raw264.7 cells co-cultured with the OCSCs was altered following treatment with GW9662. GW9662 led to an increase in NF-κB expression in the Raw264.7 cells co-cultured with the OCSCs.

Inhibition of PPARγ attenuates the promoting effects of OCSCs on the M2 polarization of macrophages. To examine the role of PPARγ in mediating the promoting effects of OCSCs on the M2 polarization of macrophages, PPARγ was inhibited by

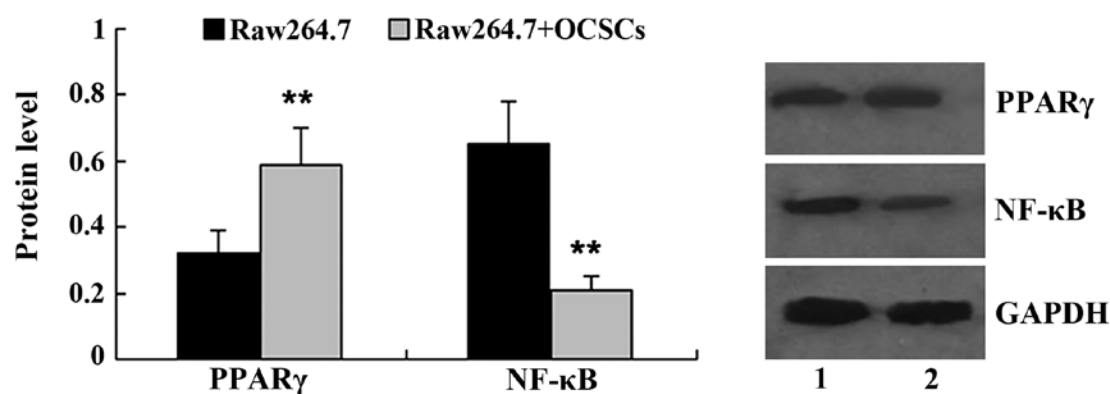


Figure 5. Expression of PPAR γ and NF- κ B in Raw264.7 cells cultured alone or co-cultured with the OCSCs determined by western blot analysis. Lane 1, Raw264.7 group; lane 2, Raw264.7+OCSCs group. ** $P < 0.01$, compared with the Raw264.7 group. OCSCs, ovarian cancer stem cells; PPAR γ , peroxisome proliferator-activated receptor γ ; NF- κ B, nuclear factor- κ B.

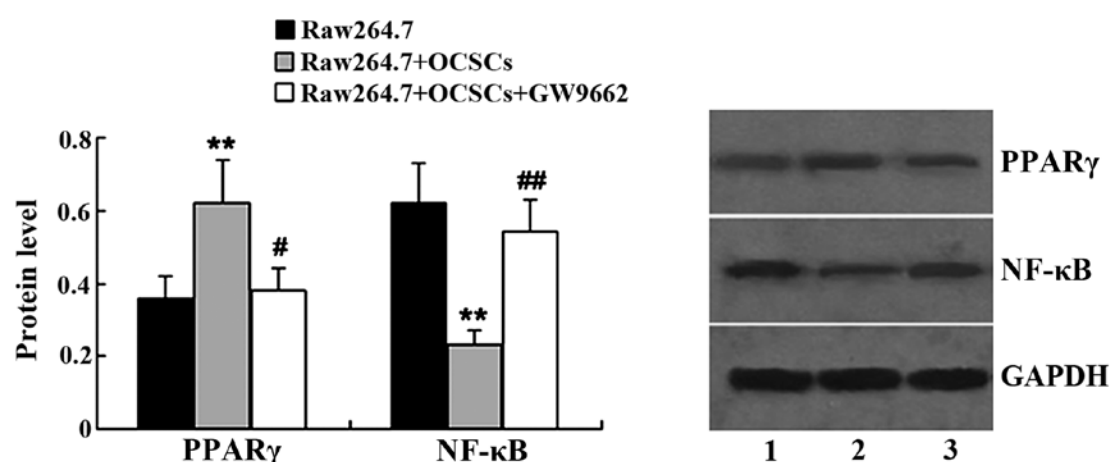


Figure 6. Expression of PPAR γ and NF- κ B in the Raw264.7 cells co-cultured with the OCSCs following treatment with GW9662 (PPAR γ inhibitor). Lane 1, Raw264.7 group; lane 2, Raw264.7 + OCSCs group; lane 3, Raw264.7 + OCSCs + GW9662 group. ** $P < 0.01$, compared with the Raw264.7 group; # $P < 0.05$ and ## $P < 0.01$, compared with the Raw264.7 + OCSCs group. OCSCs, ovarian cancer stem cells; PPAR γ , peroxisome proliferator-activated receptor γ ; NF- κ B, nuclear factor- κ B.

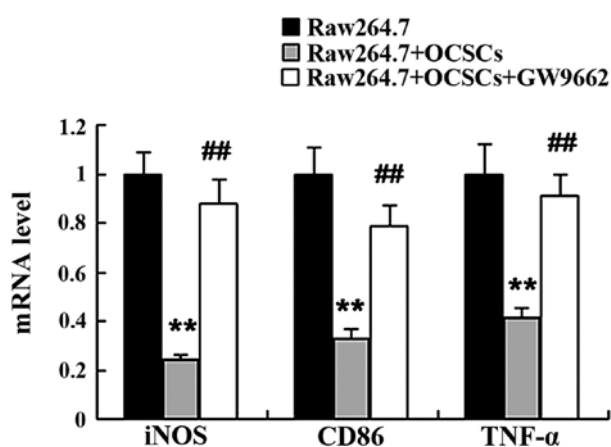


Figure 7. Expression of M1 macrophage markers in the Raw264.7 cells co-cultured with the OCSCs following treatment with GW9662 (PPAR γ inhibitor). ** $P < 0.01$, compared with the Raw264.7 group; ## $P < 0.01$, compared with the Raw264.7 + OCSCs group. OCSCs, ovarian cancer stem cells; iNOS, inducible nitric oxide synthase; CD86, CD86 molecule; TNF- α , tumor necrosis factor- α .

expression of MR, IL-10 and Arg-1, and a decreased mRNA expression of iNOS, CD86 and TNF- α . However, this effect was reversed by treatment with GW9662. Compared with the Raw264.7 cells co-cultured with the OCSCs, the expression of markers of the M2 macrophage phenotype was significantly decreased, while that of markers of the M1 macrophage phenotype was significantly increased following treatment with GW9662 (Figs. 7 and 8).

Discussion

The tumor microenvironment is a complex cellular environment in which the tumor exists; it consists of cancer cells, fibroblasts, immune cells, surrounding blood vessels, cytokines, chemicals and the extracellular matrix (20-25). The interaction between cancer cells and the tumor microenvironment plays an important role in tumor progression.

Macrophages are a heterogeneous cell population which exists in abundance in the tumor microenvironment, adapting and responding to a large variety of tumor-secreted cytokines (26). Tumor-associated macrophages (TAMs) can influence tumor growth, invasion, angiogenesis and metas-

treatment with GW9662. We found that the OCSCs promoted the M2 polarization of macrophages with an increased mRNA

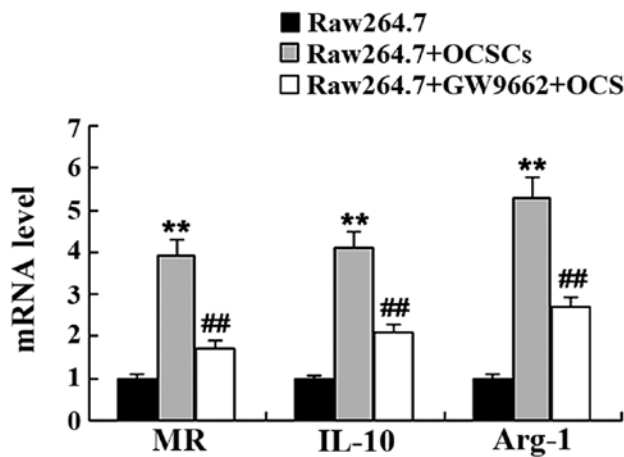


Figure 8. Expression of M2 macrophage markers in the Raw264.7 cells co-cultured with the OCSCs following treatment with GW9662 (PPAR γ inhibitor). **P<0.01, compared with the Raw264.7 group; ##P<0.01, compared with the Raw264.7 + OCSCs group. OCSCs, ovarian cancer stem cells; MR, mannose receptor; IL-10, interleukin 10; Arg-1, arginase I.

tasis. There are mainly two types of polarized macrophages, termed M1 and M2 macrophages. Th1 cytokines, such as lipopolysaccharide (LPS), IL-1 β and interferon γ (IFN γ), promote monocyte differentiation into a 'classical' M1 macrophage phenotype, while Th2 cytokines, such as IL-4 and IL-13, lead to an 'alternative' M2 macrophage phenotype. M1 macrophages are generally anti-tumoral and they primarily produce pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-12 (27). M2 macrophages on the other hand exert pro-tumoral effects and they produce anti-inflammatory factors, including transforming growth factor (TGF)- β , IL-10 and IL-1 receptor antagonist, as well as promote angiogenesis and tissue remodeling (27-29). Moreover, upon specific signals, macrophages can switch from an activated M1 state back to an M2 state and vice versa (30).

Raw264.7 macrophages have been identified as 'innate' macrophages that can differentiate into M1 or M2 macrophages (31). In the present study, we demonstrated the interplay between OCSCs and macrophages. Following co-culture with OCSCs, the macrophages acquired an M2 phenotype characterized by an increased expression of MR, IL-10 and Arg-1, while the expression of M1 macrophages markers, including TNF- α , iNOS and CD86, was suppressed.

NF- κ B is one of the major transcription factors responsible for the development of immune responses. NF- κ B activation has been associated with the enhanced transcriptional activity of chemokines, cytokines and adhesion molecules, which can facilitate the recruitment and activation of inflammatory cells to the site of NF- κ B activation. Gao *et al* found that mouse mesenchymal stem cells induced macrophage M2 activation through NF- κ B (32). As expected, in the present study, the expression of NF- κ B in the Raw264.7 cells was inhibited by OCSCs.

PPAR γ , a ligand-activated nuclear receptor, is abundantly expressed in macrophages. PPAR γ can be activated by natural and synthetic ligands, such as 15d-PGJ2 (33), antidiabetic thiazolidinediones (TZDs) and the GW1929 compound (34). During the differentiation of monocytes into macrophages,

the expression of PPAR γ is rapidly induced (35). PPAR γ modulates the immune inflammatory response with anti-inflammatory properties (36). It has been suggested that PPAR γ inhibits the activation of inflammatory response genes (such as IL-2, IL-6, IL-8, TNF- α and metalloproteases) by negatively interfering with NF- κ B (37). In the present study, we further investigated the effects of OCSCs on PPAR γ activation in macrophages. The results revealed that the expression of PPAR γ was significantly increased in the Raw264.7 cells co-cultured with OCSCs.

To determine whether PPAR γ and NF- κ B play a role in mediating the effects of OCSCs on the M2 polarization of macrophages, GW9662, a potent and selective antagonist of full-length PPAR γ was added to block PPAR γ activation. Our results revealed that GW9662 effectively suppressed PPAR γ activation in the Raw264.7 cells co-cultured with OCSCs, while the expression of NF- κ B increased. Furthermore, treatment with GW9662 influenced M1 and M2 marker expression in the Raw264.7 cells co-cultured with OCSCs. These results support the view that the effects of OCSCs on the M2 polarization of macrophages may be attenuated by treatment with PPAR γ inhibitor.

Taken together, the results of the present study suggest a potential functional association between OCSCs and the M1/M2 polarization of macrophages. OCSCs are capable of promoting the M2 polarization of macrophages by affecting the PPAR γ /NF- κ B pathway.

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