

SHORT COMMUNICATION

Tumour cell-derived exosomes endow mesenchymal stromal cells with tumour-promotion capabilities

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Mesenchymal stromal cells (MSCs) are a major component of the tumour microenvironment. A plethora of elegant studies focusing on tumour-derived MSCs have shown that they, unlike normal MSCs in other tissue, exhibit a strong ability to promote tumour progression. However, the mechanisms underlying the conversion of normal MSCs into tumour-associated MSCs are unknown. We report here a critical role of tumour cell-derived exosomes in endowing bone marrow-derived MSCs (BM-MSCs) with a tumour-favourable phenotype. Tumour cell-derived exosomes affected neither the growth factor production nor the immunosuppressive property of MSCs; rather, they endowed MSCs with a strong ability to promote macrophage infiltration into B16-F0 melanoma or EL-4 lymphoma. Ablation of macrophages by clodronate liposome administration reversed the tumour-promoting effect of MSCs educated by tumour cell-derived exosomes (TE-MSCs) on the tumour growth. By comparing the chemokine profile of BM-MSCs with that of TE-MSCs, we found that TE-MSCs produced a large amount of CCR2 ligands, CCL2 and CCL7, which are responsible for macrophage recruitment. CCR2-specific inhibitor was found to block the tumour-promoting effect of TE-MSCs. Thus, our investigations demonstrated that tumour cell-derived exosomes confer BM-MSCs the ability to enhance tumour growth. Therefore, we uncovered a novel mechanism underlying the conversion of normal MSCs to tumour-associated MSCs.

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INTRODUCTION

Tumour microenvironment, variably composed of endothelial cells, immune cells, mesenchymal stromal cells (MSCs), fibroblasts, extracellular matrix and soluble factors, has a critical role in orchestrating tumour growth.^{1,2} The nonmalignant cellular and acellular components provide a fertile bed for tumour initiation, progression and metastasis.^{1,2} Recent studies demonstrated that tumour resident MSCs had an important role in coordinating the tumour microenvironment.^{3–5} After mobilized and integrated into the tumour stroma upon sensing the cues of a tumour, MSCs acquire a series of properties to promote tumour growth, including producing growth factors, inhibiting anti-tumour immune responses, shaping tumour inflammatory environment and building the niche for tumour cells.^{6–12} Importantly, recent studies revealed that the enhanced tumour promotion ability of tumour-derived MSCs is not intrinsic, rather acquired upon arrival at the tumour microenvironment.^{3,4,7,11} However, the underlying mechanisms of the conversion of normal MSCs into tumour-favourable MSCs remain unclear.

Exosomes are microvesicles (30–100 nm) originating from the multivesicular bodies and can be isolated from diverse body fluids and cell culture supernatant.¹³ The contents of exosomes are complex, including various types of proteins, RNAs and lipids and they can act as messengers for cell communication.^{13–15} Interestingly, a series of studies have identified exosomes as one of the important mediators of the interaction between tumour cells and their surrounding cells in the tumour microenvironment.^{16–20} It

has been reported that exosomes from tumour-associated MSCs participate into tumour promotion by transducing micro-RNAs into tumour cells.^{21,22} However, the influence of tumour cell-derived exosomes on MSCs is still largely unknown. In the present study, we found that, in comparison with bone marrow-derived MSCs (BM-MSCs), tumour cell-derived exosome-educated MSCs (TE-MSCs) could significantly promote tumour growth. This tumour-promoting effect of TE-MSCs was independent of their growth factor production and the suppression on T cells. Instead, this effect was relied on a vigorous production of CCR2 ligands by TE-MSCs and the resulting recruitment of macrophages into tumour sites. Administration of clodronate liposomes or a CCR2-specific inhibitor could abolish the tumour-promoting effect of TE-MSCs. Therefore, our investigation demonstrated a critical role of tumour cell-derived exosomes in endowing BM-MSCs with tumour promotion capability.

RESULTS AND DISCUSSION

Various investigations have shown that tumour-associated MSCs can promote tumour growth through released exosomes. However, it is unknown whether tumour cell-derived exosomes could endow BM-MSCs with the capability to promote tumour growth. To this end, we employed the mouse B16-F0 melanoma model. We first isolated exosomes from the culture medium of tumour cells using a commercially available total exosomes isolation kit following the provided protocol. The diameter of the harvested

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particles was determined by transmission electron microscopy and found to be within range of 30–100 nm (Figure 1a), consistent with the reported diameters of exosomes.^{13,15} In addition, these particles were positive for CD9, TSG101 and CD61 (Figure 1b), while the intracellular proteins like CYC1, Grp94 and CANX were absent, verifying that the isolated particles were exosomes.^{13,15}

Although exosomes have been identified in different experimental systems, the mechanisms that exosomes exert their biological effects are different. Recent studies demonstrated that endocytosis is a critical process for exosomes to deliver messages to target cells.^{19,23} To test whether tumour cell-derived exosomes could enter MSCs, we labelled exosomes isolated from tumour cells with red fluorescent lipid dye PKH26 and cultured these labelled exosomes with GFP-MSCs. As shown in Figure 1c, the exosomes were effectively internalized by MSCs.

On the basis of the newly recognized role of exosomes in mediating horizontal transfer of information between cells,^{20,21} we hypothesized that tumour cell-derived exosomes are the driving force for the formation of tumour-associated MSCs. To test this hypothesis, we cultured BM-MSCs with or without B16-F0

melanoma cells derived exosomes for 6 days and then inoculated these MSCs together with B16-F0 melanoma cells to the hind limbs of C57BL/6 mice intramuscularly. Strikingly, MSCs cultured with tumour cell-derived exosomes (TE-MSCs) were found to significantly promote the growth of B16-F0 melanoma, while BM-MSCs had little effect (Figure 1d).

Previous studies have revealed that MSCs are rich sources of growth factors, which play an important role in facilitating tumour growth.^{7,8,12} We wondered whether the tumour-promoting effect of TE-MSCs was dependent on the production of growth factors. We thus compared the changes in the expression of growth factors in BM-MSCs and TE-MSCs by employing a growth factor PCR array. Little difference was observed in growth factor expression between TE-MSCs and BM-MSCs (Supplementary Table). In addition, conditioned medium of TE-MSCs exhibited similar effects on B16 melanoma growth as with conditioned medium of BM-MSCs (Supplementary Figure 1a).

Immunoregulation is one of the most prominent properties of MSCs.^{9,24} MSC-mediated immunosuppression has been demonstrated to promote tumour growth in many models, such as B16

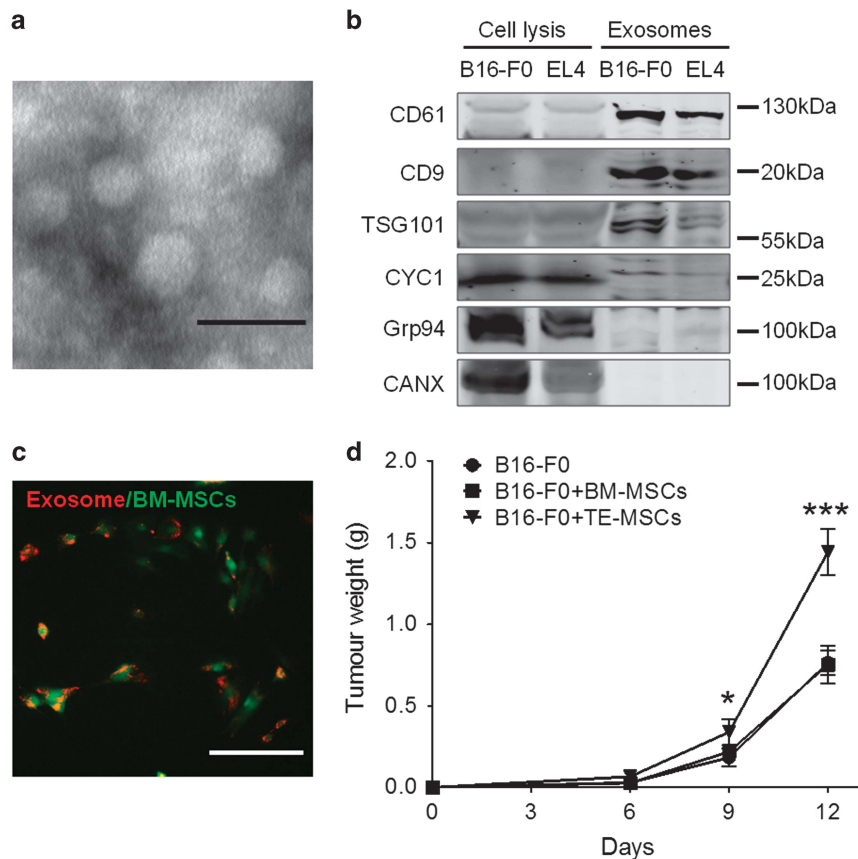


Figure 1. Tumour cell-derived exosomes endowed BM-MSCs with the ability to promote tumour growth *in vivo*. **(a)** A representative electron microscope (PHILIPS CM120, Eindhoven, Netherlands) image of tumour cell-derived exosomes. Scale bar, 50 nm. Tumour cell-derived exosomes were isolated from tumour conditioned medium using an exosomes isolation kit (Invitrogen Carlsbad, CA, USA). **(b)** Tumour cells and tumour cell-derived exosomes were lysed with RIPA lysis buffer (Millipore, Darmstadt, Germany) containing a protease inhibitor cocktail (Sigma, St Louis, MO, USA). Protein concentration was quantified by protein assay (Bio-rad, Hercules, CA, USA), and analysed the exosomes markers CD61 (Cell Signaling, Danvers, MA, USA), CD9 (Cell Signaling), TSG101 (Abcam, Cambridge, MA, USA), CYC1 (Cell Signaling), Grp94 (Cell Signaling) and CANX (Santa Cruz, Dallas, TX, USA). **(c)** Exosomes derived from tumour cells labelled with PKH26 Dye (Sigma) were cultured with BM-MSCs isolated from GFP transgenic mouse for 24 h. The uptake of exosomes by BM-MSCs was observed under fluorescent microscope. Scale bar, 200 μ m. **(d)** B16-F0 melanoma cells (1×10^6) were inoculated into the hind legs of C57BL/6 mice intramuscularly alone or with BM-MSCs or TE-MSCs (2×10^5), which were derived by culturing BM-MSCs with B16-F0-derived exosomes for 6 days (exosomes were added daily at a final protein concentration of 5 μ g/ml). The fetal bovine serum used in our experiments was depleted of exosomes by 18 h ultracentrifugation. Tumours were excised and weighed on days 6, 9 and 12 post tumour inoculation. All procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Health Sciences, Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences. Data was shown as mean \pm s.d. ($n \geq 4$). Statistical significance was assessed by nonpaired two-tailed Student's *t*-test, with significance set as $P < 0.05$; ns, no significance, * $P < 0.05$, *** $P < 0.001$.

melanoma and EL-4 lymphoma.²⁵ Therefore, it is possible that TE-MSCs harbour stronger inhibitory effects on T-cell-mediated anti-tumour immune responses. We analysed the types and prevalence of tumour-infiltrated T lymphocytes and found that the percentages of CD4⁺, CD8⁺ T cells and Tregs in tumours were not influenced by TE-MSCs (Supplementary Figures 2a-d). We also found in *in vitro* experimental system that no enhanced

suppression on T-cell response was observed in TE-MSCs (Supplementary Figure 1b). Thus, the tumour-promoting effect of TE-MSCs does not rely on their immunosuppressive effect on T cells.

In addition to their potential to regulate T-cell responses, tumour-associated MSCs have been demonstrated to facilitate tumour development through recruiting macrophages and

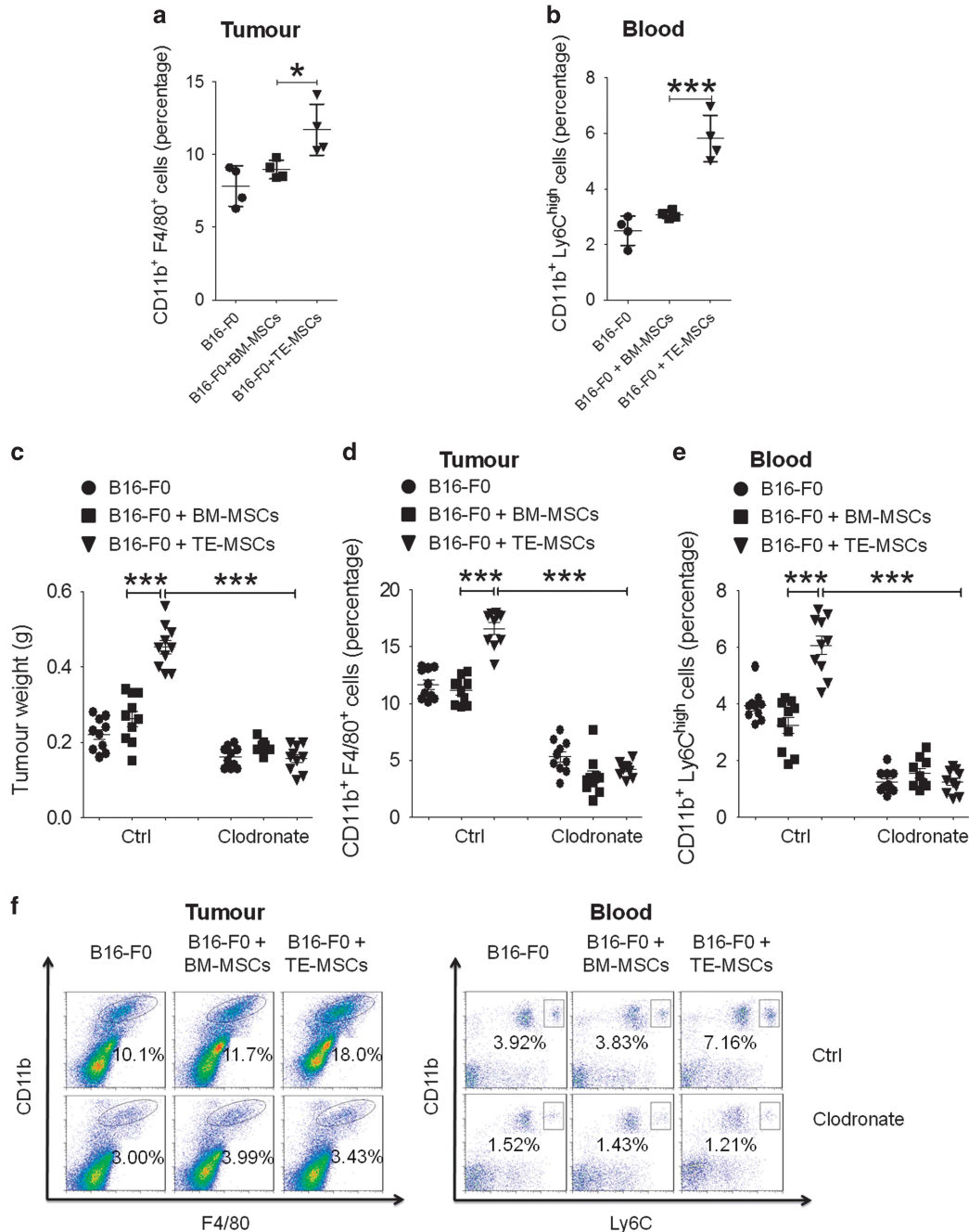


Figure 2. The effect of TE-MSCs on tumour growth was dependent on macrophage recruitment. (a and b) B16-F0 melanoma cells (1×10^6) were inoculated into the hind legs of C57BL/6 mice intramuscularly with or without BM-MSCs or TE-MSCs (2×10^5). Nine days post tumour inoculation, single-cell suspensions were prepared from tumour and peripheral blood and analysed for the cell population of CD11b⁺F4/80⁺ cells and CD11b⁺Ly6C^{high} cells. All these fluorescence conjugated antibodies were purchased from eBioscience (La Jolla, CA, USA). (c-f) Clodronate liposomes (Encapsula, NanoSciences, Brentwood, TN, USA) and control (Ctrl) liposomes were intravenously injected to B16-F0-bearing mice with BM-MSCs or TE-MSCs injection every other day. Nine days post tumour inoculation, tumours were excised and weighed. Single-cell suspensions prepared from tumour were analysed for the presence of CD11b⁺F4/80⁺ cells and CD11b⁺Ly6C^{high} cells by flow cytometry (BD calibur, BD Bioscience, Shanghai, China). Results were representative of three independent experiments. Data was shown as mean \pm s.d. (more than nine mice in each group). Statistical significance was assessed by nonpaired two-tailed Student's *t*-test, with significance set as ns, no significance, **P* < 0.05, ****P* < 0.001.

monocytes into tumours.^{3,4} We, thus determined the percentage of macrophages in tumour sites and monocytes in peripheral blood from mice with established B16-F0 melanoma by melanoma cell alone, or together with BM-MSCs or TE-MSCs. Flow cytometrical analysis revealed that tumours from mice with TE-MSCs administration exhibited significantly more macrophage (CD11b⁺F4/80⁺) accumulation than that from tumours with BM-MSCs inoculation (Figure 2a). As monocytes are considered as precursors of macrophages, we further examined CD11b⁺Ly6C^{high} monocytes in the peripheral blood. Consistently, the higher percentage of CD11b⁺Ly6C^{high} monocytes in the peripheral blood of mice bearing TE-MSCs co-inoculated tumour strongly suggests that these circulating monocytes could contribute to the accumulation of macrophages in the tumour microenvironment

(Figure 2b). To verify whether the tumour-promoting effect of TE-MSCs was due to the enhanced macrophage recruitment, we depleted macrophages using clodronate liposomes. We found that following clodronate liposomes administration, the percentage of tumour resident macrophages and circulating monocytes dramatically decreased to the comparable level of mice treated with either TE-MSCs or BM-MSCs alone (Figures 2d-f). Interestingly, depletion of macrophages completely eliminated the promoting effect of TE-MSCs on tumour growth (Figure 2c), suggesting a critical role of macrophages in TE-MSC-mediated tumour promotion. Consistence with previous study,²⁶ clodronate administration was shown to reduce tumour weight in mice injected B16-F0 melanomas alone, further emphasizing the role of macrophages in tumour development.²⁶

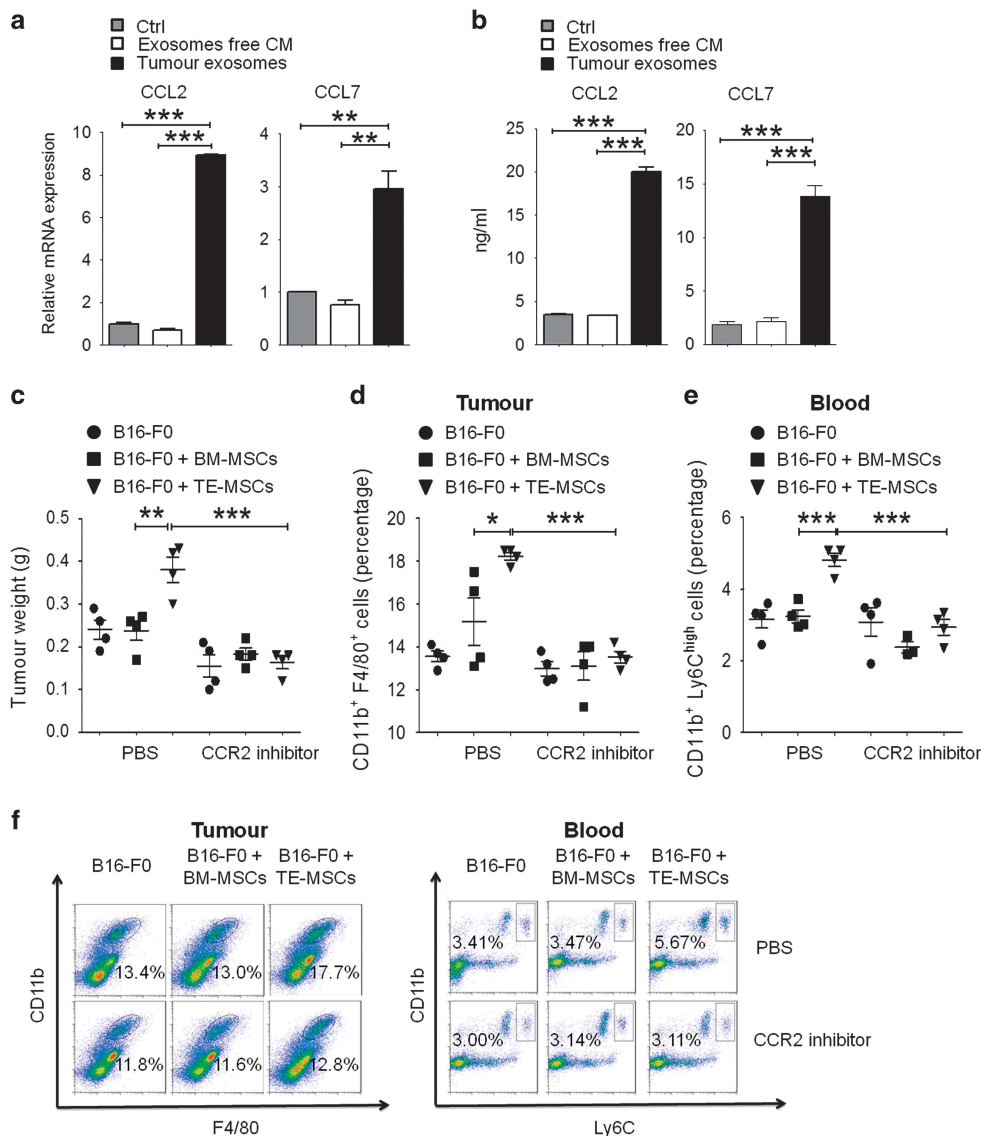


Figure 3. The dependence of CCR2 ligand production during TE-MSC-mediated recruitment of macrophages. **(a)** A total of 1×10^3 BM-MSCs or TE-MSCs were seeded in a six-well plate, with the addition of exosomes (at a final concentration of $5 \mu\text{g/ml}$) every day. After 6 days incubation, MSCs were collected and analysed for the mRNA expression of CCL2 and CCL7. Data was shown as mean \pm s.e.m. **(b)** To analyse the protein levels of CCL2 and CCL7 in BM-MSCs or TE-MSCs, 1×10^5 MSCs were seeded in six-well plate, supernatant was collected after 24 h for ELISA analysis. CCL2 ELISA kit was purchased from R&D (Minneapolis, MN, USA), CCL7 ELISA kit was purchased from eBioscience (La Jolla, CA, USA). Data was shown as mean \pm s.e.m. **(c–f)** RS504393 (Sigma), a selective CCR2 inhibitor, was intraperitoneally injected daily (2.5 mg/kg) to B16-F0 melanoma bearing mice with or without BM-MSCs or TE-MSCs coadministration. Tumours were excised and weighed 9 days post tumour inoculation. Single-cell suspensions prepared from tumour were analysed for CD11b⁺F4/80⁺ cells and CD11b⁺Ly6C^{high} cells by flow cytometry. Results were representative of three independent experiments. Data was shown as mean \pm s.d. (more than four mice in each group). Statistical significance was assessed by nonpaired two-tailed Student's *t*-test, with significance set as ns, no significance, ***P* < 0.01, ****P* < 0.001.

The CCR2 chemotaxis axis is important for macrophage accumulation at tumour sites.^{4,27} In comparison with BM-MSCs, we found that TE-MSCs produced higher levels of CCL2 and CCL7 (Figures 3a and b), supporting the observation that more macrophages were recruited to B16-F0 melanoma when injected with TE-MSCs. And the regulation of tumour-derived exosomes on CCL2 and CCL7 expression showed a dose-dependent manner (Supplementary Figure 3). To further test the role of these CCR2 ligands, we employed RS504393, a highly selective CCR2 antagonist, to verify the role of CCR2 ligand-mediated macrophage recruitment in the tumour promotion by TE-MSCs. Interestingly, the tumour-promoting effect and the increased macrophage recruitment by TE-MSCs were both diminished upon RS504393 treatment (Figures 3c-f). Besides, blockade of CCR2 by its inhibitor can reduce the tumour weight in mice with tumour cell injection alone. These results strongly suggest that TE-MSCs recruit more macrophages to tumour sites via producing high level of CCR2 ligands and subsequently facilitate tumour growth.

The role MSCs in tumour progression has been investigated in many studies. MSCs are believed to promote tumour growth through various mechanisms, including growth factor production, immunomodulation and pro-angiogenesis.^{8,9,12} Our study reveals a novel mechanism for the establishment of the tumour-favourable microenvironment by MSCs. In this process, BM-MSCs are enticed by tumour cells released exosomes and evolve into tumour-associated MSCs, with the tumour-promoting capability. This phenomenon can be extended to the EL-4 lymphoma model (Supplementary Figures 4a-c). Therefore, our work provides a new insight into the understanding of the complexity of the interaction between tumour cells and MSCs.

ABBREVIATIONS

BM-MSCs, bone marrow-derived mesenchymal stromal cells; TE-MSCs, tumour cell-derived exosomes-educated MSCs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

LD and LL designed this project, performed experiments, analysed the data and prepared the manuscript; KC and YH performed experiments and prepared the manuscript; PY, LZ and FL performed experiments; and YW and YS conceived the project, analysed and interpreted the data and drafted the manuscript.

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