

Original Article

Decreased MACC1 expression in osteosarcoma cells reverses the decline of dendritic cells activation and the cytotoxicity

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Abstract: Objective: Metastasis-associated in colon cancer-1 (MACC1), a novel protein highly expressed in a variety of cancers, is closely related to tumor progression, metastasis and prognosis. Dendritic cells (DCs) can be activated by exogenous tumor antigens to kill tumor cells directly. The purpose of this study was to investigate the effect of MACC1 in osteosarcoma cells on DCs activation and cytotoxicity. Methods: MACC1 gene expression was determined by RT-qPCR in four osteosarcoma cell lines, in which 143B cells expressed the highest level of MACC1, and were selected as the target cell line. By lentivirus infection generating MACC1 shRNA, MACC1 was knocked down in 143B cells (143B-i cells). Human DCs, isolated from PBMCs, were divided into four groups: i) control group (no stimulation); ii) DCs group (with stimulation); iii) DCs/143B group (DCs and 143B cells were co-cultured); and iv) DCs/143B group (DCs and 143B-i cells were co-cultured). Results: Co-culture of DCs with 143B cells inhibited DCs activation and decreased DCs induced cytotoxicity as evidenced by reduced DCs activation markers expression, IL-12 secretion and LDH release. However, MACC1 knockdown reversed the above-mentioned effects. Furthermore, the immunofluorescence indicating the endocytosis of 143B cell fragments into DCs was decreased by MACC1 knockdown. Conclusion: This study suggests the recovered activation and cytotoxicity of DCs by MACC1 knockdown in osteosarcoma cells.

Keywords: Metastasis-associated in colon cancer-1, dendritic cells, osteosarcoma cell lines, maturation, cytotoxicity

Introduction

Osteosarcoma is the most common solid bone cancer and the second most dangerous cause of cancer mortality in pediatric patients [1]. With increased understanding for the pathomechanism of osteosarcoma, the identification of harmful molecules has produced many therapeutic benefits for an increasing number of osteosarcoma patients [2]. Metastasis-associated in colon cancer-1 (MACC1), as a novel gene, is first detected in colon cancer [3]. MACC1 is located on the minus strand of human chromosome 7 (7p21.1) [4]. Recent studies have shown that this gene is highly expressed in a wide range of cancers [5]. Zhang et al. have reported that the high expression of MACC1 is correlated with the poor prognosis in patients with osteosarcoma, as well as with the metastasis and malignancy of osteosarcoma cells [6, 7]. Cancer stem cells (CSCs) with abili-

ties of self-renewal and survival of immune clearance are the seeds leading to tumor metastasis and occurrence. Several studies have suggested the promoting impact of LASP-1 on CSCs-such as the capacities, including chemoresistance and enhanced survival, in other cancers [8]. Therefore, we suspected whether MACC1 functions in immune system in osteosarcoma.

Kurose et al. have indicated that the drug tolerance of tumor is related to the clearance inability of immune system, whereas the functional deficit of dendritic cells (DCs) contributes to the immune escape of tumor cells [9]. Maturation of DCs can be inhibited by tumor-derived signals, apoptotic tumor cells, as well as the direct contact of DCs with tumor cells [10]. Immunosuppression can be promoted by molecules produced in tumor microenvironment, such as pro-angiogenic factors, which includes

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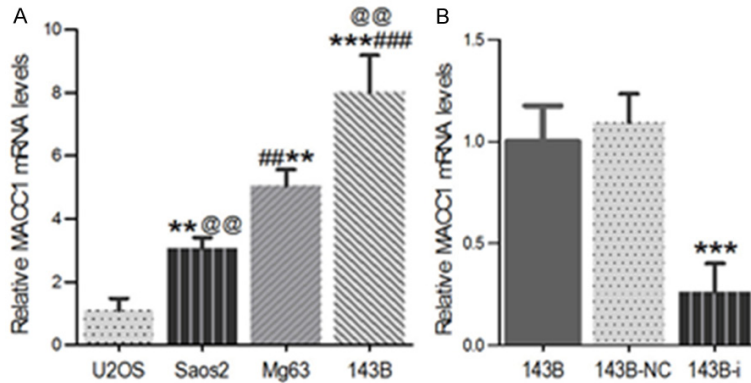


Figure 1. MACC1 mRNA detection in four cell lines. A. MACC1 mRNA expression in four representative osteosarcoma cell lines as determined by RT-qPCR. **P<0.01, ***P<0.001 vs. U2OS, ##P<0.01 and ###P<0.001 vs. Saos2, @@P<0.01 vs. Mg63. B. MACC1 mRNA levels after lentiviral transfection of MACC1 shRNA in 143B cells. ***P<0.001 vs. 143B. Data were presented as mean \pm sd. The relative amount of mRNA was normalized to GAPDH mRNA.

vascular endothelial growth factor A (VEGF-A) and placental growth factor (PIGF) [11]. In addition, there have been various studies suggesting that human DCs in co-culture can disrupt tumor cells proliferation and induce tumor cells apoptosis directly [12, 13]. Trinite and Chauvin et al. found that DCs can recognize and kill target cells, as well as phagocytose cell fragments to present tumor antigens for inducing adaptive immune response [14, 15].

In this present study, we investigated the effects of MACC1 on DCs maturation and cytotoxicity.

Materials and methods

Cell culture

Human osteosarcoma cell lines, Mg63, U2OS, Saos2 and 143B, were purchased from Shanghai Biofort Co. Ltd., and were cultured in DMEM with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air [16].

Building MACC1-shRNA-expressing lentivirus and cell transfection

Lentiviral vector containing shRNA sequence can deliver and integrate a substantive quantity of viral RNA into the DNA of the host cell to continually express the small/short interfering RNAs (siRNAs) by Dicer process. In the study, we designed several MACC1 siRNA sequences and selected the best one: 5'-GCCACC AUUUGGGAUUUAUATT-3' (data not shown). The sequence was subcloned into lentivirus to form

LV-MACC1-shRNA which was packaged and titered into 293T cells in 10 cm tissue culture dishes for 48 h. The collected cell supernatant was gradually diluted at a fold range of 10⁻¹-10⁻⁶ for titer determination. Triplicates were used in 96-well plates. After 48 h, the number of infected fluorescent 293T cells was recorded and the lentiviral titers were calculated. The negative control lentivirus (LV-NC-shRNA) was also constructed and titered as MACC1 shRNA and the sequence was 5'-CCTAAGGTTAAGTCGCCCTCG-3'. The 143B cells in logarithmic growth were cultured

into 50% confluence and infected with LV-MACC1-shRNA or LV-NC-shRNA (MOI=50), which were called 143B-i or 143B-NC cells respectively. Meanwhile, 8 μ g/mL polybrene was added to enhance the transfer efficiency. After 48 h of transduction, cells were lysated for detection of MACC1 mRNA level using fluorescence quantitative PCR.

Fluorescence quantitative PCR

Total RNA was isolated using the PureLink RNA Mini Kit (Ambion) from 4 human osteosarcoma cell lines, followed by reverse transcription with PrimeScript™ RT-PCR Kit (Takara). Primers were designed by using Primer Express 4 (Applied Biosystems) and synthesized by Invitrogen. qPCR was determined in 25 μ l per well, including 1 μ l cDNA sample, 100 nM each of forward and reverse primers, and 12.5 μ l SYBR Premix Ex Taq II (Takara). No-RNA and no-primer control were included in each experiment. The quantitative PCR was carried out on a 7500 ABI Prism Sequence Detection System under the following cycling conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 20 sec. Melting curves were determined at the end of the run for verifying the specificity of the detected SYBR signal. The PCR primers for MACC1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: MACC1 forward, 5'-CCTTCGTGGTAATAATGCTTCC-3'; and MACC1 reverse, 5'-AGGGCTTCCATTGTATTGAGGT-3'; GAPDH forward, 5'-GCCTCAAGATCATCAGCAAT-3'; GAPDH reverse 5'-AGGTCCACCACTGACACGTT-3'. Data

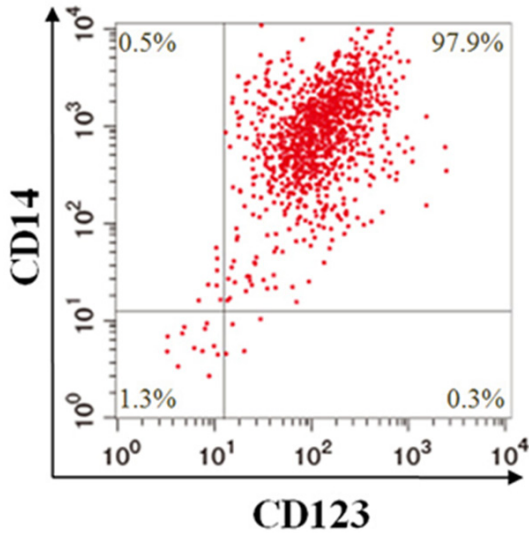


Figure 2. Purity of isolated DCs determined by flow cytometry DCs was co-stained with CD123 and CD14 antibodies.

were normalized to GAPDH RNA, and the fold-change for RNA was calculated using the $2^{-\Delta\Delta CT}$ method. Results were analyzed with three independent repeats.

Flow cytometry of isolated DCs

PBMCs (peripheral blood mononuclear cell) for DCs separation were obtained from heparinized blood (healthy donors) using Ficoll density gradient centrifugation. DCs were isolated by using immunomagnetic anti-CD14 beads according to the manufacturer's instruction (Miltenyi Biotec, MA, USA). Briefly, PBMCs in separation buffer (10^8 cells/ml) were incubated with FcR-blocking reagent (100 μ l) and anti-CD14 microbeads (100 μ l) for 20 min at 4°C. After incubation, PBMCs were washed with 20 volumes of cold separation buffer followed by filtration through a magnetic column. The purity of DCs was measured by flow cytometry (BD Bioscience, CA, USA) with percentage of CD14⁺ and CD123⁺. The DCs with purity above 95% were selected for further stimulation, and cells preparations with lower purity were discarded.

Cell stimulation and co-culture with 143B

Isolated DCs were cultured on 6-well plates in RPMI 1640 (Invitrogen, MA, USA) supplemented with 10% FBS. DCs could be induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) and other cytokines, such as IL-4 [17]. DCs were divided into four groups based on different types of treatment: stimulated with

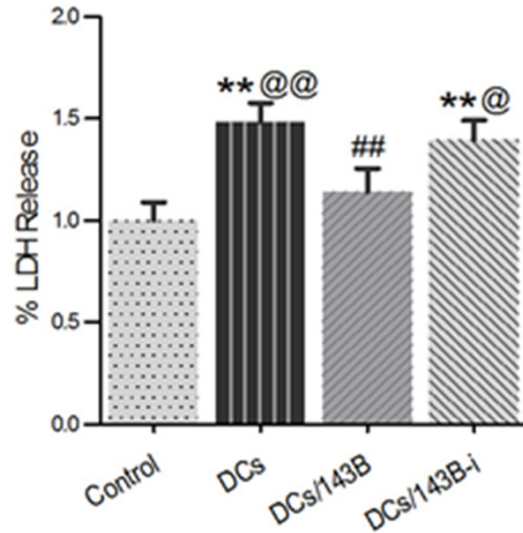


Figure 3. The effect of MACC1 knockdown in 143B cells on DCs cytotoxicity Purified DCs were stimulated with GM-CSF and IL-4 in the presence of 143B or 143B-i cells. The level LDH release to supernatant was determined. Data were shown as mean \pm sd of three independent experiments. *P<0.05 and **P<0.01 vs. control, ##P<0.01 vs. DCs, @P<0.05 and @@P<0.01 vs. DCs/143B.

GM-CSF and IL-4 (eBioscience) (DCs group), co-cultured with 143B cell line (143B group), stimulated with GM-CSF and IL-4 and co-cultured with 143B cell line (DC/143B group), and stimulated with GM-CSF and IL-4 and co-cultured with 143B/i cell line (DC/143B-i group). The stimulation time was 72 h [18].

Flow cytometry analysis of surface markers

In brief, 2×10^6 DCs were harvested, washed and resuspended in the staining buffer for 20 min at 4°C with various fluorescently labeled antibodies (BD Bioscience). Surface markers were determined by flow cytometer. Results were presented as percentage of positive cells [19]. The following antibodies with fluorescent group were used: CD40, CD80, C83 and CD86 from R&D systems (MN, USA).

Lactate dehydrogenase (LDH) assay

After treatments, the culture supernatants were collected and centrifuged for 10 min at 1000 \times g, and the extracellular LDH activity was measured according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) to detect DC induced cytotoxicity. Results were presented as percentage of LDH release and were normalized to control group.

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Table 1. Percentages of cell surface markers in DCs stimulated with GM-CSF and IL-4 in the presence of 143B or 143B-i

	CD40	CD80	CD83	CD86
Control	3.3±0.8 ^{b,c}	0.5±0.2 ^{b,c}	0.6±0.1 ^{b,c}	1.4±0.6 ^{b,c}
DCs	78.7±2.4 ^{a,c}	83.6±3.3 ^{a,c}	71.8±2.1 ^{a,c}	70.2±2.2 ^{a,c}
DCs/143B	33.3±2.3 ^{a,b}	46.3±3.2 ^{a,b}	55.0±5.6 ^{a,b}	37.6±3.7 ^{a,b}
DCs/143B-i	56.7±1.8 ^{a,b,c}	61.3±0.6 ^{a,b,c}	65.7±0.1 ^{a,b,c}	55.5±2.7 ^{a,b,c}

Note: Data were shown as mean ± sd. ^aP<0.05 vs. control, ^bP<0.05 vs. DCs, and ^cP<0.05 vs. DCs/143B.

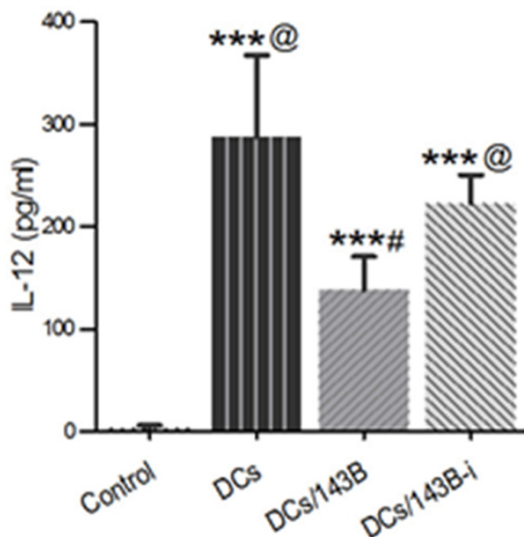


Figure 4. The effect of MACC1 knockdown in 143B cells on IL-12 secretion by DCs. Purified DCs were stimulated with GM-CSF and IL-4 in the presence of 143B or 143B-i cells. The level of IL-12 in supernatants was determined by ELISA. Data were presented as mean ± SD of three independent experiments. ***P<0.001 vs. control, #P<0.05 vs. DCs, and @P<0.05 vs. DCs/143B.

ELISA

IL-12 secretion increases significantly during DCs maturation. The IL-12 concentration was analyzed from supernatants in various DCs by ELISA, according to directions of manufacturer (R&D System). The concentration (pg/ml) was calculated by standard curve method.

DCs endocytosis

DCs can present antigens and clear apoptotic debris by phagocytosis. 143B and 143B-i cells were incubated with 5 μM carboxyfluorescein succinimidyl ester (CFSE) for 20 min at 37°C, followed by complete washing. Then, cells were incubated with mitomycin C (20 μg/ml) for 3 h at 37°C, followed by washing. DCs stimulated

with GM-CSF and IL-4 were mixed with CFSE-labeled 143B or 143B-i in a volume ratio of 1:3 for 4 h. CFSE⁺ DCs were determined by flow cytometer. Results were presented as percentage of CFSE⁺ DCs.

Statistical analysis

The data were presented as mean ± standard deviation and processed by SPSS13.0 software. Data were analyzed by one-way ANOVA with post hoc Bonferroni's test. P<0.05 was considered as statistically significant.

Results

MACC1 expression of four colon cancer cell lines

In order to find the cell line with highest expression of MACC1, the level of MACC1 mRNA was detected using qRT-PCR in four cell lines, Mg63, U2OS, Saos2 and 143B. **Figure 1A** shows that MACC1 mRNA was expressed in all four cell lines with highest expression in 143B. Therefore, 143B was chosen for the following experiments.

MACC1 expression was reduced by its lentivirus expressing shRNA

By lentivirus infection, MACC1 mRNA expression of 143B-i group was significantly reduced, as compared with that in non-transfection group (P<0.001). However, the difference between 143B-NC group and non-transfection group was not obvious (P=0.653). This result (**Figure 1B**) proved that LV-MACC1-shRNA successfully inhibited MACC1 mRNA expression of 143B and the infected cell could be used for the next detection.

Characterization of plasmacytoid DCs

The percentage of CD123⁺CD14⁺ DCs was detected by FACS after the selection by immunomagnetic beads. In **Figure 2**, the percentage of CD123⁺CD14⁺ cell was 96.3%±15.3%. The value of more than 95% proved the successful isolation of plasmacytoid DCs.

DCs cytotoxicity

After GM-CSF and IL4 stimulation (**Figure 3**), the supernatant showed an increased LDH con-

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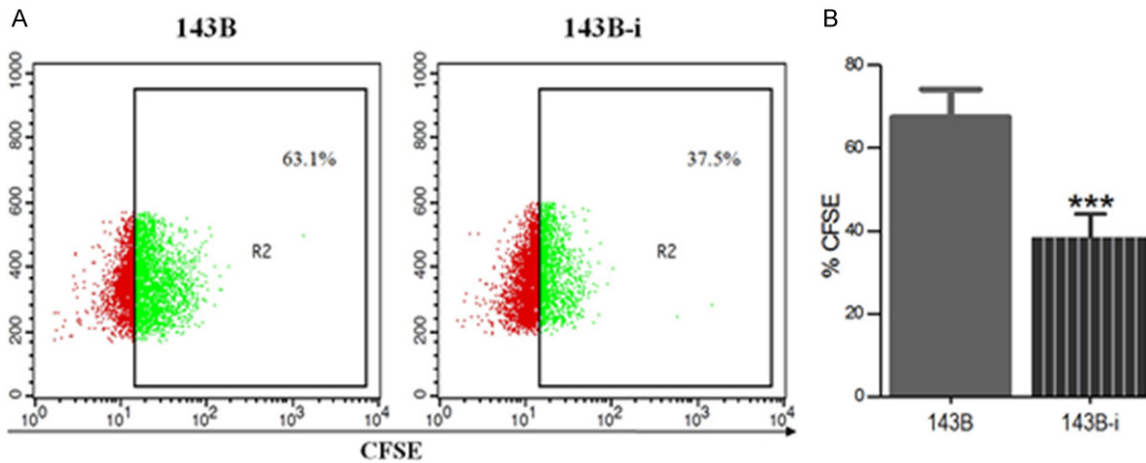


Figure 5. The effect of MACC1 knockdown in 143B cells on endocytosis of 143B fragments into DCs. 143B or 143B-i cells were labeled by CFSE, followed by mitomycin C incubation, and were then mixed with GM-CSF and IL-4 stimulated DCs for 4h, and CFSE⁺ DCs were detected. Representative FACS results (A) and the mean \pm sd of the CFSE⁺ DCs percentage (B) as calculated from three independent experiments were shown. ***P<0.001 vs. 143B group.

centration in DCs as compared to that in control group ($p=0.002$). Compared to DCs group, co-culture treatment with 143B cells significantly decreased LDH concentration ($P=0.004$), whereas DCs/143B-i group enhanced LDH release which differed significantly from DCs/143B group ($p=0.041$).

Expression of cell activation markers of DCs

Flow cytometry results showed that the freshly isolated PBMCs expressed very low level of cell surface antigen (CD40, CD80, CD83, or CD86). The expressions were upregulated by GM-CSF and IL-4 stimulation but decreased by 143B co-culture (Table 1), whereas 143B-i with MACC1 knockdown significantly reversed the decline of cell surface antigens ($P=0.020$).

IL-12 secretion of DCs after MACC1 knockdown in 143B cells

It can be seen that GM-CSF and IL-4 stimulated DCs activation, and made DCs secrete high level of IL-12 ($P<0.001$). The co-culture with 143B cells (Figure 4) inhibited IL-12 secretion ($P=0.021$), and the inhibition could be reversed by MACC1 knockdown in 143B-i group, which was different from DCs/143B group ($P=0.017$).

MACC1 knockdown inhibited the endocytosis of 143B into DCs

To verify the endocytosis of 143B debris into DCs, CFSE was labeled on cells and presented by flow cytometry. Figure 5 presents that the amount of CFSE⁺ DCs, representing endocytosis

level of cell debris into DCs, was more in DCs/143B co-cultured group ($67.4\% \pm 6.8\%$) than in DCs/143B-i co-cultured group ($38.5\% \pm 5.6\%$). It indicated that MACC1 knockdown inhibited DCs endocytosis ($P<0.001$).

Discussion

In the present study, we demonstrated that the osteosarcoma cell line 143B could express the highest level of MACC1 and was selected as target cell line [20]. Co-culture of DCs with MACC1-knockdown 143B cells reversed the decline of DCs cytotoxicity, enhanced the inhibition of specific markers for cell activation, and increased IL-12 secretion. Furthermore, the CFSE assay indicated that MACC1 inhibition lowered endocytosis of 143B debris into DCs. These results support the idea that MACC1 has a significant inhibitory effect on DCs maturation and killing activity, whereas the knockdown MACC1 could reverse the inhibitory effect.

Current researches of antitumor drugs have shown that the therapy targets also include the host immune system in addition to the tumor itself. It was known that therapy, recurrence and tumor cells clearance would depend on the immune state of host [21]. DCs have been increasingly acknowledged as an important factor of cancer immunotherapy, which are based on their potent abilities to present antigens, including tumor associated antigens [22]. Osteosarcoma is type of highly malignant tumor with incidence of metastasis in the early phase. Although there have been numerous studies

and huge technical advances, drug resistance and immune escape still make the survival rate limited within 20% [23, 24]. Escape of cancer cells from immune system control may be due to the deficiency of DC function, such as immaturation. Muraro et al. found that both expression of specific biomarkers and IL-12 secretion decrease by co-culture treatment with human osteosarcoma cell, indicated the blunt DCs maturation [25]. MACC1 has been verified to be in correlation with metastasis and malignancy of osteosarcoma and drug resistance of other tumors [5-8]. Hence, it is necessary to explore the link of MACC1 to immune cells in osteosarcoma.

In the present study, we found that DCs maturation was negatively correlated with MACC1 expression levels in 143B cells. High expression of MACC1 in 143B could inhibit DC maturation, whereas the inhibited expression of MACC1 by shRNA knockdown could reverse this phenomenon. This observation leads to the conclusion that MACC1 can inhibit DC activation and maturation, thus might potentially lead to the immune escape of tumor cell lines. IL-12 secretion represents the main immune function of DCs [26]. It is an important cytokine in mediating cellular immunity, especially in inducing Th1 differentiation both in vivo and in vitro [27]. Some researchers also found that DCs maturation can increase IL-12 secretion [28]. In this study, **Figure 4** shows that GM-CSF and IL4 could upregulate IL-12 levels, and co-culture with 143B could inhibit IL-12 secretion at the present of GM-CSF and IL4. However, the inhibition of MACC1 significantly reversed IL-12 decline. Therefore, MACC1 is involved in 143B cells-induced deficiency of DCs immune functions by inhibiting IL-12 secretion. DCs can also directly recognize and kill target cells, except for antigen-presenting cells, and phagocytose cell debris. Phagocytosis of the target cells could present tumor antigens for inducing anti-tumor response [15]. In the present study, 143B cells with high expression level of MACC1 could inhibit DCs' cytotoxicity, while MACC1 knockdown could recover this cytotoxicity. This result indicates that MACC1 is involved in the inhibitory effect of 143B against DCs' cytotoxicity. Furthermore, MACC1 knockdown could inhibit the uptake of cell fragments into DCs, which supports the potential of MACC1 in inhibiting DCs immune functions.

This study offers a preliminary discussion about the reversed effects on DCs activation by inhib-

itory MACC1 in osteosarcoma cells. However, due to the lack of MACC1 overexpression, the generalizability of the authors' results is limited. Furthermore, it would also be necessary to explore the time period effect of MACC1 on DCs activation. The work suggests inhibitory effect of MACC1 on DCs activation, but it still needs more evidence for support.

In sum, the study provides evidences of the recovered activation and cytotoxicity of DCs by MACC1 knockdown in osteosarcoma cells, which indicates the needs for further research of immune-regulatory function of MACC1 in osteosarcoma cell.

Disclosure of conflict of interest

None.

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