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Celastrol inhibits cancer metastasis by suppressing M2-like polarization of macrophages

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ABSTRACT

In recent years, a large amount of clinical and experimental data has shown that M2-like polarized tumor-associated macrophages (TAMs) play an important role in cancer metastasis. Therefore, TAMs, especially M2-like TAMs is a promising target for anti-tumor metastasis therapy. Here, we found that celastrol dose-dependently suppressed IL-13 induced CD206 expression both in RAW264.7 and in primary macrophages. Consistently, celastrol also inhibited the expression of M2-like specific genes, including MRC1, Arg1, Fizz1, Mgl2 and CD11c. Further, by the employment of 4T1 breast cancer model, we found that celastrol significantly prevented cancer metastasis *in vivo*. Mechanistically, celastrol completely ameliorated STAT6 phosphorylation, which is the key signal molecule responsible for M2 polarization. Our research puts forward a new application of celastrol in anti-cancer metastasis, by intervening M2-like polarization through inhibiting STAT6.

1. Introduction

Among the numerous infiltrating inflammatory cells in the tumor microenvironment, tumor-associated macrophages (TAMs) are the most abundant, accounting for 30–50% of the total [1]. Based on original results, TAMs are involved in anti-tumor immunity. However, recent clinical and experimental data shed light on their tumor-promoting effect [2,3]. A strong correlation was found between increased macrophage infiltration and tumor malignancy, poor prognosis and tumor recurrence [4,5]. Analysis of the diversity of TAMs has revealed their facilitating role in cancer progression while attenuating antitumor immunity at the same time [6]. A large number of macrophage colony-stimulating factors (M-CSF) after resection treatment has provided evidence for pool survival associated with macrophages [7]. Mediating macrophages depletion with clodronate-contained liposomes inhibits growth in several tumor models including ovarian, melanoma, Lewis lung, rhabdomyosarcoma, teratocarcinoma and prostate tumor [8]. These studies indicate that macrophages are advantageous for cancer progression.

TAMs are highly plastic and flexible, which means that they can be polarized into subgroups with different phenotypes, functions and even opposite effects under the influence of diverse environmental factors [9]. According to the diversity of their functions and activated microenvironment, macrophages can be divided into two categories: classic activation of macrophage (M1-like) or alternative activation of macrophage (M2-like) [10,11]. The major populations of TAMs are classic activated macrophages (M1-like) in nonmalignant tumors, exhibiting pro-inflammatory activity and promoting immune responses. In contrast, TAMs in malignant tumors are inclined to polarize into alternative activated macrophages (M2-like), which inhibit the anti-tumor immune response, promote cancer progression and tumor-associated angiogenesis. Usually, M2-like TAMs exert their cancer-promoting effect by secreting the corresponding cytokines like angiogenesis factors and extracellular matrix regulatory factors [12]. It has been recognized that TAMs release numerous compounds ranging from mutagentic oxygen and nitrogen radicals to angiogenic factors, which initiate cancer and make it invasive. A.P. Cardoso et al. also uncovered a stimulating effect of macrophages in cancer invasion and migration by producing epidermal growth factor (EGF) [13]. Thus, modulation of M2-like TAMs can be potential approach for anticancer treatment therapeutically.

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By far, several strategies have been proved efficiently in cancer therapy by interfering M2 polarization of macrophages. After inhibiting CSF-1 receptor, which greatly contributes to macrophage differentiation, the tumor-promoting function of TAMs was significantly suppressed, consistent with M2-like TAM reduction [14]. HRG prevents cancer progression by skewing TAM polarization away from the M2-like phenotype, which suppresses antitumor immunity, accompanied by vessel normalization and decreasing cancer growth and metastasis [15]. Furthermore, macrophages showed a significant shift from M2 to M1 quantitatively after treatment with Rapamycin [16]. In a laser-injury model of choroidal neovascularization, doxycycline impaired the survival of M2-like macrophages and induced M1-like inflammatory response with subsequent neovascularization *in vivo* [17]. Our previous study found that metformin is able to attenuate Lewis lung cancer metastasis by inhibiting the M2-like polarization of macrophages partially through the activation of AMPKα1 [18]. Given the above experiments, finding compounds that could interfere M2 polarization of macrophages would be promising in improving clinical therapy of cancer. Here we found that celastrol is able to efficiently suppress M2 polarization of macrophages and prevent cancer metastasis, suggesting its new application in cancer therapy.

2. Materials and methods

2.1. Cell culture

The murine cell lines for breast cancer cells (4T1) and macrophages (RAW264.7) were purchased from the Cell Bank of the China Science Academy (Shanghai, China). Both cells were cultured in DMEM medium with 10% fetal bovine serum and incubated at 37 °C in a 5% CO_2 humidified incubator.

2.2. Animal experiment

Female BALB/c mice (4–6 weeks old) were purchased from National Rodent Laboratory Animal Resource (Shanghai, China). The animal experiments were approved by Zhejiang University Animal Research Committee. Animal care was provided in accordance with the guidelines approved by the IACUC.

4T1 cells (5×10^4 cells) in 0.1 ml of medium were injected into the mammary fat pad. After 5 days, mice were divided into two groups according to the mean tumor volume and celastrol was given at 10 mg/kg via intraperitoneal injection once a day for prolonged treatments. Animals were sacrificed 14 days after celastrol treatment. Tumors were weighed and lungs were fixed in Bouin solution.

2.3. Isolation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were obtained according to protocol [19]with slight modification. Six-week-old C57BL/6 mice were sacrificed and soaked in 75% ethanol. Then bone marrow cells were cultured in DMEM medium with 50 ng/ml M-CSF and 10% FBS for three days to obtain BMDMs.

2.4. Reagents and antibodies

Celastrol was obtained from Cayman chemical company (Ann Arbor, MI, USA). Recombinant murine M-CSF was obtained from Cell Signaling Technology (Beverly, MA, USA). Recombinant murine IL-13 was obtained from PeproTech (Rocky Hill, NJ, USA). Antibodies against GADPH, phospho-MEK1, phospho-ERK1/2 and phospho-STAT6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PE-conjugated anti-mouse CD206 antibody and FITC-conjugated anti-mouse F4/80 antibody for flow cytometry were obtained from Biolegend (San Diego, CA, USA).

2.5. Preparation of conditioned medium

M2 polarized macrophages were induced by cultivating cells in DEME containing 10 ng/mL IL-13 and 10% FBS for three days. After that, the cells were transferred to serum-free medium for another 24 h. Then the medium was collected and stored at −80 °C as a conditioned medium (CM) after being centrifuged at 3000 rpm to filter the debris.

2.6. Quantitative real-time PCR

All RNAs were collected and purified in the use of Easy Pure Viral DNA/RNA Kit (Transgen Biotech Co., Ltd). After cDNAs were synthesized, quantitative real-time RT-PCR analysis was performed using Bio-Rad SYBR Premix. The combinations, including SYBR Green, were composed according to the manufacturer's instructions. The murine primers to MRC1, IL-1β, Mgl2, Arg1, Fizz1, CD11c, Actin are listed in Table 1. Relative target genes expression levels were standardized with the control gene Actin.

2.7. Flow cytometry

RAW264.7 cells and BMDMs were collected with a scraper and blocked with 3% BSA for 45mins. Pursuant to the manufacturers' instructions, cells were incubated with FITC-conjugated anti-mouse F4/80 (1:200) and PE-conjugated anti-mouse CD206 (1:100) antibody. 1×10^4 cells were evaluated by BD Biosciences FACSCalibur flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA).

2.8. Western blot analysis

1×10^6 RAW264.7 cells per well were plated in 6-well plates, and then the cells were treated by 62.5 nM celastrol, 10 ng/ml IL-13 or both of them in 2 h. Medium was removed and cells were washed twice with PBS before we collected cellular and nuclear extracts. Then those extracts were analyzed following the protocol [20]. The protein lysates were quantified and analyzed by 8% SDS-PAGE and blotted onto PVDF membranes. The membrane was probed with 1:1000 primary antibodies, including p-STAT6,p-MEK1,p-ERK1/2 and GAPDH at 4 °C overnight. A 1:5000 dilution of HRP-conjugated secondary antibodies was added and incubated on the membrane at room temperature for 1 h. The

Table 1
Primers used for qRT–PCR analysis.

Genes	Primer sequence (5′→3′)	
MRC1	Forward primer:	AGGGACCTGGATGGATGACA
	Reverse primer:	TGTACCGCACCCTCCATCTA
Mgl2	Forward primer:	CTCTGGTCTGAGGGAGAGGT
	Reverse primer:	CAAGGTAGAGGGGAGCAAGC
CD11c	Forward primer:	TTGCTTAGCAGTCTCTGGTGG
	Reverse primer:	TTCTGGGTCATAGGCTTGGC
Fizz1	Forward primer:	CCCTGCTGGGATGACTGCTA
	Reverse primer:	TGCAAGTATCTCCACTCTGGATCT
Arg1	Forward primer:	AACACGGCAGTGGCTTTAAC
	Reverse primer:	GTCAGTCCCTGGCTTATGGTT
IL-1β	Forward primer:	GGGGAAGAGGCTATTGCTACC
	Reverse primer:	ATGCCCATTTCCACCACGAT
Actin	Forward primer:	GGTCATCACTATTGGCAACG
	Reverse primer:	ACGGATGTCAACGTCACACT

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