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# Methionine enkephalin (MENK) mounts antitumor effect *via* regulating dendritic cells (DCs)

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ABSTRACT

MENK, an endogenous opioid peptide has been reported to have many immunological and antitumor activities. So far the detailed mechanisms of antitumor through regulating DCs by MENK have not been elucidated yet. The aim of this work was to investigate the antitumor mechanisms of MENK *via* regulating DC. The monitoring methods, such as ELISA, MTS assay, CFSE, Real-time PCR and Western blot were included in our research. We found bone marrow derived dendritic cells (BMDCs) in 36 female C57BL/6 mice treated with MENK enhanced expression of key surface molecules, increased production of critical cytokines reduced endocytosis of FITC-dextran, upregulated TLR4 through MyD88/NF-κB signaling pathway and mounted higher antitumor activity. These observations were further supported by an enhancement of nuclear translocation of the p65NF-κB subunit involved in this process. Surprisingly, mu-opioid receptors were the main participants of this kind of activation, not delta-opioid receptors nor kappa-opioid receptors, and these interactions could be partly blocked by Naltrexone (a kind of opioid antagonist). *In vivo* study the activated $CD4^+$, $CD8^+$T cells and decreased ability to induce differentiation of $Foxp3^+$ regulatory T cells were detected post treatment of MENK. Thus, it is concluded that MENK could exert antitumor effect through precisely regulating opioid receptor mediated functions of DCs. In addition, MENK treated DCs may serve as a new immunotherapy approach against tumor.

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## 1. Introduction

Endogenous opioids are neuropeptides [1] with multi-functionality and widespread distribution. Opioid effects are mediated by three major types of opioid receptors: μ-, δ-, and κ-opioid receptors (MORs, DORs, and KORs, respectively). These peptides [2,3], distributed in the digestive system of higher animals, play a role in neurotransmission and pain modulation *via* coupling to opioid receptors (ORs). MENK, a member of opioid neuropeptides family, derived from both proenkephalin or pro-opiomelanocortin is a tonically active, inhibitory penta-peptide, whose action in both normal and abnormal cells is receptor mediated.

Opioid receptors have been detected out on the surface of immune cells such as: NK cells, macrophage, T cells and some tumor cells. MENK could bind to these receptors either to stimulate activity of immune cells, mounting antitumor activity or to inhibit cancer cells directly. Also MENK binding to receptors is an inhibitory pathway that has been shown to retard cell proliferation in multiple human cancer cell lines, including breast, soft tissue, gastrointestinal, brain and liver cell cultures [4].

Tumor transplantation studies of small cell carcinoma of the head and neck, pancreatic adenoma and colon adenoma have also demonstrated that administration of MENK can arrest tumor growth by inhibiting cell proliferation as well as decrease toxicity when MENK is combined with paclitaxel (taxol), cisplatin, or gemcitabine [5–7]. These data support MENK gene function as a regulator of cell proliferation that impacts immunologic factors expressed in the tumorigenic state.

Quite some of published articles from our research team have provided convincible evidence for the effect of MENK on the cells in immune system. MENK could intensify the pathway between DC and $CD4^+$T cell, mount special cytotoxicity to kill the tumor through activating $CD8^+$T cell, inhibiting regulatory T cells (Tregs), turning M2 type macrophage into M1 type within tumor microenvironment and inducing maturation of DC progenitor [8–15].

Dendritic cells (DCs) were first described in the mid-1970s by Ralph Steinman. DC upon maturation will present antigen to initiate Ag-

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specific T lymphocyte responses and may be essential for the development of human vaccines that rely on T cell immunity [16,17]. Despite considerable data on MENK, however, mechanisms of antitumor by MENK are still not well understood. Especially the precise mechanisms of MENK mounts antitumor activity through regulating DCs remain unclear. Therefore, we conducted the following exploration trying to elucidate these. In addition, the opioid antagonist naltrexone (NTX) was applied in this research for detecting the function and mechanism of MENK after being blocked.

## 2. Materials and methods

### 2.1. Chemicals

MENK were provided by Penta Biotech, Inc. USA (≥98% in purity). RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin was used. Recombinant murine GM-CSF (Peprotech, USA) and recombinant murine IL-4 (Peprotech, USA) were used to stimulate bone marrow dendritic cells to DC lineage. GoScript™ Reverse Transcription System and GoTap qPCR Master Mix were obtained from Promega Corporation (Madison, USA). FITC-conjugated rat anti-mouse CD11c, PE-conjugated mouse anti-mouse MHC-II, PE-conjugated rat anti-mouse CD86 were purchased from BD bioscience. The other antibodies for FCM analysis were products of Biolegend (USA). Primers were all prepared by Invitrogen (Life technology, USA). Specific antibodies against β-actin, myeloid differentiation factor 88 (MyD88), NF-κB, phospho-NF-κB (p-NF-κB), phosphor-mu opioid receptor (p-MOR), Toll like receptor (TLR)4 and TLR7 were purchased from Cell Signaling (Cell Signaling Technology, Inc. USA). Specific antibodies against delta opioid receptor (DOR), kappa opioid receptor (KOR), p-KOR, MOR, TLR2 and TLR9 were purchased from Abcam (Abcam, Inc. USA). Specific antibodies against p-DOR were from Biomathematics and Statistics Scotland, Scotland. LPS (lipopolysaccharides from *E.coli* 055:B5) was purchased from Sigma Aldrich, Inc. (St. Louis, MO). ELISA kits for murine IL-12p70, IL-23, IP-10 and TNF-α were purchased from eBioscience (USA). Other chemicals frequently used in our laboratory were either products of Sigma-Aldrich or e-Bioscience.

### 2.2. Mice and cells

Female C57BL/6 mice (4–6 weeks) were obtained from China Medical University. Mice were maintained in specific pathogen-free conditions and housed in a regulated environment (22 ± 1 °C, relative humidity 55 ± 5%) under a 12 h light/dark cycle. All mice were treated nicely according to the guidelines of the Animal Welfare Committee of China medical university and acclimatized for at least one week prior to the experiments, and all surgical procedures were approved by the committee of experimental animals of China medical university. At the time of experiment, animals were anesthetized with sodium thiamylal, 10 mg/kg, and then euthanized by dislocation of cervical vertebra.

Sarcoma 180 (S180) cells were tumor from an outbred mouse strain obtained from China Medical University [18,19].

### 2.3. Generation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were induced from bone marrow (BM) cells obtained from 4 to 6 week-old C57BL/6 mice. Briefly, a single cell suspension was prepared from femurs and tibias. After red blood cells were lysed, whole BM cells ($2 \times 10^6$ cells/ml) were cultured in RPMI 1640 medium in six-well flat bottom plates at 37 °C under an atmosphere of 5%(v/v) $CO_2$ and 80% humidity atmosphere, supplemented with 10% heat-inactivated FBS, 10 ng/ml recombinant murine GM-CSF and 10 ng/ml recombinant murine IL-4. The cells were incubated for 24 h. Plates were then gently swirled; the medium containing non-adherent cells was removed and replaced with nutrient medium as described above. Every 2 days, the culture media were replaced. On day 6, non-adherent and loosely adherent DCs were purified and used in subsequent experiments. $CD11c^+$ BMDCs were isolated by positive selection using a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. The purity of BMDCs was >96% as determined by CD11c staining. The gating strategy employed for data analysis was to restrict the analysis to live singlet of CD11c-positive cells (Fig. 1). Then the cells were counted, seeded and cultivated in tissue culture plates 12 h before further experimental procedure.

### 2.4. Determination of cytokine concentrations in culture supernatants

After induction, the BMDCs were purified and divided into 4 groups: i) control group, ii) LPS treated group, iii) MENK treated group, iv) MENK+ naltrexone group. The four 96 μ well plates were coated with the coating antibodies at 4 °C temperature for 12 h. The samples or the standard were added in triplicate to separate columns of the plates. The plates were incubated at room temperature for 2 h. After incubation, the contents of the wells were aspirated, washed and blot dried before adding the secondary antibodies. 100 μl of the HRP-conjugated detection antibodies and the plates were incubated for 1 h, washed and dried and 100 μl tetramethylbenzidine substrate solution was added. Finally, the plate was incubated in dark for 20 min for color development, after which 100 μl of 1 N Sulphuric acid was added to stop the reaction. The amounts of cytokines were measured at 450 nm (A450) using a 96-well bichromatic microplate reader (BIO-BAD).

### 2.5. Allogeneicly mixed lymphocyte reaction (MLR) using carboxy-fluorescein succinimidyl ester (CFSE)

To generate allogeneic lymphocytes, spleens were obtained from C57BL/c mice (n = 6) and labeled with 2.5 μM CFSE for 15 min at 37 °C in the dark, quenched by addition of cold RPMI 1640 with 10% FBS, followed by 4 washes with the same solution to removed excess CFSE. $2 \times 10^5$ cells BMDCs of the C57BL/6 mice were seeded in 6 wells with lymphocytes at the ratio of 1:1, 1:5, 1:50 and 1:100 for 3 days. After co-cultured, CFSE labeled lymphocytes were harvested, washed and stained with APC-anti-CD4 McAb (Biolegend, USA). Cells were then analyzed by FACSCalibur flow cytometry (BD Biosciences, CA, USA) and was evaluated on gated $CD4^+$ lymphocytes.

### 2.6. Signal pathway confirmation

The purified BMDC lysates were centrifuged. Total proteins were extracted and protein content was determined using UV–Vis Spectrophotometer (ThermoFisher, USA). An equal amount of total protein lysates for each sample were subjected to 8%–12% SDS-PAGE separation and transferred on PVDF membranes (Millipore, Italy) by standard Western blotting. Membranes were blocked for 2 h, washed three times and incubated overnight at 4 °C with different primary antibodies, phosphor-specific antibodies of opioid receptors, TLRs and NF-κB signaling pathway or anti-β actin antibodies in 5% low fat milk in PBS-T buffer at 1:1000. Immuno-detection were performed by adding specific goat anti rabbit HRP-linked secondary antibody (anti-rabbit IgG, HRP-linked Antibody, CST, USA) (1:1000) for 1 h at room temperature, followed by visualization by means of Pierce™ ECL Plus Western Blotting Substrate (ThermoFisher Scientific, USA) on ECL chemi-luminescence film.

### 2.7. Confirmation of signal protein by qRT-PCR

Total RNA was isolated from BMDCs in culture *in vitro* experiment. The gene expression of corresponding proteins was quantified by qRT-PCR. Primers were designed by Primer-BLAST program (NCBI) using nucleotide sequences from the NCBI database. Primer sequences for analysis were described in Table 1. The relative quantity of gene

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