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Novel modulation on myeloid-derived suppressor cells (MDSCs) by methionine encephalin (MENK)

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ABSTRACT

The purpose of this study was to identify the modulatory effect of MENK on MDSCs and to investigate the relationship between this modulation and the expression of opioid receptors. Our results showed that MENK could inhibit the proliferation of MDSCs from both marine bone marrow and spleen. MENK also could promote the expression of opioid receptors MOR and DOR. MENK suppressed the PMN-MDSCs generated from splenocyte while up-regulated M-MDSCs. The stimulation of MENK increased the production of IL-4 secreted by MDSCs from slpenocytes. Our currently data indicated that MENK could suppress the accumulation of MDSCs in tumor-bearing mice via binding to and up-regulating expressions of subunits of opioid receptors. Therefore, it is concluded that MENK, through triggering opioid receptors could exert inhibiting modulation on MDSCs.

1. Introduction

Opioid receptors have been identified on the membranes of diverse immune cells including T-cells, NK-cells, macrophages, and dendritic cells [1]. Mu, delta and kappa are three subtypes of opioid receptors. Activating these receptors can inhibit the activity of adenylate cyclase, L-type and N-type Ca_2^+ channels, and then play a significant role of signal transduction and neuroregulation [2]. Methionine encephalin (MENK), originated from pre-enkephalin, as a penta-peptide consists of Tye-Ala-Ala-Phe-Met. In blood circulation, the concentration of MENK is low. The physiological concentration of MENK shows the positive immunomodulatory effect on manifold types of immune cells via regulation of opioid receptors expression. Binding to opioid receptors MENK can inspire the cytotoxicity of CD8+ T cells [3], induce macrophages polarized to M1phenotype, increase the antigen presentation via inducing phenotypic and functional differentiation [4,5]. The data from published study also indicates that MENK could inhibit the expansion and activity of regulatory T cells [6].

Myeloid-derived suppressor cells (MDSCs) are immune-suppressive cells existing in cancer and in pathologic conditions such as chronic inflammation or stress. MDSCs consist of undifferentiated immature heterogeneous cells derived from myeloid system, including dendritic cells, macrophages and granulocytes. Multiple cytokines secreted by cancer cells can induce the accumulation, movement and activation of MDSCs [7]. MDSCs can be divided into two populations: cells termed granulocytic or polymorph nuclear (PMN-MDSCs) and monocytic (M-MDSCs). In mice, PMN-MDSCs were recognized as $CD11b^+Ly6G^+Ly6C^{dim}$ and M-MDSCs were recognized as $CD11b^+Ly6G^-Ly6C^{high}$ [8]. The levels of MDSCs in bone marrow, spleen, peripheral blood, or tumors of mice were higher. Although phenotypically and morphologically similar to monocytes and neutrophils, MDSCs have different biological functions. It can inhibit antigen-specific and nonspecific T cell response by promoting the production of nitric oxide (NO) and reactive oxygen species (ROS) [9,10].

Abbreviations: MENK, methionine enkephalin; MDSCs, myeloid-derived suppressor cells; NTX, naltrexone hydrochloride; mAbs, monoclonal antibodies; MACS, magnetic activated cell sorting; MOR, μ-opioid receptor; DOR, δ-opioid receptor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TCCM, tumor cell- conditioned medium; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage colony stimulating factor; IL-6, inrerleukin-6; IL-4, inrerleukin-4; IRF4, interferon regulatory factor 4; S180, Sarcoma180; CCK-8, cell counting kit-8

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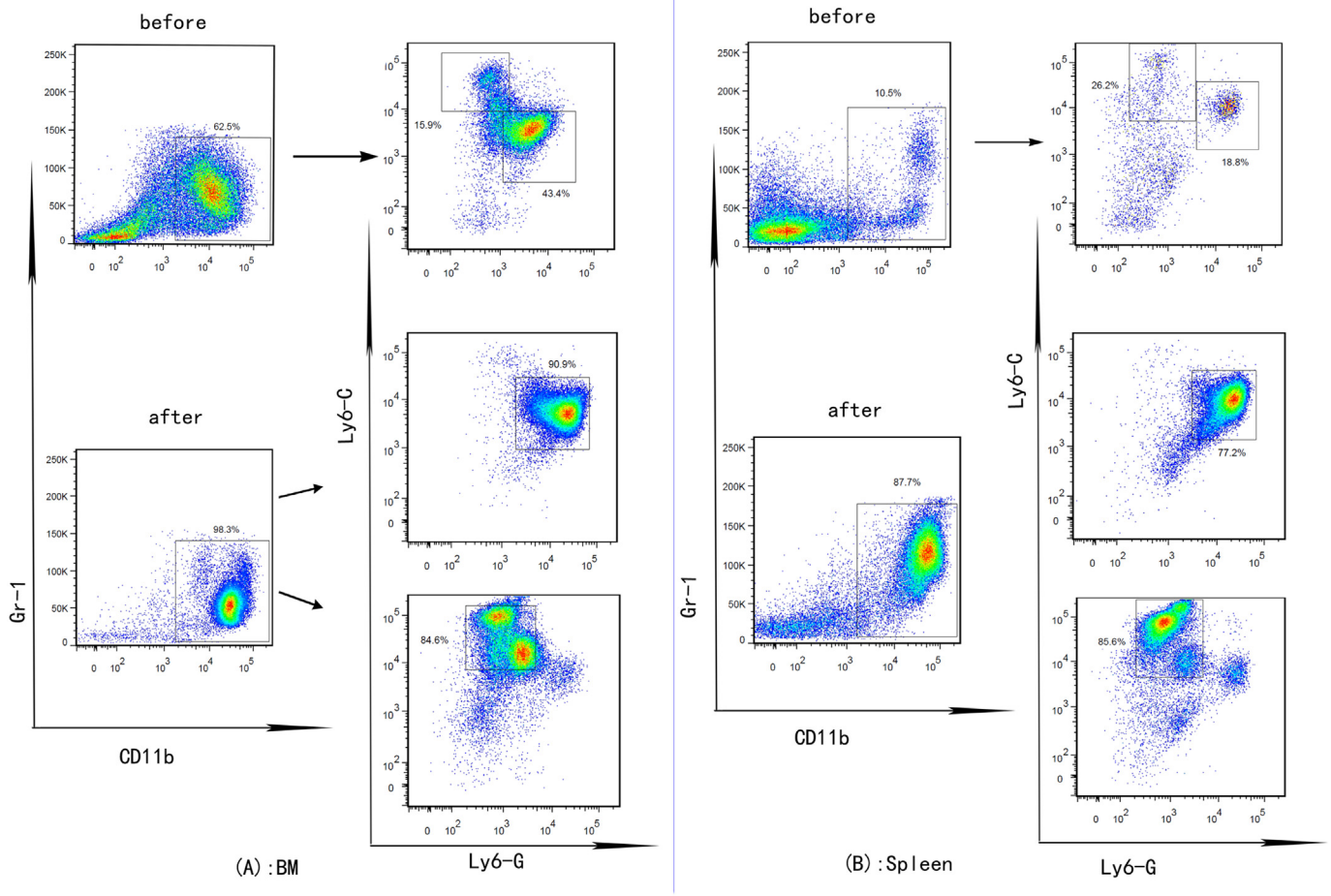

Fig. 1. Purification of MDSCs from bone marrow cells and splenocytes. Purity of MDSCs was up to 80%. (A) Purification of MDSCs from bone marrow cells. The initial MDSCs suspension was separated from bone marrow of tumor-bearing mice. MDSCs isolation kit was used for positive magnetic separation twice to purify MDSCs. The results represent mean ± SD. After the process of magnetic separation, the purity of ly6-G$^+$ cells was up to 91.35 ± 0.45 and ly6-G$^-$ cells was up to 84.95 ± 1.75. (B) Purification of MDSCs from splenocytes. The initial MDSCs cell suspension was separated from splenocytes of tumor-bearing mice. MDSC isolation kit was used for positive magnetic separation twice to purify MDSCs. After the process of magnetic separation, the purity of ly6-G$^+$ cells was up to 80.2 ± 3.0 and ly6-G$^-$ cells was up to 83.05 ± 2.55.

Table 1
The absolute number of MDSCs from spleen and peripheral blood.

	Normal mice	Tumor bearing mice	*p* value
In spleen	$(2.07 \pm 0.36) \times 10^6$	$(1.27 \pm 0.27) \times 10^7$	< 0.01
In blood	$(6.62 \pm 3.51) \times 10^3$	$(6.43 \pm 1.79) \times 10^5$	< 0.01

MDSCs from both spleen and peripheral blood were harvested from normal mice and tumor bearing mice. The absolute number of MDSCs was analyzed by flow cytometry. Results represent the mean ± SD of three independent experiments.

Meanwhile MDSCs play a significant role in the progress of tumor including angiogenesis, metastases and drug resistance [11,12]. Therefore, targeting these cells may provide a potential choice for cancer immunotherapy. Despite that, MENK can exert positive role in immune system, however, the influence of MENK on MDSCs remains unclear. We conducted the following investigation trying to gain insight to understand this.

2. Materials and methods

2.1. Mice and cell line

8–10 weeks old female C57BL/6 mice were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd. According to Care and Use of Laboratory Animals (NIH issued), the mice were raised in specific pathogen-free condition. Animal Use and Care Committee of China Medical University approved all related experiments.

Sarcoma180 (S180) cell line was obtained from cell resource center of Shanghai institute of life science, Chinese Academy of Science. The cells were injected intravenously into syngeneic C57BL/6 mice to cause ascites.

2.2. Reagents

MENK was a product of Chinese Peptide Company (> 98% in purity). The mAbs, required for flow cytometry included: APC anti-mouse/human CD11b Antibody (BioLegend Cat. No. 101212); FITC anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody (Bio Legend Cat. No. 108406) and Mouse MDSC Flow Cocktail 2 with Isotype Ctrl (BioLegend Cat. No 147003). The mouse MDSCs isolation kit was obtained from Miltenyi Biotec (Germany). Naltrexone hydrochloride (NTX) was purchased

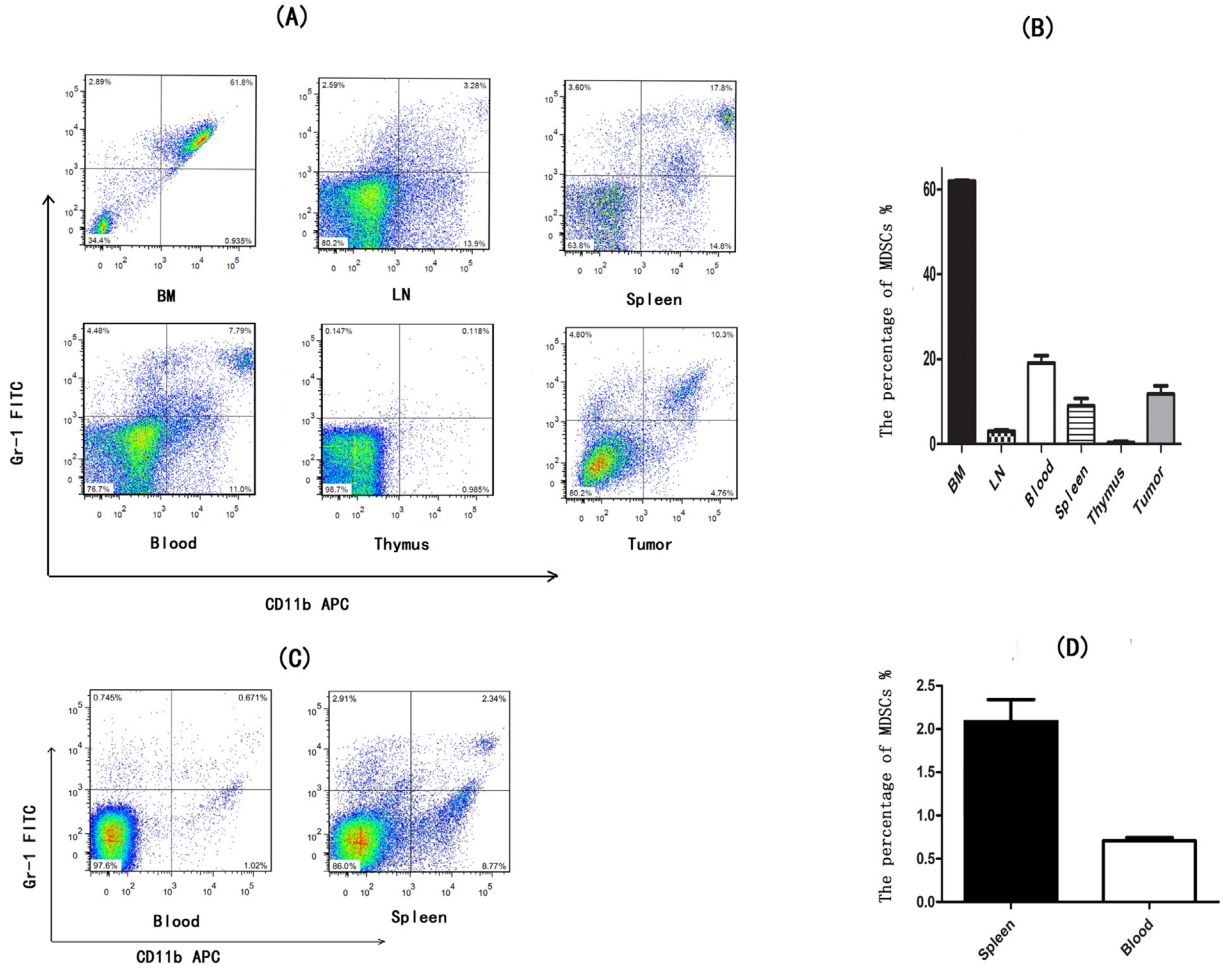

Fig. 2. Distribution of MDSCs in tumor-bearing mice and normal mice. (A) MDSCs from bone marrow, lymph node, spleen, peripheral blood, thymus and tumor of tumor bearing mice were collected and the percentage of MDSCs in these organs was measured by flow cytometry. (B) The results represented mean ± SD of three independent experiments. The accumulation of MDSCs from high level to low level in different organs of tumor bearing mice was: bone marrow > peripheral blood > tumor > spleen > lymph node > thymus. (C) MDSCs from both spleen and peripheral blood of normal mice were collected and the percentage of MDSCs was measured by flow cytometry. (D) The results represented mean ± SD of three independent experiments. The accumulation of MDSCs from spleen was higher than that from peripheral blood in normal mice.

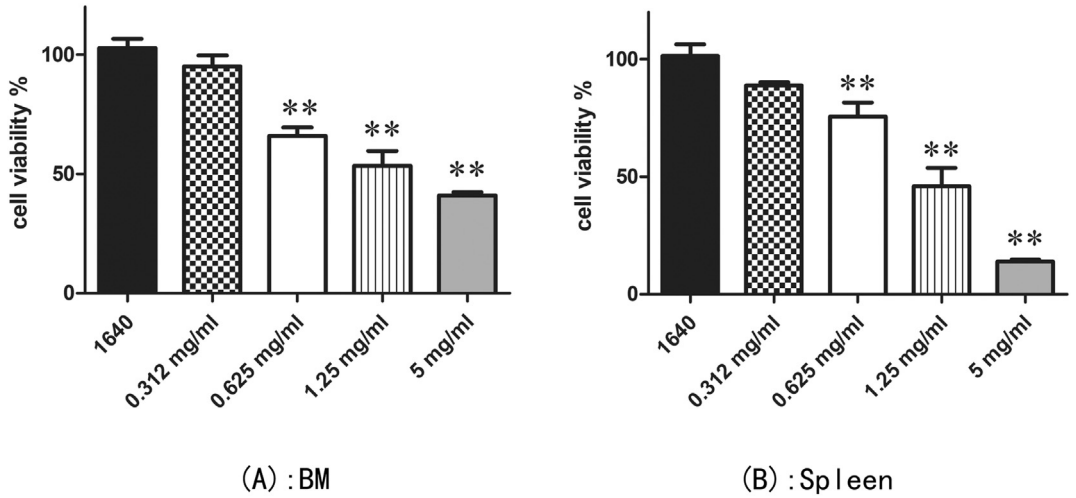

Fig. 3. The effect of MENK on proliferation of MDSCs in vitro. Purified MDSCs were cultured with MENK at a range of concentrations of 0.312 mg/ml, 0.625 mg/ml, 1.25 mg/ml and 5 mg/ml for 48 h in vitro and the proliferation was determined by CCK8. The data indicated that the proliferation of MDSCs was inhibited in bone marrow (A) and in spleen (B) by MENK. The optimal concentration was 1.25 mg/ml with the about 50% ratio of cell viability (**$p < 0.01$). The results represent mean ± SD of three independent experiments.

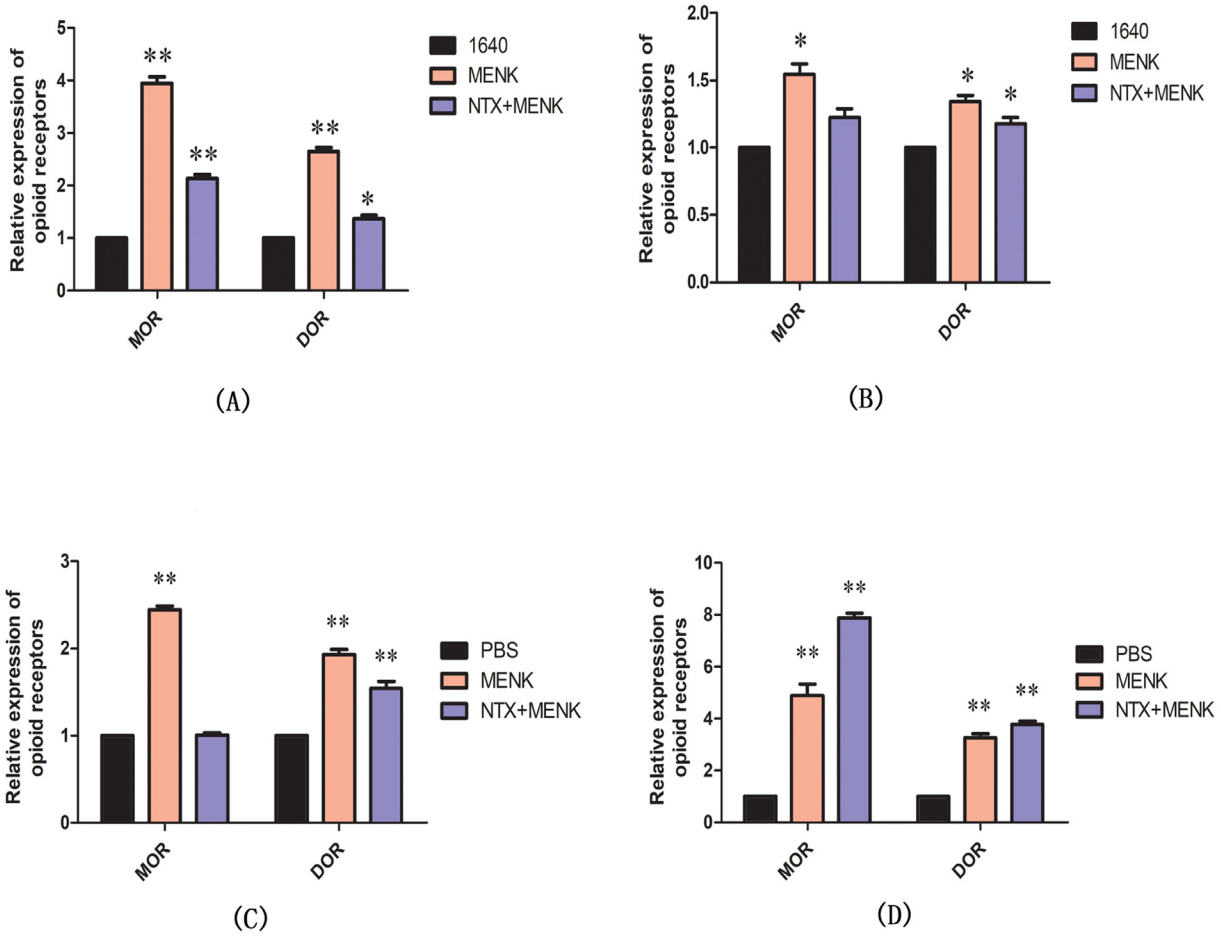

Fig. 4. The administration of MENK increased the expression of opioid receptors in MDSCs in vitro and in vivo. To identify the expression of opioid receptors, total RNA was extracted from purified MDSCs and reverse transcription-quantitative polymerase chain reaction were performed. The results represent mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$. The data indicated that after the administration of MENK the relative expressions of MOR and DOR on MDSCs from spleen increased in vitro (A). After the administration of MENK the relative expressions of MOR and DOR on MDSCs from bone marrow increased in vitro (B). After the administration of MENK the relative expressions of MOR and DOR on MDSCs from spleen increased in vivo (C). After the administration of MENK the relative expressions of MOR and DOR on MDSCs from bone marrow increased in vivo (D).

Table 2
The absolute number of MDSCs from spleen in tumor bearing mice after treatment with MENK.

Group	Number of MDSCs	*p* value
MENK	$(6.69 \pm 2.10) \times 10^6$	
PBS	$(1.27 \pm 0.27) \times 10^7$	< 0.05
MENK + NTX	$(1.38 \pm 0.17) \times 10^7$	< 0.05

After administration of MENK alone or with NTX, the absolute number of MDSCs from spleen in tumor bearing mice was detected by flow cytometry. Results represent the mean ± SD of three independent experiments.

from Amquar Bio Co., Ltd (USA). The E.Z.N.A.® total RNA kit II was purchased from Omega bio-tek (USA). PrimeScriptTMRT reagent kit and SYBR® Premix Ex TaqTM II were acquired from TaKaRa (Japan). The enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-1b, IL-4, IL-6, IL-10, and TGF-β were products of eBioscience Inc. (San Diego, CA, USA). Other chemicals frequently used in our laboratory are all from Sigma or Aldrich.

2.3. Establishment and treatment of mice models

S180 cells in logarithmic growth phase were inoculated into the abdominal cavity of C57BL/6 mice. As ascites grew, the ascites fluid in mice was extracted and supplemented with physiological saline. The tumor cell suspension ($2.0 * 10^7$/ml) was transplanted subcutaneously into the bilateral axilla of mice at 0.2 ml per mouse aseptically. For in vitro experiments, bone marrow, spleen, tumor, lymph node and peripheral blood were collected on 14th day. For in vivo experiment, the mice were randomly divided into three groups (8 mice/group): model control group, MENK group and MENK + NTX group. The mice in MENK group were treated with 20 mg/kg MENK. The mice in MENK + NTX group were treated with 20 mg/kg NTX first and then with 20 mg/kg MENK. The mice in model control group were treated with the same amount of PBS (Gibco, Thermo Fisher Scientific, USA). From the second day of tumor bearing, the mice in all groups were given intraperitoneal injection once a day for 14 successive days.

2.4. Isolation and purification of MDSCs

Highly purified and untouched MDSCs were isolated from spleen, bone marrow of tumor bearing mice by positive immunomagnetic selection according to the manufacturer's protocol. Firstly, under sterile condition splenocyte and bone marrow cells were separated respectively from mice, supplemented with red blood cell lysis buffer (Solarbio R1010) for 10 min at 4 °C and rinsed with PBS to stop the reaction. The cells were centrifuged at 1000*g* for 5 min, discarded supernatant and treated with culture medium. With use of Myeloid-Derived Suppressor Cell Isolation Kit, Gr-1^{high}Ly-$6G^+$ cells of single cell suspension was indirectly magnetically labeled with anti-Ly-6G,

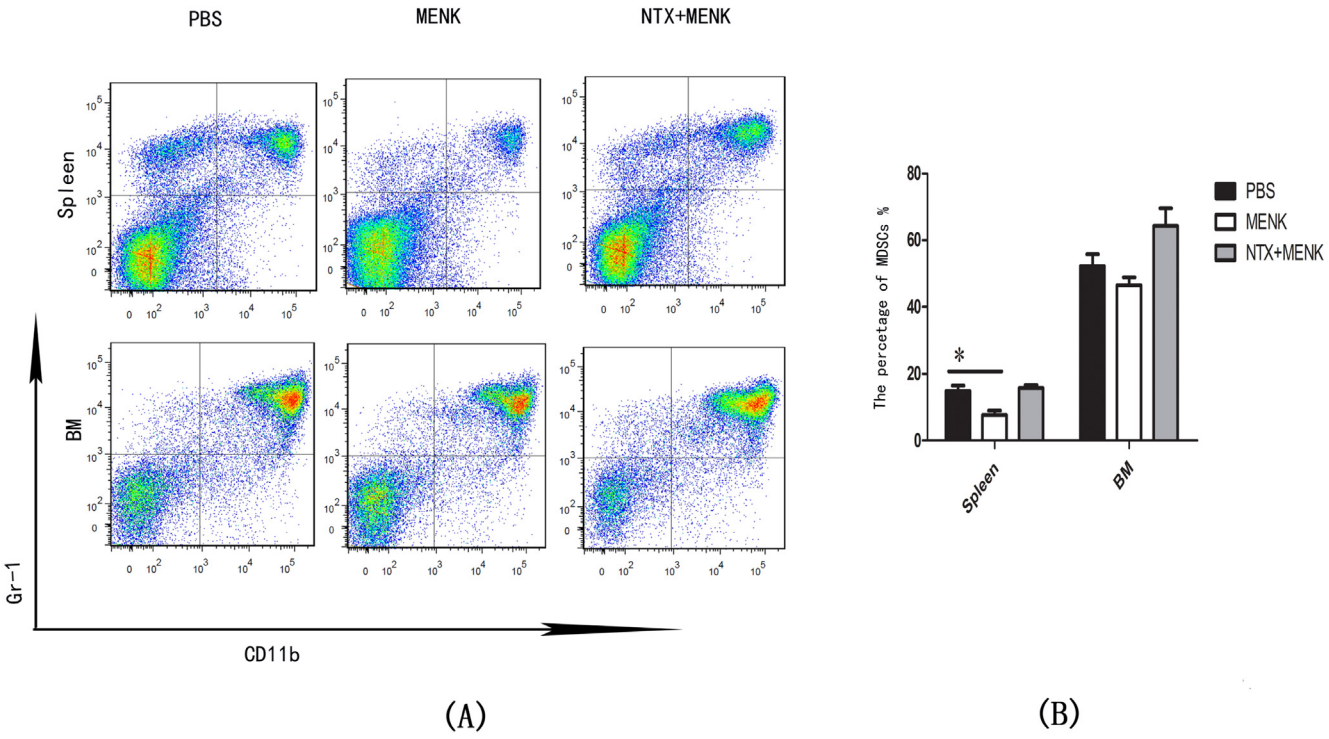

Fig. 5. Effect of MENK on MDSCs in vivo. The S180 cell suspension ($2.0 * 10^7$/ml) was transplanted subcutaneously into the bilateral axilla of mice at 0.2 ml per mouse aseptically to establish the animal models. The mice were randomly divided into three groups (8 mice/group): model control group (treated with same volume of PBS buffer), MENK group (treated with 20 mg/kg MENK) and MENK + NTX group (treated with 20 mg/kg NTX first and 1 h later associated with 20 mg/kg MENK). From the second day of tumor bearing the mice in all groups were given intraperitoneal injection once a day for 14 successive days. The MDSCs were harvested from splenocytes and bone marrow cells separately and flow cytometry was used to detect the proportion of MDSCs (A). The data represents mean ± SD of three independent experiments. *$p < 0.05$. The result showed that after the administration of MENK, the MDSCs from splenocytes were significantly inhibited and NTX could block this effect. There was no statistical significance among the three groups in bone marrow (B).

positively select Gr-1^{high}Ly-$6G^+$ cells in the process of magnetic separation to acquire pre-enriched Gr-1^{dim}Ly-$6G^-$ cells (flow-through fraction) and enriched Gr-1^{high}Ly-$6G^+$ cells (positive selected fraction). To increase the purity, Gr-1^{dim}Ly-$6G^-$ cells were indirectly labeled with anti-Gr-1 and positively select Gr-1^{dim}Ly-$6G^-$ cells in the process of magnetic separation. The purity of MDSCs was > 80% and the purified MDSCs were collected for subsequent experiments.

2.5. Administration of MENK in vitro

The purified MDSCs derived from tumor bearing mice bone marrow and spleen were collected separately for further analysis with CCK8 test, ELISA and quantitative real-time PCR and they were co-cultured with different chemicals for 48 h. For stimulation of MDSCs the primarily purified cells were cultured with the culture medium supplemented with GM-CSF (20 ng/ml), IL-6 (20 ng/ml), and 50% volume TCCM and stimulated with different concentrations of MENK in the presence or absence of NTX (200 nM) at 37 °C for 48 h with 5% CO_2. The cell in control group were treated with RPMI 1640 Medium (Glibo Thermo Fisher Scientific, USA). The treated cells were then used for subsequent experiments.

2.6. mRNA analysis by RT-qPCR

Total RNA was extracted from purified MDSCs and the reverse transcription-quantitative polymerase chain reaction were performed according to the instructions of OMEGA bio-tek total RNA kitII, TaKaRa SYBR® Premix Ex Tap™II and PrimeScript™ RT reagent kit. All primers were designed and synthesized by TaKaRa biomedical technology. Primer sequences for analysis were: Forward (5′-CCC AGT TCT TTA TGC GTT CCT-3′) and reverse (5′-ATT AGC CGT GGA GGG GTG T-3′) for MOR; forward (5′-ATG TAA AGA GGG CTG GGA ATG TAG-3′) and reverse (5′-GGG TTG GTT TTG TTG TTT GGA-3′) for DOR; Forward (5′-AGT TCT GGG AAT CTG CAT GG-3′) and reverse (5′-ATG TAC ACG ATG TCT TTG GCA GAT A-3′) for Arg-1; Forward (5′-CAA GCT GAA CTT CGA GGA-3′) and reverse (5′-TTT ACT CAG TGC CAG AAG CTG GA-3′) for iNOS; Forward (5′-CAT CCG TAA AGA CCT CTA TGC CAA C-3′) and reverse (5′-ATG GAG CCA CCG ATC CAC A-3′) for *Mus musculus* beta-actin. Applied biosystems 7500 Real-Time PCR system was used to detect the relative expression levels of MOR and DOR in vivo and in vitro, and the relative expression levels of Arg-1 and iNOS in vivo. The qPCR reactions were carried out as following: 1 cycle of pre-degeneration at 95 °C for 30 s, followed by40 cycles of PCR reaction at 95 °C for 5 s, and dissociation stage at 60 °C for 34 s, the last step was a melting curve. The relative mRNA expression levels were analyzed by the comparative $2-\Delta\Delta Ct$ formula and data were normalized by a housekeeping gene (β-Actin).

2.7. MDSCs proliferation assay in vitro

The 10^6 purified MDSCs suspension was inoculated into 96-well plate (100 μl/well). The plates were placed in the incubator for 4 h (37 °C, 5% CO_2) and the suspended lymphocytes were removed. Culture medium containing GM-CSF (20 ng/ml), IL-6(20 ng/ml) and 50% volume TCCM were added to culture for 48 h. 10 μl CCK8 solution was added to each well. Finally the cell activity in each well was detected at 450 nm (OD number, A450) by microplate reader.

2.8. Flow cytometry analysis in vivo

MDSCs were collected from tumor-bearing mice and normal mice. The proportion and absolute number of MDSCs were analyzed by flow

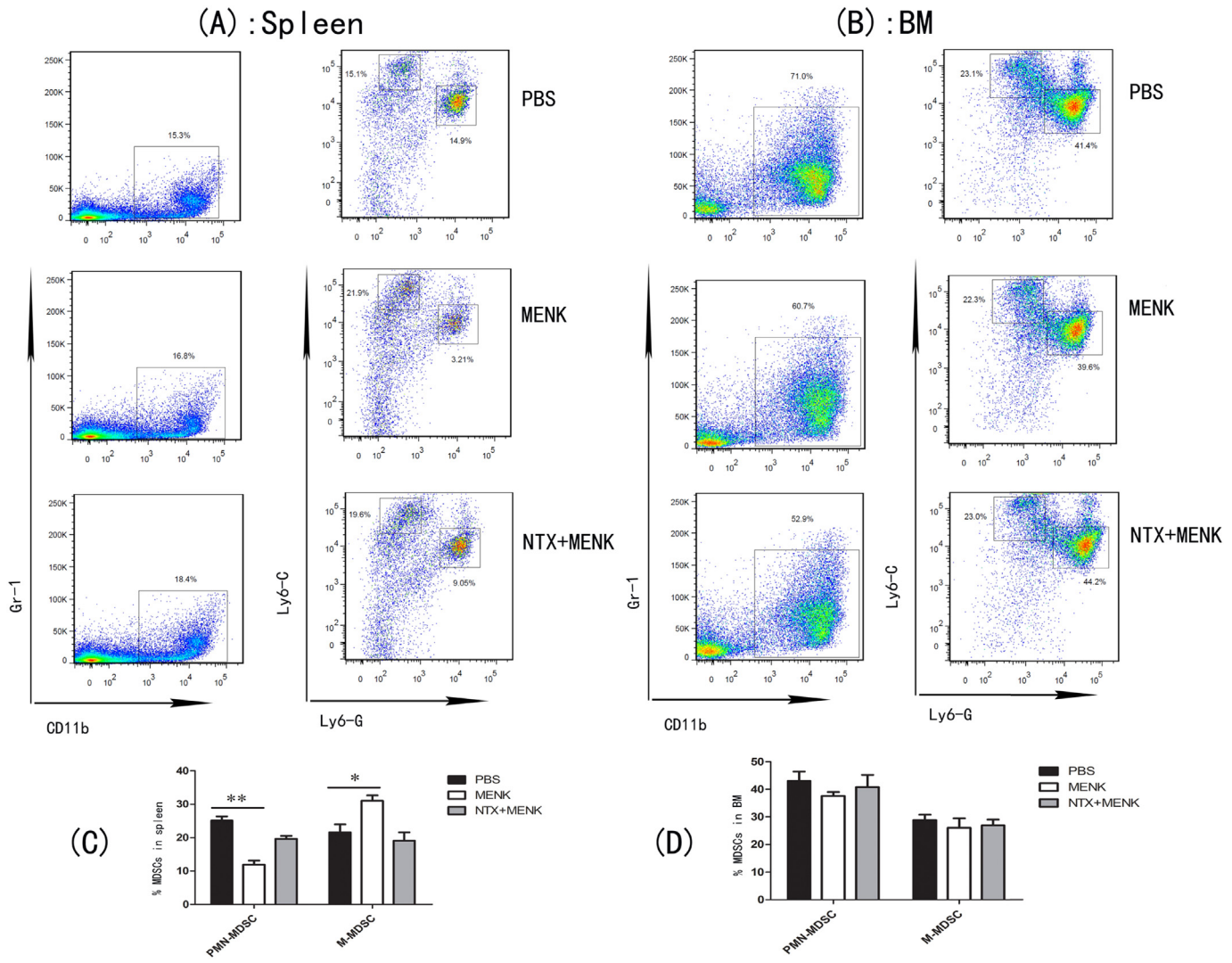

Fig. 6. The effect of MENK on subpopulations of MDSCs in tumor bearing mice. The method to establish animal models and the group situation were as same as mentioned before (in Fig. 5). The MDSCs were harvested from splenocytes and bone marrow cells separately and flow cytometry was used to detect the proportion of MDSCs. Mouse MDSC Flow Cocktail 2 with Isotype Ctrl (Cat. No 147003) was used to identify the subpopulations of MDSCs in spleen (A) and in bone marrow (B). The data represents mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$. The result showed that after the administration of MENK, PMN-MDSCs generated from splenocytes were significantly inhibited and NTX could block this effect. The level of M-MDSCs generated from splenocytes was up-regulated and NTX could block this effect (C). There was no significant difference of among the three groups in bone marrow (D).

cytometry. The staining was performed in accordance with the manufacturer's instructions and following Abs: anti-mouse/human CD11b (APC) (Cat. No. 101212); and anti-mouse Ly-6G/Ly-6C (Gr-1) (FITC) (Cat. No. 108406) were used. To identify the subpopulations of MDSCs, after administration of MENK alone or with NTX, the MDSCs were collected from bone marrow and spleen of tumor-bearing mice. Mouse MDSCs Flow Cocktail 2 with Isotype Ctrl (Cat. No 147003) was used for staining. The samples were acquired with a FACS Calibur (BDBiosciences) and data was analyzed with Flow Jo 7.6.1.

2.9. Elisa assay for cytokine production

The supernatant of MDSCs culture after administration of different drugs for 48 h was collected and productions of IL-1b, IL-4, Il-6, IL-10 and TGF-beta were measured by ELISA according to the manufacturer's instruction. The absorbance at 450 nm was determined by microplate reader.

2.10. Statistical analyses

All data represent the mean ± SD. Independent-samples *t*-test was used between two groups and in the occasion of more than three groups one-way ANOVA was used. p values < 0.05 ($^{*}p < 0.05$, $^{**}p < 0.01$) were considered statistically significant.

3. Results

3.1. Purification of MDSCs by MACS

The MDSCs were collected and purified from splenocyte and bone marrow cells separately of C57BL/6 mice by immune-magnetic positive selection. Before the process of purification the purity of ly6-G$^+$ cells was up to (16.45 ± 2.35) and ly6-G$^-$ cells was up to (24.20 ± 1.0) in spleen, after the process of purification the purity of ly6-G$^+$ cells was up to (80.2 ± 3.0) and ly6-G$^-$ cells was up to (83.05 ± 2.55). Before process of purification the purity of ly6-G$^+$ cells was up to (43.35 ± 0.85) and ly6-G$^-$ cells was up to (15.85 ± 1.45) in bone marrow, after the process of purification the purity of ly6-G$^+$ cells was

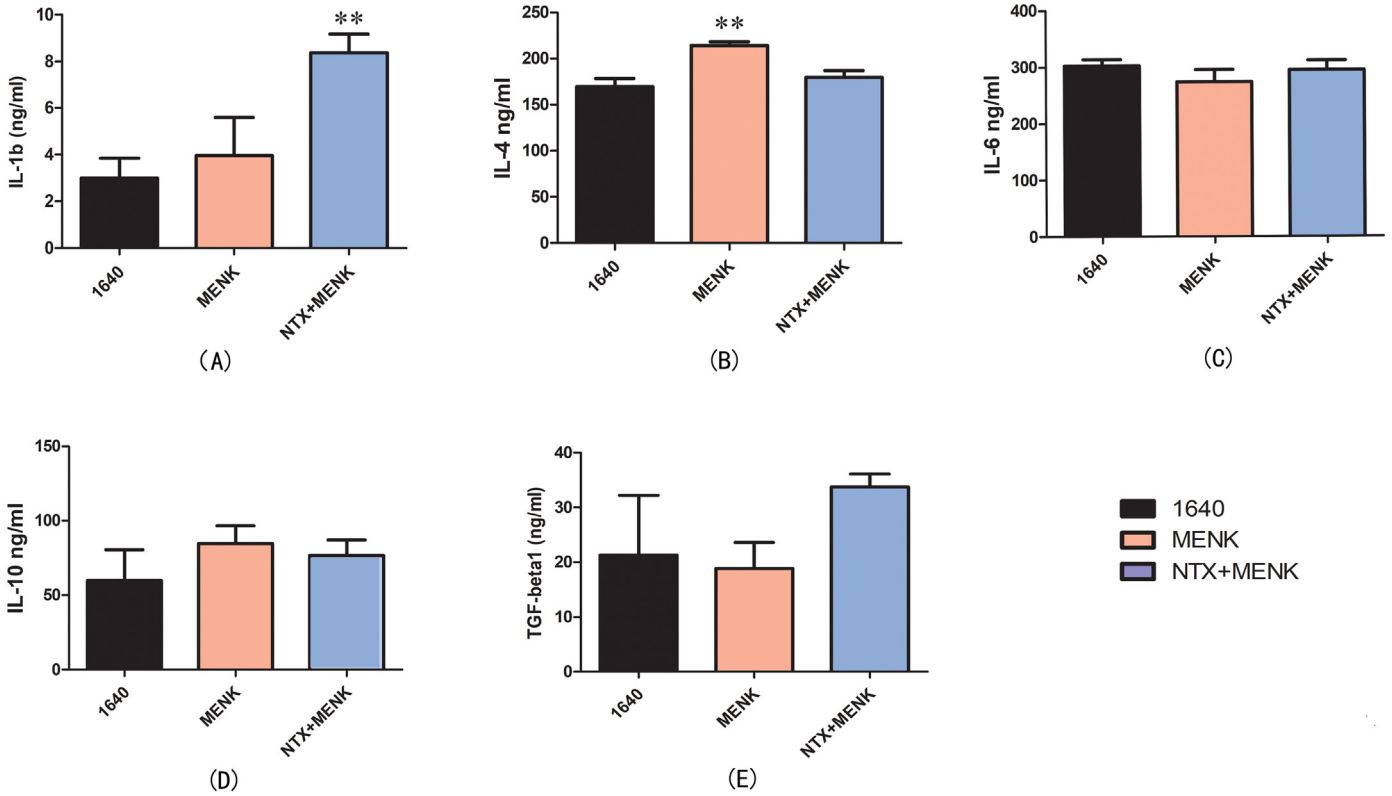

Fig. 7. Effect of MENK on cytokine production in MDSCs from bone marrow cells of tumor bearing mice in vitro. To detect the levels of cytokine productions in MDSCs generated from bone marrow of tumor bearing mice, after the administration of MENK present or absent with NTX, the supernatant of MDSCs were collected and ELISA was used to test the levels of IL-1β, IL-4, IL-6, IL-10, and TGF-β. The data represents mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$. The results showed that the level of IL-6 increased with the administration of MENK alone or accompanied with NTX (C). The level of IL-4 increased with the administration of MENK accompanied with NTX (B). There was no significant difference for IL-1β (A), IL-10 (D) and TGF-β (E).

up to (91.35 ± 0.45) and ly6-G$^-$ cells was up to (84.95 ± 1.75). After the process of purification, the purity of MDSCs was > 80%. The subsequent experiments were carried out with the purified cells (Fig. 1).

3.2. Distribution of MDSCs

Determined by flow cytometry the absolute number of MDSCs from spleen was $(2.07 \pm 0.36) \times 10^6$ in normal mice and was $(1.27 \pm 0.27) \times 10^7$ in tumor bearing mice. The difference was statistically significant ($p < 0.01$). The absolute number of MDSCs from peripheral blood was $(6.62 \pm 3.51) \times 10^3$ in normal mice and was $(6.43 \pm 1.79) \times 10^5$ in tumor bearing mice. The difference was statistically significant ($p < 0.01$) (Table 1).

Determined by flow cytometry the contents of MDSCs in various organs of tumor-bearing mice from high to low were: 61.95 ± 0.21 in bone marrow, 19.05 ± 1.76 in peripheral blood, 11.70 ± 1.97 in tumor, 8.99 ± 1.70 in spleen, 3.00 ± 0.38 in lymph node and 0.34 ± 0.31 in thymus (Fig. 2A, B). The percentage of MDSCs from normal mice was 2.13 ± 0.17 in spleen and 0.71 ± 0.04 in peripheral blood (Fig. 2C, D).

3.3. The viability of MDSCs post treatment with MENK

Purified MDSCs were treated with MENK at a range of concentrations of 0.312 mg/ml, 0.625 mg/ml, 1.25 mg/ml and 5 mg/ml for 48 h in vitro and the proliferation was determined by CCK8. The data showed that MENK inhibited the viability of MDSCs at a dose dependent way. The optimal concentration was found to be 1.25 mg/ml, with the 46.05 ± 7.70 ratio of cell viability of MDSCs in spleen and 53.45 ± 6.29 ratio of cell viability of MDSCs in bone marrow. When the concentration was 0.625 mg/ml, the ratio of cell viability was 75.65 ± 6.01 in spleen and 65.95 ± 3.74 in bone marrow as shown in Fig. 3.

3.4. MENK up-regulated the expressions of opioid receptors in vitro and in vivo

To explore the relation between MENK and opioid receptors on MDSCs, the expressions of opioid receptors were detected at RNA level in vitro and in vivo (Fig. 4). According to the current data, MENK could augment the relative expressions of mu and delta opioid receptors at RNA level both in spleen and bone marrow. Fig. 4A showed expressions of opioid receptors from spleen in vitro: MENK group yielded the fold-change of MOR (3.94 ± 0.21) and DOR (2.64 ± 0.13), MENK + NTX group yielded the fold-change of MOR (2.12 ± 0.14) and DOR (1.36 ± 0.11), separately compared with those in the control group. The difference was statistically significant ($p < 0.05$). Fig. 4B showed expressions of opioid receptors from bone marrow in vitro: MENK group yielded the fold-change of MOR (1.54 ± 0.14) and DOR (1.34 ± 0.07), MENK + NTX group yielded fold-change of DOR (1.18 ± 0.014) compared with those in the control group. The difference was statistically significant ($p < 0.05$), however, MOR (1.22 ± 0.0.11) was with no significant difference from that in the control group. Fig. 4C showed expressions of opioid receptors from spleen in vivo: MENK group yielded the fold-change of MOR (2.44 ± 0.07) and DOR (1.93 ± 0.10), MENK + NTX group yielded the fold-change of DOR (1.54 ± 0.14), separately compared with those in the control group. The difference was statistically significant ($p < 0.05$), however, MOR (1.01 ± 0.03) was with no significant difference from that in the control group. Fig. 4D showed expressions of opioid receptors from bone marrow in vivo: MENK group yielded the fold-change of MOR (4.86 ± 0.80) and DOR (3.25 ± 0.26),

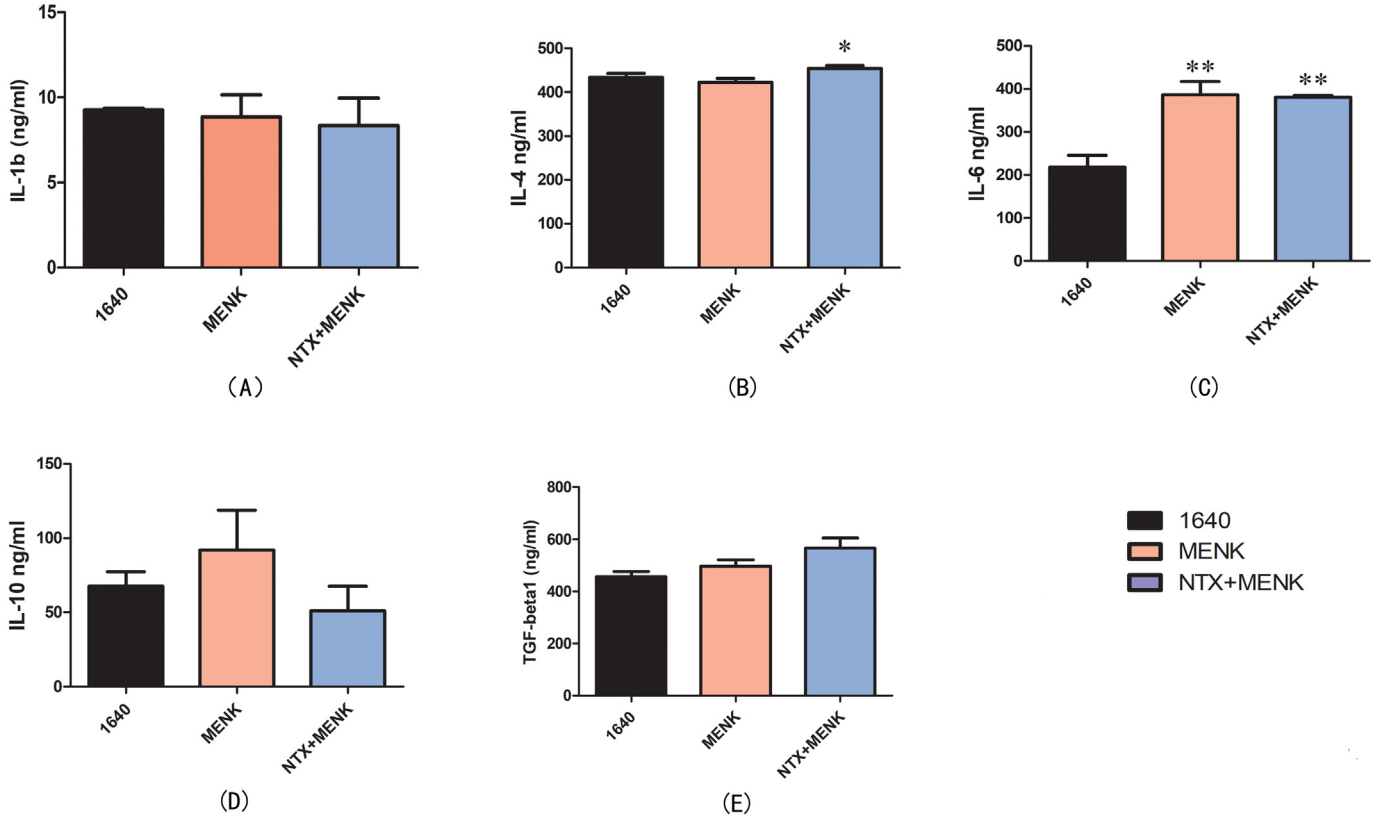

Fig. 8. Effect of MENK on cytokine production in MDSCs in spleen of tumor bearing mice in vitro. To detect the levels of cytokine productions in MDSCs generated from spleen of tumor bearing mice, after the administration of MENK present or absent with NTX, the supernatant of MDSCs were collected and ELISA was used to test the levels of IL-1β, IL-4, IL-6, IL-10, and TGF-β. The data represents mean ± SD of three independent experiments. $^{**}p < 0.01$. The results showed that the level of IL-4 increased with the administration of MENK (B). The level of IL-1β increased with the administration of MENK (A). There was no significant difference for IL-6 (C), IL-10 (D) and TGF-β (E).

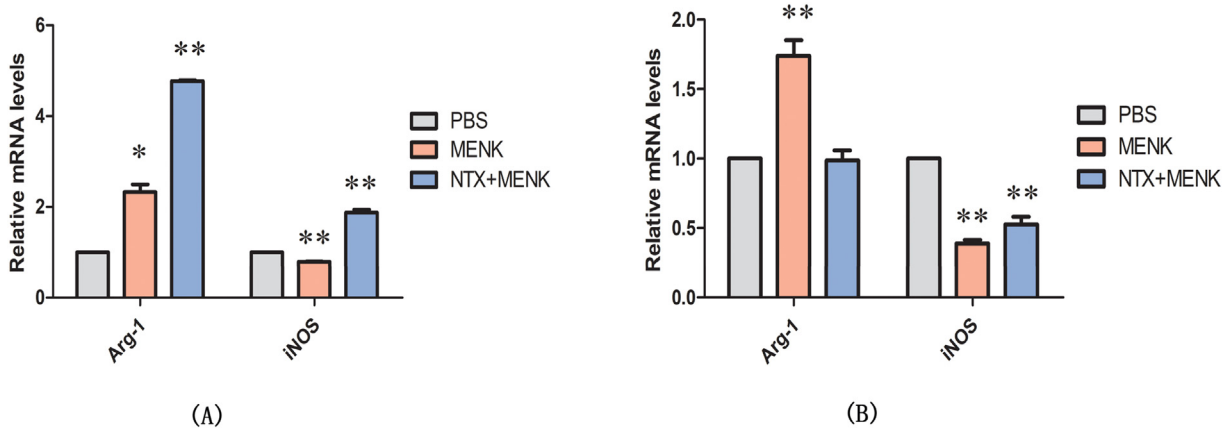

Fig. 9. The administration of MENK reduced the expression of iNOS while it increased the expression of Arg-1 in MDSCs in vivo. After treatment with MENK with or without NTX, the total RNA was extracted from the purified MDSCs from splenocytes and myeloid cells separately in tumor bearing mice. RT-qPCR was used to identify the relative expression of Arg-1 and iNOS. The data represents mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$. The results indicated that MENK reduced the expressions of iNOS in bone marrow (A) and in spleen (B). MENK increased the expressions of Arg-1 in bone marrow (A) and in spleen (B).

MENK + NTX group yielded the fold-change of MOR (7.86 ± 0.3) and DOR (3.76 ± 0.21), separately compared with those in the control group. The difference was statistically significant ($p < 0.01$).

3.5. Effect of MENK on subpopulations of MDSCs in vivo

After administration with MENK in the presence or absence of NTX, the influence of MENK on subpopulations of MDSCs was identified with flow cytometry. The absolute number of total MDSCs in the spleen was $(6.69 \pm 2.10) \times 10^6$ in the MENK group, compared with $(1.27 \pm 0.27) \times 10^7$ in the tumor bearing control group and in the MENK + NTX group was $(1.38 \pm 0.17) \times 10^7$. The reduction in the number of total MDSCs in the MENK group was statistically significant ($p < 0.05$) (Table 2).

The frequency of total MDSCs in the spleen was 7.62 ± 2.4 in the MENK group, compared with 14.83 ± 2.92 in the tumor bearing control group and in the MENK + NTX group was 15.93 ± 1.0. The decrease in the frequency of total MDSCs in the MENK group was

statistically significant ($p < 0.05$) (Fig. 5). While examining the MDSC subsets, the frequency of PMN-MDSCs was 11.90 ± 2.2 in the MENK group, which was significantly different from the frequency 25.16 ± 2.1 in the tumor bearing control group and the frequency in the MENK + NTX group, which was 19.60 ± 1.65 ($p < 0.05$). In contrast to the decreased frequency of PMN-MDSCs the frequency of M-MDSCs was significantly increased to 31.06 ± 2.70 in the MENK group relative to the 21.60 ± 4.07 in the tumor bearing control group, which was reversed by the co-administration of NTX to 19.06 ± 4.39 ($p < 0.05$) (Fig. 6A, C). These data indicate that in spleen, after the administration with MENK, the frequency of MDSCs decreased in tumor bearing mice, which was due to a significant reduction in PMN-MDSCs; however, the frequency of M-MDSCs increased.

The frequency of total MDSCs in the bone marrow cells was 46.53 ± 63.93 in the MENK group, compared with 52.36 ± 6.34 in the tumor bearing control group and in the MENK + NTX group was 15.93 ± 1.0 (Fig. 5). While examining the MDSC subsets, the frequency of PMN-MDSCs was 37.56 ± 2.55 in the MENK group, which was 43.03 ± 5.92 in the tumor bearing control group and the frequency in the MENK + NTX group was 40.08 ± 7.76. The frequency of M-MDSCs was 22.76 ± 3.46 in the MENK group, which was 28.80 ± 3.46 in the tumor bearing control group and the frequency in the MENK + NTX group was 26.96 ± 3.52 (Fig. 6B, D). In bone marrow, the frequency of total MDSCs and two subsets in the tumor bearing mice was decreased after the administration of MENK despite there was no statistical significance.

3.6. Effect of MENK on cytokine production in MDSCs

Post treatment with MENK the productions of IL-1β, IL-4, IL-6, IL-10, and TGF-β secreted by MDSCs in tumor bearing mice were assayed by ELISA. As illustrated in (Fig. 7), MENK treatment triggered IL-4 secretion (214.0 ± 4.25) vs. that in control group (169.53 ± 8.92) ($p < 0.05$) in spleen only. IL-6 secretion in bone marrow increased as 386.51 ± 30.97 in MENK group and 380.94 ± 4.66 in MENK + NTX group, compared with those in control group (218.05 ± 27.75) ($p < 0.05$, all) (Fig. 8). However there was not significant effect on the expressions of IL-1b, IL-10 and TGF-β either in spleen or in bone marrow ($p > 0.05$ for all).

3.7. MENK up-regulated the expression of Arg-1and down-regulated the expression of iNOS in MDSCs

To identify the suppressing potential of MDSCs in tumor bearing mice, the expressions of Arg-1 and iNOS were measured at RNA level (Fig. 9). According to the current data, the expressions of iNOS reduced after treatment with MENK while the expressions of Arg-1 increased both in spleen and bone marrow. As shown in Fig. 9A, MENK group yielded the fold-change of Arg-1 (2.33 ± 0.28) and iNOS (0.078 ± 0.01) in bone marrow, MENK + NTX group yielded the fold-change of Arg-1 (4.76 ± 0.04) and iNOS (1.87 ± 0.011), separately compared with those in the control group. The difference was statistically significant ($p < 0.05$). As shown in Fig. 9B, MENK group yielded the fold-change of Arg-1 (1.74 ± 0.19) and iNOS (0.39 ± 0.04) in spleen, MENK + NTX group yielded the fold-change of iNOS (0.52 ± 0,08), separately compared with those in the control group. The difference was statistically significant ($p < 0.05$), however, there was no significant difference for Arg-1 (0.98 ± 0.12) from that in the control group.

4. Discussion

It is reported that MDSCs could accumulate in chronic inflammation and tumor environment and have broad immune-suppressive effect, which have significant relationship with immune escape of tumors. They comprise a variety of myeloid-derived immature and highly heterogeneous cellular populations, including dendritic cells, macrophages and/or granulocytes [13]. Tumor-derived biological factors are responsible for the expansion, mobilization and activation of MDSCs. Published data reveal that the immune-suppressive mechanisms of MDSCs include amounts of inducible nitric oxide syntheses and arginase1 (Arg-1), which directly inhibit T cell functions by stimulating NO and reactive oxygen species (ROS) [14,15]. Also TCR CD3ζsynthesis was blocked by consumption of arginine by Arg-1 that will cause T cells signal transduction obstacle. MDSCs in tumor tissues differentiated into tumor-associated macrophage (TAM) and vascular endothelial cells to exert immune-suppressive effect [16,17]. Inhibitory cytokines such as TGF-β and IL-10 secreted by MDSCs could induce CD4 + CD25 + Foxp3 + regulatory T cells (Treg), which will suppress tumor immunity [18,19].

Immuno-regulatory and anti-cancer effects of MENK have gradually attracted increasing attention. According to the published literatures, MENK could regulate polarization of macrophage to M1 phenotype, stimulate natural killer cells response, inducing the production of cytokine, such as IL-2, IL-12, TNF-α and enhancing target killing activities of NK cells [20]. In vivo and in vitro administration of MENK could up-regulate the frequency of CD8 + T cells as well as induce markers of T-cell activation, enhance cytotoxicity against mouse S180 tumor cells and increase secretion of IFN-γ [21]. MENK could also promote the differentiation and maturation of dendritic cells and boost Ag processing and presentation. There is positive modulation of MENK on the interactions between DCs and CD4 + T cells. MENK can inhibit Treg activity and down-regulate level of Treg to impede tumor development in mice [22–24].

MDSCs were similar to granulocyte or macrophage in morphology and phenotype. In humans, MDSCs were mostly identified in blood and tumors in various organs. In mice, MDSCs accumulate in peripheral blood, spleen, liver, lung, or tumors of various organs.

Our current study indicated that: (1) compared with that in normal mice the number of MDSCs from both spleen and peripheral blood in tumor bearing mice increased significantly. In tumor-bearing mice the distribution of MDSCs from high to low was: bone marrow, peripheral blood, tumor, spleen, lymph node and thymus; (2) as concentration of MENK increased, the proliferation of MDSCs generated from both splenocyte and bone marrow cells was suppressed at a dose dependent manner and the optimal concentration was 1.25 mg/ml (inhibition rate was up to 50%); (3) there were MOR and DOR receptors on MDSCs and MENK could up-regulate the expressions of both MOR and DOR, to which MENK would bind to and trigger suppression of MDSCs; (4) MENK could regulate the functions of MDSCs as evidenced by changes of secretion of a spectrum of cytokines by MDSCs, such as higher level of IL-4; (5) MENK could significantly reduce the number and the frequency of total MDSCs in spleen of tumor bearing mice. Although there was no statistical difference, after administration of MENK, the frequency of total MDSCs was decreased in bone marrow, which was consistent with the phenomenon found in spleen; (6) The decrease in frequency of total MDSCs in spleen was due to the significant reduction of PMN-MDSCs. However, the frequency of M-MDSCs increased. This was consistent with the results of the increased arginase1. Arg-1 and iNOS, contributing to the immune-regulatory activity of MDSCs was mainly found in M-MDSCs [25]. In our study, the proportion of M-MDSCs enhanced after the treatment of MENK, which was consistent with the increased expression of Arg-1 in RNA level, however, the expression of iNOS decreased. The present results did not figure out whether different changes either iNOS or Arg-1 affect the suppressing ability of MDSCs. Biochemical analyses of downstream metabolites (such as NO, kinurenines, ornithine, urea and polyamines) may solve this problem (under separate research).

It should be further noted that there were two main populations of MDSCs: PMN-MDSCs ($CD11b^+Ly6G^+Ly6C^{dim}$) and M-MDSCs ($CD11b^+Ly6G^-Ly6C^{high}$) in mice. In addition to these two groups, MDSCs include a small group (< 3%) of cells with myeloid colony-

forming activity, representing a mixture of myeloid progenitors and precursors. Maybe this can explain that after purification of MDSCs there were still two groups' cells in $CD11b^{+}Ly6G^{-}Ly6C^{+}$ cells.

Although PMN-MDSCs and M-MDSCs were activated by different mechanism, via the production of iNOS, NO, ROS, and arginase1, both groups have immune suppressive functions. The tumor cells and host cells secrete various cytokines into the tumor microenvironment, such as IL-1β, IL-6, VEGF and STAT1, which would help the accumulation of M-MDSCs. STAT1 induces downstream factors, including iNOS and NO [26], which can inhibit the proliferation and MHC class II expression on T cells [27] resulting in T cell apoptosis [28]. In other hand, induction of ER stress and STAT3 are associated with activation of PMN-MDSCs [29]. STAT3 can induce the production of ROS and NADPH [30]. ROS has been known to suppress T cell function through modification of the TCR and develop in many cancers [31]. It has been proven in published papers that the percentage of CD8+ T cells in spleen of tumor-bearing mice was up-regulated by MENK [3]. Meanwhile in our research that the number and the frequency of total MDSCs in spleen of tumor bearing mice decreased after treatment with MENK. We inferred that MNEK could suppress the inhibitory effect of MDSCs on T cells through interfering with the mechanisms mentioned above (This issue needs to be solved by separate research).

Mu and delta receptors are the two most studied and best established opioid receptor types though several types of opioid receptors have been described [32,33]. These receptors have all been found on the surface of various immune cells and tumor cells. Through binding to opioid receptors on immune cells, MENK at the appropriate concentration can act as a messenger to bridge between the immune system and the central nervous system, and play a significant role in immune regulation. NTX was used as a non-selective opioid receptor antagonist to block the binding of endogenous opioid peptides to opioid receptors. The current results manifested that both MOR and DOR on MDSCs were up-regulated by the treatment of MENK accompanied with the reduction in number and frequency of MDSCs in spleen of tumor bearing mice. In some extent NTX could partially block this action. This indicated that the inhibition of MDSCs by MENK could be the result from the up-regulation of opioid receptors and binding to the receptors by MENK. There were no statistically significant results in bone marrow. It will be meaningful work to explore other mechanisms of MENK in regulating MDSCs.

Additionally, IL-4 is with antitumor activity and is associated with the proliferation and differentiation of myeloid progenitor cells. It is reported that IL-4 could induce IRF4 expression in MDSCs, and recover the expression of IRF4 decreased by tumor induction. Thus, IL-4 induced IRF4 expression may positively affect the immune response by inhibiting MDSCs generation [34]. In the present experiment, after the treatment with MENK the level of IL-4 in the supernatants increased. This result indirectly proved the inhibition effect of MENK on MDSCs.

The data obtained above are preliminary and there is still more work to do in depth in order to unravel the detailed mechanisms through which, MENK regulate MDSCs.

We believe it is the first time that published results prove that MENK could exert inhibiting effect on MDSCs and this work provides a more meaningful clue to the application of MENK as immune modulator in immune regulation and cancer therapy.

In conclusion, our study has proven that MENK could suppress the proliferation of MDSCs both in vivo and in vitro. This inhibition effect is mainly achieved by reducing the proportion of PMN-MDSCs. The mechanism of this effect may associate with MENK up-regulating the expressions of MOR and DOR on MDSCs.

Conflict of interest

Noreen Griffin is CEO for Immune Therapeutics Inc. USA, Fengping Shan is Chief Science Officer for Immune Therapeutics Inc. All of the Authors formally inform editorial office of that there is no conflict of interest for this work.

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