

Original Article

Anti-CD40 antibody and toll-like receptor 3 ligand restore dendritic cell-mediated anti-tumor immunity suppressed by morphine

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Abstract: The influence of morphine on host immunity and the underlying mechanism are still unclear. In the current study, we investigated the influence of morphine on dendritic cells (DCs), its possible mechanism of action, and the molecules that could reverse these effects. Morphine suppressed DC maturation, antigen presenting abilities, and the ability to activate antigen-specific CD8⁺ T cells. Morphine-treated DCs also secreted higher concentrations of IL-10, but lower IL-6 and TNF- α . Morphine-treated DCs showed decreased ERK1/2 phosphorylation and reduced p38 dephosphorylation. The *in vivo* administration of immuno-modulators, anti-CD40 Ab and TLR3 ligand-poly(I:C), enhanced antigen-specific immunity, promoted the anti-tumor effects, and prolonged the survival of morphine-treated, tumor-bearing mice by promoting the maturation and function of BMM-derived DCs by enhancing ERK1/2 phosphorylation and p38 dephosphorylation. We concluded that morphine can inhibit DC-mediated anti-tumor immunity by suppressing DC maturation and function. Immuno-modulators, such as anti-CD40 Abs and TLR agonists, can restore the DC-mediated anti-tumor immunity. Use of immuno-modulators could serve as a useful approach to overcome the immunocompromised state generated by morphine.

Keywords: Morphine, dendritic cells, antigen processing, antigen presenting cells, ERK1/2 transduction pathway, p38 transduction pathway, immunosuppression, immune-modulator, anti-tumor effects

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells, and are required for the initiation of immune responses by stimulating naïve, memory, and effector T cells [1]. Besides linking innate and adaptive immune responses, DCs also control immunity based on their ability to induce T-regulatory cells to promote antigen-specific unresponsiveness of lymphocytes in primary and secondary lymphoid tissues [2]. Thus, the possibility of harnessing the power of DCs to fight infectious diseases and cancer, and to regulate inflammatory diseases has received a lot of attention in immunology research.

DCs exist in two functional states, immature and mature, with only mature DCs having the ability to prime an immune response. Immature DCs capture foreign antigens, undergo maturation, and migrate to secondary lymphoid organs, such as lymph nodes. Immature DCs have a reduced capacity to capture antigens and stimulate the proliferation and secretion of IFN- γ by T-lymphocytes in immunocompromised or cancer patients. Mature DCs show high surface expression of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86) but a decreased capacity to internalize antigens [3]. Previous studies have also demonstrated a reduced number of immature DCs in peripheral blood and tumors, correlating with the progno-

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sis for some cancers, including non-small cell lung cancer, and head and neck and breast cancers [4]. Tumor-derived soluble factors, such as vascular endothelial growth factor (VEGF) and transforming growth factor- β (TGF- β), have been shown to be potent stimulators of immature DC migration from bone marrow to the tumor microenvironment [5]. However, the nature of DCs in tumors, the interaction between the tumor environment and the interruption in DC function, and the related mechanisms affecting DC-mediated immunity by tumors are unclear.

Immature DCs can also increase the expression of anti-inflammatory cytokines, such as IL-10 and TGF- β , and decrease the expression of pro-inflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF)- α [6]. Thus, investigating the existence of a constitutively increased number of immature DCs and subsequent production is important in ascertaining the immune privilege in tumor progression and the development of cancer therapy.

Morphine is used for the management of post-operative pain after surgery [7] and cancer-related pains [8]. Sacerdote et al. [9] reported that morphine is highly associated with the risk of immunomodulatory and immunosuppressive effects. Morphine has been reported to alter mitogen-stimulated T and B cell responses [10], to attenuate antibody production [11], and to reduce NK cell activity [12]. However, the molecular events involved in the differentiation and generation of immature DCs after morphine treatment are still poorly characterized.

Our previous study showed that morphine promoted tumor growth and suppressed host immunity by decreasing the viability of CD4⁺ helper and CD8⁺ cytotoxic T cells in a dose-dependent manner [13]. In the present study, the possible biological effects of morphine on antigen-presenting DCs were further evaluated. We first identified that morphine delayed the differentiation and maturation of bone marrow monocyte (BMM)-derived DCs. The secreted cytokines of morphine-treated DCs, such as IL-6, IL-10, and TNF- α , also changed. The morphine-treated BMM-derived DCs showed decreased ERK1/2 dephosphorylation but not decreased p38 phosphorylation, when compared with PBS-treated BMM-derived DCs that were stimulated with lipopolysaccharide. The maturation status and function of the BMM-

derived DCs could be restored by activating the ERK1/2 phosphorylation pathway and reducing the p38 phosphorylation pathway in morphine-treated mice after administering anti-CD40 Abs and/or poly(I:C). In addition, anti-CD40 Abs combined with poly(I:C) could enhance mesothelin antigen-specific immunity, generate more potent anti-tumor effects, and prolong the survival of morphine-treated, tumor bearing mice. In summary, morphine resulted in an immunocompromised status and promoted tumor formation by inhibiting the maturation and function of DCs. Therefore, using immunomodulators could serve as a useful approach to overcome the immunocompromised status generated by morphine treatment.

Materials and methods

Mice

Six- to 8-week-old female C57BL/6J mice were purchased and kept in the animal facility at the school of Medicine, National Taiwan University. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the College of Medicine, National Taiwan University.

Generation of bone marrow monocyte (BMM)-derived immature DCs

BMM-derived DCs were generated by culturing bone marrow mononuclear cells (BMMCs) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), as described previously [14, 15]. In brief, BMMCs were acquired from the femurs of C57BL/6J mice and cultured in Dulbecco's modified Eagle's minimal essential medium, supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT), 10^{-2} mM 2-mercaptoethanol (2-ME; Sigma Chemicals Co., St Louis, MO), 2 mM L-glutamine, 1 mM vitamins, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 100 μ g/ml gentamicin (all purchased from Gibco BRL, Rockville, NY), in 24-well plates (Sarstedt, Newton, NC) at 1×10^6 cells/ml in a total volume of 2 ml/well with 10 ng/ml of recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) for 6 days. For the morphine experiments, morphine was added on the first day of culture. Fresh GM-CSF- and/or morphine-containing medium was replaced every other day, and the cells were collected on day 6 for further study.

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BMM-derived DCs induced by lipopolysaccharide (LPS)

To examine whether LPS could induce the maturation of BMM-derived DCs, the DCs treated with or without morphine were cultured as described earlier, and LPS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added on days 2, 4, or 6. These LPS-stimulated BMM-derived DCs were collected 24 hours later for further study.

Monoclonal antibodies (mAbs) and flow cytometric analysis

The antibodies used for the flow cytometric analysis of the BMM-derived DCs included Armenian hamster anti-mouse CD11c (clone HL3; phycoerythrin (PE)- or allophycocyanin-conjugated), Armenian hamster anti-mouse CD40 (clone HM40-3; fluorescein isothiocyanate (FITC)-conjugated), rat anti-mouse CD86 (clone GL1; unlabeled), and rat anti-mouse major histocompatibility complex (MHC) class II (clone AF6-120.01; biotinylated). All mAbs were purchased from BD PharMingen (Heidelberg, Germany). The secondary reagents used for the detection of purified or biotinylated primary mAbs were Cy5-conjugated goat anti-rat IgG (Dianova, Hamburg, Germany) and peridinin chlorophyll protein-conjugated streptavidin (BD PharMingen).

Multicolor flow cytometry was performed as described previously [14, 16]. The cells were electronically gated according to their light scatter properties and the expression of the DC marker- CD11c [14]. Data were collected using a FACS Calibur flow cytometer, and analyzed with the CellQuest software (BD PharMingen).

ELISA for secreted cytokines from the BMM-derived DCs treated with morphine

To further confirm the changes in cytokine secretion by the morphine-treated BMM-derived DCs after LPS stimulation, BMM-derived DCs (10^6 /well) were seeded on a 24-well plate, cultured with GM-CSF and morphine, followed by LPS stimulation as described earlier. The concentrations of murine IL-2, IL-4, IL-6, IL-10, and TNF- α in the cell supernatants were determined using ELISA kits (BD Biosciences). All measurements were performed in triplicate.

In vitro antigen processing ability of BMM-derived DCs treated with morphine

To analyze the influence of morphine on antigen processing ability, the BMM-derived DCs were treated with different concentrations of morphine or PBS, and 1 μ g/ml FITC-conjugated OVA long peptide (OVA₃₂₃₋₃₃₉ [ISQAVHAAHAEI-NEAGR]) (Molecular Probes, Eugene, OR) for 6 days, as described previously [14]. The cells were then washed and stained with PE-conjugated (BD Biosciences) anti-CD11c Ab, and assessed by flow cytometry as described earlier.

In vitro antigen presentation ability of BMM-derived DCs treated with morphine

To further analyze the influence of morphine on antigen presentation abilities, the BMM-derived DCs were treated with different concentrations of morphine or PBS, and 50 μ g/ml FITC-conjugated OVA short peptide (OVA₂₅₇₋₂₆₄ [SIINFEKL]) (Molecular Probes) for 6 days. The cells were then washed and stained with PE-conjugated (BD Biosciences) anti-CD11c Ab and assessed by flow cytometry as described earlier.

Antigen presentation ability of the BMM-derived DCs treated with morphine in the activation of antigen-specific cytotoxic CD8⁺ T lymphocytes analyzed by flow cytometric analysis

The influence of morphine on BMM-derived DCs mediated antigen-specific cytotoxic CD8⁺ T lymphocytes activation was analyzed as described previously, with some modifications [17]. Briefly, the BMM-derived DCs (1×10^5 cells/well) were pulsed with 1 μ g/ml D^b compatible MHC I E7 peptide (amino acids (aa) 49-57; Kelowna International Scientific Inc.) at day 7 and then co-cultured with the E7-specific CD8⁺ T cell line (1:10 ratio) overnight. Protein transport inhibitor BD GolgiPlugTM (BD Biosciences) was added 6 h before collecting the cells. The co-cultured cells were then stained with PE-conjugated anti-CD8 Ab (BioLegend) and FITC-conjugated anti-IFN- γ Ab (BioLegend), and analyzed by flow cytometry.

In vitro anti-tumor activity of antigen-specific cytotoxic CD8⁺ T lymphocytes activated by BMM-derived DCs treated with morphine

In vitro effects of morphine on BMM-derived DC-mediated antigen-specific cytotoxic CD8⁺ T

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lymphocytes was analyzed as described previously, with some modifications [17]. Briefly, the BMM-derived DCs (1×10^5 cells/well) were pulsed with $1 \mu\text{g/ml}$ D^b compatible MHC I E7 peptide (aa 49-57) on day 7 and then co-cultured with the E7-specific CD8⁺ T cell line (1:5 ratio) overnight. The co-cultured cells were then co-cultured with the irradiated TC-1-LG (1:8 ratio) in a 96-well plate (1×10^4 cells/well) for 24 h. Luciferin (Promega) was added and the total flux (p/s) from each well was measured using the IVISR Imaging Systems.

Western blot analysis of BMM-derived DCs treated with morphine

Western blot analysis was used to detect the phosphorylated forms of ERK1/2, Akt, and p38, as compared to the non-phosphorylated forms, in the progression of maturation in the DCs treated with morphine or PBS. Briefly, BMM-derived DCs were cultured and collected at the indicated intervals, and then further treated with morphine and/or LPS as described earlier. These BMM-derived DCs were then lysed in immunoprecipitation assay buffer and analyzed, as described previously [18]. The protein extracts were quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL), and 50 μg of each cell lysate was then resolved by SDS/PAGE (12% gel), transferred onto a PVDF/nylon membrane (Millipore, Billerica, MA), and probed with antibodies specific to ERK1/2, phospho-ERK1/2, Akt (Upstate Biotechnology, Lake Placid, NY), phospho-Akt (Ser⁴⁷³, Chemicon International, Temecula, CA), p38, phospho-p38 (Cell Signaling, Beverly, MA) or β -actin (Chemicon International). The membrane was then probed with either horseradish peroxidase-conjugated goat anti-mouse (Promega, Madison, WI) or goat anti-rabbit (Promega) antibodies. The specific bands were visualized by an ECL® (enhanced chemiluminescence) Western blotting system (GE Healthcare, Little Chalfont, UK).

Tumorigenesis in mice treated with morphine

To investigate whether morphine enhanced tumorigenesis by suppressing the maturation and function of the BMM-derived DCs, an ovarian cancer tumorigenesis animal model was established with morphine, as described in our previous studies [19, 20]. C57BL/6J mice were intraperitoneally injected with 5×10^4 WF-3/Luc tumor cells. The mice were then injected

with PBS, 10 or 40 mg/kg of morphine daily for 28 days starting on the first day of tumor injection. The tumor burden was detected and measured by tumor imaging using an IVIS Imaging System Series 200 (Xenogen, Alameda, CA). Bioluminescence tumor images were taken 3 days after WF3/Luc challenge and every 4 days thereafter. To detect the bioluminescence signals, the mice were injected intraperitoneally with 300 μl of 15 mg/ml luciferin (Xenogen, Alameda, CA), and imaged 10 minutes later. The bioluminescence signals were acquired for 3 minutes. The survival of the mice in each group was also determined and monitored twice a week.

The rescue effect of anti-CD40 antibodies with or without poly(I:C) in the tumor-bearing mice that were treated with morphine

We further investigated whether anti-CD40 Abs with or without poly(I:C) could delay tumor growth by rescuing the function of DCs in mice treated with morphine. Briefly, the mice were injected with WF-3 tumor cells and morphine (40 mg/kg) daily at day 0, as described previously [21]. They were then injected intraperitoneally with either PBS, anti-CD40 Ab (FGK4.5; BioExpress; 50 $\mu\text{g}/\text{mouse}$) and/or poly(I:C) (Invitrogen; 100 $\mu\text{g}/\text{mouse}$) on days 7, 14, 21, and 28 after tumor challenge. The mice were monitored twice every week, and their survival was recorded from 3 days after tumor challenge.

The possible *in vivo* mechanisms of anti-CD40 Ab combined with poly(I:C) were further evaluated. The BMM-derived DCs from the morphine-treated mice followed by anti-CD40 Ab and/or poly(I:C) treatment were acquired, cultured and generated as described earlier. The antigen processing, presentation activity, cytokine secretion, and signal transduction pathways of the BMM-derived DCs were then analyzed as described earlier.

IFN- γ ELISPOT assays to detect the numbers of IFN- γ -secreting CD8⁺ T cytotoxic lymphocytes in tumor-bearing mice treated with morphine and/or anti-CD40 Ab with poly(I:C)

Mice were injected with WF-3 tumor cells and morphine (40 mg/kg) on day 0, and injected intraperitoneally with either PBS, anti-CD40 Ab and/or poly(I:C) on days 7, 14, 21, and 28 after tumor challenge as described earlier. Mice

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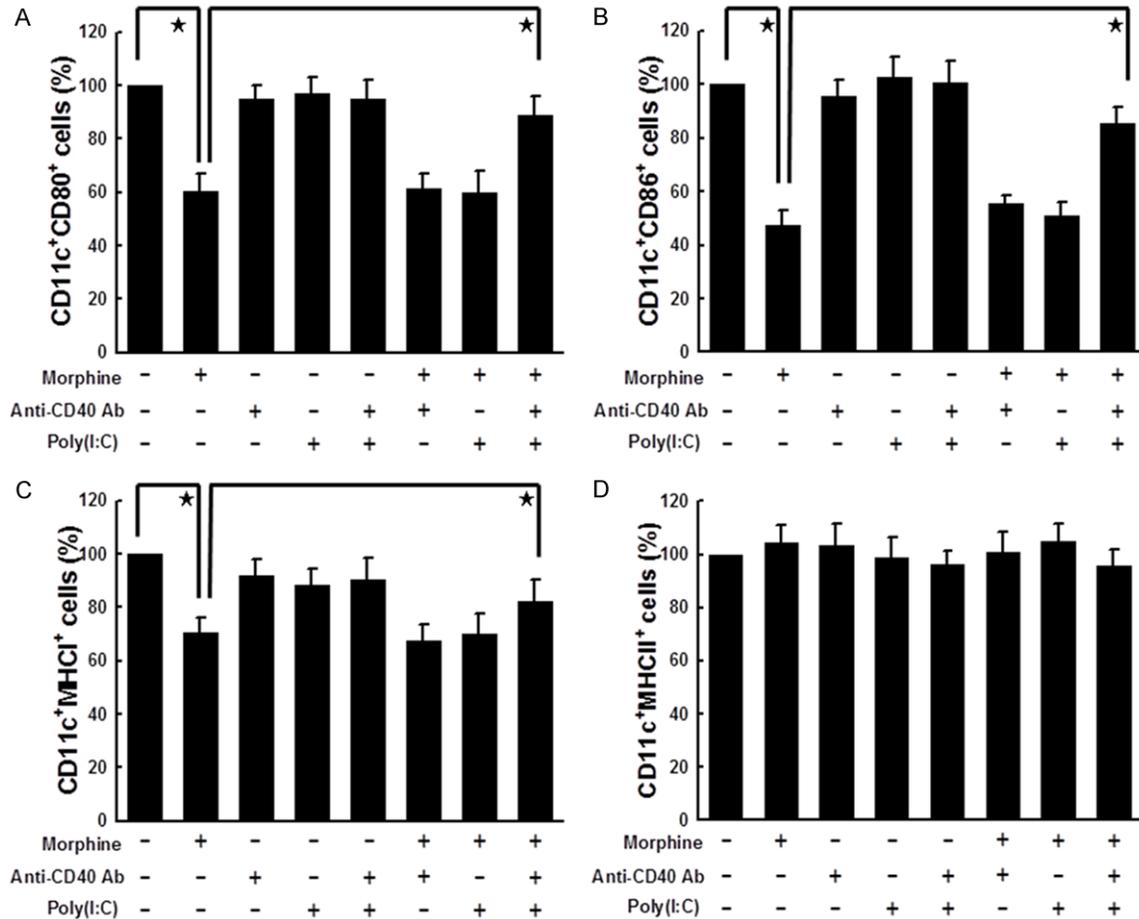


Figure 1. The effects of morphine on the expression of surface markers in BMM-derived DCs. A. The percentage of CD80 in CD11c⁺ cells in BMM-derived DCs (*: P < 0.05, one-way ANOVA). B. The percentage of CD86 in CD11c⁺ cells in BMM-derived DCs (*: P < 0.05, one-way ANOVA). C. The percentage of MHC I in CD11c⁺ cells in BMM-derived DCs (*: P < 0.05, one-way ANOVA). D. The percentage of MHC II in CD11c⁺ cells in BMM-derived DCs. All experiments were performed in at least three separate experiments.

were sacrificed on day 35 to get the splenocytes. ELISPOT assays on mesothelin antigen-specific CD8⁺ T cytotoxic lymphocytes in the splenocytes of the mice were performed, as described in our previous report [20]. Briefly, 96-well filtration plates (Millipore, Bedford, MA) were coated with 5 mg/ml of anti-mouse INF- γ antibody (BD Biosciences) in 100 μ l PBS. After incubating overnight at 4°C, the wells were washed and blocked with culture medium containing 10% FBS. Different groups of splenocytes were serially cultured with 10 μ g/ml mesothelin peptide (aa 406-414; GQKMNAQAI) for 48 hours at 37°C in 5% CO₂. The plates were then washed and incubated with 2 μ g/ml biotinylated anti-mouse IFN-antibody (BD Biosciences) in 100 μ l PBS at 4°C overnight. After subsequent washing, 1.2 μ g/ml avidin-alkaline phosphatase (Sigma-Aldrich) in 100 μ l PBS was

added, and the plates were incubated for 2 h at room temperature. The spots were subsequently developed by adding 100 μ l 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Boehringer Mannheim, Indianapolis, IN) with incubation at room temperature for 20 min. The reactions were stopped by discarding the substrate and washing the plates under tap water. The plates were then air-dried and the colored spots were counted using a dissecting microscope.

ELISA for the detection of anti-mesothelin Abs in tumor-bearing mice treated with morphine and/or anti-CD40 Ab with poly(I:C)

To determine the amount of anti-mesothelin Abs in various groups, the sera of the tumor-bearing mice treated with morphine and/or

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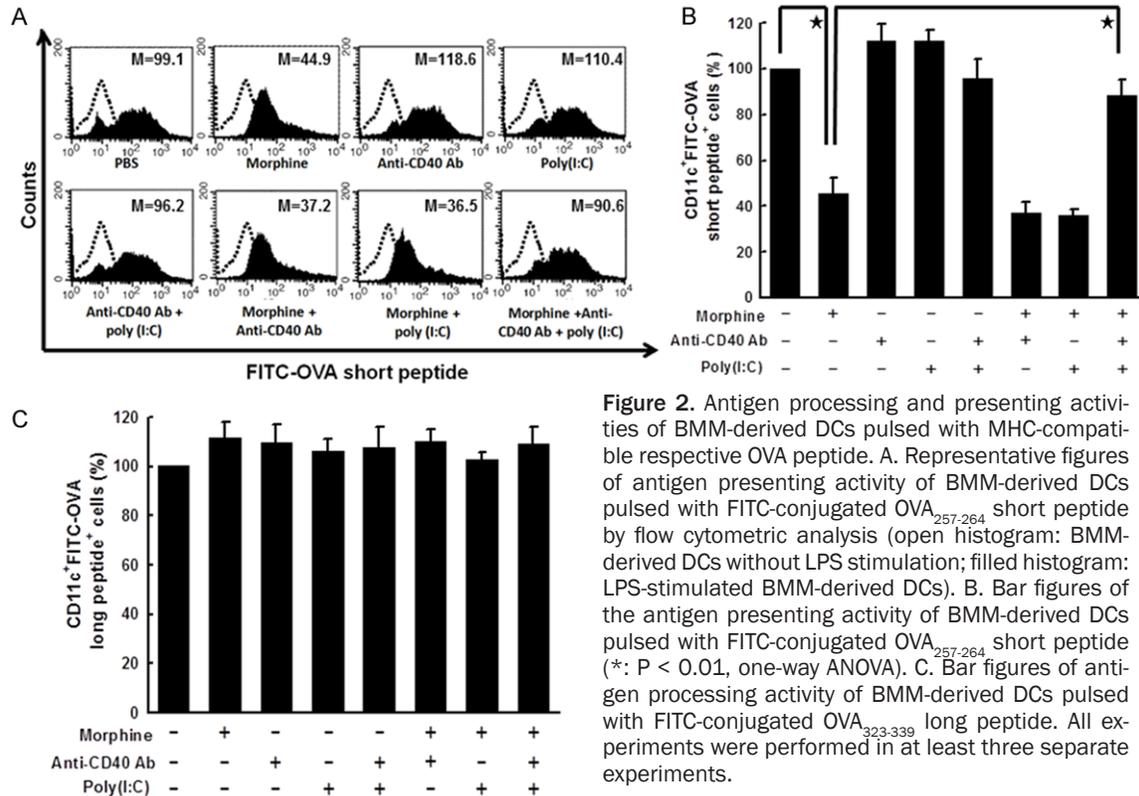


Figure 2. Antigen processing and presenting activities of BMM-derived DCs pulsed with MHC-compatible respective OVA peptide. A. Representative figures of antigen presenting activity of BMM-derived DCs pulsed with FITC-conjugated OVA₂₅₇₋₂₆₄ short peptide by flow cytometric analysis (open histogram: BMM-derived DCs without LPS stimulation; filled histogram: LPS-stimulated BMM-derived DCs). B. Bar figures of the antigen presenting activity of BMM-derived DCs pulsed with FITC-conjugated OVA₂₅₇₋₂₆₄ short peptide (*: P < 0.01, one-way ANOVA). C. Bar figures of antigen processing activity of BMM-derived DCs pulsed with FITC-conjugated OVA₃₂₃₋₃₃₉ long peptide. All experiments were performed in at least three separate experiments.

anti-CD40 Ab and poly(I:C) were collected, and the detection of anti-mesothelin Abs was performed, as described previously [22]. The ELISA plate was read with a standard ELISA reader at 450 nm.

Statistical analysis

All data were expressed as mean \pm SEM and were representative of at least two independent experiments. The data for surface marker staining in flow cytometric analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). Comparisons between individual data points were made using Student's t-tests. The survival data was analyzed by Log rank test. A *p* value of less than 0.05 was considered statistically significant.

Results

Immune modulator anti-CD40 Ab combined TLR-3 ligand-poly(I:C) can reverse the maturation status of BMM-derived DCs suppressed by morphine

The percentages of CD80⁺CD11c⁺ BMM-derived DCs in morphine-treated groups were lower than those of the PBS-treated group (morphine

group 60.5 \pm 6.5% as compared with PBS-treated group [as reference 100%], P = 0.002, one-way ANOVA; **Figure 1A**). The percentages of CD86⁺CD11c⁺ BMM-derived DCs in the morphine-treated group were also significantly lower than those of the PBS-treated group (P < 0.001, one-way ANOVA; **Figure 1B**). The percentages of MHC class I-positive CD11c⁺ BMM-derived DCs in morphine-treated groups were also lower than those of the PBS-treated group (morphine group 70.5 \pm 5.6% as compared with PBS-treated group [as reference 100%], P = 0.004, one-way ANOVA; **Figure 1C**). However, the expression of MHC class II-positive BMM-derived DCs were not significantly different between the morphine- and PBS-treated groups (P = 0.86, one-way ANOVA; **Figure 1D**).

We then examined whether immune-modulators, anti-CD40 Ab and poly(I:C), could reverse the suppressive effects of morphine on the expression of the surface markers on BMM-derived DCs. The percentages of CD80⁺ (P = 0.002, one-way ANOVA; **Figure 1A**), CD86⁺ (P = 0.006, one-way ANOVA; **Figure 1B**), and MHC class I-positive (P = 0.043, one-way ANOVA; **Figure 1C**) CD11c⁺ BMM-derived DCs in the morphine-treated group increased when treat-

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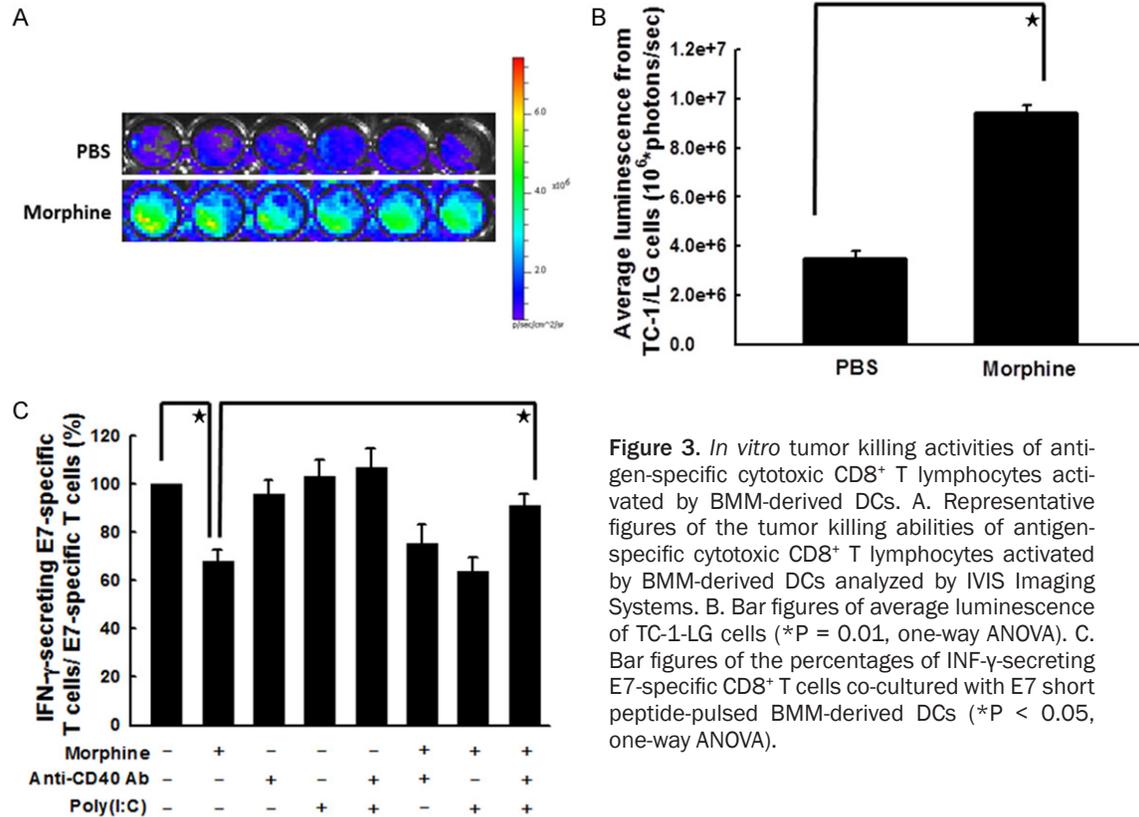


Figure 3. *In vitro* tumor killing activities of antigen-specific cytotoxic CD8⁺ T lymphocytes activated by BMM-derived DCs. A. Representative figures of the tumor killing abilities of antigen-specific cytotoxic CD8⁺ T lymphocytes activated by BMM-derived DCs analyzed by IVIS Imaging Systems. B. Bar figures of average luminescence of TC-1/LG cells (*P = 0.01, one-way ANOVA). C. Bar figures of the percentages of INF-γ-secreting E7-specific CD8⁺ T cells co-cultured with E7 short peptide-pulsed BMM-derived DCs (*P < 0.05, one-way ANOVA).

ed with anti-CD40 Abs and poly(I:C). Besides, the percentages of CD80⁺ (Figure 1A), CD86⁺ (Figure 1B), or MHC class I-positive (Figure 1C) CD11C⁺ BMM-derived DCs in the morphine-treated group was not statistically different as compared with the control group when treated with anti-CD40 Abs and poly(I:C) (all *p* values > 0.05, one way ANOVA). However, the anti-CD40 Ab or poly(I:C) alone could not enhance the percentages of any surface markers in CD11C⁺ BMM-derived DCs that were treated with morphine.

Our results revealed that anti-CD40 Abs combined TLR-3 ligand- poly(I:C) could enhance the maturation of BMM-derived DCs that were suppressed by morphine.

Anti-CD40 Abs combined poly(I:C) can rescue the antigen-presenting abilities of the BMM-derived DCs that were suppressed by morphine

The representative figures of the percentages of FITC-OVA₂₅₇₋₂₆₄ short peptide loaded-DCs in flow cytometric analysis are shown in Figure 2A. The percentages of FITC-OVA₂₅₇₋₂₆₄ short

peptide loaded-DCs treated with morphine were significantly lower than those treated with PBS alone (*P* < 0.001, one-way ANOVA; Figure 2B). We then examined whether anti-CD40 Abs and poly(I:C) could rescue the suppressive effect of the morphine on the antigen presenting ability of the BMM-derived DCs. When treated with anti-CD40 Abs and poly(I:C), the percentages of FITC-OVA₂₅₇₋₂₆₄ short peptide loaded-DCs in the morphine-treated group was significantly increased as compared with the no treatment group, treated with anti-CD40 Abs or poly(I:C) alone groups (*P* < 0.001, one-way ANOVA; Figure 2B).

We further evaluated whether morphine could influence the antigen processing ability of the BMM-derived DCs. The percentages of FITC-OVA₂₅₇₋₂₆₄ short peptide loaded-DCs treated with morphine were significantly lower than those treated with PBS alone (*P* < 0.001, one-way ANOVA; Figure 2B). The percentages of FITC-conjugated OVA₃₂₃₋₃₃₉ long peptide loaded-DCs treated with morphine were not statistically different as compared to those treated with PBS control (*P* = 0.32, one-way ANOVA; Figure 2C) or the other groups.

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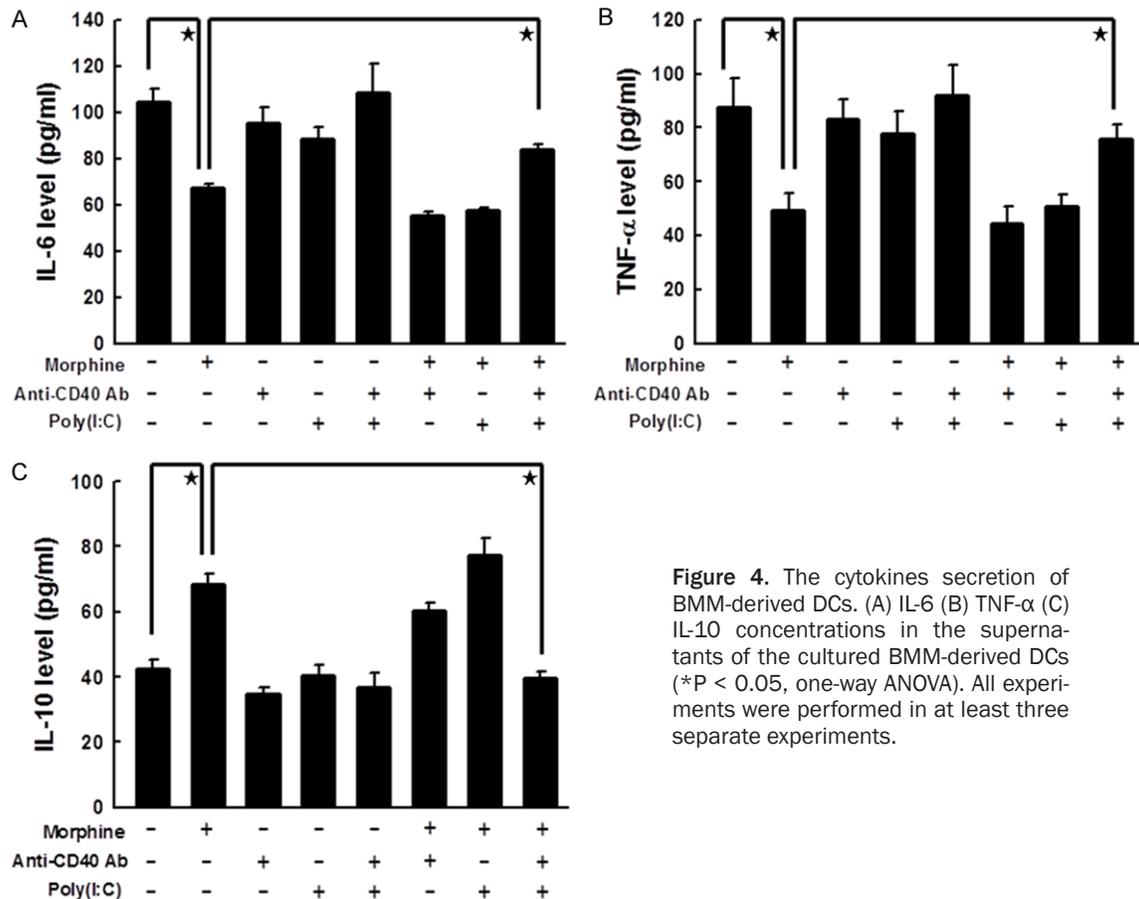


Figure 4. The cytokines secretion of BMM-derived DCs. (A) IL-6 (B) TNF- α (C) IL-10 concentrations in the supernatants of the cultured BMM-derived DCs (*P < 0.05, one-way ANOVA). All experiments were performed in at least three separate experiments.

Our results indicate that morphine could suppress antigen presentation, but not antigen processing, of the BMM-derived DCs. Anti-CD40 Abs combined with poly(I:C) could rescue the antigen presenting ability of the BMM-derived DCs, which was suppressed by morphine.

In vitro anti-tumor activities of antigen-specific cytotoxic CD8⁺ T lymphocytes were lower when activated by BMM-derived DCs that were treated with morphine

We next evaluated whether the *in vitro* tumor killing activities generated by antigen-specific CD8⁺ cytotoxic T lymphocytes could be reduced when activated by morphine-treated BMM-derived DCs. The representative figures of the luminescence of TC-1-LG cells when detected by the IVIS imaging system are shown in **Figure 3A**. The PBS-treated BMM-derived DCs had the lowest average luminescence in TC-1-LG cells as compared with the morphine-treated group (PBS group $3.5 \times 10^6 \pm 2.7 \times 10^5$ [p/s]; mor-

phine-treated group $9.4 \times 10^6 \pm 1.2 \times 10^6$ [p/s], P = 0.010, one-way ANOVA; **Figure 3B**).

Our results revealed that antigen-specific CD8⁺ T cells activated by morphine-treated BMM-derived DCs had lower killing activities than those activated by PBS-treated DCs.

Anti-CD40 Abs combined with poly(I:C) could restore the activating ability of the morphine-treated BMM-derived DCs to stimulate IFN- γ secretion of antigen-specific CD8⁺ cytotoxic T lymphocytes

We then determined whether morphine-induced, immature BMM-derived DCs had a reduced ability to stimulate the IFN- γ secretion of antigen-specific CD8⁺ cytotoxic T lymphocytes. The percentages of IFN- γ -secreting E7-specific CD8⁺ T lymphocytes were significantly lower in morphine-treated BMM-derived DCs than in PBS-treated BMM-derived DCs (P = 0.006, one-way ANOVA; **Figure 3C**). However, morphine-treated BMM-derived DCs, when fur-

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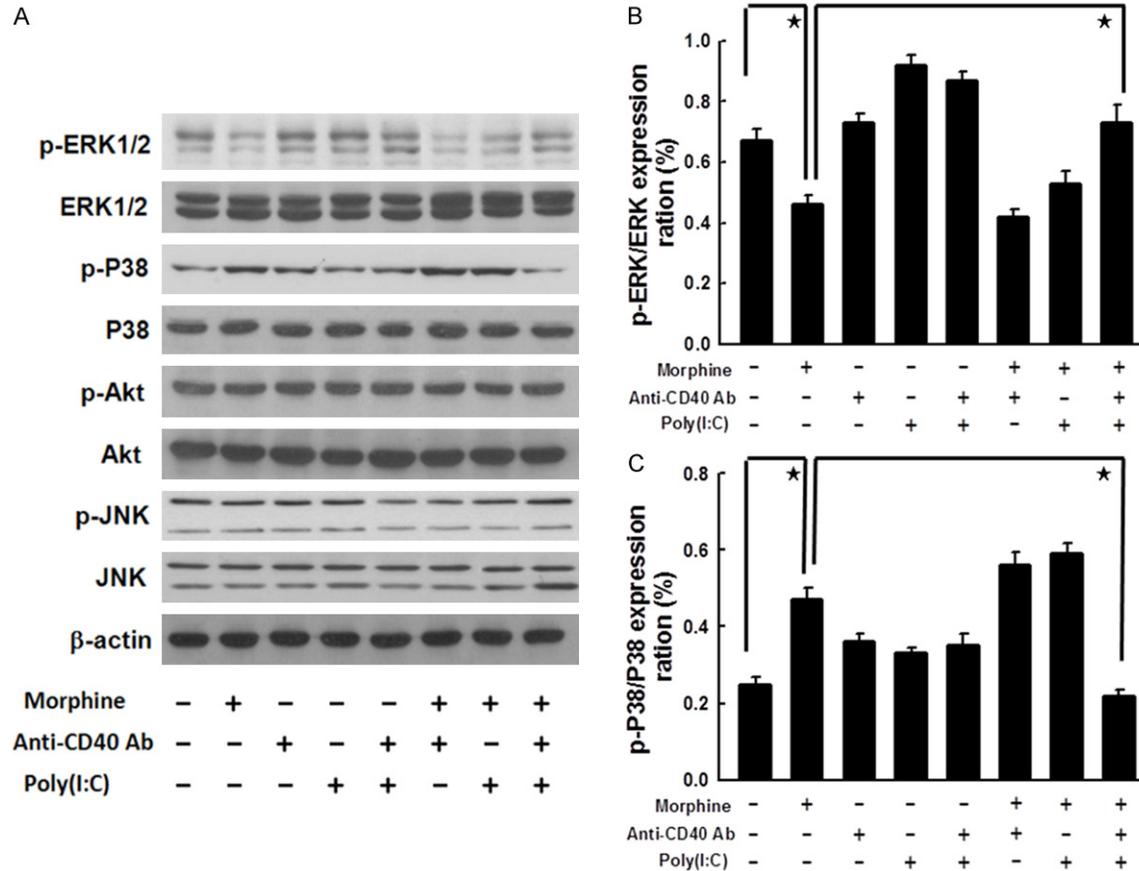


Figure 5. Western blotting of signaling pathways of BMM-derived DCs. A. Representative figures of the phosphorylation of p38, ERK1/2, Akt, and JNK of LPS-induced BMM-derived DCs in various groups by immunoblotting. B. The ratios of phospho-ERK to total ERK (pERK/ERK) (* $P < 0.01$, one-way ANOVA). C. Phospho-p38 to total p38 (pp38/p38) (* $P < 0.05$ one-way ANOVA). All experiments were performed in at least three separate experiments.

ther treated with a combination of anti-CD40 Abs and poly(I:C), enhanced higher percentages of IFN- γ -secreting E7-specific CD8⁺ T lymphocytes than those treated with anti-CD40 Abs or poly(I:C) alone ($P = 0.020$, one-way ANOVA; **Figure 3C**).

Our results indicate that morphine could lower the activating capability of BMM-derived DCs to stimulate the IFN- γ -secreting antigen-specific CD8⁺ T lymphocytes. Anti-CD40 Abs combined with poly(I:C) can rescue the suppressing effects.

Anti-CD40 Abs combined with poly(I:C) could reverse morphine regulated cytokine secretion of the BMM-derived DCs

The influence of morphine on the secreting cytokines of the BMM-derived DCs was further evaluated by ELISA. The concentrations of IL-2, IL-4, or IL-12 were too low to be detected with or

without LPS stimulation (data not shown). The IL-6 ($P = 0.017$, one-way ANOVA; **Figure 4A**) and TNF- α ($P = 0.015$, one-way ANOVA; **Figure 4B**) concentrations of the morphine-treated group were significantly lower than those of PBS-treated group. Whereas, the IL-10 concentrations were significantly higher in the morphine-treated group as compared with those in the PBS-treated group ($P = 0.020$, one-way ANOVA; **Figure 4C**).

We then examined whether anti-CD40 Abs and poly(I:C) could change the effect of the morphine on the cytokine secretion of the BMM-derived DCs. When treated with anti-CD40 Abs combined poly(I:C), the IL-6 ($P = 0.007$, one-way ANOVA; **Figure 4A**) and TNF- α ($P = 0.002$, one-way ANOVA; **Figure 4B**) concentrations were higher in the morphine-treated group as compared with those treated with anti-CD40 Abs or poly(I:C) alone. However, IL-10 concentrations in the morphine-treated group were lower when

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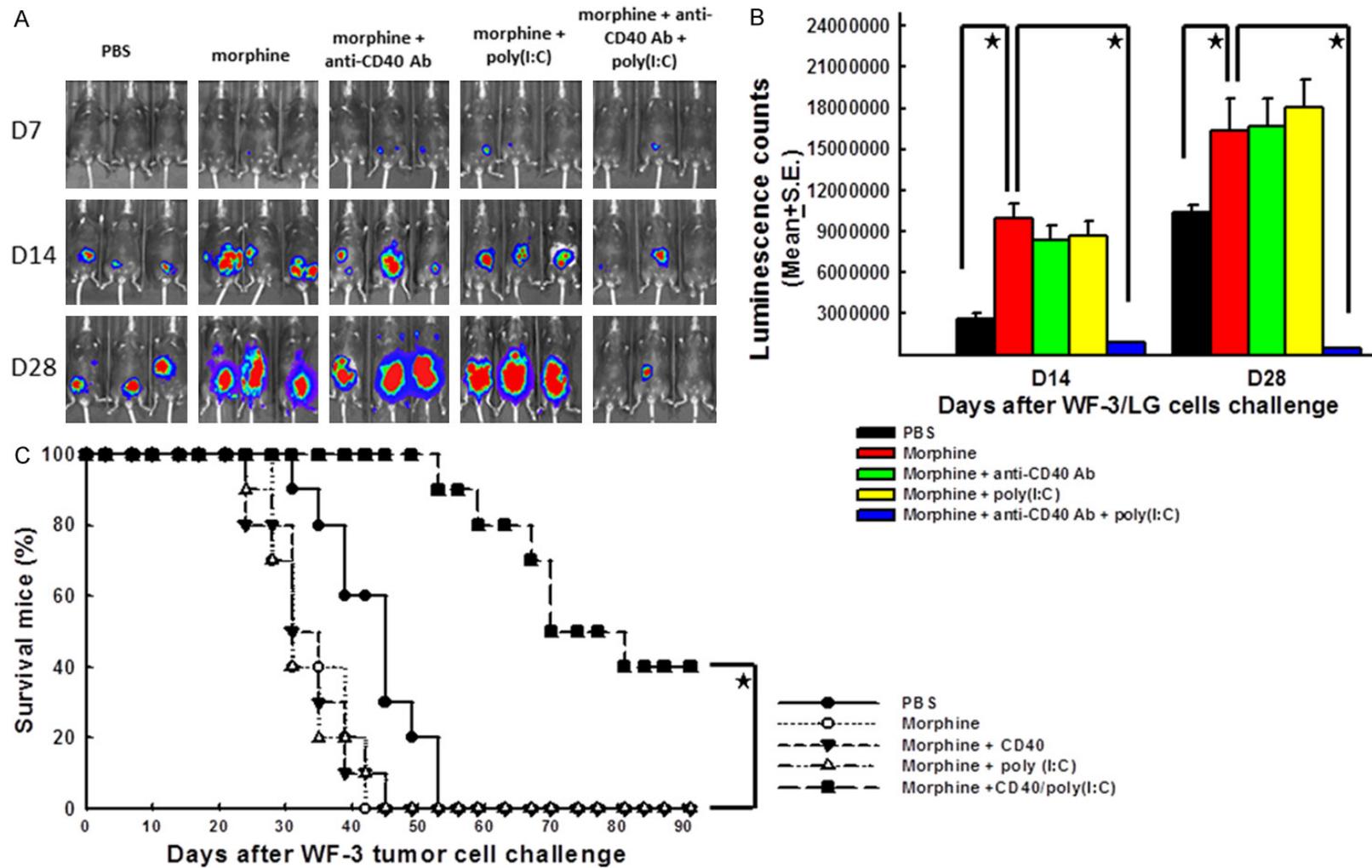


Figure 6. Anti-tumor effects of morphine-treated mice further administered with anti-CD40 Abs and/or poly(I:C). A. Representative figures of the luminescence images of WF-3/LG-challenged mice in various groups at the indicated time points. B. Bar figures of the tumor densities in the mice after 14 and 28 days of WF-3/LG tumor challenge. C. Survival curves of the mice in various groups. The morphine-treated mice, when further administered with the combination of anti-CD40 Abs and poly(I:C) had a significantly longer survival than the other groups ($P < 0.001$, log-rank test). All experiments were performed in at least three separate experiments.

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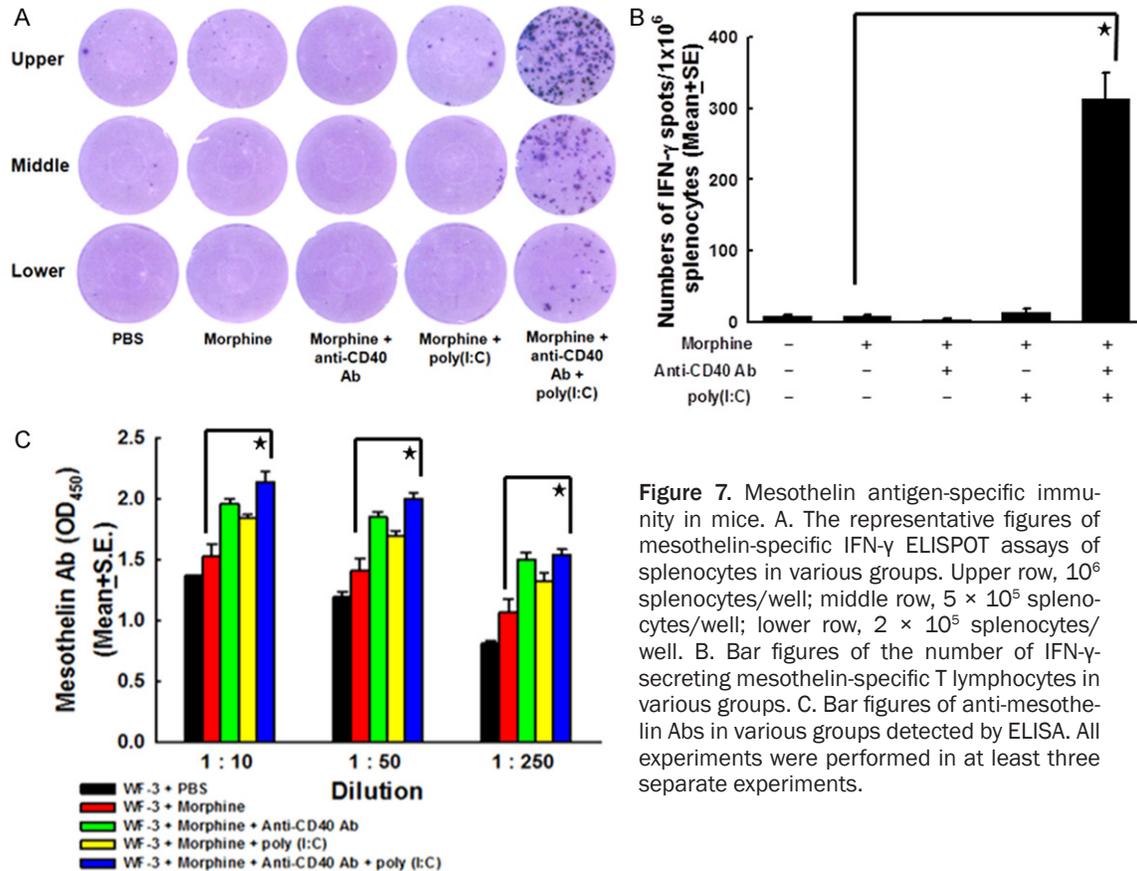


Figure 7. Mesothelin antigen-specific immunity in mice. A. The representative figures of mesothelin-specific IFN- γ ELISPOT assays of splenocytes in various groups. Upper row, 10^6 splenocytes/well; middle row, 5×10^5 splenocytes/well; lower row, 2×10^5 splenocytes/well. B. Bar figures of the number of IFN- γ -secreting mesothelin-specific T lymphocytes in various groups. C. Bar figures of anti-mesothelin Abs in various groups detected by ELISA. All experiments were performed in at least three separate experiments.

treated with anti-CD40 Abs and poly(I:C) than those treated with anti-CD40 Abs or poly(I:C) alone ($P < 0.001$, one-way ANOVA; **Figure 4C**).

Morphine inhibited the maturation of BMM-derived DCs by suppressing ERK1/2 phosphorylation and p38 dephosphorylation

We further investigated the signaling pathway involved in the inhibitory effects of morphine on the maturation of BMM-derived DCs. The representative figures for Western blotting various molecules are shown in **Figure 5A**. ERK1/2 phosphorylation of morphine-treated BMM-derived DCs was significantly reduced as compared with that of the PBS-treated group (**Figure 5B**). However, the ERK1/2 phosphorylation of the morphine-treated BMM-derived DCs increased when treated with anti-CD40 Abs and poly(I:C). Besides, the dephosphorylation of p38 observed in the PBS-treated BMM-derived DCs was not observed in the morphine-treated group (**Figure 5C**); dephosphorylation of p38 was observed in the morphine-treated BMM-derived DCs when further treated with anti-CD40 Abs and poly(I:C) as compared to

those treated with anti-CD40 Abs or poly(I:C) alone (**Figure 5C**). There were no differences in Akt phosphorylation or JNK phosphorylation between the PBS- and morphine-treated groups.

Our results revealed that morphine could inhibit the maturation of BMM-derived DCs by suppressing ERK1/2 phosphorylation and p38 dephosphorylation and anti-CD40 Abs and poly(I:C) could reverse these phenomena.

Anti-CD40 Abs and poly(I:C) can enhance the anti-tumor effects and prolonged the survival of morphine-treated tumor-bearing mice

We further determined whether anti-CD40 Abs and poly(I:C) could enhance the anti-tumor effects and extend the survival of morphine-treated, tumor-bearing mice *in vivo*. The representative figures for the luminescence of WF-3/LG tumor-bearing mice as detected by the IVIS imaging system are shown in **Figure 6A**. The morphine-treated mice had higher luminescence counts than the PBS-treated group. However, the morphine-treated mice had the lowest

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luminescence counts when further treated with anti-CD40 Abs and poly(I:C) as compared with the other groups (**Figure 6B**). The morphine-treated mice when further treated with anti-CD40 Abs and poly(I:C) had significantly longer survival than the other groups ($P < 0.001$, log-rank test; **Figure 6C**). Forty percent of the morphine-treated mice when further treated with anti-CD40 Abs and poly(I:C) were still alive after 90 days of tumor challenge.

Anti-CD40 Abs combined poly(I:C) enhanced in vivo antigen-specific immunity in the morphine-treated, tumor-bearing mice

To elucidate whether the host immunity could be enhanced in the morphine-treated, WF-3-bearing mice when administered with anti-CD40 Abs and/or poly(I:C), antigen-specific immunity against mesothelin, a novel ovarian tumor-associated antigen, was further evaluated. Representative figures of the mesothelin-specific IFN- γ ELISPOT assays of splenocytes are shown in **Figure 7A**. The combination of anti-CD40 Abs and poly(I:C) generated the highest number of IFN- γ -secreting, mesothelin-specific T lymphocytes (314.0 ± 32.1 spots/ 10^6 splenocytes) as compared to the other groups (PBS 8.6 ± 2.4 spots, morphine only 8.3 ± 2.8 spots, morphine with anti-CD40 Abs 4.0 ± 1.0 spots, and morphine with poly(I:C) 13.0 ± 5.6 spots, $P < 0.001$, one-way ANOVA) (**Figure 7B**).

We further evaluated the titers of anti-mesothelin Abs in each group. The titers of anti-mesothelin Abs in the anti-CD40 Abs group as combined with poly(I:C) group were significantly higher than those of the other groups (1.4 ± 0.002 in the PBS group, 1.5 ± 0.01 in the morphine group, 1.8 ± 0.03 in the morphine with anti-CD40 Abs group, 2.0 ± 0.04 in the morphine with poly(I:C) group, and 2.1 ± 0.10 in the morphine with anti-CD40 Abs and poly(I:C) group, 1:10 dilution, $P < 0.01$, one-way ANOVA) (**Figure 7C**).

Discussion

Morphine is regarded as a safe medication with great analgesic efficacy. Nonetheless, evidence suggests that morphine is highly associated with the risk of immunosuppression as revealed by both *in vitro* and *in vivo* studies [13, 21]. Sabita et al. [23] reported that morphine treatment *in vivo* could increase the Th2 differentiation of CD4⁺ T cells. In contrast to the activation

of CD4⁺ helper T lymphocytes, the activity of CD8⁺ cytotoxic T lymphocytes has been shown to be suppressed in the cellular immune response with morphine administration [13]. However, the cellular and molecular mechanisms by which morphine modulates DC maturation and DC-mediated immunity have not been clearly delineated. In this study, we demonstrated that morphine could inhibit DC generation from BMM-derived CD11c⁺ cells. In addition, the attenuation of antigen presentation activity was also noted in the maturation process of morphine-treated BMM-derived DCs.

Morphine decreased the cellular markers of DCs from BMM-derived cells, including CD11c⁺CD80⁺, CD11c⁺CD86⁺ and CD11c⁺MHCI⁺ in this study. Morphine abuse is known to impair host defenses in both humans and animals [13, 21, 24]; however, the immunosuppressive mechanisms of chronic morphine abuse are not completely understood. Multiple mechanisms have been proposed to contribute to the immunosuppressive effects of morphine, and acute morphine use has been shown to cause apoptosis of endothelial and immune effector cells [13, 25]. The results of this study provide an additional potential mechanism underlying the impaired adaptive immune function seen in morphine-inhibited host immunity to diminish BMM-derived CD11c⁺ cell-differentiated DCs (**Figure 1**). However, only a few investigations have focused on the differentiation process in morphine-mediated BMM-derived CD11c⁺ cells. Messmer et al. [26] suggested that morphine-treated *peripheral blood mononuclear cells*-derived DCs could increase the expression of HLA-DR, CD86, and CD83. The differences in the findings may be the result of the different species and culture conditions used in these studies.

Antigen processing and antigen presentation ability of DCs changed when treated with morphine, which influenced their maturation status. Immature DCs are very effective in antigen uptake in antigen processing activities, whereas mature DCs show an enhanced capacity for antigen presentation to stimulate potent immune responses. Our results showed that the DCs were more immature after being treating with morphine (**Figures 1 and 2**). Increasing expressions of co-stimulatory molecules, including CD80 and CD86, have been shown to be a unique feature that is correlated with the func-

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tional enhancement of DC antigen presentation [27]. Our results are consistent with these findings and showed lower expressions of co-stimulatory molecules in the morphine-treated BMM-derived DCs (**Figure 1**). Another important feature of DCs is their capacity to stimulate the proliferation of T lymphocytes and to induce antigen-specific immunity. The morphine-treated BMM-derived DCs also generated weaker antigen presenting abilities than the PBS-treated group in this study (**Figure 2**).

Morphine inhibited the production of proinflammatory cytokines, including TNF- α and IL-6 in this study. Cytokines, such as IL-2, IL-4, IL-6, IL-10, and TNF- α , play an important role in the pathogenesis of many immune-mediated diseases. Eisenstein et al. [28] suggested that the suppression of immune function by morphine could contribute to the disruption of cytokine expression, including that of IL-2. Lugo-Chinchilla et al. [29] also reported an association between morphine treatment and IL-2 expression in peripheral blood mononuclear cells. Morphine did not increase BMM-derived, DC-mediated IL-2 secretion in the current study; however, the expressions of proinflammatory cytokines, including IL-6 and TNF- α , decreased after treatment with morphine (**Figure 4**). Wang et al. [30] also showed that levels of IL-6 and TNF- α significantly decreased in both bronchoalveolar lavage fluids and lung tissue in morphine-treated mice. The inhibition of the production of TNF- α and IL-6 by morphine has been proposed to decrease early innate immunity responses [31]. Proposed mechanisms by which morphine inhibits the production of TNF- α and IL-6 include the suppression of extracellular signal-regulated kinase pathways [32]. The inhibition of extracellular signal-regulated kinase pathways not only suppresses host immunity but also enhances tolerance to morphine treatment [33]. Our results also showed IL-6 and TNF- α inhibition in the BMM-derived DCs treated with morphine (**Figure 4**). Taken together, morphine may act as an immune suppressor in proinflammatory cytokine-mediated immunity.

Morphine also stimulated the secretion of immunosuppressive cytokines in this study. IL-10 has been shown to suppress the maturation and activation of Th1 cells during their development [34], and IL-10 is considered an inhibitor of DCs and an immunosuppressor. Sacerdote

et al. [35] observed that mice receiving long-term morphine treatment showed significantly increased production of IL-10 in splenocytes. IL-10 is known to stimulate the differentiation of regulatory T lymphocytes and thus induce anergy of T lymphocytes, characterized by tolerogenic antigen presenting cells [36]. Our results showed that morphine could stimulate IL-10 secretion from DCs after transient or long-term treatment (**Figure 2**). Numerous growth factors and cytokines, such as VEGF, TGF- β and IL-10, can be secreted by cancer cells to hamper DC maturation [37]. However, morphine did not change the TGF- β secretion of DCs in this study.

Signal transduction pathways play an important role in the maturation of DCs. The process of DC maturation is a well-coordinated series of events tightly controlled by the balance of particular intracellular signaling pathways [38]. Ardeshtna et al. [39] demonstrated that the three major intracellular MAPK signaling pathways, p38, ERK1/2, and JNK, are involved in the process of DC maturation. Our previous investigation found that the ERK1/2, Akt, and p38 pathways were involved in the DC maturation process [14]. In the current study, morphine was found to suppress DC maturation by inhibiting ERK1/2 phosphorylation and eliminating p38 de-activation (**Figure 5**), resulting in a greater amount of immature BMM-derived DCs.

The stimulation of CD40 can be significantly enhanced by an additional stimulus. Pilon et al. [40] reported that CD40L-stimulated DCs could increase the expression of CD80/86 molecules and the production of IL-12p40, and enhance T cell-mediated IFN- γ secretion. Scarlett et al. [41] also reported that synergistic CD40/TLR activation could induce DC migration to lymphatic tissues and promote their capacity to present antigens in ovarian carcinomas. Morphine treatment can give rise to immature DCs, which may suppress host immunity through antigen presentation deficiency, cytokine control, and the killing of effector cells. However, in this study, the combination of anti-CD40 Abs and the TLR3 agonist poly(I:C) generated anti-specific immunity (**Figure 7**) and enhanced proinflammatory cytokines, including IL-6 and TNF- α (**Figure 6**). Antibodies against CD40 have also been reported to eradicate established breast tumors by enhancing the expression of

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MHC class II, CD54, CD86, and CD23 in human B cells [42]. Ahonen et al. [43] reported that the combination of CD40 and TLR agonists can be used in cancer vaccines to elicit higher frequencies of tumor-specific CD8⁺ T cells and to generate more potent anti-tumor effects than CD40 or TLR alone. Our results also revealed that the combination of the TLR3 ligand poly(I:C) and anti-CD40 Abs could restore the function of DCs, elicit higher frequencies of tumor-specific (mesothelin-specific) CD8⁺ T lymphocytes, and generate more potent anti-tumor effects (**Figure 7**).

In conclusion, morphine resulted in an imbalance between mature and immature BMM-derived DCs, and then suppressed the host anti-tumor immunity. However, the combination of anti-CD40 Abs and TLR-3 agonists reversed the effects of morphine on the maturation and function of DCs, thereby recovering host immunity and generating potent anti-tumor effects. The development of other new strategies to generate effective DCs for potent anti-tumor effects is important for morphine-mediated immune-suppression.

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Disclosure of conflict of interest

None.

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