Inducible expression of endomorphins in murine dendritic cells

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Abstract
Bone marrow precursor cells were extracted from C57BL/6J mice aged 7–8 weeks, and dendritic cells were purified using anti-CD11c (a specific marker for dendritic cells) antibody-coated magnetic beads. Immunofluorescence staining revealed that the expression levels of endomorphin-1 and endomorphin-2 were upregulated in dendritic cells activated by lipopolysaccharide. An enzyme immunoassay showed that lipopolysaccharide and other Toll-like receptor ligands promoted the secretion of endomorphin-1 and endomorphin-2 from activated dendritic cells. [3H]-thymidine incorporation demonstrated that endomorphin-1 and endomorphin-2 both inhibited the proliferation of T lymphocyte induced by activated dendritic cells. Furthermore, this immunosuppressive effect was blocked by CTOP, a specific antagonist of µ-opioid receptors. Our experimental findings indicate that activated dendritic cells can induce the expression and secretion of endomorphins, and that endomorphins suppress T lymphocyte proliferation through activation of µ-opioid receptors.

Key Words
dendritic cells; endomorphin; µ-receptor; antigen-presenting cell; nerve immunization; neural regeneration

Research Highlights
This study, for the first time, showed that activated dendritic cells can induce the expression and secretion of endomorphins, and that endomorphins can suppress T lymphocyte proliferation through activation of the µ-opioid receptor.

Abbreviations
EM, endomorphin; DCs, dendritic cells; APC, antigen-presenting cell; TLR, Toll-like receptor; LPS, lipopolysaccharide

INTRODUCTION
Endomorphin (EM) is a newly discovered opioid tetrapeptide with a specific structure, which is more resistant to enzymatic degradation than other opioid peptides such as enkephalin, and is considered to be an endogenous ligand with a high affinity for µ-opioid receptors[1]. EM-1 and EM-2 have been reported to be widely distributed in central nervous system cells that are rich in µ-receptors, and increasing evidence is revealing their involvement in analgesia[2-3]. Recent findings have shown that endogenous opioid peptides including EM-1 and EM-2 are produced by immune cells and potentially modulate the cellular immune response[4]. Elevated levels of EM-1 and EM-2 were observed in synovial tissue...
from the hind paws of rats, in which arthritis had been induced, and in inflamed paw tissue and lymph nodes in a rat model of localized inflammation, with concomitant increased expression of µ-opioid receptors\[5-6\]. EM-1 and EM-2 were also detected in macrophages/monocytes and B cells in the medullary region of the popliteal lymph nodes\[6\] and spleen\[7\]. Furthermore, EM-1 and EM-2 can alter macrophage functions such as cytokine production, as well as functions related to innate immunity\[8-10\].

Dendritic cells (DCs) are uniquely well-equipped antigen-presenting cells. During the bi-directional interaction of DCs with T cells, DCs provide not only antigen-specific stimulatory and co-stimulatory signals, but also cytokines and chemokines that can polarize the differentiation of T cells\[11\]. DCs originate from hematopoietic precursors in the bone marrow. After sensing various pathogens including some Toll-like receptor (TLR) ligands, DCs undergo phenotypic and functional alterations, referred to as maturation. Mature DCs lose their capacity to process antigens, but increase their immunomodulatory potential and antigen-presentation capacity\[12\]. Thus, DCs have a central and crucial role in determining the fate of immune responses toward either immunity or tolerance\[11-12\]. This dichotomous function of DCs renders them attractive therapeutic targets for immune modulation\[13-14\].

To our knowledge, it is not known whether DCs express EM-1 and EM-2, nor whether EM-1 and EM-2 alter DC immune function. In the present study, we first studied whether DCs produce EM-1 and EM-2. Immunofluorescence staining was used to detect the expression of EMs in DCs. An enzyme immunoassay was then used to measure the EM content. Finally, \[^{[3]}\text{H}\]-thymidine incorporation was used to detect the proliferation of T lymphocytes, to study the effects of EM-1 and EM-2 on DC immune functions.

RESULTS

Culture and identification of DCs

To determine the expression of EM-1 and EM-2 on DCs, DCs from bone marrow were first cultured in the presence of granulocyte macrophage colony stimulating factor. After 7 days of culture, DCs (Figure 1) were purified by immunomagnetic sorting using anti-CD11c antibody-coated magnetic beads. Fluorescence-activated cell sorting analysis indicated that the purity of DCs was > 95% (Figure 2). The purified DCs were activated by 100 ng/mL lipopolysaccharide (LPS) for 24 hours. Flow cytometry analysis revealed that these mature (activated) DCs expressed the DC-specific surface markers CD80 and CD86 along with major histocompatibility complex class II molecules (Figure 3).

![Figure 1](image1.jpg) Morphology of cultured dendritic cells (DCs) (arrows; inverted microscopy, × 400).
(A) Resting DCs; (B) DCs activated by 100 ng/mL lipopolysaccharide for 24 hours.

![Figure 2](image2.png) Fluorescence-activated cell sorting analysis shows a high purity of sorted dendritic cells. Dendritic cells were purified by CD11c\(^+\) beads and the purity was > 95%.

![Figure 3](image3.png) Phenotypes of dendritic cells by flow cytometry analysis. Mature dendritic cells expressed the dendritic cell-specific surface markers major histocompatibility complex class II (A) and the co-stimulatory molecules CD80 (B) and CD86 (C).

Activated DCs expressed EM-1 and EM-2

Next, we examined whether EM-1 and EM-2 are expressed in mature DCs by double immunofluorescence staining. As shown in Figure 4, no co-expression of EM-1 or EM-2 with CD11c was observed in non-activated resting DCs, although a few EM-1- and EM-2-positive cells were detected.
However, DCs activated by LPS displayed significant immunoreactivity for EM-1 and EM-2, and this was co-localized with immunoreactivity for CD11c. These results indicate that activated mature DCs show significant upregulation of the expression of EM-1 and EM-2.

Secretion of EM-1 and EM-2 from TLR ligand-activated DCs

To determine whether activated DCs secrete EM, the DCs were cultured with various TLR ligands, including Pam3Cys (TLR2 ligand, 1 µg/mL), poly I:C (TLR3 ligand, 12.5 µg/mL), LPS (TLR4 ligand, 1 µg/mL), and cytosine-phosphate-guanosine oligodeoxynucleotide (TLR9 ligand, 6 µg/mL). TLR ligands are known to induce maturation of DCs and trigger the release of proinflammatory cytokines\(^1\). After 48 hours of culture, all of the TLR ligands used in the experiment could activate DCs and induce release of EMs (\(P < 0.05\) or \(P < 0.01\)). Among them, the releasing effect of LPS was the most significant (\(P < 0.01\)). The concentration of EMs released into supernatant attained 190 ± 50 pg/mL for EM-1 and 250 ± 70 pg/mL for EM-2, respectively (Figure 5). These data showed that EM-1 and EM-2 are released during the maturation of DCs.

EMs inhibited T lymphocyte proliferation induced by activated DCs

To study the effect of EM-treated DCs on T lymphocyte proliferation, dendritic cells activated by LPS and pre-treated with EMs were co-cultured with purified T lymphocytes in the presence of \(^{3}H\)-thymidine for 3 days. DCs not activated by LPS were used as controls. As shown in Figure 6, the proliferation of T lymphocytes was suppressed in a concentration-dependent manner when they were co-cultured with EMs.

![Figure 4](image_url)  
**Figure 4**  Endomorphin (EM) expression on dendritic cells (DCs) (confocal microscopy, × 200).  
Double fluorescence staining showing the co-expression of EM-1 (A) or EM-2 (B) with CD11c in DCs. DCs were activated by lipopolysaccharide (100 ng/mL) for 24 hours. Resting DCs were not activated by lipopolysaccharide.  
(a–c) No co-expression of EM with CD11c was detected in non-activated resting DCs, although a few EM-positive cells (indicated by arrows) were observed.  
(d–f) Co-expression of EM-1 or EM-2 (green, labeled with Alexa Fluor 488) with CD11c (red, labeled with Alexa Fluor 546) was detected in the lipopolysaccharide-activated group. Inset at the left corner of (f) shows co-expression of EM and CD11c in DCs (arrow) with a high magnification (× 600).

![Figure 5](image_url)  
**Figure 5**  Secretion of endomorphin (EM)-1 and EM-2 from Toll-like receptor (TLR) ligand-treated dendritic cells (DCs).  
(A) Concentration of EM-1 released from DCs in the presence of TLR ligands.  
(B) Concentration of EM-2 released from DCs in the presence of TLR ligands.  
DCs were cultured in the presence of TLR ligands for 48 hours. The concentrations of EMs in supernatant were measured using an enzyme immunoassay kit. Non-activated DCs were used as controls. Data are expressed as mean ± SD and calculated from triplicate independent experiments. \(^a P < 0.05\), \(^b P < 0.01\), vs. control group (Mann-Whitney U test).

LPS: Lipopolysaccharide; CpGODN: cytosine-phosphate-guanosine oligodeoxynucleotide.
The suppression of T lymphocyte proliferation was significant at a concentration of $10^{-6}$ M for EM-1 (Figure 6A) and at a concentration of $10^{-6}$ M for EM-2 (Figure 6B) compared with that induced by LPS ($P < 0.01$). In both cases, the suppressive effect of EMs was abolished by the specific μ-opioid receptor antagonist CTOP (Figure 6), indicating that the effect of EMs was mediated by μ-opioid receptors.

**DISCUSSION**

The main results of the present study showed that DCs co-cultured with LPS can produce and secrete EM-1 and EM-2. Besides LPS, other TLR ligands also promote the production and secretion of EMs. Functionally, EM-treated DCs can inhibit the proliferation of splenic T lymphocytes.

EM-1 and EM-2 were first isolated from bovine and human brains[1, 16]. Thereafter, EMs have been shown to be also present in the cells and tissues of the immune system, including macrophages, T and B cells[4, 6-7, 17]. We extended these studies by demonstrating for the first time that both EM-1 and EM-2 are expressed and secreted from DCs activated by LPS, as revealed by double immunofluorescence staining and an enzyme immunoassay, respectively.

Our findings showed that EM-treated DCs had an inhibitory effect on T lymphocyte proliferation, consistent with the immunosuppressive effect of EMs reported in previous literature. In fact, Azuma and Ohura have shown that both EM-1 and EM-2 suppress LPS-induced cytokine production (of interleukin-12 and interleukin-10) in a human macrophage cell line and in rat peritoneal macrophages[9-10]. Further, EM-1 has been shown to inhibit interleukin-8 production in an intestinal cell line[18], while EM-2 was shown to inhibit tumor necrosis factor-alpha production in rats[9]. Recently Anton et al[19], using a plaque-forming cell assay, revealed that EMs in vitro inhibited formation of antibodies to sheep red blood cells in murine spleen cells. On the contrary, there are also reports that EM-2 potentiates interleukin-1β secretion in rat cells[10], and that EM-1 increases HIV replication in human microglia in vitro[20]. In our previous study, we also observed that treatment of DCs with EM-1 altered cytokine production by increasing production of interleukin-10 and decreasing production of interleukin-12 and interleukin-23[21]. Taken together, all these data show that DC-derived EMs have an important immunomodulatory effect: suppressive in some cases and potentiating in others.

With regard to the mechanisms underlying these actions of EMs, the results of the present study showed that the immunosuppressive effects of EM-1 and EM-2 on T cell proliferation were abolished by the specific μ-opioid receptor antagonist CTOP. This finding indicated that μ-opioid receptors are similarly involved in the immunosuppressive effects of both EM-1 and EM-2. However, a differential antinociception effect induced by spinally administered EM-1 and EM-2 has been reported[22]. The antinociception effect induced by EM-1 was blocked by a μ-opioid receptor antagonist, but not by a κ-opioid receptor antagonist, while the antinociception effect induced by EM-2 was blocked by a μ-opioid receptor antagonist, as well as by a κ-opioid receptor antagonist. It was therefore proposed that there are at least two different subtypes of μ-opioid receptor for EM-1 and EM-2 to produce antinociception in the spinal cord. It would be worthwhile studying whether similar differential
immunomodulatory effects exist for EM-1 and EM-2. The µ-opioid receptor is widely distributed throughout immune cells[23]. Our previous study also demonstrated inducible expression of µ-opioid receptor in DCs[21]. It is well known that DCs are mobile sentinels that bring antigens to T cells and express co-stimulators for the induction of immunity. Therefore, the finding of the present study that EM-1 and EM-2 can be produced by and secreted from mature DCs suggested that DC-derived EMs might act on DCs bearing µ-opioid receptors themselves, as well as on other immune cells in autocrine and paracrine manners, such that immune responses ensue.

MATERIALS AND METHODS

Design
An in vitro cytological, comparative, and observational study.

Time and setting
The experiments were performed in the Anhui Key Laboratory of Infection & Immunity, Bengbu Medical College, China from December 2008 to August 2010.

Materials
Five healthy female C57BL/6J mice aged 7–8 weeks, weighing 15–22 g, were obtained from the Animal Center of Peking Union Medical College (license No. SCXK (Jing) 2002-2003) and housed in cages lined with ground corncob bedding. Mice were kept in a climate- and light-controlled room. All procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[24].

Methods

Bone marrow-derived DC preparation and maturation
The procedure of Inaba et al[25] was adopted for generation of DCs from mouse bone marrow culture. Bone marrow cells were flushed from the femurs and tibiae of normal female C57BL/6J mice and then depleted of erythrocytes using ammonium chloride. The remaining marrow cells were cultured in 100-mm Petri dishes with 10 mL RPMI 1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum and 200 U/mL of mouse granulocyte macrophage colony stimulating factor (PeproTech, Rocky Hill, NJ, USA) at 37°C in 5% humidified CO₂. Non-adherent granulocytes were removed after 48 hours of culture and fresh medium was added. After 7 days of culture, CD11c+ DCs were purified by immunomagnetic sorting using anti-CD11c antibody-coated magnetic beads and the autoMACS system according to the manufacturer’s instructions (Miltenyi Biotech, Bergish-Gladbach, Germany). The purity of the sorted cells was determined by fluorescence-activated cell sorting analysis and flow cytometry analysis (Beckman Dickson, San Jose, CA, USA). Purified DCs were replated at 5 × 10^5 cells/mL in their conditioned media and were stimulated with the following TLR ligands: 12.5 μg/mL poly (I:C); 6 μg/mL cytosine-phosphate-guanosine oligodeoxynucleotide, 1 μg/mL Pam3Cys (all from InvivoGen, San Diego, CA, USA), or 1 μg/mL LPS (Sigma, St. Louis, MO, USA).

Double immunofluorescence staining for EM-1 and EM-2 expression
To investigate the expression of EM-1 and EM-2 on DCs, double immunofluorescence staining was performed. DCs were cultured in four-well chambers and matured in the presence of LPS (100 ng/mL for 24 hours). Then, the slides were fixed with acetone and immunostained with rabbit polyclonal antibodies against EM-1 (1:200; Chemicon, Santa Cruz, CA, USA) and EM-2 (1:100; Chemicon) and a rat anti-mouse monoclonal antibody against CD11c (1:30; clone: HL3; BD-Pharmingen, San Diego, CA, USA) at 4°C, overnight. After washing in PBS, secondary antibody (Alexa Fluor 488-labeled goat anti-rabbit and Alexa Fluor 546-labelled donkey anti-rat antibodies, 1:1 000; Invitrogen, Carlsbad, CA, USA) was added and sections were incubated for 2 hours at room temperature. Sections were viewed under a Nikon Eclipse 600 fluorescent microscope (Tokyo, Japan). Negative controls including omitting primary antibody, or isotype controls were utilized in all double-labeling experiments. No positive staining was detected in any of these negative control experiments.

Enzyme immunoassay assay for EM production
EM-1 and EM-2 were measured in the supernatant of DCs using EIA kits (Wuhan ElAab Science Co., Ltd., Wuhan, China) that employed highly specific antibodies against EM-1 and EM-2. A standard curve was constructed by plotting the known concentrations of standard peptide on the log scale, and their corresponding absorbance was read on the linear scale. Bovine serum albumin (0.1%) was included in the assay buffer to minimize nonspecific adherence to the tube surface. To extract peptide from the culture supernatant, the supernatant was acidified by adding an equal amount of buffer A. The acidified samples were loaded
through a separator-column containing 200 mg of C18. The eluant was collected in a polypropylene tube, and evaporated using Speedvac. The immunoplate in the kit was pre-coated with secondary antibody, which can bind to the Fc fragment of the primary antibody. The Fab fragment of the primary antibody can be competitively bound by both biotinylated peptide and standard peptide or peptide in samples. The biotinylated peptide is able to interact with streptavidin-horseradish peroxidase, which can catalyze the substrate solution composed of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide to produce a blue-colored solution. The enzyme-substrate reaction was stopped by hydrogen chloride and the solution color turned to yellow. The intensity of the yellow color is directly proportional to the amount of biotinylated peptide-streptavidin-horseradish peroxidase complex, but inversely proportional to the amount of peptide in the standard solution or in samples. The amount of peptide in samples of unknown concentration could be determined by extrapolation to the standard curve.

\[ ^{3}H \text{-thymidine incorporation test} \]

One of the most important functions of DCs is to promote T lymphocyte activation and proliferation, leading to the development of adaptive immunity[11]. To study the functional significance of EMs for T lymphocytes, a \[ ^{3}H \text{-thymidine incorporation test} \] was conducted. Purified T cells were extracted from the spleens of normal C57BL6 mice. Because T lymphocytes are known to also express \( \mu \)-receptors[23], to exclude a direct effect of EMs on T lymphocytes, DCs were pre-exposed to LPS (100 ng/mL) and different concentrations of EMs or EM (10^{-6} M; Sigma), a specific antagonist of \( \mu \)-receptors, for 24 hours, and washed thoroughly with PBS thereafter. Treated DCs were co-cultured with purified T lymphocytes for 3 days in 96-well U-bottomed culture plates (5 000 DCs per well and 10^{5} purified T cells per well). Then, 0.5 \( \mu \)Ci \[ ^{3}H \text{-thymidine} \] (China Atomic Energy Research Institute, Beijing, China) was added and cells were incubated for 18 hours. Cells were harvested and counted by scintillation counter (Beckman Coulter Inc, Brea, CA, USA).

**Statistical analysis**

Data were statistically analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and are expressed as mean \( \pm \) SD. The Mann-Whitney \( U \) test was used for comparisons of the differences among different experimental groups. \( P \) values < 0.05 were considered statistically significant.

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**Author contributions:** This study was designed by Xiaohuai Yang, Hui Xia, and Yong Chen, performed by Xiaohuai Yang, Xiaofan Liu and Cheng Zhou, and results were evaluated by Qin Gao and Zhenghong Li, who were blinded to the experimental design. Xiaohuai Yang wrote the paper. Qin Gao and Zhenghong Li revised the paper. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Committee of Laboratory Animal Management, Bengbu Medical College in China.

**Author statements:** The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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