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ORIGINAL ARTICLE

Morphine Suppresses T helper Lymphocyte Differentiation to Th1 Type Through PI3K/AKT Pathway

Mao Mao,[1] Yanning Qian,[2] and Jie Sun[2,3]

Abstract—To investigate the effect of morphine on T helper lymphocyte differentiation and PI3K/AKT pathway mechanism, CD4+ lymphocytes were treated by phorbol-myristate-acetate (25 ng/ml) (PMA) plus ionomycin (1 μg/ml) in the presence of various concentrations of morphine (25, 50, 100, 200 ng/ml) for 4 h. Th1 and Th2 subsets, supernatant cytokines, and PI3K, AKT, and protein kinase C-theta (PKC-θ) levels were detected. The Th1 cell percentage, Th1-derived cytokines, and ratio of Th1/Th2 decreased in the presence of morphine in a concentration-dependent manner. However, Th2 cell percentage kept stable after morphine treatment. The phosphorylation of PI3K and AKT decreased, but the phosphorylation of PKC-θ did not change in the presence of morphine. The decreased percentage of Th1 cells and ratio of Th1/Th2 was recovered by naloxone concentration-dependently. Morphine can inhibit the differentiation of Th1 lymphocytes and decrease the ratio of Th1/Th2 via the pathway of PI3K/AKT. The effect can be inhibited by naloxone.

KEY WORDS: T helper lymphocyte; morphine; Th1 cell; PI3K/AKT; PKC-θ.

INTRODUCTION

Activated T helper cells can divide into two major subsets, Th1 and Th2, which will produce cytokines and mediate cellular immunity [1, 2]. Th1 cells produce interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-2; Th2 cells secrete interleukin (IL)-4, IL-5, IL-6, IL-10, and IL-13. These two subsets of Th cells inhibit each other and keep a relative balance during differentiation [3, 4]. Multiple stimuli, including activation through the TCR plus CD3/CD28 costimulatory molecules, the external cytokine environment, and nonspecific activators (phorbol-myristate-acetate (PMA) + ionomycin), can promote Th cell differentiation [5, 6]. It has been well documented that Th cell imbalance, especially the decrement of Th1/Th2 ratio, is associated with increased mobility and mortality.

Morphine, a widely used drug for surgical patients and cancer patients, can induce immunological suppression. Studies investigated the effect of morphine on Th cells and demonstrated that morphine treatment can induce Th cell imbalance. Our previous work found that morphine could decrease the ratio of Th1/Th2 in CD4-positive cells [7]. However, the exact mechanism is still to be determined.

There are opioid receptors on the surface of T lymphocytes [8]. Morphine can bind opioid receptors on the cell surface. Phosphoinositide 3 kinase/akt (PI3K/AKT) and protein kinase C-theta (PKC-θ) are important intracellular downstream phosphate kinases following opioid receptor excitation [9, 10]. In addition, PI3K, AKT, and PKC-θ are upstream incidences of T-bet and GATA3 activation, which are the key transcriptional factors of T helper lymphocyte differentiation [11, 12].

Therefore, in this study, we examined the effect of morphine and its antagonist on Th differentiation and intracellular kinase pathway mechanism.

[1] Department of Anesthesiology, Nanjing Maternal and Child Health Hospital, Nanjing, China
[2] Department of Anesthesiology, the First Affiliated Hospital with Nanjing Medical University, No. 300, Guangzhou road, Nanjing, 210029, People's Republic of China
[3] To whom correspondence should be addressed at Department of Anesthesiology, the First Affiliated Hospital with Nanjing Medical University, No. 300, Guangzhou road, Nanjing, 210029, People's Republic of China. E-mail: dgsunjie@hotmail.com

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MATERIAL AND METHODS

Our study protocol was approved by the Ethics and Research Committee of Nanjing Medical University. All the participants signed written informed consents before the beginning of this study.

Twenty milliliters of peripheral venous blood was collected in the morning from ten fasted volunteers, and peripheral blood mononuclear cells (PBMC) were isolated in cell separation medium (Ficoll, Shanghai Bioscience and Technology Co., Ltd, China).

Purification of CD4-Positive Cells

As it was described in our previous research [7], human T lymphocytes were purified from PBMC by anti-CD4 magnetic microbeads (Dynal, China). Briefly, PBMCs were added to Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) supplemented with 0.1 % BSA and 2 mM EDTA. CD4-positive lymphocytes were separated by magnetic microbeads binding the antibody CD4. Purified CD4-positive T cells were detached and suspended (1×10^6 cells) in RPMI Medium 1640 (Thermo Fisher Scientific Co., Ltd, China), supplemented with 10 % calf serum (Hanyang Biologicals Technology Co., Ltd. china) and incubated for 24 h for later use. Trypan blue dye test assured the cell viability at >95 %. The purity of CD4-positive T cells was more than 90 % which was confirmed by flow cytometer (BD, New Jersey, USA).

Groups Protocol

According to experiment groups, cells were stimulated by 25 ng/ml PMA + ionomycin (1 μg/ml) (Sigma Chemical Co., USA) with or without the presence of morphine (Shenyang Pharmacy, China) (final morphine concentrations of 25, 50,100, 200 ng/ml) in 95 % humidified atmosphere containing 5 % CO_2 at 37 °C. Cells and supernatants were collected for further analysis 4 h later. To investigate the relationship between morphine and μ-opioid receptor on Th cell differentiation, we used naloxone (Wuzhong Pharmacy, China) to antagonize μ-opioid receptor. Cells were exposed to morphine (200 ng/ml) and different concentrations of naloxone (100, 200 ng/ml, respectively) for 4 h in the presence of PMA and ionomycin. In addition, the incubation system osmotic pressure was kept constant in each group.

Flow cytometer

The Th cell subsets were calculated by flow cytometer (BD, New Jersey, USA) as soon as they were harvested. Th1 or Th2 cells in the CD4-positive cells were detected by intracellular cytokines IFN-γ or IL-4. In brief, CD4+ cells were fixed, permeabilized, and subsequently stained with APC-Mouse anti-human IFN-γ or PE-Mouse anti-human IL-4 (APC-Mouse anti-human IFN-γ and PE-Mouse anti-human IL-4 antibodies from BD Biosciences). The cell counts were presented as percentage to total CD4-positive cells.

Supernatant Cytokine Analysis

The supernatant levels of IFN-γ and IL-4 were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendation (Ray Biotech, Inc., for IFN-γ ELISA kit and Pierce Biotechnology Inc., for IL-4 ELISA kit).

Western Blotting

The total and phosphated protein levels of PI3K, AKT, and PKC-θ of CD4+ cells were detected by western blotting methods. After 4 h incubation following by experiment groups, protein extracts were separated by electrophoresis on 15 % acrylamide, transferred to polyvinylidene fluoride sheets, and then blocked for 1 h at room temperature with 5 % nonfat milk dissolved in TBS-T buffer (Tris 50 mM, NaCl 1.5 %, Tween 20 0.05 %, pH = 7.5). The membranes were incubated with the related antibody (Jingmei, Beijing) followed by anti-human polyclonal antibody (1:1 000). After 4 h incubation, the blots were washed and incubated with a peroxidase-conjugated IgG antibody for another hour. The blots were quantitated with densitometry by the Bandleader 3.0 software (Magnitec Ltd., Israel).

Statistical Analysis

All data are expressed as mean ± SD. The percentages of T helper cell subsets, the levels of cytokines, and the level of cytoplasmic kinases were compared with one-factor analysis of variance followed by LSD or Dunnett's T3 post hoc test according to the variance homogeneity. $P < 0.05$ was considered to be significant.

RESULT

Cells Viability and CD4-Positive Cells Before PMA and Ionomycin

The viability of cells in all groups was more than 95 % before and after incubation, which was identified by trypan blue experiment. The number of CD4+ cell was kept constant and at baseline level before PMA and ionomycin in each group (data not shown).

Effect of Morphine on Th1 and Th2 Subsets

There were very few Th1 and Th2 cells in CD4-positive cells before PMA and ionomycin, but they were significantly increased after PMA and ionomycin. Morphine decreased the percentage of Th1 cells and also decreased the Th1/Th2 ratio concentration-dependently in the presence of PMA and ionomycin. But morphine did not change Th2 percentage at any concentration. Morphine alone had no effect on Th1, Th2, or Th1/ Th2 (Fig. 1; $^{**}P<0.01$ compared to control group, $^{\#\#}P<0.01$ compared to PMA + ionomycin alone group)

Effect of Morphine on IFN-γ and IL-4 Levels in the Supernatants

IFN-γ and IL-4 maintained at low levels before PMA and ionomycin; however, they were significantly increased after PMA and ionomycin. Morphine decreased the level of IFN-γ concentration-dependently in the presence of PMA and ionomycin. But morphine did not change supernatant IL-4 level at any concentration. Morphine alone had no effect on IFN-γ or IL-4 (Fig. 2; $^{**}P<0.01$ compared to control group, $^{\#\#}P<0.01$ compared to PMA + ionomycin alone group)

Kinase Protein Levels in CD4-Positive Cells

p-PI3K/PI3K, p-AKT/AKT, and p-PKC-θ/PKC-θ levels in cells maintained at low levels before PMA and ionomycin; however, they were significantly increased after PMA and ionomycin. Morphine decreased the levels of p-PI3K/PI3K and p-AKT/AKT concentration-dependently in the presence of PMA and ionomycin. But morphine did not change p-PKC-θ/PKC-θ level at any concentration. Morphine alone had no effect on p-PI3K/PI3K, p-AKT/AKT, or p-PKC-θ/PKC-θ levels (Fig. 3; $^{**}P<0.01$ compared to control group, $^{\#\#}P<0.01$ compared to PMA + ionomycin alone group)

Effect of Morphine on Th1 and Th2 Subsets in the Presence of Naloxone

Th1 and Th2 cells in CD4-positive cells were significantly increased after PMA and ionomycin. Morphine decreased the percentage of Th1 cells and also decreased the Th1/Th2 ratio. However, naloxone suppressed morphine's effect concentration-dependently. Naloxone alone had no effect on Th1, Th2 or Th1/Th2 (Fig. 4; $^{**}P<0.01$ compared to control group, $^{\&}P<0.05$ and $^{\&\&}P<0.01$ compared to PMA + ionomycin + morphine group)

Effect of Morphine on Supernatants IFN-γ and IL-4 Levels in the Presence of Naloxone

IFN-γ and IL-4 maintained at low levels before PMA and ionomycin; however, they were significantly increased after PMA and ionomycin. Morphine decreased the level of IFN-γ in the presence of PMA and ionomycin. However naloxone suppressed morphine's effect concentration-dependently. Naloxone alone had no effect on IFN-γ or IL-4 (Fig. 5; $^{**}P<0.01$ compared to control group, $^{\&}P<0.05$ and $^{\&\&}P<0.01$ compared to PMA + ionomycin + morphine group.)

Kinase Protein Levels in CD4-Positive Cells in the Presence of Morphine and Naloxone

p-PI3K/PI3K, p-AKT/AKT, and p-PKC-θ/PKC-θ levels in cells maintained at low levels before PMA and ionomycin; however, they were significantly increased after PMA and ionomycin. Morphine decreased the levels of p-PI3K/PI3K and p-AKT/AKT in the presence of PMA and ionomycin. However, naloxone suppressed morphine's effect concentration-dependently. Naloxone alone had no effect on p-PI3K/PI3K, p-AKT/AKT, or p-PKC-θ/PKC-θ levels (Fig.6; $^{**}P<0.01$ compared to control group, $^{\&}P<0.05$ and $^{\&\&}P<0.01$ compared to PMA + ionomycin + morphine group)

DISCUSSION

In our study, we demonstrated that morphine could suppress Th cell differentiating to Th1 cell type, decrease the ratio of Th1/Th2, and suppress subsequent cytokine production after PMA and ionomycin challenge. Also, this effect was acting through the μ-opioid receptor, which was in accordance with our previous studies. However, we found for the first time that intracellular PI3K/AKT kinase,

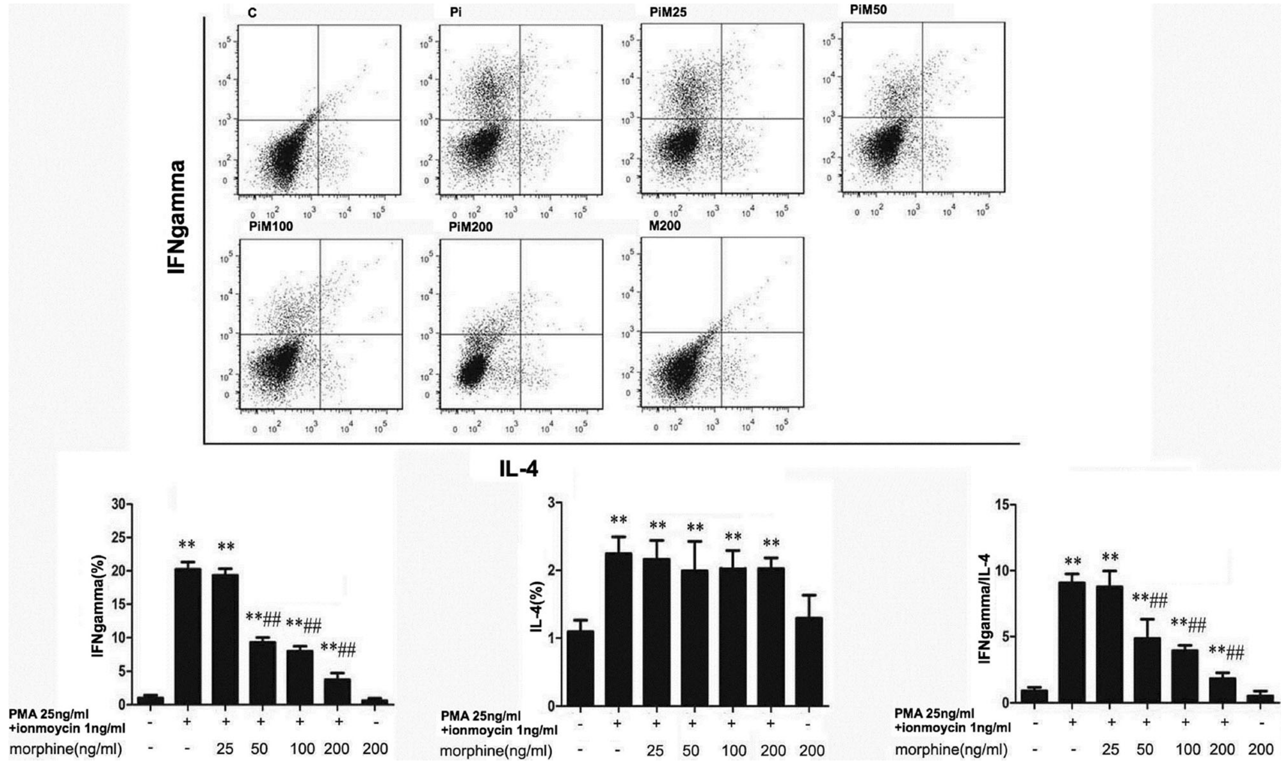

Fig 1. Effect of morphine on Th1 and Th2 subsets. Group *C*: control group. Group *Pi*: cells were incubated with PMA and ionomycin. Group PiM_{25}: cells were treated with morphine (25 ng/ml) in the presence of PMA and ionomycin. Group PiM_{50}: cells were treated with morphine (50 ng/ml) in the presence of PMA and ionomycin. Group PiM_{100}: cells were treated with morphine (100 ng/ml) in the presence of PMA and ionomycin. Group PiM_{200}: cells were treated with morphine (200 ng/ml) in the presence of PMA and ionomycin. Group M_{200}: cells were treated with morphine (200 ng/ml) alone. $^{**}P < 0.01$ compared to control group. $^{\#\#}P < 0.01$ compared to PMA + ionomycin alone group.

but not PKC-θ, was an important signaling pathway during morphine's effect on Th cell differentiation.

In our previous study, we used PBMCs as experimental cells [13]. However, PBMCs are mixed cells containing many other cell types: monocytes, B lymphocyte, and T lymphocyte. CD4+ cells are regarded as Th cells. Therefore, in this study, we purified experimental cells with anti-CD4 magnetic microbead methods in order to obtain Th cells. Th cells will differentiate to Th1 or Th2 cell types after multiple challenges, such as cytokines, TCR, and

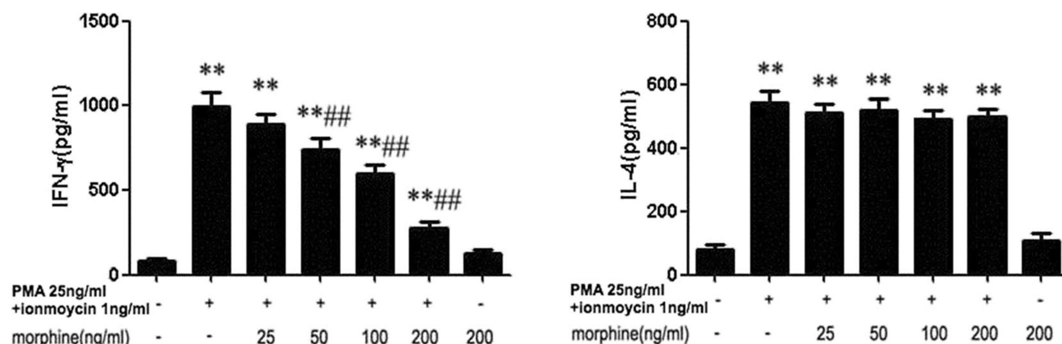

Fig 2. Effect of morphine on IFN-γ and IL-4 levels in the supernatants. $^{**}P < 0.01$ compared to control group. $^{\#\#}P < 0.01$ compared to PMA + ionomycin alone group.

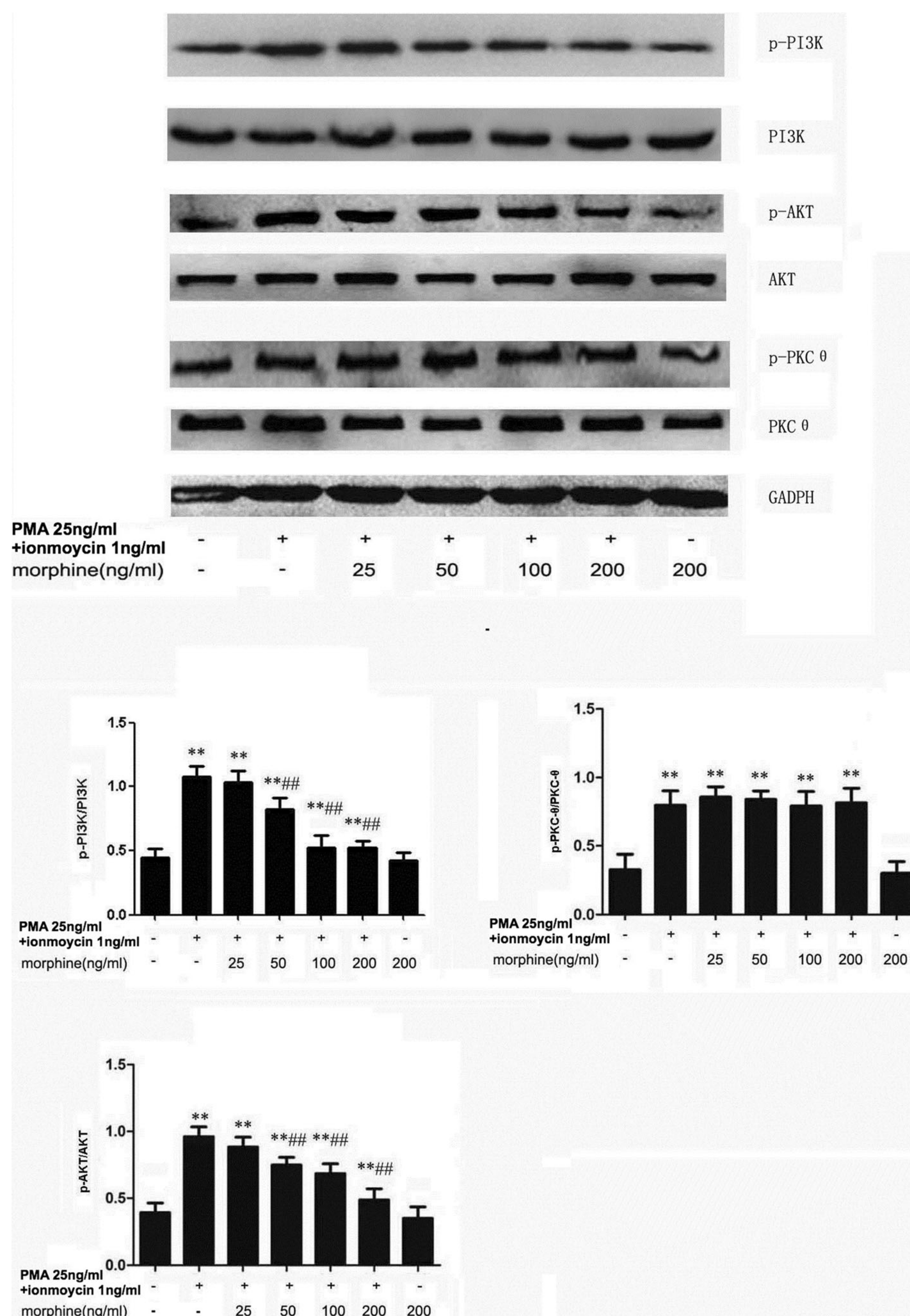

Fig 3. Kinase protein levels in CD4-positive cells. p-PI3K/PI3K, p-AKT/AKT, and p-PKC-θ/PKC-θ levels in cells were detected by western blot analysis. Cells were treated with PMA and ionomycin in the presence of various concentration of morphine (25, 50, 100, 200 ng/ml). $^{**}P < 0.01$ compared to control group. $^{\#\#}P < 0.01$ compared to PMA + ionomycin alone group.

lymphocyte stimulus. PMA plus ionomycin were used in our study since they can promote Th cell differentiation within 4 to 5 h in our experimental settings without obvious impaired cell viability.

The PI3K/AKT pathway is an important and complicated intracellular kinase pathway leading to cell growth, differentiation, regulation, aging, cell mutation, and death. In addition, the PI3K/AKT pathway is an upstream

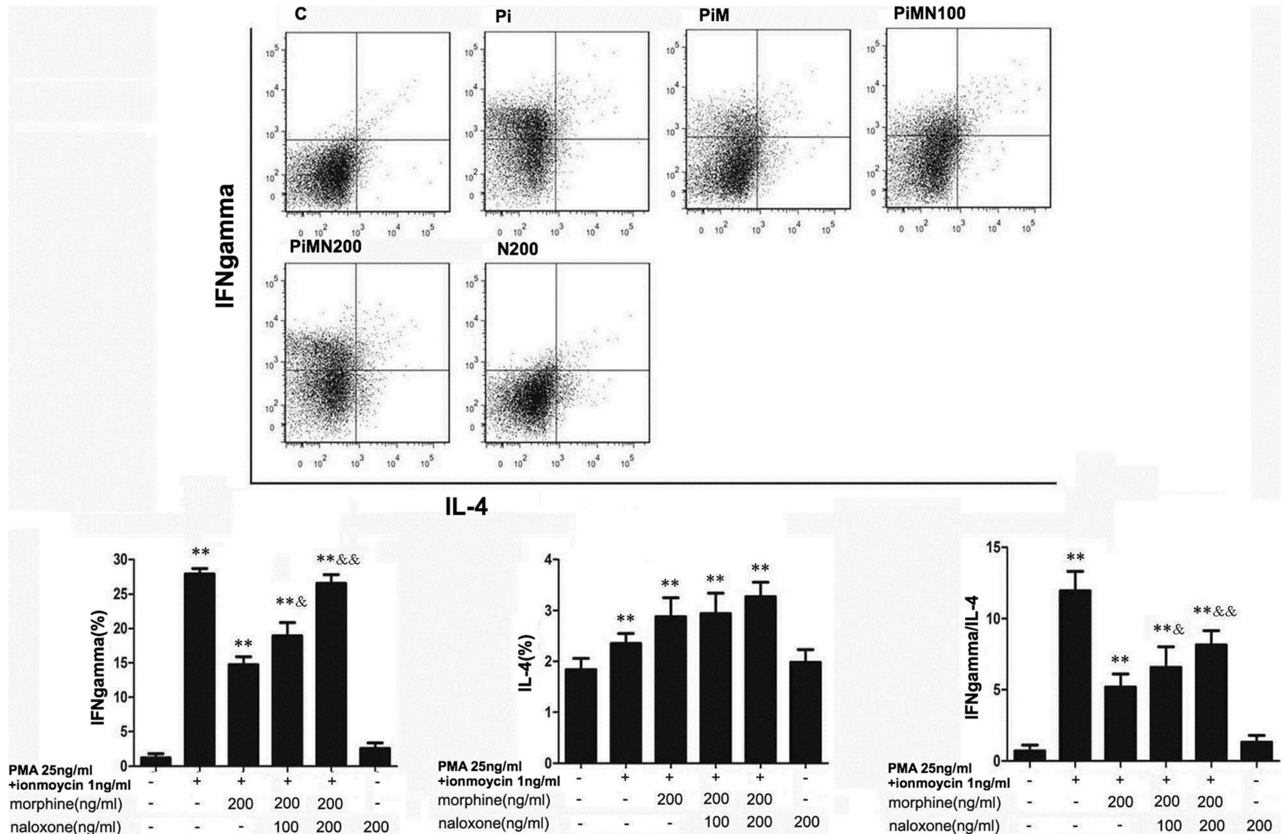

Fig 4. Effect of morphine on Th1 and Th2 subsets in the presence of naloxone. Group *C*: control group. Group *Pi*: cells were incubated with PMA and ionomycin. Group *PiM*: cells were treated with morphine (200 ng/ml) in the presence of PMA and ionomycin. Group $PiMN_{100}$: cells were treated with morphine (200 ng/ml) and naloxone (100 ng/ml) in the presence of PMA and ionomycin. Group $PiMN_{200}$: cells were treated with morphine (200 ng/ml) and naloxone (200 ng/ml) in the presence of PMA and ionomycin. Group N_{200}: cells were treated with naloxone (200 ng/ml) alone. $^{**}P < 0.01$ compared to control group. &$P < 0.05$, &&$P < 0.01$ compared to PMA + ionomycin + morphine group.

mechanism and has potent effect on Th cells mainly on Th1 differentiation [14]. PKC-θ is a serine/threonine-specific protein kinase, which plays a central role in the activation of T cells after T lymphocyte cell activation.

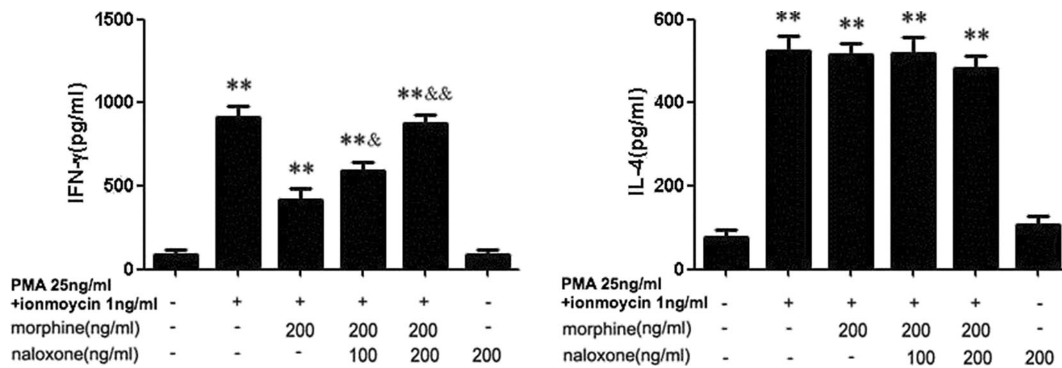

Fig 5. Effect of morphine on supernatants IFN-γ and IL-4 levels in the presence of naloxone. $^{**}P < 0.01$ compared to control group. &$P < 0.05$, &&$P < 0.01$ compared to PMA + ionomycin + morphine group.

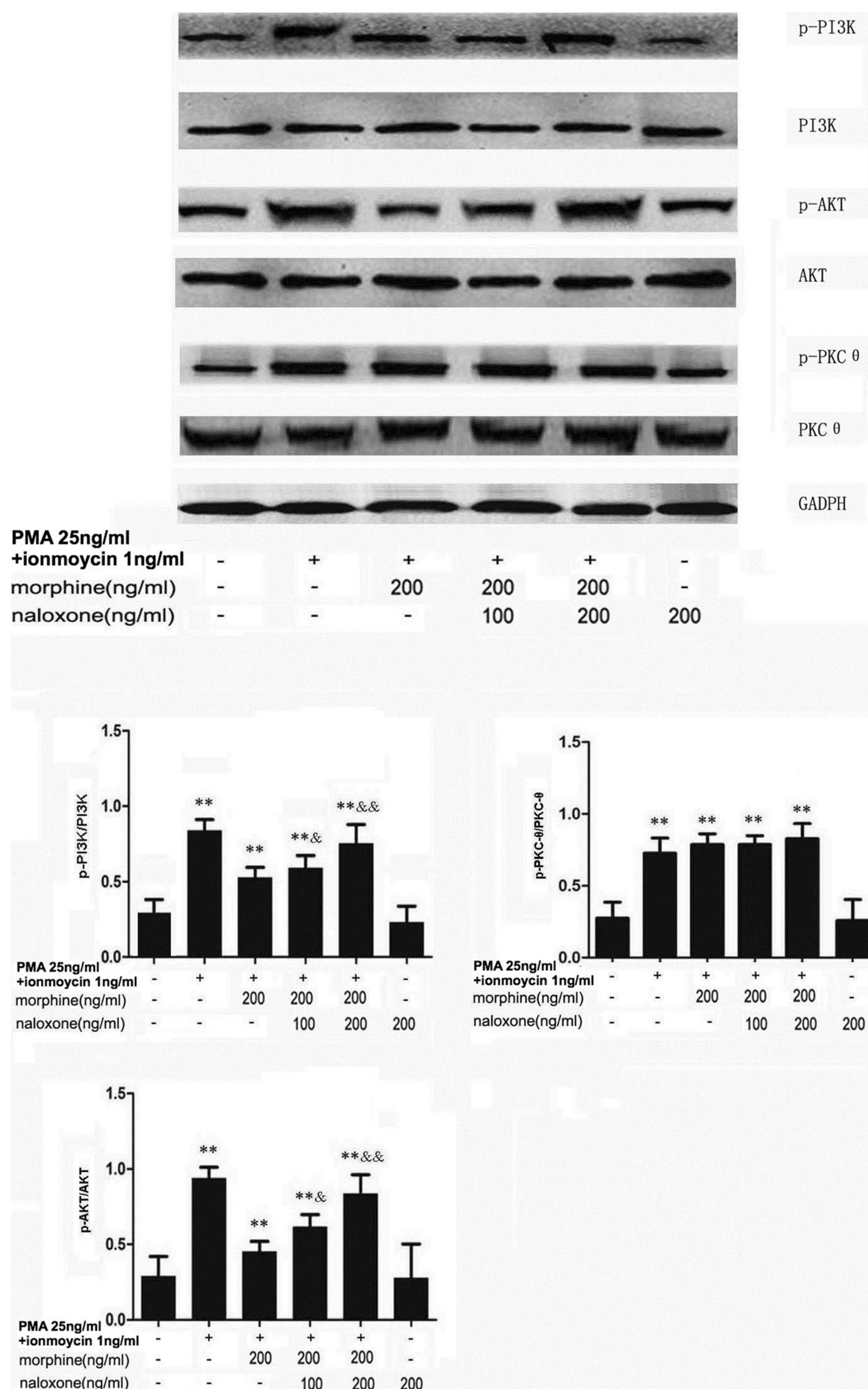

Fig 6. Kinase protein levels in CD4-positive cells by naloxone treatment. p-PI3K/PI3K, p-AKT/AKT, and p-PKC-θ/PKC-θ levels in cells were detected by western blot analysis. Cells were treated with PMA and ionomycin in the presence of morphine and naloxone. $^{**}P < 0.01$ compared to control group. $\&P < 0.05$, $\&\&P < 0.01$ compared to PMA + ionomycin + morphine group.

Also, in recent years, PKC-θ signaling is regarded to be differentially required for Th2 cell type differentiation [15]. In our study, we demonstrated that morphine suppressed Th1 cell differentiation and PI3K/AKT kinase levels, which indicated that the suppressive effect of morphine on Th1 cell differentiation was acting at least in part through the PI3K/AKT kinase pathway. Accordingly, the percentage of Th2 cells and PKC-θ level was not changed by morphine treatment with or without the presence of PMA challenge, which also proved that Th2 cell differentiation might be acting through PKC-θ in spite of the presence of morphine or its antagonist.

It was reported that opioid receptors were constitutively expressed in T cells. Moreover, morphine treatment had influences on T lymphocytes *in vitro* and *in vivo* [8, 16, 17]. We found that morphine suppressed Th cell differentiation and would be antagonized by naloxone dose-dependently, which also indicated that there was an opioid receptor on the surface of T lymphocyte. However, because our study was an *in vitro* investigation, we cannot exclude that morphine does not have an indirect effect on Th cells *in vivo*. The concentrations of morphine used in our study were 25–200 ng/ml, which included its analgesic range [18]. It was indicated that our study was applicable in a clinical setting to some extent. Besides, morphine decreases the percentage of Th1 cells and breaks the balance of Th1/Th2. It means that morphine increases the percentage of Th2 subsets. It has been reported that a chronic morphine treatment enables the promotion of the Th2 differentiation [19, 20], but in our study, the increase of Th2 cell percentage is not obvious in an acute morphine (about 4 to 5 h) treatment.

In conclusion, morphine can suppress Th cell differentiating to Th1 cell type, decrease the ratio of Th1/Th2, and suppress subsequent cytokine production after PMA and ionomycin challenge. This effect is acting through the μ-opioid receptor and intracellular PI3K/AKT kinase, but not the PKC-θ kinase pathway.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The study was supported by the National Natural Science Foundation of China (no. 30801068). This study was also supported by A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We do not have any other competing interest.

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