Morphine and ketamine treatment suppress the differentiation of T helper cells of patients with colorectal cancer *in vitro*

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Abstract. There have been conflicting reports regarding the effects of anesthetic and analgesic drugs on immune function in patients with cancer. The aim of the present study was to investigate changes to T helper (Th) cell populations in patients with colorectal cancer (CRC) and to assess the effects of morphine and ketamine on the differentiation of Th cells harvested from patients with CRC *in vitro*. Peripheral blood samples were extracted from 20 patients with CRC and 20 healthy participants. Peripheral blood mononuclear cells were isolated and incubated in a solution containing phorbol-myristate-acetate (PMA) and ionomycin in the presence or absence of morphine or various ketamine concentrations (25, 50, and 100 μM). Samples were analyzed 4 h later. Th1 and Th2 cells were significantly increased by PMA and ionomycin stimulation; however, Th1 cells and the Th1/Th2 ratio in PMA and ionomycin treatments were significantly decreased in the CRC group compared with the control group. Following incubation with PMA and ionomycin, morphine significantly decreased Th1 cells and the Th1/Th2 ratio in the CRC group. Ketamine did not significantly affect levels of Th1 or Th2 cells or the Th1/Th2 ratio at a concentration of 25 μM; however, a significant increase in the Th1/Th2 ratio was observed at a concentration of 50 μM and, at 100 μM, a significant decrease in Th1 and Th2 cells and an increase in the Th1/Th2 ratio were observed. The present study suggests that CRC may shift the balance of Th1/Th2 towards Th2 by inducing an immunological response, morphine is able to suppress the differentiation of Th cells and decreases the Th1/Th2 ratio, and ketamine may affect the differentiation of Th cells in a dose-dependent manner.

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Introduction

Colorectal cancer (CRC) is one of the most prevalent malignancies in the world, and is a primary cause of tumor-associated mortality. Previous data has indicated that incidence of CRC in China increased from 12.8 in 2003 to 16.8 per 100,000 in 2011, while the mortality rate increased from 5.9 to 7.8 per 100,000 and is expected to reach 8.6 per 100,000 in 2020 (1). Pain is one of the most typical symptoms in patients with cancer, and malignant tumor-associated pain occurs throughout all stages and courses of treatment, including surgery, radiotherapy and chemotherapy (2).

The use of opioid analgesics in clinical practice for the management of cancer-related pain is widely accepted (3). Morphine is one of the most frequently used opioid analgesics in the treatment of various pains, including cancer-associated pain; however, morphine may induce detrimental side effects such as opioid-induced hyperalgesia or may result in patients developing a tolerance to opioids (3). Supplementation of morphine with adjuvant agents is the preferred method of providing adequate pain relief and may also reduce the occurrence of adverse side effects (4,5). As a noncompetitive N-methyl-D-aspartate-receptor antagonist, ketamine has previously been shown to be synergistic with morphine (6). When co-administered with morphine, ketamine is able to reduce hyperalgesia and delay the development of tolerance to opioids via enhancing opioid-induced antinociception and decreasing morphine consumption (6). The co-administration of ketamine and morphine has been described in a number of clinical trials, and ketamine is usually administered off-label in combination with opioids at subanesthetic doses to treat pain associated with cancer (7).

Patients with cancer typically exhibit immunosuppression (8,9). It is widely reported that host immunosuppression may influence anti-tumor immune responses (8-10).

Given that cluster of differentiation (CD)4$^+$T cells serves a crucial role in the regulation of all antigen specific immune responses, their potential involvement in antitumor immunity is of interest to tumor immunologists (11). T-helper (Th)1 and Th2 cells are the classical subsets of CD4$^+$ T cells. Th1 cells produce interferon (IFN)-γ and favor cell-mediated immune responses. Th2 cells produce interleukin(IL)-4 and/or IL-10 and are associated with humoral immunity in terms of control of antibody production (12). The imbalance of Th cells, particularly decreased Th1/Th2 ratios, has been associated with mortality and complications in patients with gastrointestinal tumors (13,14).

It has previously been demonstrated that anesthetic and sedative agents exhibit immunomodulatory activity (15). For example, the effects of morphine and ketamine on the differentiation of Th cells have been demonstrated in previous studies in healthy volunteers *in vitro* (16,17). To the best of our knowledge, no previous studies have investigated whether morphine and ketamine are able to alter the differentiation of Th cells in patients with tumors; therefore, this study was designed to assess the effects of morphine and ketamine on the differentiation of CD4$^+$ T cells induced by phorbol-myristate-acetate (PMA) and ionomycin in patients with CRC.

Materials and methods

Ethics approval. The present study was approved by the Ethics and Research Committee of Shandong Academy of Medical Sciences (Jinan, Shandong). All participants included in the study gave their informed consent for the tests to be performed, and the present study was conducted in adherence with the Declaration of Helsinki.

Study population. A total of 20 patients with primary CRC (10 males, 10 females) and 20 healthy subjects (10 males, 10 females), with an age range of 45-65 years and body mass indices from 18-25 kg/m^2, were enrolled as research subjects in the present study between October 2014 and May 2015 at Shandong Cancer Hospital affiliated to Shandong University (Jinan, China). Routine blood tests were performed on patients in the CRC group including lymphocyte counts and calculation of these as a proportion of total cells. No patients had a history of long-term medication use, drug abuse, transfusion, diabetes mellitus, recent infection, systemic inflammatory disease or immunological deficiency, and patients did not have any other tumors. None of the patients had previously been treated using immunosuppression, radiotherapy or chemotherapy. All patients in the normal group were either healthy or had benign noninflammatory conditions of the large bowel which were diagnosed via barium enema or colonoscopy.

Reagents. Ketamine (Shanghai Hengrui Pharmaceutical Co., Ltd., Shanghai, China) was diluted to different concentrations (25, 50, and 100 μM) with distilled water. Morphine (Shenyang Pharmaceutical University, Shenyang, China) was diluted to 50 ng/ml with distilled water.

Peripheral blood mononuclear cell (PBMC) isolation. PMBCs were isolated from the blood samples harvested from CRC and normal groups as previously described (18). Briefly, peripheral blood from the ulnar vein (5 ml) was placed in a heparinized tube and layered using density gradient sedimentation. Following centrifugation (500 x g; 20˚C for 20 min) PBMC were collected from the interface and washed three times in culture medium. Atrypan blue dye test (17) was conducted to ensure that cell viability >95%. Qualifying cells were suspended (1x10^6 cells) in RPMI Medium1640 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) and incubated for 30 min. PBMCs were then stimulated using 2 μl/ml of a leukocyte activation cocktail containing PMA and ionomycin (P550583; 1X; BD Biosciences, Franklin Lanes, NJ, USA) in the presence or absence of ketamine and morphine, in an atmosphere containing 5% CO_2 at 95% humidity and 37˚C for 4 h, prior to analysis.

Study groups. Cells isolated from healthy subjects were assigned to one of the following groups: Group 0, healthy, untreated control group; or group 1, healthy group, treated with PMA and ionomycin but not ketamine or morphine. Cells isolated from patients with CRC were assigned to one of the following groups: Group 2, CRC control group not treated with PMA and ionomycin; group 3, CRC group treated with PMA and ionomycin without ketamine or morphine; group 4, CRC group treated with ketamine (25 μM), PMA and ionomycin; group 5, CRC group treated with ketamine (50 μM), PMA and ionomycin; group 6, CRC group treated with ketamine (100 μM), PMA and ionomycin; or group 7, CRC group treated with morphine (50 ng/ml), PMA and ionomycin.

Th cell subset analysis. Cells were harvested and subsequently counted using a FACS Caliburflow cytometer (BD Biosciences). Subsets of Th1 and Th2 cells were detected via the surface antigen CD3, CD8 and intracellular cytokines IFN-γ or IL-4. Briefly, the cells (1x10^6) were stained with fluorescein isothiocyanate-mouse anti-human CD3 (561806) and phycoerythin (PE)-Cy5 mouse anti-human CD8antibodies (561946; both BD Biosciences), fixed, permeabilized, and stained with PE-mouse anti-human IFN-γ (557074) or PE-mouse anti-human IL-4 antibodies (551774; both BD Biosciences). Th1 cells were marked as CD3$^+$CD8$^-$IFN-γ$^+$ and Th2 cells were marked as CD3$^+$CD8$^-$IL-4$^+$. The cell counts were presented as the percentage of total CD3-positive cells.

Statistical analysis. SPSS19.0 statistical software (IBM SPSS, Armonk, NY, USA) was used for all data analysis. Data are presented as the mean ± standard error of the mean. The Shapiro-Wilk test was performed and the percentages of T helper cell subsets were found to be normally distributed. Tests of variant homogeneity were followed by Bartlett's test (when data were normally distributed) or Levene's test (when data were not normally distributed). The percentages of T helper cell subsets were compared using one-way analysis of variance followed by least-significant difference or Dunnett's T3 post hoc test based on the homogeneity of variance. As the Th1/Th2 ratio did not follow a normal distribution, the data were presented as medians (range). Friedman tests were

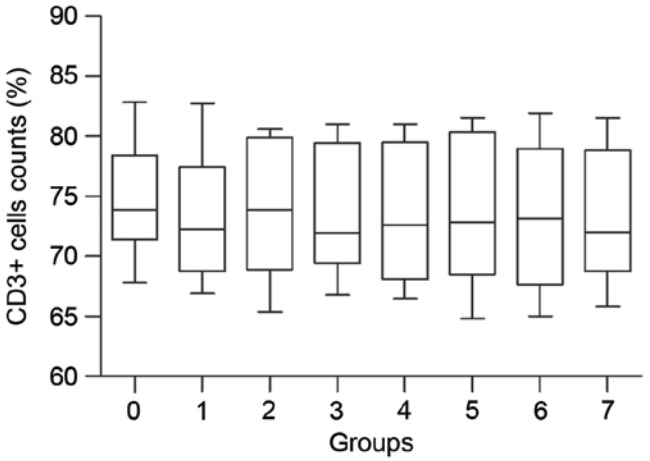

Figure 1. Percentages of CD3$^+$ cells in eight groups. Group 0, healthy control group without PMA and ionomycin treatment; group 1, healthy patient group treated with PMA and ionomycin but not ketamine or morphine; group 2, CRC control group without PMA and ionomycin; group 3, CRC group treated with PMA and ionomycin but not ketamine or morphine; group 4, CRC group treated with ketamine (25 μM), PMA and ionomycin; group 5, CRC group treated with ketamine (50 μM), PMA and ionomycin; group 6, CRC group treated with ketamine (100 μM), PMA and ionomycin; group 7, CRC group treated with morphine (50 ng/ml), PMA and ionomycin. CD3$^+$, cluster of differentiation 3$^+$; PMA, phorbol-myristate-acetate; CRC, colorectal cancer.

performed to establish Th1/Th2 ratio. The significant effects were investigated post hoc using Wilcoxon-signed-ranks tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability. Cell viability in all groups was >95% with or without PMA and ionomycin treatment, as confirmed via trypan blue staining.

CD3$^+$ cell counts. There was no significant difference in the number of CD3$^+$ cells in the eight groups prior to PMA and ionomycin stimulation (Fig. 1).

Th cell differentiation in normal subjects and patients with CRC. In the absence of PMA and ionomycin stimulation, there were few Th1 cells [<0.10% in the healthy subject group (group 0) compared to 0.12% in the CRC group (group 2); P>0.05] and Th2 cells [<0.10% in the healthy subject group (group 0) compared to 0.13% in the CRC group (group 2); P>0.05]. However, the proportion of Th1 and Th2 cells were significantly increased following stimulation with PMA and ionomycin in the healthy and CRC group cell populations [Th1 cells from <0.10 to 9.69±1.31%, and from 0.120 to 6.38±1.00% in the healthy subject and CRC groups (group 1 and group 3), respectively, P<0.001; Fig. 2); Th2 cells from <0.10 to 3.99±0.60% and from 0.13 to 3.93±0.91% in the healthy subject and CRC groups (group1 and group 3), respectively, P<0.001; Fig. 3]. Following PMA and ionomycin stimulation, the number of Th1 cells in group 1 compared with group 3 were significantly different (9.69±1.31 and 6.38±1.00%, respectively; P<0.001; Fig. 2), whereas the number of Th2 cells were not significantly different between group 1 and group 3 (3.99±0.60 vs. 3.93±0.91%; P=0.82, Fig. 3). The Th1/Th2 ratio therefore significantly differed in group 1 compared with the group 3 (2.48 and 1.63, respectively; P<0.001) following stimulation with PMA and ionomycin (Fig. 4).

Effects of ketamine on Th1 and Th2 subsets following PMA and ionomycin stimulation in the CRC groups. Ketamine treatment of CRC group cells at a concentration of 100 μM (following PMA and ionomycin treatment) significantly decreased the proportion of Th1 cells from 6.38±1.00% in group 3 CRC patient cell populations to 5.14±0.80% (P<0.001; Fig. 2) and Th2 cells from 3.93±0.91% in group 3 CRC patient cell populations to 2.61±0.64% (P<0.001; Fig. 3); however, these measures were not significantly altered by 50 μM ketamine treatment.

Ketamine at 50 and 100 μM significantly increased the Th1/Th2 ratio in CRC groups from 1.62 (group 3) to 1.71 (group 4; P<0.001) and to 2.03 (group 6; P<0.001), respectively (Fig. 4), acting in a dose-dependent manner. Ketamine at a concentration of 25 μM did not significantly affect the proportion of Th1 cells, Th2 cells or the Th1/Th2 ratio in the presence of PMA and ionomycin.

Effects of morphine on Th1 and Th2 subsets following PMA and ionomycin stimulation in the CRC groups. Morphine significantly decreased the number of Th1 cells from 6.38±1.00 (in group 3 CRC cell populations) to 5.04±0.94% (P<0.001; Fig. 2), and the Th1/Th2 ratio from 1.62 to 1.35 (group 7; P<0.001) (Fig. 4) following PMA and ionomycin stimulation in the CRC group; however, no significant difference was observed in the number of Th2 cells (3.93±0.60% in group 3 vs. 3.70±0.98%; P=0.374; Fig. 3).

Discussion

It is established that Th cells modulate immune responses and serve an important role in immune protection (19). Furthermore, it has recently been demonstrated that CD4$^+$Th cells are important for effective antitumor immunity (20). According to their cytokine synthesis profile, CD4$^+$Th cells maybe classified as Th1 and Th2 subsets. The Th1 subset, which was the first identified group of Th cells, selectively expresses IFN-c, tumor necrosis factor (TNF)-α, TNF-β and other proinflammatory cytokines (21). Th1 cells are therefore important for regulating innate and T-cell-mediated immune responses, and protecting the host from obligate intracellular pathogens. Th2 cells were identified at the same time as Th1 cells in the early 1980s. An important function of Th2 cells is the production of IL-4, IL-5, IL-9, IL-10 and IL-13. Th2 cells also produce immunoglobulins by inducing differentiation in B cells (22). Therefore, Th2 cells are important in the humoral response and in resistance against extracellular pathogens. It is generally believed that polarization of Th cells toward either Th1 or Th2 typing may significantly influence the later immune responses during carcinogenesis (22).

In the present study, counts were performed *in vitro* to assess the number of Th1 and Th2 cells in the peripheral blood of patients with CRC. The results demonstrated that the

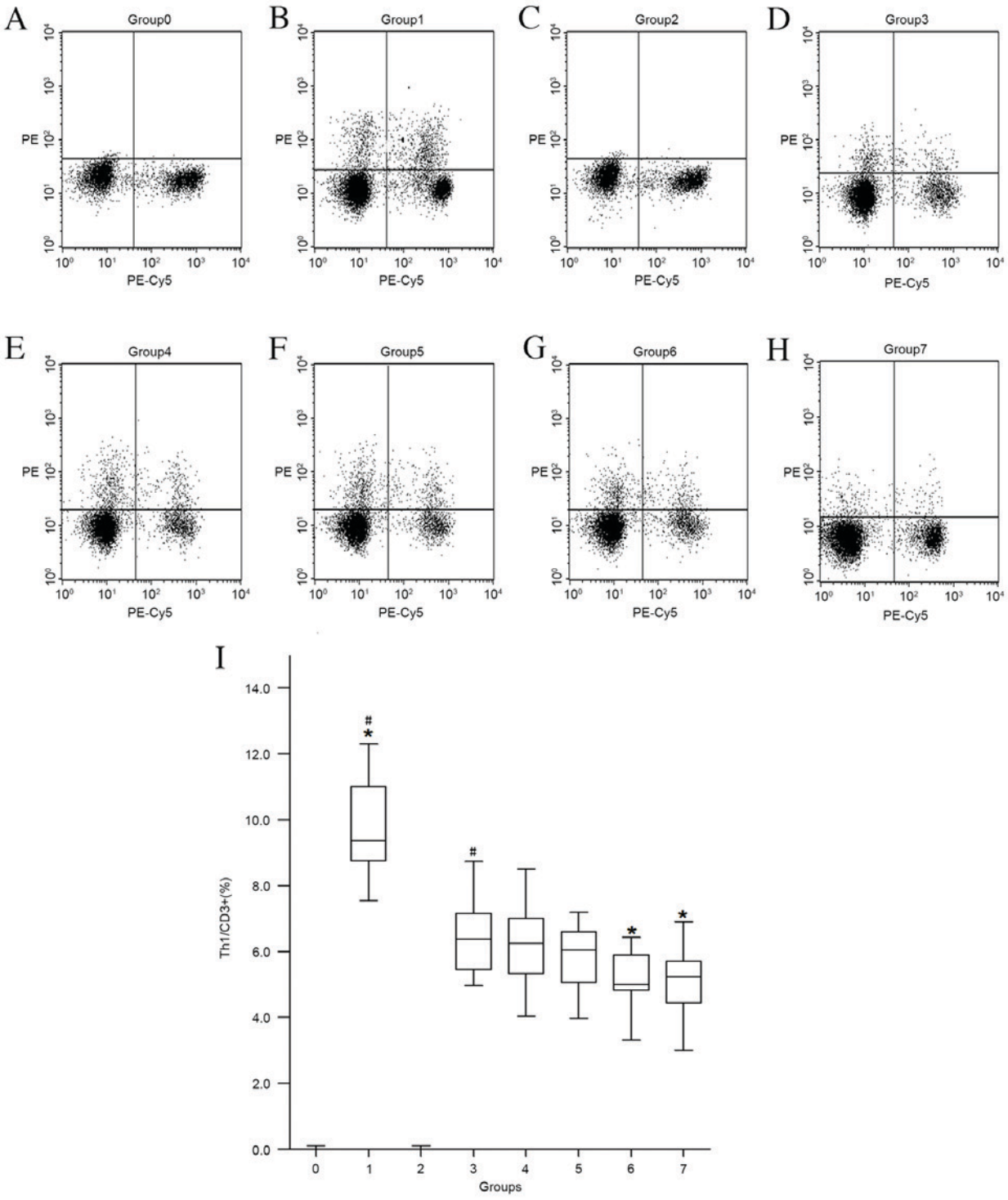

Figure 2. Th1 cell counts in the presence of ketamine or morphine following PMA and ionomycin stimulation in healthy control and CRC groups. (A) Group 0, healthy control group without PMA and ionomycin treatment. (B) Group 1, healthy patient group treated with PMA and ionomycin but not ketamine or morphine. (C) Group 2, CRC control group without PMA and ionomycin. (D) Group 3, CRC group treated with PMA and ionomycin but not ketamine or morphine. (E) Group 4, CRC group treated with ketamine (25 μM), PMA and ionomycin. (F) Group 5, CRC group treated with ketamine (50 μM), PMA and ionomycin. (G) Group 6, CRC group treated with ketamine (100 μM), PMA and ionomycin (H) Group 7, CRC group treated with morphine (50 ng/ml), PMA and ionomycin. (I) $CD3^+CD8^-IFN\text{-}\gamma^+$cells, labeled as Th1 cells, in dot plots. Data are presented as the mean ± standard error of the mean. $^{\#}P<0.001$ vs. group 0; $^{*}P<0.001$ vs. group 3. Th1, T helper 1; PMA, phorbol-myristate-acetate; CRC, colorectal cancer; CD, cluster of differentiation; IFN-g^+, interferon γ.

number of Th1 cells and the Th1/Th2 ratio were significantly lower in patients with CRC compared with healthy subjects following administration of PMA and ionomycin, whereas there was no significant difference in the number of Th2 cells. These results are supported by the findings of previous studies, in which the cytokines produced by Th1 cells, such as IFN-c, TNF-α and IL-2, were significantly reduced in CRC patients, whereas the cytokines produced by Th2 cells, such as IL-6 and IL-4 showed no marked change (23). Furthermore, Kanazawa *et al* (24) demonstrated that patients

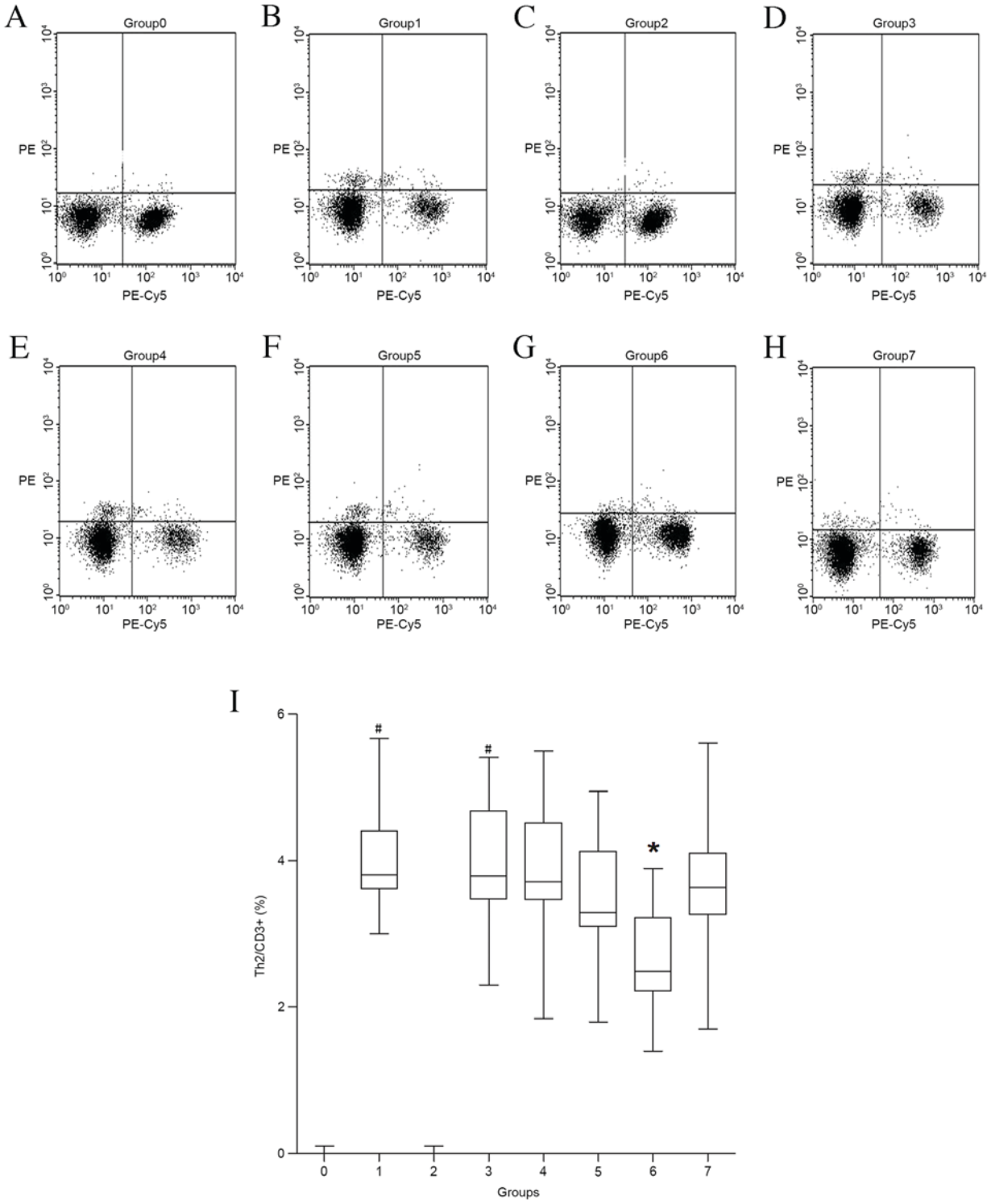

Figure 3. Th2 cell counts in the presence of ketamine or morphine following PMA and ionomycin stimulation in healthy control and CRC groups. (A) Group 0, healthy control group without PMA and ionomycin treatment. (B) Group 1, healthy patient group treated with PMA and ionomycin but not ketamine or morphine. (C) Group 2, CRC control group without PMA and ionomycin. (D) Group 3, CRC group treated with PMA and ionomycin but not ketamine or morphine. (E) Group 4, CRC group treated with ketamine (25 μM), PMA and ionomycin. (F) Group 5, CRC group treated with ketamine (50 μM), PMA and ionomycin. (G) Group 6, CRC group treated with ketamine (100 μM), PMA and ionomycin (H) Group 7, CRC group treated with morphine (50 ng/ml), PMA and ionomycin. (I) $CD3^+CD8^-IL\text{-}4^+$cells, labeled as Th2 cells, in dot plots. Data are presented as the mean ± standard error of the mean. $^{\#}$P<0.001 vs. group 0; *P<0.001 vs. Group 3. Th2, T helper 2; PMA, phorbol-myristate-acetate; CRC, colorectal cancer; CD, cluster of differentiation; IFN-g^+, interferon γ.

with gastric or colorectal cancer have a lower Th1/Th2 ratio compared with healthy subjects, and Tabata *et al* (25) demonstrated Th2 dominance in patients with gastrointestinal tract cancer.

Domino *et al* (26) demonstrated that, following intravenous administration of 2 mg/kg ketamine, the blood concentration of ketamine may reach 27 μg/ml (100 μM), and it may therefore provide an analgesic effect *in vivo* at a concentration of

0.5 mg/kg (26). It has been suggested that the strength of this effect would be dose-dependent (27); therefore the following serial concentrations of ketamine were used in the present study: 6.25 μg/ml (25 μM), 12.5 μg/ml (50 μM) and 25 μg/ml (100 μM). It has previously been demonstrated that a morphine plasma concentration of 50 ng/ml is within the analgesic range (28); therefore, a morphine concentration of 50 ng/ml was used in the present study. Additionally, the culture conditions including temperature, osmotic pressure and pH value were kept in normal ranges for all groups to ensure that the results would not be affected by differences in culture.

The results of the present study indicated that morphine had a negative effect on Th cell balance as it decreased the counts of Th1 cells and the ratio of Th1/Th2 in the CRC group. Gao *et al* (17) previously demonstrated that morphine is able to suppress the differentiation of Th cells and the subsequent secretion of cytokines, and decrease the ratios of Th1/Th2 and IFN-γ/IL-4. Given that patients with CRC are Th2 dominant, it may be hypothesized that analgesia with morphine may result in a further imbalance of the Th1/Th2 ratio. These changes may inhibit the immunological response and hasten tumor invasion, recurrence and metastasis of cancer in patients with CRC. However, in the present study, ketamine shifted the balance of Th1/Th2 toward Th1, suggesting that it may have a beneficial immunoregulatory effect in patients with CRC. This supports the findings of a previous study in healthy participants, in which ketamine suppressed the differentiation of Th cells and secretion of cytokines, whereas the Th1/Th2 ratio was increased in the presence of PMA and ionomycin (16).

The results of the present study demonstrate that ketamine affects the differentiation of Th cells in a concentration-dependent manner, as with increased concentrations, the effect of ketamine on the differentiation of Th cells was increased. However, at a concentration of 25 μM, ketamine did not induce any significant changes in the number of Th1 and Th2 cells or the Th1/Th2 ratio. This suggests that a low dose of ketamine, combined with morphine, may provide sufficient pain relief without increasing immune suppression in patients with CRC.

There are numerous cytokine analysis methods available, such as ELISA, reverse transcription-polymerase chain reaction, and immunohistochemistry (29,30). ELISA is widely used due to the ease with which it is performed; however, it is unable to identify the cellular source of cytokines in the plasma (29). Intracellular cytokine staining, a flow cytometry method, is currently the only technique that can enumerate antigen-specific T cells and determine their phenotype simultaneously (31). It has previously been used to investigate cytokine production at the single-cell level following polyclonal stimulation with mitogens with a short incubation time, which depending on the retention of cytokines in cells, typically peaks between 4 (for TNF-α) to 8 h (for IFN-γ and IL-2) and up to 12 h for IL-12 (31,32). Furthermore, two or more cytokines may be simultaneously detected within a single cell by multiparameter flow cytometry in the presence of cytokine secretion inhibitors; therefore, it maybe used to determine the Th1/Th2 ratio directly (33). A modified method using whole blood has also been developed, which requires less time as PBMC isolation is not required (34); however, some components of the serum may interfere with the results, and therefore PBMCs isolated from patients with CRC were used in the present study.

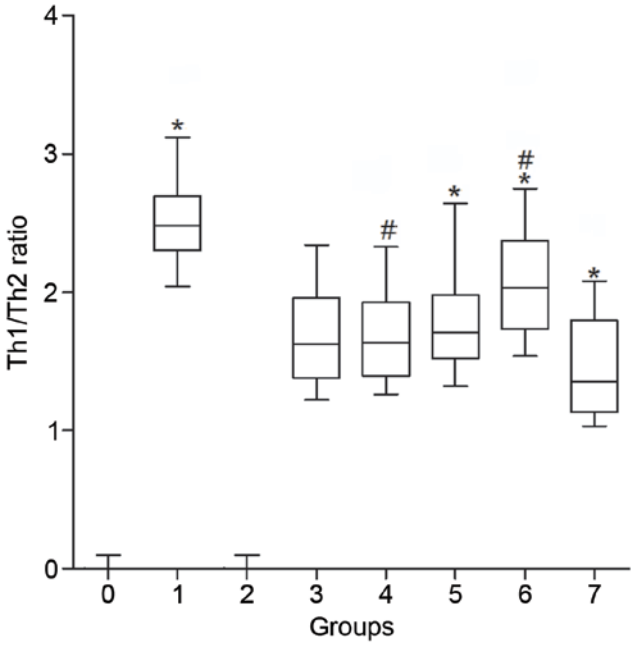

Figure 4. Th1/Th2 ratio in the presence of ketamine or morphine following PMA and ionomycin stimulation in the normal control and CRC groups. Group 0, healthy control group without PMA and ionomycin treatment; group 1, healthy patient group treated with PMA and ionomycin but not ketamine or morphine; group 2, CRC control group without PMA and ionomycin; group 3, CRC group treated with PMA and ionomycin but not ketamine or morphine; group 4, CRC group treated with ketamine (25 μM), PMA and ionomycin; group 5, CRC group treated with ketamine (50 μM), PMA and ionomycin; group 6, CRC group treated with ketamine (100 μM), PMA and ionomycin; group 7, CRC group treated with morphine (50 ng/ml), PMA and ionomycin. Data are presented as the mean ± standard error of the mean. *P<0.001 vs. group 3; #P<0.001 vs. group 5. PMA, phorbol-myristate-acetate; CRC, colorectal cancer; Th, T helper.

It has been reported that phytohaemagglutinin is able to activate Th cells; however, it takes 48 h for this to occur (16). As PMA and ionomycin are able to activate Th cells within 4-6 h, they were selected in the present study to minimize incubation time of PBMCs *in vitro* and maintain cell viability. As PMA and ionomycin are able to down regulate CD4 expression, Th1 and Th2 lymphocytes with $CD3^+$ and $CD8^-$ were labeled in the present study, a detection method that has been used in previous studies (16,35). Additionally, trypan blue staining was performed to assess the extent of cell death over the course of the present study. The results indicated that there was no significant cell death in the presence or absence of PMA and ionomycin. In preliminary experiments, there were few Th1 and Th2 cells in the presence of morphine or ketamine. Therefore, in the CRC groups, there was no subgroup in which only morphine or ketamine were administered without incubating with PMA and ionomycin.

In the present study, patients with CRC exhibited Th2 dominance. Furthermore, morphine and ketamine with concentrations over the subanesthestic level (<100 μM) suppressed the differentiation of Th cells *in vitro*. Morphine induced a decrease in the Th1/Th2 ratio, whereas ketamine increased the Th1/Th2 ratio and did not affect the differentiation of Th cells at the subanesthestic concentration. With increasing concentration, the effect of ketamine on the differentiation of Th cells was increased. These findings suggest that in clinical practice,

combinatorial treatment with morphine and a low dose of ketamine may reduce morphine consumption and the risk of adverse reactions, alleviate immune inhibition and improve the quality of life in patients with CRC.

In conclusion, the present study demonstrated that CRC shifts the balance of Th1/Th2 towards Th2 by inducing an immunological response. Morphine is able to suppress the differentiation of Th cells; however, it induces a decrease in the Th1/Th2 ratio. Furthermore, ketamine is able to affect the differentiation of Th cells in a dose-dependent manner; therefore, the findings of the present study may provide a novel clinical approach for treatment of patients with CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MH designed and implemented the current study, acquired data, analyzed and interpreted, results and drafted the manuscript. KW and FX conceived and designed the present study, and provided their assistance and critical review when drafting the manuscript. NZ, HL, BW, XiuW, XinW and TJ acquired and interpreted the data.

Ethics approval and consent to participate

The present study was approved by the Ethics and Research Committee of Shandong Academy of Medical Science. All patients included in the current study provided their informed consent and the current study was performed in accordance with the Declaration of Helsinki.

Patient consent for publication

All patients included in the study gave their informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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