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Original Article

Effects of fentanyl anesthesia and sufentanil anesthesia on regulatory T cells frequencies

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Abstract: Background: $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) can inhibit anti-tumor immune responses and opioids were also immunosuppressive. We set out to compare the effects of sufentanil and fentanyl on Tregs frequencies both in vitro and in breast cancer (BC) patients undergoing eradicative operation. Methods: PBMCs from 12 BC patients were activated in vitro in the presence of fentanyl or sufentanil. The percentage of Tregs was detected by flow cytometry after seven days culture. Other 38 patients who underwent eradicative operation were prospectively randomized to sufentanil anesthesia and fentanyl anesthesia. Blood samples were collected for Tregs quantification by flow cytometry analysis and for Foxp3 mRNA expression by RT-PCR, at 10 min before anesthesia (D0), 24h (D1), and 168 h (D7) after the operation respectively. Results: Activation of PBMCs in the presence of either fentanyl or sufentanil increased the Tregs number, and the effect of sufentanil was more significant under the same analgesic effect with fentanyl. In the 38 operated cases, both the Tregs frequencies and Foxp3 mRNA expression on D1 decreased in comparison to those on D0, but then recovered on D7. By comparing SF and F group, there ware no significant differences in Tregs frequencies and Foxp3 mRNA expression on D0, D1 and D7. Conclusion: With the same analgesic potency, sufentanil is more powerful in increasing the Tregs quantity than fentanyl in vitro. But there are no significant differences as to Tregs frequencies between sufentanil anesthesia and fentanyl anesthesia perioperatively. Further studies are needed to determine the differences in the Tregs function and long-term outcome of these patients.

Keywords: $CD4^+CD25^+Foxp3^+$ regulatory T cell, breast cancer, recurrence, fentanyl, sufentanil

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and it is also the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths [1]. As metastatic disease is regarded as one of the most important factors inducing cancer-related deaths, about 30-40% of patients will die from metastatic disease despite radical surgery [2]. In addition, the immune response in cancer patients is epidemiologically associated with an increased incidence of tumor and its recurrence. Thus, activation of humoral and cellular immunity may predispose the patients to risk cancer development.

Extensive interests have recently been aroused in the role of a naturally occurring population of $CD4^+CD25^+$ regulatory T cells (Tregs) that are characterized by expression of the forked-head transcription factor Foxp3 [3]. Specialized T-cells are able to inhibit the activation of the immune system and even to shut off the normal immune system. Hence, they may play a role in controlling anti-tumor immune responses. Interestingly, some relationships have been discovered between Tregs and breast cancer recurrence. For breast cancer patients, it has been reported that the immune systems were dysfunctional. And Tregs are in relation to a more advanced disease in breast cancers and possibly promote immunologic tolerance to tumors. Moreover, a recent study demonstrated a significant intratumoral infiltration of the $Foxp3^+$Tregs in high-risk breast cancer patients and those at risk of late relapse [4]. More recently, it has been observed an increased frequency of Tregs in the peripheral blood of breast cancer patients [5]. In addition, a linear variation of intratumoral Foxp3 expression with invasion, size, and vascularity suggested a use for Foxp3, an indicator of Treg activity, as a marker of tumor progression and metastasis in breast carcinoma [6].

Opioid peptides have long been used as the mainstay of treatment of cancer-related pain and also as an important modality for the prevention of perioperative pain. Apart from its analgesic action, opioid peptides appear to be of importance in the regulation of neoplastic tissue. A retrospective investigation on the patients undergoing breast cancer surgery, demonstrated that the prominent difference in cancer recurrence depends on whether the patients received a combined general anesthetic with paravertebral block or a combined general anesthetic with opiate analgesia [7]. And the patients receiving an epidural had gained a 65% reduction in recurrence as defined by prostate-specific antigen levels compared with the control group [8]. More and more evidence indicates that opiate analgesia is capable of accelerating the dissemination of malignant cells through restraining the immune function. Furthermore, it has been shown that the opioids have broad immunomodulatory activity, both on cellular and on humoral immune responses and are able to modulate inflammatory cytokine production [9]. That immunomodulation is mediated by μ, δ or κ opioid receptors either in nervous system [10] or immunocompetent cells such as neutrophils, NK cells, macrophages and equally in T cells [11]. Particularly, different concentrations of opioid peptides might to some extent exert influences on the body's immune system. It is suggested that morphine and tramadol could direct human T-helper cell to Th2 and that the effect of morphine was apparently more powerful than tramadol. Both drugs presented a dose-dependent Th2 differentiation response [12]. What's more, Riss GL et al have found that the frequencies of immune-suppressive $CD4^+CD25^{high}$ Tregs are increased within the $CD4^+$ T cell compartment of peripheral blood in heroin user [13]. So far, however, it has been rarely reported about the effect of different concentrations of opioid peptides on Tregs in vitro. As we know, fentanyl and its N-4 thienyl derivative, sufentanil, are two very potent opioids commonly used in anesthesia. Although both are classified as pure μ agonists, there are significant differences in their potencies [14], receptor affinities [15], lipophilicity [16] and pharmacokinetics [17]. Whether sufentanil (SF) and fentanyl (F) have different properties in respect to Tregs for breast cancer patients after eradicative operation is unknown. Therefore, we set out to compare the influence of sufentanil and fentanyl on the quantity of Tregs in vitro and in the breast cancer patients after eradicative operation.

Materials and methods

Patients with breast cancer and matched volunteers

From December 2011 to June 2012, peripheral blood was obtained from 12 breast cancer (BC) patients of Xiangya Hospital in accordance with local ethical committee approval. Other 38 BC patients were also recruited from Xiangya Hospital and they underwent eradicative operation between August 2012 and August 2013. All of them gave informed consent. All these 50 patients were on their first visit and diagnosed with breast cancer. Exclusion criteria included smoking, any history of impairment of the immune system, allergy, immunosuppressive therapy, signs of preexisting infection (white blood cell count >12 000/μl, body temperature >38°C, C-reactive protein >5 mg/dl), liver insufficiency (> Child B) or end-stage renal disease.

Cell purification, cultures and experimental grouping

From December 2011 to June 2012, peripheral blood obtained from 12 breast cancer patients were separated by density-gradient centrifugation over Lymphoprep (GE). PBMCs from healthy subjects or breast cancer patients were activated with plated-bound anti-CD3 (OKT3 clone, 0.5 μg/ml) and soluble anti-CD28 (CD28.2 clone, 1 μg/ml) mAbs (Biolegend). Cells at a concentration of 1×10^6 cells/well were cultured in the 24 well plates for 7 days in the presence of RPMI-1640 (Hyclone), 10% fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Hyclone) and 100 U/ml IL-2 (Peprotech) in the presence of sufentanil (Sufentanil Citrate Injection, Yichang Humanwell Pharmaceutical CO.) or fentanyl (Fentanyl Citrate Injection, Yichang Humanwell Pharmaceutical CO.) at 37°C in a humidified 5% CO_2 atmosphere. The

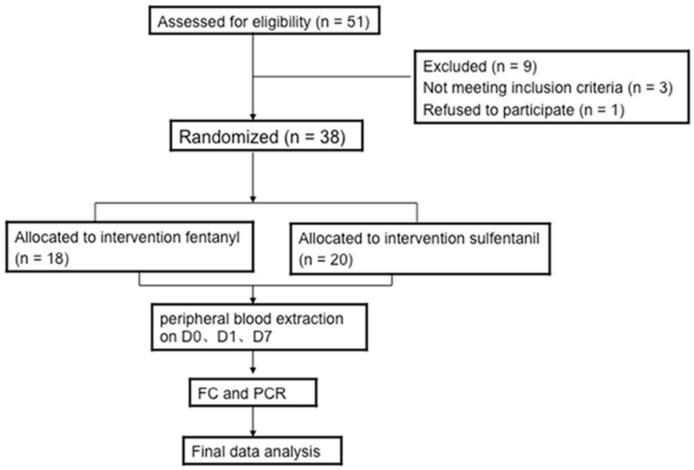

Figure 1. Flow diagram showing progress through the various stages of the trial for breast cancer surgery patients selected and then randomized to receive fentanyl or sufentanil anesthesia.

Table 1. Characteristics of the study participants

Category	Recruiting time	
	Dec. 2011 to Jun. 2012	Aug. 2012 to Aug. 2013
Number of patients	12	38
Age (yr)	41.88±5.682	46.53±2.211
Gender	Female	Female
Body weight (kg)	55.5±3.61	50.1±2.35
TNM stage	I–III	I–III
ASA	I–III	I–III

clinical plasma concentration of both sufentanil and fentanyl was used. The PBMCs from each subject can be grouped as follows: 1) Control group: Phosphate buffered saline (PBS) and IL-2, anti-CD3/28 group; 2) sufentanil 0.3 ng/ml and IL-2, anti-CD3/28 group (SF group); 3) fentanyl 3 ng/ml and IL-2, anti-CD3/28 group (F group).

Patients underwent eradicative operation under general anesthesia

The 38 patients who underwent eradicative operation were premedicated with intramuscular diazepam 0.2 mg/kg and atropine 0.01 mg/kg 30 minutes before arrival in the operating room. They were randomly assigned into two anesthetic technique groups: 18 patients of them were anesthetized using fentanyl anesthesia (F group), but the rest using sufentanil anesthesia (SF group). Anaesthesia was induced with midazolam (0.1-0.15 mg/kg), propofol (1.5-2 mg/kg), fentanyl (5-8 μg/kg) or sufentanil (0.5-0.8 μg/kg), and cisatracurium (0.15 mg/kg) for muscle relaxation. Anaesthesia was maintained with propofol (4-14 mg/kg per h) and cisatracurium (0.06-0.1 mg/kg per h) in all the patients. For the two groups, fentanyl (2-4 μg/kg per h) or sufentanil intravenous infusions (0.2-0.4 μg/kg per min) were started after induction of anesthesia. Both fentanyl and sufentanil infusion were stopped at the end of the surgery. All the patients remained intubated and mechanically ventilated for transfer.

During the surgery, normal monitoring was used. Intra-operatively patients were ventilated with a tidal volume of 6-8 ml/kg, with the respiratory rate adjusted to achieve an end-tidal carbon dioxide level of 36-44 mmHg. Patients were weaned from mechanical ventilation as soon as hemodynamic stability and normothermia were established and blood loss was satisfactory (<50 ml/h), in accordance with our published standard operating procedures. Patients were extubated once awake. Heart rate, mean arterial pressure and oxygen saturation were monitored continuously. The same team of anesthetists and surgeons performed all the procedures.

Venous blood samples (15 mL) were collected at 10 min before anesthesia, and at 24 and 168 h after the operation, respectively. After the surgery, the use of any hormones and immunosuppressive drugs, antibiotics and so on were avoided. PBMCs were isolated from these samples and then were suspended in fetal calf serum containing 10% dimethyl sulphoxide and gradient cooling and cryopreservation at -70°C until used.

Flow cytometry

Cells obtained from December 2011 to June 2012 were stained with the indicated surface Abs: PE/Cy5 anti-human CD4, PE anti-human CD25 and Alexa Fluor488 anti-human FOXP3, all from Biolegend and were analyzed with a FAC Scan flow cytometer equipped with CellQuest software (Beckman FC500). Intra-

Table 2. Demographic, morphometric, operative characteristics, and total propofol dose of breast cancer patients receiving sulfentanil anesthesia and fentanyl anesthesia

Category	Participants		
	Fentanyl group	Sulfentanil group	*P*
Number of patients	18	20	
Age (yr)	46.30±2.314	47.63±2.283	0.7127
Gender	Female	Female	
Body weight (kg)	50.30±1.970	48.33±2.281	0.3291
ASA	I-III	I-III	
TNM stage	I-III	I-III	
Operation time (min)	93.00±1.431	88.56±2.011	0.0749
Time to extubation (min)	43.00±1.694	47.22±2.120	0.1251
Intraoperative infusion of solutions (mL)	847.5±35.44	766.7±35.54	0.1126
Total propofol dose (mg)	564.6±15.04	583.2±35.44	0.098
Blood loss (ml)	72.00±7.348	68.00±9.695	0.6351

Values given are the median and 25-75% quartiles (except for where n is shown). There are no statistically significant between-group differences (Unpaired t-test $P > 0.05$).

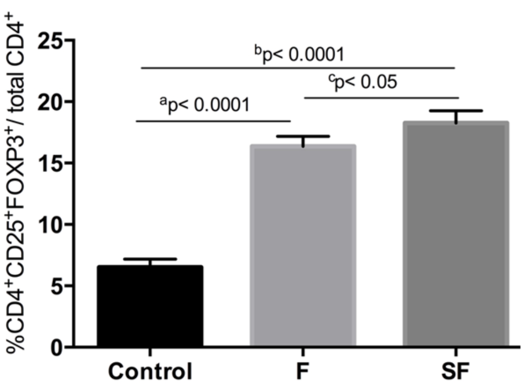

Figure 2. Statistics of Tregs frequencies in PBMCs cultured in vitro in the SF and F group by FC. The percentage of $CD4^+CD25^+Foxp3^+$Tregs by flow cytometry analysis in PBMCs from the BC patients increased notably after culturing either with fentanyl or sufentanil ($^aP < 0.0001$, $^bP < 0.0001$). And with the same analgesic effect, there is a higher ratio of $CD4^+CD25^+Foxp3^+$Tregs to $CD4^+$ T cells in SF group compared with F group ($^cP < 0.05$).

cytoplasmic staining for human FOXP3 was performed using the FOXP3 Fix/Perm Buffer set, according to the manufacturer's instructions. Likewise, cells obtained from August 2012 and August 2013 were stained with the indicated Abs: BD Pharmingen™ PE Mouse anti-human FoxP3 clone: 259D/C7, PE Mouse IgG1 Isotype, CD25PE-Cy7, CD4FITC and analyzed with a FAC Scan flow cytometer (BD FACSCanto™ II).

Real-time PCR

Total RNA was extracted from PBMCs of patients using Trizol reagent (Gibco BRL) according to the manufacture's instructions, then reverse transcribed to obtain cDNA (Transcription Factor Buffer Set BD Pharmingen™). Reverse transcription was carried out with the Superscript preamplification system (Gibco BRL). Primer sequences for FoxP3 were: FoxP3 forward: 5'-CTGACCAAGGCTTCATCTG-TG-3' and FoxP3 reverse: 5'-GA-ACTCTGGGAATGTGCTGTT-3'; after 10 min 'hot start' at 95°C, 40 cycles of amplification were followed by 2 min extension at 50°C. Each cycle included denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and final extension at 72°C for 10 minutes. The PCR products were visualized using an Eagle Eye analyzer (Stratagene, La Jolla, CA, USA). As control, mRNA content for β-actin was analyzed using the following primers: β-actin forward 5'-ACCGAGCGCGGCTACAG-3' and β-actin reverse: 5'-CTTAATGTCACGCACGATTTCC-3'. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (Ct). The amount of gene expression was then calculated as the difference (ΔCt) between the Ct value of Foxp3 and the Ct value of β-actin. Fold changes in Foxp3 mRNA were determined as $2^{-\Delta Ct}$.

Statistical analysis

All data were presented as mean ± SE (X ± s). Student's *t* tests were used to compare quantitative variables, and Fisher's exact tests were used for categorical variables. Statistical analysis was implemented with SPSS 15.0 (SPSS, Inc., Chicago, IL), during which a significant level of 0.05 was adopted.

Results

Various stages of the trial in the flow diagram

A total of 51 patients were enrolled in the study between August 2012 and August 2013. Nine

Table 3. Tregs frenquencies in BC patients detected by RT-PCR

	D0	D1	D7	*P* (D0/D1)	*P* (D0/D7)	*P* (D1/D7)
SF group	1.033±0.07869	0.7937±0.08158	1.327±0.1050	0.0418	0.0307	0.0003
F group	1.069±0.09909	0.7483±0.07424	1.526±0.1810	0.014	0.0338	0.0004

Table 4. Comparison of regulatory T cell frequencies between SF and F groups by RT-PCR

	SF	F	*P*
D0	1.033±0.07869	1.069±0.09909	0.7719
D1	0.7937±0.08158	0.7483±0.07424	0.6856
D7	1.327±0.1050	1.526±0.1810	0.337

of these patients were subsequently excluded due to the abnormal of hepatic and renal function; three patients were not meeting inclusion criteria; and one refused to participate. Consequently, 38 patients were included in the final analysis. In the 38 patients enrolled in the study, 20 patients were randomized to sufentanil anesthesia, and the other 18 patients were randomized to fentanyl anesthesia. All the patients completed the study according to the protocol. The same team of anesthetists and surgeons performed all the procedures. The operations and anesthetic treatments of these 38 patients were all successful (**Figure 1**).

Demographic data of breast cancer patients in sufentanil and fentanyl groups

12 BC patients were recruited from December 2011 to June 2012. Compared with 38 patients between August 2012 and August 2013, no significant differences were found in average age, gender, ASA, body weight, and TNM stages (**Table 1**).

Other 38 BC patients were enrolled between August 2012 and August 2013. Both sufentanil Anesthesia and fentanyl Anesthesia treatment groups were reasonably well balanced regarding age, body weight, TNM stage, operation time, ASA, time to extubation, intraoperative infusion of solutions and propofol dosage (**Table 2**).

Effects of fentanyl and sufentanil on the percentage of $CD4^+CD25^+Foxp3^+$Tregs in PBMCs and in vitro

Our hypothesis was that co-culturation of fentanyl (or sufentanil) and PBMCs inhibits anti-tumor immunity. To document this hypothesis, the percentage of $CD4^+CD25^+Foxp3^+$Tregs by flow cytometry analysis in PBMCs from the BC patients was tested. The number of $CD4^+$ $CD25^+Foxp3^+$Tregs increased notably after culturing either with fentanyl or sufentanil. And with the same analgesic effect, there is a higher ratio of $CD4^+CD25^+Foxp3^+$Tregs to $CD4^+$ T cells in SF group compared with F group (**Figure 2**).

Measurement of $CD4^+CD25^+Foxp3^+$Tregs on D0, D1 and D7 by PCR and FC analysis after surgery in both sufentanil and fentanyl groups

Statistics of the Tregs contents measured by Foxp3 mRNA expression and the Tregs frequencies measured by FC are shown in **Tables 3**, **5** and **Figure 3**. It can be seen that in both groups, both of the mRNA expression levels of Foxp3 and the Tregs percentages on D1 decreases compared to that on D0, and then recovers on D7. Moreover the comparison of Foxp3 mRNA expression by quantitative real-time PCR between SF group and F group in **Table 4** does not show significant differences in D0, D1 and D7. **Figure 4** shows the FC pictures of $CD4^+CD25^+Foxp3^+$Tregs in the SF and F group. Consistent with the RT-PCR results, Treg frequencies detected by FC declines on D1 and increases on D7 in both F and SF groups. But the difference of the Tregs percentage in $CD4^+$ T cells is not statistically significant between SF group and F group on D0, D1 and D7, respectively (**Table 6**).

Discussion

We found that when the culturing was conducted in vitro, activation of human peripheral blood mononuclear cells in the presence of fentanyl or sufentanil increased the quantity of the $CD4^+CD25^+Foxp3^+$Tregs, and the effect of sufentanil was much stronger when the analgesic effect was the same. However, during the eradicative operation, there were no remarkable discrepancies between the effect of sufentanil anesthesia and the effect of fentanyl anesthe-

Table 5. Tregs frequencies in BC patients detected by FC

	D0	D1	D7	*P* (D0/D1)	*P* (D0/D7)	*P* (D1/D7)
SF	5.685±0.2794	4.755±0.2998	7.265±0.3731	0.029	0.0016	<0.0001
F	5.511±0.2456	5.261±0.2646	7.206±0.4890	0.4933	0.0039	0.0013

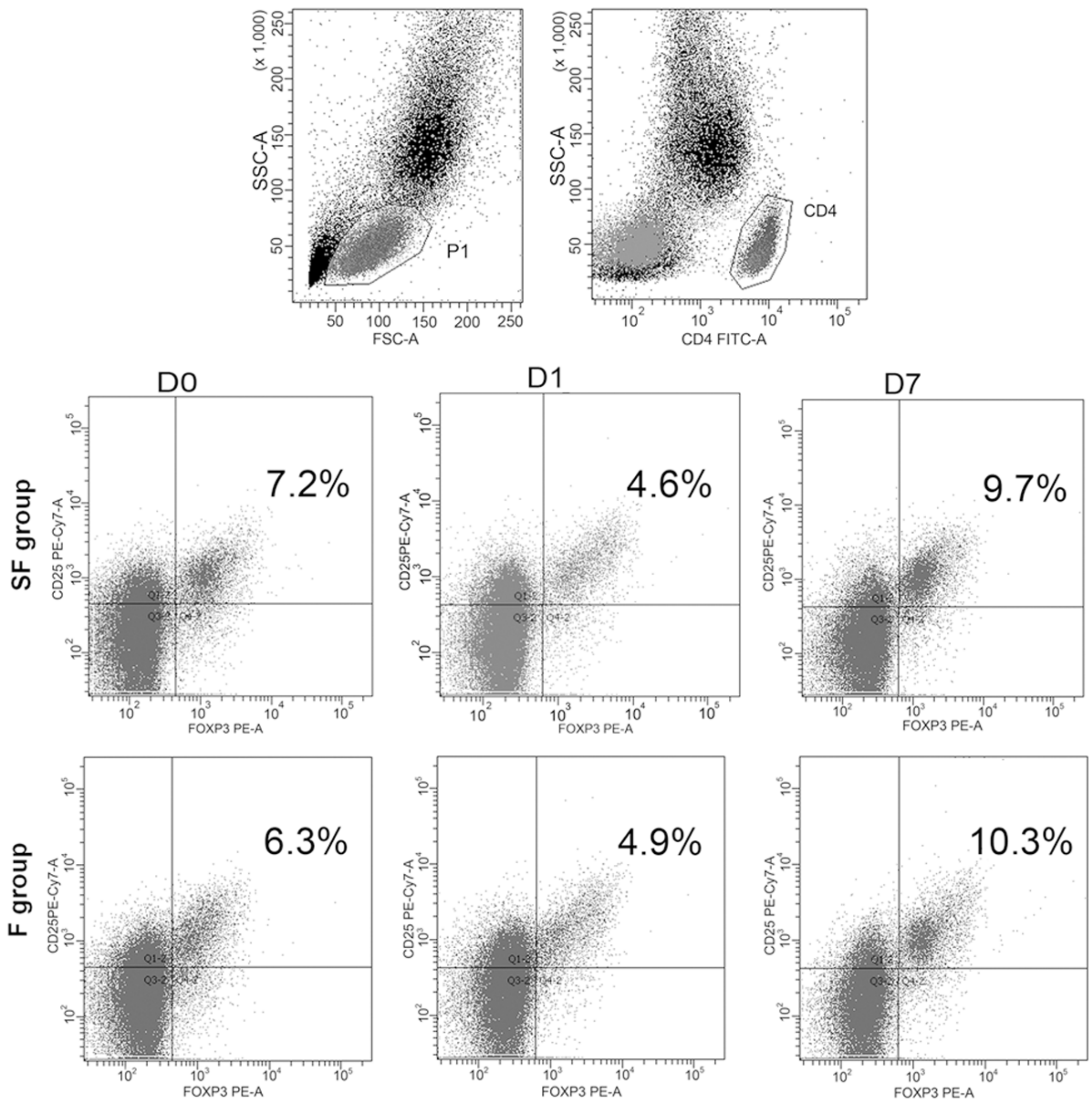

Figure 3. The Tregs contents measured by Foxp3 mRNA expression decreased on D1 (D0 vs. D7, $P < 0.05$), and then increased on D7 (D1 vs. D7, $P < 0.01$) in both SF group and F group. Circulating Tregs frequencies detected by FC decreased on D1 (D0 vs. D7, $P < 0.05$), and then recovered on D7 (D1 vs. D7, $P < 0.01$) in both SF group and F group. Data are mean ± SEM.

sia on Tregs frequencies and Foxp3 mRNA expressions perioperatively.

Recently, the effect of anesthetics, especially opioids, on tumor prognosis has received widespread attention. When the immune system plays a key role in the control of tumor formation and metastasis, a vast majority of investigations indicate that morphine and other exogenous opioids are immunosuppressive [18, 19]. It is found that the administration of exogenous opioids inhibits components of the cel-

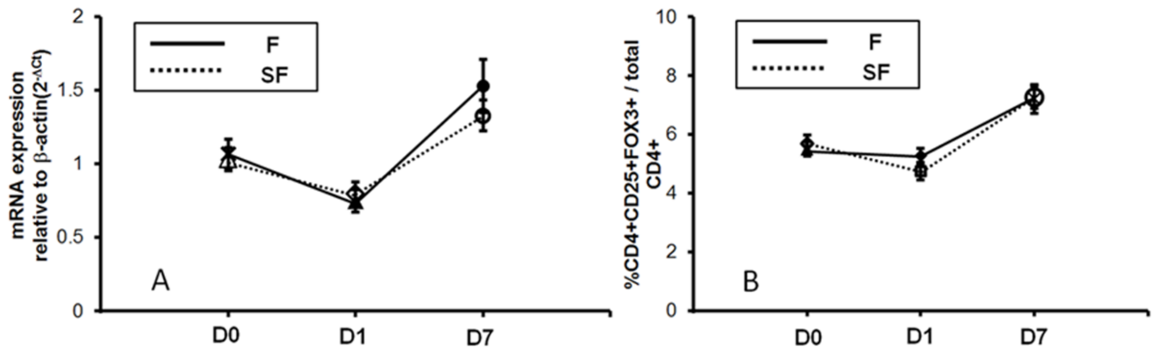

Figure 4. Representative FC pictures of Tregs in the SF and F group on D0, D1 and D7.

Table 6. Comparison of regulatory T cell frequencies between SF and F groups by FAC

	SF	F	*P*
D0	5.685±0.2794	5.511±0.2456	0.6462
D1	4.755±0.2998	5.261±0.2646	0.218
D7	7.265±0.3731	7.206±0.4890	0.9227

Values given are the median and 25-75% quartiles. There are no statistically significant between-group differences (Unpaired t-test $P > 0.05$).

lular and humoral immune function such as antibody production, NK cell activity, cytokine expression, blood lymphocyte proliferative responses to mitogen, and so on [20, 21]. Similarly, we found that activation of human peripheral blood mononuclear cells in the presence of fentanyl or sufentanil increased the Tregs number when culturing in vitro. It should be noted that Tregs are an important contributor to the suppression of anti-tumor immunity [22]. The higher proportion of Tregs was a significant unfavorable prognostic factor for breast cancer, non-small-cell lung cancer, and so on [23, 24]. What's more, the report we hypothesized before [25] may help to explain the effect of sufentanil and fentanyl on Treg frequencies. We proposed that opioids affect Tregs, possibly through vascular endothelial growth factor receptor 2 and opioid receptors on immune cells. And other immune cells or cytokines, such as tumor growth factor-β and interleukin-2 may also involve in. Therefore, regarding the Treg population, both fentanyl and sufentanil showed an immunosuppressive response in vitro.

It is well known that analgesic potency ratios for sufentanil to fentanyl are in the range of 5:1 to 10:1 [26]. For the purposes of our study, we used a potency ratio of sufentanil 10:1 to fentanyl. For the purposes of our study, we used a potency ratio of sufentanil 10:1 to fentanyl. Just as Schneemilch CE, et al [27] did in vitro, the clinically administrated concentrations of fentanyl or sulfentanil were added into the PBMCs culture liquid in vitro. Interestingly in our study, with the same analgesic effect, the effect of sufentanil was apparently more significant than fentanyl in increasing Treg population. Therefore, it seems that sufentanil is inferior to fentanyl in breast cancer patients, while the effect of sufentanil is relatively more significant in increasing Tregs. To testify this, clinical trials were conducted. However, different outcomes were presented.

During the perioperative and postoperative periods, a complex biologic response takes place in response to surgical stress. And it is well known that the activation of endocrine and sympathetic nervous systems during surgery leads to a transient period of immunosuppression [28]. However, opiates can inhibit the activity of the HPA axis, resulting in lower levels of adrenocorticotropic hormone and cortisol. In addition, the suppressed activity of the HPA axis is usually followed by a rebound of its activity, resulting in significantly increased levels of cortisol, still evident 6 days after surgery [29]. Indeed, we observed that both the Tregs frequencies and Foxp3 mRNA expression on D1 decreased compared to that on D0 and then recovered on D7. During the breast cancer surgery, the SF and F groups were comparable in all variables except for the pharmacological intervention. Although we did not monitor the anesthesia depth, the propofol consumption was similar in both groups. And intraoperative infusion of solutions, blood loss and so on were also similar in two groups. Therefore, the comparison of Treg population between the two groups were observed in the present study was an accurate reflection of the effects of fentanyl and sufentanil. However, by comparing SF group and F group, there ware no significant differences in Tregs frequencies and Foxp3 mRNA expression on D0, D1 and D7, although sufentanil was found more effective in increasing Tregs number in vitro. As we know, serum expression levels of particular cytokines and immune cell population vary much in surgery and trauma circumstances. In patients undergoing major surgery, the depressed postoperative immune response is mainly attributed to surgery. Previous reports had demonstrated that anaesthetics can influence various aspects of lymphocyte function in vitro and in vivo, but the results of these were often contradictory [30, 31]. Thus, the effect of pharmacological intervention on Treg population is probably very weak in comparison with the surgical stress during the perioperative period. Although a different story was told in clinical trial, it at least provided a rough indicator of relationship between opiates and Tregs. And an increased percentage of Tregs was first found with the presence of fentanyl or sufentanil when cultured in vitro. More specific mechanism is to be studied.

Nevertheless, one limitation of our study is that we cultured PBMCs instead of $CD4^+$ T cells in vitro. Therefore, the ratio of Tregs to $CD4^+$ T cells in PBMCs may not accurately reflect the Treg number. But the PBMCs cultured better mimic in vivo environments. In addition, although no significant differences were found in Tregs frequencies between SF group and F group on D0, D1 and D7, no other clinical outcomes were compared between the two groups except for the hospital length of stay. And thus, further work, focusing on clinical outcomes, would be required to investigate this possibility. Another limitation is that only the Tregs frequencies were detected. And further researches about the change in the Tregs function are also needed.

In summary, it indicates that clinical concentration of opiate anesthesia could exacerbate immunosuppression via expansion of $CD4^+ CD25^+ Foxp3^+$ Tregs population in vitro, suggesting a need for a careful use of these anesthesia drugs, particularly in cancer patients. Furthermore, even though sufentanil is more powerful in increasing the quantity of Tregs in vitro, there are no significant differences between the effects of sufentanil anesthesia and fentanyl anesthesia on Tregs frequencies and Foxp3 mRNA expressions perioperatively. Further studies are needed to determine differences in the Tregs function and long-term outcome of these patients.

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Disclosure of conflict of interest

None.

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