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Intraoperative Flurbiprofen Treatment Alters Immune Checkpoint Expression in Patients Undergoing Elective Thoracoscopic Resection of Lung Cancer

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Highlights of the Study

- $CD4^{(+)}$ and $CD8^{(+)}$ T cells in the peripheral blood of lung cancer patients express higher levels of PD-1, which indicates a poorer clinical outcome.
- PD-1 expression levels were markedly induced in the peripheral blood of lung cancer patients following surgery.
- Perioperative administration of flurbiprofen attenuated the postoperative increase in PD-1 levels on circulating $CD8^{(+)}$ T cells for up to 72 h.

Keywords

Immune cells · Lung cancer · Flurbiprofen · Programmed death 1

Abstract

Objectives: This study aimed to determine the effect of intraoperative administration of flurbiprofen on postoperative levels of programmed death 1 (PD-1) in patients undergoing thoracoscopic surgery. ***Materials and Methods:*** In this prospective double-blind trial, patients were randomized to receive intralipid (control group, $n = 34$, 0.1 mL/kg, i.v.) or flurbiprofen axetil (flurbiprofen group, $n = 34$, 50 mg, i.v.) before induction of anesthesia. PD-1 levels on T cell subsets, inflammation, and immune markers in peripheral blood were examined before the induction of anesthesia (T_0) and 24 h (T_1), 72 h (T_2), and 1 week (T_3) after surgery. A linear mixed model was used to determine whether the changes from baseline values (T_0) between groups were significantly different. ***Results:*** The increases in the percentage of $PD\text{-}1^{(+)}CD8^{(+)}$ T cells observed at T_1 and T_2 in the control group were higher than those in the flurbiprofen group (T_1: 12.91 ± 1.65 vs. 7.86 ± 5.71%, $p = 0.031$; T_2: 11.54 ± 1.54 vs. 8.75 ± 1.73%, $p = 0.004$), whereas no differences were observed in the changes in the percentage of $PD\text{-}1^{(+)}CD4^{(+)}$ T cells at T_1 and T_2 between the groups. Moreover, extensive changes in the percentage of lymphocyte subsets and inflammatory marker concentrations were observed at T_1 and T_2 after surgery and flurbiprofen attenuated most of these changes. ***Conclusions:*** Perioperative administration of flurbiprofen attenuated the postoperative increase in PD-1 levels on $CD8^{(+)}$ T cells up to 72 h after surgery, but not after this duration. The clinical relevance of changes in PD-1 levels to long-term surgical outcome remains unknown.

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Introduction

Lung cancer is a major cause of cancer-related deaths globally and approximately 80% of lung cancers occur as non-small cell lung cancer (NSCLC) [1–3]. Some well-established immune-checkpoint molecules expressed on activated T cells, such as programmed death 1 (PD-1 or CD279) and cytotoxic T lymphocyte-associated antigen 4 (CLTA-4 or CD152), functionally suppress T-cell-mediated immunity against tumors and are considered a hallmark of exhausted T cells following persistent stimulation with tumor antigens [4–8]. Increasing evidence indicates that higher levels of immune-checkpoint molecules predict poorer outcomes in cancer patients [9, 10]. Furthermore, immune-checkpoint blockade (e.g., anti-PD-1 therapy) elicits persistent and significant therapeutic responses in multiple tumor types, including lung cancer [1, 11–16]. However, it remains unclear whether perioperative use of analgesic drugs can directly influence these immune-checkpoint molecules in cancer patients undergoing surgery.

Interestingly, PD-1 and its ligands are markedly inducible by cyclooxygenase (COX) enzymes and downstream prostaglandins (PGs), which are prominent tumor-sustaining inflammatory mediators [17, 18]. Additionally, nonsteroidal anti-inflammatory drugs (NSAIDs), which have anti-inflammatory and analgesic activities, including inhibiting COX enzymes, pharmacologically cooperate with anti-PD-1 treatment to increase its efficacy in a preclinical cancer model [19]. In clinical settings, the perioperative administration of flurbiprofen, which is regularly prescribed as a perioperative analgesic, efficiently elicits a short-term increase in the number of innate and adaptive immune cells in postoperative peripheral blood [20]. Since less is known about how NSAIDs synergize with anti-PD-1 therapy in clinical settings, we aimed to determine whether flurbiprofen had a direct effect on postoperative PD-1 levels in circulating T cell subsets, in patients undergoing elective thoracoscopic resection of NSCLC.

Materials and Methods

Study Sample

This study was approved by the Biomedical Research Ethics Committee of Anhui Medical University and was registered with the Chinese Clinical Trial Registry (No. ChiCTR-IPR-15006482). All participating patients provided written informed consent. This study was a prospective, double-blind, randomized, controlled clinical trial. Patients were screened at the outpatient department or inpatient wards and underwent randomization between October 15, 2016, and May 10, 2017, at Anhui Provincial Hospital, Hefei, China. Patients were eligible for participation in this study if they met the following criteria: (1) adults undergoing elective thoracoscopic resection of lung cancer, (2) American Society of Anesthesiologists (ASA) status of I–II, (3) aged 40–65 years, and (4) weighing 45–80 kg for both genders. Patients were excluded if they met any of the following criteria: (1) allergy or contraindication to NSAIDs; (2) history of peptic ulceration; (3) blood coagulation disorder; (4) severe cardiac, hepatic, or renal dysfunction; (5) perioperative blood transfusion; (6) bronchial asthma; (7) current or recent use of radiotherapy, chemotherapy, immune depressant, or glucocorticoid; (8) autoimmune disease or acute inflammation; (9) severe hypertension or diabetes mellitus; (10) pregnancy; (11) use of enoxacin, lomefloxacin, or norfloxacin; and (12) duration of operation <120 min.

Anesthesia and Analgesia

General anesthesia was induced with 0.05 mg/kg midazolam, 2 mg/kg propofol, and 0.4 μg/kg sufentanil, and double-lumen endobronchial tube insertion was facilitated with 1 mg/kg rocuronium. The site of the tube was confirmed by fiberoptic bronchoscopy after intubation and by changing the position of the patient prior to surgery. All patients received target-controlled infusion to maintain anesthesia (i.e., propofol and remifentanil with an effective concentration of 3.5–4.5 μg/mL and 2–4 ng/mL, respectively). Patients were mechanically ventilated with suitable ventilation parameters to maintain end-tidal carbon dioxide in the range of 35–45 mm Hg. In addition, the appropriate depth of anesthesia was monitored using a Narcotrend monitor (MonitorTechnik, Bad Bramstedt, Germany). Postoperatively, all patients received the same regimen of patient-controlled intravenous analgesia with sufentanil (100 μg, diluted to a total volume of 100 mL with 0.9% sodium chloride). Patient-controlled intravenous analgesia was performed with a loading dose of 2 mL, a background infusion of 2 mL/h, a bolus of 2 mL, and a lockout time of 15 min.

Intervention and Randomization

Using a computer-generated random number sequence, patients were allocated in a 1:1 ratio to receive treatment with either flurbiprofen (flurbiprofen axetil, 50 mg, i.v.) or placebo (intralipid, 0.1 mL/kg, i.v.) before the induction of anesthesia. Data collection was performed by an independent researcher who was not involved in the trial. In addition, another researcher was in charge of the preparing the study drugs. The drugs were placed into unmarked syringes, and treatment assignments were concealed in sealed, opaque envelopes. All of which was blinded to patients, anesthetists and other investigators involved in the study. The statistician was unaware of the assignments until all data analyses were completed. In addition, tramadol was administered postoperatively as a rescue analgesic for unbearable pain, when the Visual Analogue Scale score was ≥5.

Outcomes

Venous blood (2 mL) was collected from the noninfused peripheral vein before the induction of anesthesia (T_0) and 24 h (T_1), 72 h (T_2), and 1 week (T_3) after surgery. Blood samples were preserved in an EDTA anticoagulation tube at 4 °C for subsequent testing within 24 h. The primary outcomes were counts of circulating PD-$1^{(+)}$ CD$8^{(+)}$ and PD-$1^{(+)}$CD$4^{(+)}$ T cells at each perioperative time point. The secondary outcomes were the percentages of lymphocyte subsets in peripheral blood mononuclear cells (PBMCs)

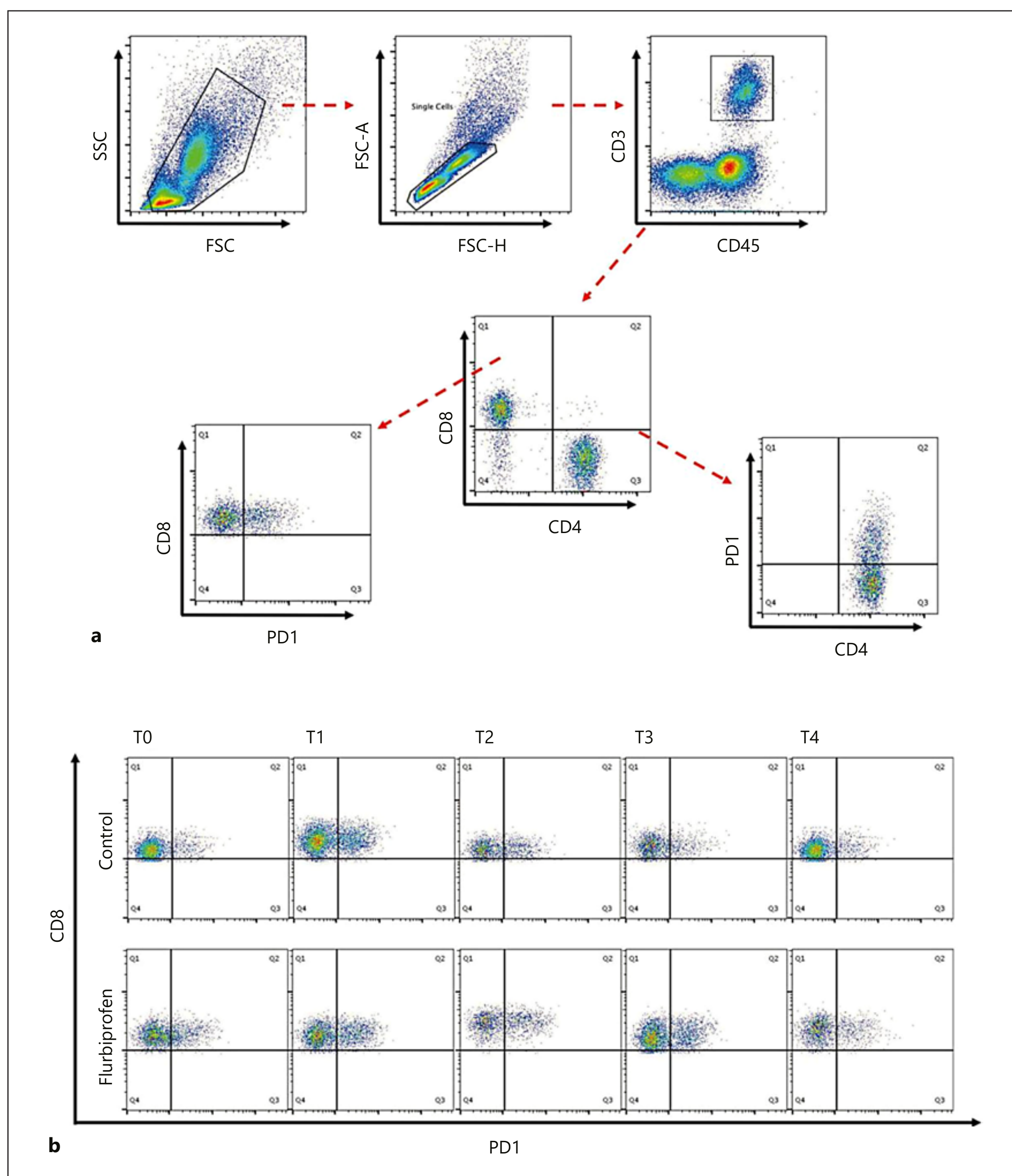

Fig. 1. Flow cytometric analysis results. **a** Flow cytometric gating strategy and analysis of PD-1 expression on $CD45^{(+)}CD3^{(+)}CD4^{(+)}$ and $CD45^{(+)}CD3^{(+)}CD8^{(+)}$ T-cell populations. **b** representative plots of PD-1 expression on the $CD8^{(+)}$ T-cell population at different time points. PD-1, programmed death 1.

and the concentrations of inflammatory markers, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-6 (IL-6), and C-reactive protein (CRP), in peripheral blood. Moreover, data from full blood count tests, including platelet count, total white blood cell count, hemoglobin concentration, neutrophil count, lymphocyte count, monocyte count, eosinophil count, and basophil count, were tracked simultaneously.

Immune-Checkpoint and Lymphocyte Subset Analyses

Circulating $PD\text{-}1^{(+)}CD8^{(+)}$ and $PD\text{-}1^{(+)}CD4^{(+)}$ T cell counts were determined as the percentage of total T lymphocytes. The percentages of total $CD3^{(+)}$ T cells, $CD4^{(+)}$ helper T cells, $CD8^{(+)}$ cytotoxic T cells, and $CD3^{(-)}CD16^{(+)}CD56^{(+)}$ NK-cells in PBMCs were measured by flow cytometry (CytoFLEX; Beckman, Brea, CA, USA). PBMCs were separated from peripheral blood by Ficoll-Hypaque density-

gradient centrifugation. PBMCs were resuspended in 0.2 mL of phosphate buffered saline (4×10^6/100 μL) and incubated for 30 min on ice with the appropriate antibody dilution. The antibodies used in this study were anti-CD45-PercpCy5.5, anti-CD3-FITC, anti-CD8-PE, anti-CD4-APC, and anti-PD-1-PE-Cy7 (Biolegend, San Diego, CA, USA). The flow cytometric gating strategy and analysis of PD-1 expression were based on the $CD45^{(+)}CD3^{(+)}CD4^{(+)}$ and $CD45^{(+)}CD3^{(+)}CD8^{(+)}$ T-cell populations (Fig. 1a).

Measurement of Inflammatory Cytokines

The concentrations of TNF-α, IFN-γ, and IL-6 were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) as per the manufacturer's protocol.

Full Blood Count, Serum Glucose, and CRP Measurement

Full blood count analysis was performed on EDTA peripheral blood samples using a hematology analyzer instrument (BC-6900; Mindray, Shenzhen, China). In addition, the concentrations of serum glucose and CRP were determined in peripheral blood samples using a clinical chemistry system (ADVIA 2400, Siemens, Munich, Germany) and anti-CHROMA laser fluorescence reader, respectively.

Calculation of Sample Size

The number of patients recruited in each group was based on the primary outcome, by testing the percentage of $PD\text{-}1^{(+)}CD8^{(+)}$ T cells in a previous pilot study. According to these pilot data, the difference in mean $PD\text{-}1^{(+)}CD8^{(+)}$ T cell percentages between the 2 groups was 2.08%, with a pooled SD of 2.57%. To detect a difference of this magnitude between study groups, 32 patients were required in each group to provide 90% power at an alpha of 0.05. However, considering a loss-to-follow-up rate of approximately 10% in our study, we aimed to enroll a total of 70 patients.

Statistical Analyses

The Shapiro-Wilk test was applied to assess the normality of continuous data. Normally distributed continuous data are presented as means (SD) and were analyzed using an independent Student *t* test. Skewed data are presented as medians (interquartile range) and were compared using a Mann-Whitney U test or Wilcoxon rank-sum test. Categorical variables are reported as frequencies and were compared using a chi-square or Fisher's exact test. Changes in the percentages of $PD\text{-}1^{(+)}CD8^{(+)}$ or $PD\text{-}1^{(+)}CD4^{(+)}$ T cells and concentrations of inflammatory markers were calculated between baseline values at T_0 and those at T_1, T_2, and T_3. Changes in both groups were analyzed using a mixed linear regression model, adjusted for age and ASA status (i.e., a random effect was introduced in an effort to account for repeated measures), in order to confirm the findings of the univariable analyses. In our model, study group and time were applied as predictors, interactions between the study group and time as fixed effects, and the patient as a random effect. An interaction term was taken into account to test whether the change over time was different between groups. In the univariable analyses, a Bonferroni correction was applied to post hoc analyses comparing changes over time. In addition, sensitivity analysis using a linear mixed model, with scale identity correlation matrices, was applied to confirm major differences between groups. All statistical analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA), and *p* values <0.05 were considered to indicate statistical significance.

Results

Study Population

The CONSORT diagram is shown in Figure 2. A total of 70 patients were screened for our study, with 68 patients ultimately participating. Of the 2 patients who were not included, one refused to participate and one did not satisfy the inclusion criteria. Four patients (2 in the flurbiprofen group, 2 in the control group) were withdrawn from the statistical analysis, despite being randomized into study groups. In the flurbiprofen group, one of these patients refused phlebotomy for laboratory testing after the operation, and one patient was withdrawn because of blood loss >1.5 L. Both patients in the control group withdrew due to tumor metastasis into the pleura, not having undergone a lung parenchyma resection procedure. Baseline and intraoperative characteristics of patients in both groups are shown in online supplementary Table 1 (see www.karger.com/doi/10.1159/000503166. No significant differences in age, body mass index, gender, or ASA status were observed between groups. There were also no significant between-group differences in surgery type, duration of surgery, the use of antibiotics, or intraoperative propofol consumption. Remifentanyl consumption was higher in the control group than in the flurbiprofen group. Outcome values are shown in Table 1. There were no significant differences in the percentages of $PD\text{-}1^{(+)}CD8^{(+)}$ T cells, $PD\text{-}1^{(+)}CD4^{(+)}$ T cells, or T lymphocyte subsets or inflammatory marker concentrations between groups at baseline and 1 week after surgery (data not shown).

Outcomes

As shown in Table 2, the increases in the percentage of $PD\text{-}1^{(+)}CD8^{(+)}$ T cells observed 24 h (T_1) and 72 h postoperatively (T_2) in the control group were higher than those in the flurbiprofen group (T_1, 12.91 ± 1.65 vs. 7.86 ± 5.71%, $p = 0.031$; T_2, 11.54 ± 1.54 vs. 8.75 ± 1.73%, $p = 0.004$). Original images of $PD\text{-}1^{(+)}CD8^{(+)}$ T cells at different time points are shown in Figure 1b. No differences were observed in the change in the percentage of $PD\text{-}1^{(+)}CD4^{(+)}$ T cells at T_1 and T_2 between the groups. However, changes in the percentages of $PD\text{-}1^{(+)}CD8^{(+)}$ T cells and $PD\text{-}1^{(+)}CD4^{(+)}$ T cells from baseline (T_0) were similar between groups 1 week after surgery (T_3; data not shown). Moreover, the decreases observed for all lymphocyte subsets at T_1 and T_2 were markedly greater in the control group than in the flurbiprofen group, with the exception of $CD8^{(+)}$ T cells, which showed a significant change only at T_1. In addition, the significant increases

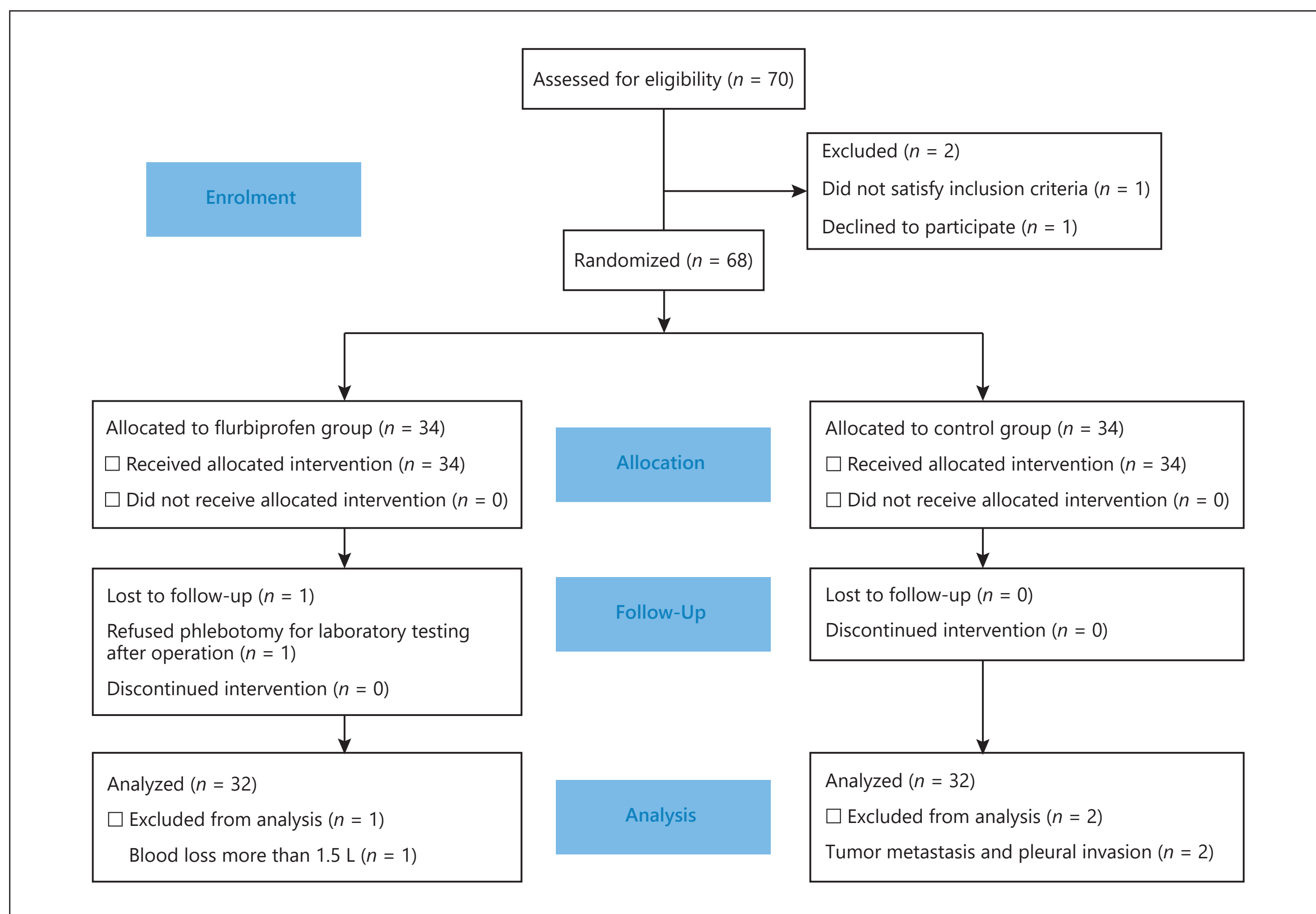

Fig. 2. CONSORT diagram of the trial process.

observed in the concentrations of TNF-α, IFN-γ, IL-6, and CRP at T_1 and T_2 were greater in the control group than in the flurbiprofen group. At a longer postoperative follow-up period (T_3), no significant differences were observed in the abovementioned data from baseline (data not shown). Samples were also collected 3 weeks (T_4) after surgery; however, at this time point, there were no significant differences in the concentration of these inflammatory markers between groups (data not shown). Figure 1b shows the expression of PD-$1^{(+)}$ on CD$8^{(+)}$ T cells in 2 groups at T_4.

There were no significant differences between groups with respect to the proportion of patients who were unexpectedly subjected to respiratory depression after surgery. Respiratory depression was defined as a respiratory rate <8 breaths per minute and oxygen saturation either below 92% or showing a decrease of >5% from baseline in patients with a baseline SPO_2 <90% [21]. There were also no significant between-group differences in the incidence of nausea or skin pruritus. Eight patients in the control group experienced vomiting and retching, compared with 1 patient in the flurbiprofen group ($p = 0.026$). Furthermore, the use of antiemetics in the control group was higher than that in the flurbiprofen group ($p = 0.011$). A significant reduction in score for cough was observed in the flurbiprofen group 24 h postoperatively, but no difference was observed between groups 72 h postoperatively. Moreover, 10 patients in the control group required postoperative rescue analgesia (tramadol) for unbearable pain, compared to three patients in the flurbiprofen group ($p = 0.030$). In addition, no differences were observed between the groups in terms of postoperative wound infection or the length of hospital stay.

Table 1. Differences in perioperative PD-1 expression on CD4$^{(+)}$ and CD8$^{(+)}$ T-cells and other inflammation and immune markers between the flurbiprofen and control groups

Variable	Control group (n = 32)	Flurbiprofen group (n = 32)	Difference (95% CI)	p value
24 h after surgery				
PD-1$^{(+)}$ on CD4$^{(+)}$ T-cells	15.74 (3.41)	18.06 (4.56)	−2.33 (−6.64 to 1.99)	0.268
PD-1$^{(+)}$ on CD8$^{(+)}$ T-cells	22.68 (1.67)	16.85 (6.83)	5.83 (0.50 to 11.16)	0.034
CD3$^{(+)}$ T-cells	33.50 (7.27)	45.38 (5.48)	−11.88 (−18.78 to −4.97)	0.002
CD4$^{(+)}$ T-cells	27.25 (3.73)	32.00 (4.78)	−4.75 (−9.35 to −0.15)	0.044
CD8$^{(+)}$ T-cells	14.13 (1.25)	17.13 (3.00)	−3.00 (−5.58 to −0.42)	0.027
CD4:CD8 ratio	0.74 (0.19)	1.16 (0.34)	−0.43 (−0.72 to −0.13)	0.008
NK cells	7.58 (0.78)	10.04 (0.93)	−2.46 (−3.39 to −1.54)	<0.001
Platelet count, 10^9/L	228.75 (60.07)	215.25 (56.74)	13.50 (−49.16 to 76.16)	0.651
Total WBC count, 10^9/L	12.85 (2.32)	12.44 (1.40)	0.41 (−1.65 to 2.47)	0.674
Hemoglobin, g/L	120.25 (8.23)	121.63 (6.63)	−1.38 (−9.39 to 6.64)	0.718
Neutrophil count, 10^9/L	12.01 (3.38)	13.20 (3.32)	−1.19 (−4.78 to 2.41)	0.490
Lymphocyte count, 10^9/L	0.84 (0.32)	0.95 (0.27)	−0.11 (−0.43 to 0.20)	0.453
Monocyte count, 10^9/L	0.66 (0.10)	0.62 (0.17)	0.04 (−0.11 to 0.18)	0.622
Eosinophil count, 10^9/L	0.183 (0.157)	0.114 (0.099)	0.069 (−0.074 to 0.212)	0.315
Basophil count, 10^9/L	0.010 (0.011)	0.011 (0.008)	−0.001 (−0.012 to 0.009)	0.798
TNF-α, pg/mL	1,680.13 (273.48)	1,350.38 (283.36)	329.75 (31.13 to 628.37)	0.033
IFN-γ, pg/ mL	44.16 (3.48)	36.71 (4.99)	7.45 (2.84 to 12.06)	0.004
IL-6, pg/ mL	178.38 (13.17)	145.50 (20.33)	32.88 (14.51 to 51.24)	0.002
CRP, mg/L	44.11 (13.46)	16.74 (3.00)	27.38 (16.05 to 38.70)	0.001
Serum glucose, mmol/L	7.78 (1.22)	7.25 (1.12)	0.53 (−0.73 to 1.78)	0.384
72 h after surgery				
PD-1$^{(+)}$ on CD4$^{(+)}$ T-cells	15.13 (2.87)	18.39 (4.64)	−3.26 (−7.40 to 0.87)	0.113
PD-1$^{(+)}$ on CD8$^{(+)}$ T-cells	21.31 (1.45)	17.74 (3.69)	3.57 (0.57 to 6.58)	0.023
CD3$^{(+)}$ T-cells	33.88 (3.87)	43.50 (7.71)	−9.63 (−16.17 to −3.08)	0.007
CD4$^{(+)}$ T-cells	20.88 (2.80)	28.38 (4.81)	−7.50 (−11.72 to −3.28)	0.002
CD8$^{(+)}$ T-cells	15.88 (1.81)	17.0 (2.0)	−1.13 (−3.17 to 0.92)	0.258
CD4:CD8 ratio	0.86 (0.36)	1.39 (0.37)	−0.53 (−0.92 to −0.13)	0.012
NK cells	7.29 (0.80)	9.12 (1.26)	−1.84 (−2.97 to −0.71)	0.004
Platelet count, 10^9/L	232.38 (75.64)	209.50 (71.30)	22.88 (−55.95 to 101.70)	0.544
Total WBC count, 10^9/L	9.60 (2.59)	8.74 (1.06)	0.86 (−1.36 to 3.09)	0.405
Hemoglobin, g/L	125.25 (5.73)	122.25 (4.27)	3.0 (−2.42 to 8.42)	0.255
Neutrophil count, 10^9/L	6.20 (1.13)	6.28 (2.43)	−0.08 (−2.11 to 1.96)	0.938
Lymphocyte count, 10^9/L	0.95 (0.26)	1.13 (0.27)	−0.18 (−0.46 to 0.11)	0.206
Monocyte count, 10^9/L	0.55 (0.09)	0.47 (0.16)	0.09 (−0.05 to 0.22)	0.210
Eosinophil count, 10^9/L	0.115 (0.068)	0.086 (0.022)	0.029 (−0.029 to 0.086)	0.284
Basophil count, 10^9/L	0.018 (0.010)	0.021 (0.016)	−0.004 (−0.018 to 0.011)	0.593
TNF-α, pg/mL	1,611.00 (298.82)	1,221.13 (187.55)	389.88 (122.35 to 657.41)	0.007
IFN-γ, pg/mL	41.43 (2.50)	35.25 (3.90)	6.18 (2.66 to 9.69)	0.002
IL-6, pg/mL	160.38 (8.18)	137.00 (14.84)	23.38 (10.53 to 36.22)	0.002
CRP, mg/L	48.68 (10.67)	24.55 (9.52)	24.13 (13.28 to 34.97)	<0.001
Serum glucose, mmol/L	6.36 (1.14)	5.58 (0.47)	0.79 (−0.15 to 1.72)	0.092

Data are presented as mean (SD) or median (IQR) and compared using an independent Student t test or Mann-Whitney U test, respectively.

PD-1, programmed death 1; NK, natural killer; WBC, white blood cells; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-6, interleukin-6; CRP, C-reactive protein; NA, not applicable; IQR, interquartile range.

Table 2. Changes in perioperative PD-1(+) expression on CD4(+) and CD8(+) T-cells and other inflammation and immune markers, after receiving flurbiprofen or placebo, at 24 and 72 h from the baseline values before induction of anesthesia

Variable	Control group (n = 32)	Flurbiprofen group (n = 32)	Difference (95% CI)	p value
24-h postoperative data compared with baseline				
PD-1(+) on CD4(+) T cells	1.76 (0.58)	1.46 (1.11)	0.30 (−0.65 to 1.25)	0.509
PD-1(+) on CD8(+) T cells	12.91 (1.65)	7.86 (5.71)	5.05 (0.55 to 9.55)	0.031
CD3(+) T-cells	−21.25 (6.71)	−9.75 (3.37)	−11.50 (−17.20 to −5.80)	0.001
CD4(+) T-cells	−7.75 (2.44)	−4.25 (1.49)	−3.50 (−5.66 to −1.34)	0.004
CD8(+) T-cells	−3.63 (2.00)	−1.25 (1.83)	−2.38 (−4.43 to −0.32)	0.026
CD4:CD8 ratio	−0.70 (0.31)	−0.35 (0.12)	−0.35 (−0.62 to −0.08)	0.016
NK cells	−3.54 (1.31)	−1.49 (0.66)	−2.05 (−3.17 to −0.93)	0.001
Platelet count, 10^9/L	16.00 (−22.50 to 20.25)	2.00 (−24.50 to 24.75)	NA	0.645
Total WBC count, 10^9/L	6.80 (4.58 to 9.08)	5.90 (4.43 to 7.58)	NA	0.161
Hemoglobin, g/L	−11.38 (2.26)	−7.25 (6.27)	−4.13 (−9.18 to 0.93)	0.102
Neutrophil count, 10^9/L	5.85 (5.55 to 11.23)	10.00 (7.10 to 12.0)	NA	0.105
Lymphocyte count, 10^9/L	−0.80 (0.17)	−0.85 (0.16)	0.05 (−0.13 to 0.23)	0.554
Monocyte count, 10^9/L	0.32 (0.09)	0.31 (0.14)	0.01 (−0.12 to 0.13)	0.933
Eosinophil count, 10^9/L	0.084 (0.145)	0.021 (0.075)	0.063 (−0.065 to 0.190)	0.302
Basophil count, 10^9/L	−0.016 (0.009)	−0.015 (0.009)	−0.001 (−0.011 to 0.009)	0.790
TNF-α, pg/mL	817.13 (131.75)	552.13 (146.24)	265.0 (115.74 to 414.26)	0.002
IFN-γ, pg/mL	25.83 (3.30)	18.96 (4.87)	6.86 (2.40 to 11.33)	0.005
IL-6, pg/mL	105.25 (10.08)	68.00 (12.74)	37.25 (24.93 to 49.57)	<0.001
CRP, mg/mL	40.34 (11.59)	13.65 (1.92)	26.69 (16.97 to 36.41)	<0.001
Serum glucose, mmol/L	3.29 (1.05)	2.26 (1.40)	1.03 (−0.30 to 2.35)	0.119
72-h postoperative data compared with baseline				
PD-1(+) on CD4(+) T-cells	1.15 (1.42)	1.79 (1.96)	−0.64 (−2.47 to 1.20)	0.469
PD-1(+) on CD8(+) T-cells	11.54 (1.54)	8.75 (1.73)	2.79 (1.04 to 4.55)	0.004
CD3(+) T-cells	−20.88 (6.45)	−11.63 (4.03)	−9.25 (−15.02 to −3.48)	0.004
CD4(+) T-cells	−14.13 (5.22)	−7.88 (1.55)	−6.25 (−10.67 to −1.83)	0.011
CD8(+) T-cells	−2.00 (1.69)	−1.38 (1.30)	−0.63 (−2.24 to 0.99)	0.421
CD4:CD8 ratio	−0.55 (−0.98 to −0.20)	−0.10 (−0.18 to −0.03)	NA	0.005
NK cells	−3.83 (1.23)	−2.40 (0.67)	−1.43 (−2.49 to −0.36)	0.012
Platelet count, 10^9/L	18.50 (−19.00 to 23.25)	−5.00 (−33.50 to 23.25)	NA	0.442
Total WBC count, 10^9/L	3.75 (2.45 to 4.58)	2.25 (0.83 to 3.95)	NA	0.105
Hemoglobin, g/L	−6.13 (3.04)	−6.63 (7.35)	0.50 (−5.53 to 6.53)	0.863
Neutrophil count, 10^9/L	2.05 (0.85 to 3.18)	2.65 (0.73 to 4.98)	NA	0.878
Lymphocyte count, 10^9/L	−0.69 (0.22)	−0.68 (0.26)	−0.01 (−0.27 to 0.25)	0.919
Monocyte count, 10^9/L	0.22 (0.08)	0.16 (0.13)	0.06 (−0.06 to 0.17)	0.338
Eosinophil count, 10^9/L	0.016 (0.034)	−0.006 (0.012)	0.023 (−0.006 to 0.051)	0.110
Basophil count, 10^9/L	−0.009 (0.015)	−0.005 (0.017)	−0.004 (−0.021 to 0.013)	0.642
TNF-α, pg/mL	748.00 (133.71)	422.88 (95.49)	325.13 (200.53 to 449.72)	0.000
IFN-γ, pg/mL	23.09 (3.08)	17.50 (3.46)	5.59 (2.07 to 9.10)	0.004
IL-6, pg/mL	87.25 (11.99)	59.50 (9.68)	27.75 (16.07 to 39.43)	<0.001
CRP, mg/mL	44.90 (9.94)	21.46 (7.26)	23.44 (14.10 to 32.77)	<0.001
Serum glucose, mmol/L	1.88 (1.26)	0.59 (1.29)	1.29 (−0.08 to 2.65)	0.062

Data are presented as mean (SD) or median (IQR) and compared using an independent Student's t test or Mann-Whitney U test, respectively.

PD-1, programmed death 1; NK, natural killer; WBC, white blood cells; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-6, interleukin-6; CRP, C-reactive protein; NA, not applicable; IQR, interquartile range.

Sensitivity Analysis

Linear mixed models confirmed the significant differences in the percentage of $CD8^{(+)}$ T cells, $CD4^{(+)}$ T cells expressing $PD\text{-}1^{(+)}$; the concentration of NK cells, TNF-α, IL-6, and CRP between patients receiving flurbiprofen and those receiving placebo; and the significant differences observed at T_1 and T_2.

Discussion

PD-1, expressed in tumor-infiltrating T cells and circulating T cells, has been shown to predict prognosis and serve as a candidate therapeutic target in several malignant tumors, including NSCLC [8, 12, 13, 16]. Recent evidence indicates that a higher level of PD-1 on circulating $CD8^{(+)}$ T cells in peripheral blood is correlated with poorer clinical outcome and shorter overall survival time [10]. In addition, recent clinical studies have shown that treatment with the PD-1 monoclonal antibody, Nivolumab, resulted in improved overall survival among NSCLC patients and cancer patients who improved significantly after receiving immune-checkpoint blockade therapy [22]. Despite the preclinical and clinical progress in immune-checkpoint blockade therapy for cancer, it remains unclear whether the perioperative use of analgesic drugs has a direct impact on these key inhibitory molecules during surgery. Interestingly, it is had been reported that COX inhibitors act synergistically with immune-checkpoint blockade therapy, implying that NSAIDs commonly used as perioperative analgesics may be a useful adjuvant for anti-PD-1/anti-CTLA-4 therapies in cancer patients [19]. To the best of our knowledge, this is the first clinical study providing direct evidence that NSAIDs alter the postoperative levels of PD-1, thus inhibiting the increase in PD-1 expression on $CD8^{(+)}$ T cells in the peripheral blood of lung cancer patients undergoing resection surgery.

Numerous studies have demonstrated that PD-1 and CTLA-4 have distinct cellular mechanisms for attenuating T cell activation [14, 23–25]. Anti-PD-1 predominantly induces expansion of tumor-infiltrating exhausted-like $CD8^{(+)}$ T cells, whereas anti-CTLA-4 engages both subsets of the $ICOS^{(+)}$ Th1-like $CD4^{(+)}$ effector population and exhausted-like $CD8^{(+)}$ T cells in the tumor microenvironment [26]. Furthermore, PD-1 and CTLA-4 are detected in peripheral blood samples under different conditions. Although both markers are constitutively expressed on lymphocytes, only PD-1 is usually detectable without any T cell stimulation in the majority of patients. By contrast, CTLA-4 is rarely expressed on peripheral blood T cells, except under certain conditions of stimulation [27]. While tumor-infiltrating lymphocytes collected by tumor biopsy offer intuitive and fundamental perspectives on the immune response for tumor site analysis, analyses of circulating lymphocytes are easier to perform, can be repeated at several time points, and may provide a more systemic view of the immune response, especially in patients with visceral tumors. Peripheral blood analysis has recently provided insights into the changes in PD-1 levels in circulating T cells after immunotherapy. Therefore, we collected peripheral blood and examined the changes in postoperative PD-1 levels in circulating T cells.

Based on the results of our pilot study, $CD4^{(+)}$ and $CD8^{(+)}$ T cells in the peripheral blood of NSCLC patients generally have an exhausted phenotype and express higher levels of PD-1 than those of healthy subjects (NSCLC patients versus healthy volunteers; $PD\text{-}1^{(+)}CD8^{(+)}$ T cell: 9.76 ± 0.67 vs. 8.61 ± 0.98%, p = 0.016; $PD\text{-}1^{(+)}CD4^{(+)}$ T cell: 16.60 ± 5.20 vs. 10.40 ± 1.37%, p = 0.012). COX activity and COX-dependent inflammatory mediators, such as PGs, facilitate the increase in the inhibitory immune-checkpoints, PD-1/CTLA-4 and low levels of PD-1/CTLA-4 have been demonstrated in $COX\text{-}2^{MEC}$ knock-out mice bearing tumors, suggesting that COX activity and downstream PGs are potentially linked with PD-1 [18]. Although NSAIDs may cooperate with anti-PD-1 blockade in inducing the eradication of tumors in preclinical studies [19], our study primarily demonstrated that flurbiprofen altered PD-1 levels on the circulating $CD8^{(+)}$ T cell population in NSCLC patients up to 72 h postoperatively, without any change in the percentage of circulating $PD\text{-}1^{(+)}CD8^{(+)}$ T cells observed after that time point. Flurbiprofen administration during the perioperative period had little influence on the postoperative percentage of $PD\text{-}1^{(+)}CD4^{(+)}$ T cells. The percentage of lymphocytes after surgery in the control and flurbiprofen groups was, in general, lower compared with hospital reference values. It is interesting to note that there were extensive changes in lymphocyte subsets and inflammatory markers following administration of flurbiprofen in the short-term postoperative period up to 72 h.

A possible explanation for the observed differences between groups is that prominent tumor-sustaining inflammatory factors are potent inducers of PD-1/CTLA-4. This indicates that the levels of PD-1 and other inhibitory checkpoints involved in $CD8^{(+)}$ T cell exhaustion were markedly enhanced, with high levels of VEGF produced

by a proangiogenic factor in the tumor microenvironment [28]. Moreover, COX-derived PGE2 promotes tumor progression by sustaining angiogenesis through the induction of VEGF. This is largely required for a stable blood supply to facilitate tumor growth [29, 30]. We speculate that the inhibition of COX and PGE2 by flurbiprofen attenuated the increase in PD-1 levels, partly by abrogating the induction of VEGF.

There are several limitations of this study. First, further studies are needed to determine the exact mechanism by which NSAIDs downregulates antitumor immunity and immune escape. Second, although the randomization of participants in our study was strict, some baseline and perioperative factors were not equal between the 2 groups. Third, we did not subdivide $CD4^{(+)}$ T cells into conventional $CD4^{(+)}$ T cell and regulatory $CD4^{(+)}$ T cell categories. Fourth, our study is a single-center investigation. A large multicenter study would be ideal to confirm our findings. Last, we have not investigated whether perioperative flurbiprofen administration affected the clinical outcome and overall survival of patients after leaving the hospital.

Conclusions

Perioperative administration of flurbiprofen attenuates the increase in levels of PD-1 on $CD8^{(+)}$ T cells up to 72 h postoperatively, with no effect identified after this time.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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