

Peer review file

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Reviewer A

General comment: The authors conducted a randomized study to investigate the immunosuppressive effects of three anesthetics in patients undergoing lung cancer surgery. Flowcytometry was used to differentiate CD4, CD8, regulatory T cells and proportion of PD-1 T cells. Their results showed that sevoflurane increases regulatory T cells and propofol decreases CD8 T cells, while none of significant differences with desflurane. General anesthetics are commonly used in cancer curative surgery with immunomodulation effects which may be detrimental or beneficial in terms of cancer recurrence or survival after surgery. The reviewed study found different mechanisms of immunosuppression, which warrant further investigations to clarify their clinical implications.

Reply: Thank you for valuable comments, we clarified the part of the anesthetic immunosuppression of mechanism in lung cancer surgery patient.

We should conduct further study in the future, and need to investigate the details.

Specific comments

Comment 1: Page 3 line 63: citation format error in reference 1.

Reply1: I am sorry the error, we corrected citation format (see Page 3 Line 63).

Comment 2: Page 4 line 108: what was the results of the mentioned preliminary study? The authors need to estimate the effect size of a specific parameter (eg CD8 T cells), so that the sample size can be calculated with an intended statistical analysis accordingly.

Reply2: Thank you for your valuable comments.

The present sample size was calculated by using preliminary study.

Sample size calculation using our preliminary study revealed that 19 patients per group would be sufficient to detect a 5.3% difference in the difference between pre- and post-surgery value of percentage of CD4 + cells in peripheral blood, based on the SD value of 5.9%, given that α is 0.05 with a two-sided test and a power of 0.8. To make sure, more than twenty patients of each group were enrolled. Since we had calculated using

CD4+, change in the methods part (see Page4 Line 110).

Change in the text: CD4+ ratio was CD4+ ratio in peripheral blood of pre- and post-surgery was calculated,

Comment 3: Page 5 line 128: 4 mL of levobupivacaine, suggest adding concentration of levobupivacaine here.

Reply 3: I added 0.25% of concentration. (see Page 5 Line130)

Comment 4: Page 7 line 195: is this paragraph misplaced or redundant? I am confused here.

Reply 4: Sorry, it was misplaced. I correct the place the last sentence of this paragraph. Also, I delete the space. (see Page 7 line 203)

Comments 5: Page 8 line 214: is there any explanation about the discrepancy with previous studies?

Reply 5: Thank you for valuable comments. We don't know the reason of discrepancy with previous study. The differences such as anesthetic method, surgical procedure, and invasiveness might have been affected on this discrepancy of the results.

Comment 6. Page 14/15/16: Figure 2/3/4, the authors adopted Wilcoxon rank sum test for data analyses. Obviously, their data were not normally distributed. I would suggest box-whisker plots to replace figures with mean+SD/CI.

Reply6: Thank you for your comments, we replace the Figure2/3/4 to box-whisker plots to replace figures with mean+SD/CI, according to your suggestion.

Comments 7. Page 14: Figure 2, it seemed the baseline value of CD8 T cells in propofol group was lower than desflurane/sevoflurane. Is there any statistical significance?

Reply 7: Thank you for valuable comment, we confirm the baseline value of each group again, there is not significant statistical difference among three groups at baseline.

Comment 8: Page 15: Figure 3. Obviously, regulatory T cells increases after surgery irrespective of choices of anesthetics. I am afraid that higher baseline value in propofol group may be a bias of the result.

Reply8: Thank you for valuable comments. We also confirm the baseline of regulatory T cells at baseline again, there was no significant difference statistically among three groups.

Reviewer B

Comment 1: Thank you for submitting your study.

It appears that the interpretation of the results and the conclusions need to be revised significantly.

It cannot be said that propofol reduced CD8+ T cells. This is because it is not known whether the stress of surgery itself reduces CD8+ T cells or propofol decreases. Based on the results of this study alone, it can be stated that "CD8+ T cells were significantly lower in the propofol group compared to sevoflurane and desflurane after surgery."

Reply1. Thank you for valuable comments, as you said, we did not investigate the effect of surgical stress on CD 8+cell. This theme should be evaluated by any other method such as animal results or a study in vitro in the future.

However, in our study, it is true that CD8 + in the end of surgery in propofol group significantly decreased compared to before surgery, NOT statistically low compared to other groups by repeated measures ANOVA. We cannot replace the phrase of the result about CD8+.

Comment 2: The mention of sevoflurane on regulatory T cells cannot say that "Sevoflurane increased the proportion of regulatory T cells", and it is correct to conclude that the proportion of regulatory T cells was significantly higher after surgery compared to other groups.

Because it is not a conclusion compared to a definitive control.

It is a pity that we cannot establish a definite control group because surgery cannot be performed without anesthesia, but as the authors mentioned at the end, it would be nice if it could be clearly demonstrated in the planned animal experiment.

Reply 2: Thank you for your valuable comment, I understand what you say that definitive control group without anesthesia should be needed, we will plan to do the animal experiment in the future. From the statistical view, the proportion of regulatory T cells in three groups was not significantly different before and at the end of surgery.

It is significantly different that the proportion of regulatory T Cells in sevoflurane at the end of surgery is higher than the one before surgery. It is why we cannot replace the conclusion in this time.

Reviewer C

General comments: In the manuscript JTD-21-878-CL “Propofol decreases CD8+ T cells and sevoflurane increases regulatory T cells after lung cancer resection: A randomized controlled trial”, the authors investigate the impact of 3 different anaesthetics on the number of blood lymphocytes. The authors find that Propofol reduces the number of CD8+ T cells and Sevoflurane increases Treg, whereas the percentage of PD-1+ T cells did not change. The authors conclude that anaesthetics may worsen the outcome of patients undergoing oncologic surgery though they cannot distinguish between the effects of surgery and of anaesthetics.

The paper has several limitations that need to be addressed by the authors.

Reply: Thank you for your valuable comments, we are to correct the points which you raised as below.

Comment 1: PBMC were prepared and stained with antibodies. This should result in relative percentages of T cells. However, Figure 2 gives absolute values of CD4+ and CD8+ T cells (10E4/ml blood). The authors have to explain in “Materials&Methods”, how these absolute numbers were generated.

Reply 1: Thank you for pointing out, Yes, we should explain how these absolute number were generated in Material and Methods part, Then, we added (Page 6 Line 157)

Change in the text: Absolute quantification of each cell was generated by the number of viable cells, which was measured by cell counter, multiplied by the percentage of each item. The percentage of each item was determined by flow cytometry with a flow of 10,000 cells after isolation of PBMC.

Comment 2: Blood was taken during surgery. The authors observed effects, such as a decrease of CD8+ T cells and deduced an immunosuppression caused by anaesthetics. However, how long does the effect last? The authors have to show a time course, e.g.

in the propofol patient's group with the CD8⁺ T cells and in the Sevo-group with Tregs.

Reply2: Thank you for valuable comments, we deduced the effect of anesthetics are lasting at the second sample collected. Because it was collected at the same time of discontinuing the anesthetics. Since we controlled general anesthesia in the range between 40 and 60 of BIS monitor, the effect of anesthetics should be clinically the same among three groups.

We added "immediately" in the phrase as below.

Change in the text: At the end of the surgery, anaesthetic administration was stopped, and immediately after that, the second blood sample was collected (see Page 5 L135)

Comment 3: The authors have to discuss, why PD1 expression of T cells should be changed by anaesthetics. Why other checkpoints are not involved in the study?

Reply 3: Thank you for your valuable comment, we investigated PD1 expression, and no change can be seen among three anesthetics groups. Certainly, as you suggested, other checkpoints except for PD-1, might have been changed depending on the anesthetics, we will plan to investigate other checkpoints in the next study referring to your suggestion. And we added the phrases as below referring your suggestion.

Change in the text: If other immune check points were investigated, the change depending on the anesthetics might have been observed. We are going to plan the measures of other checkpoints in the next study. (See page 9 line 248)

Comments 4: 600-1200 CD4⁺ T cells/ μ l blood is a normal value. Especially in the Sevo-group in Fig. 2A, there is a high standard deviation with patients with >2000 CD4⁺ T cells/ μ l blood. Do the authors have an explanation? Tumor patients normally have low number of lymphocytes.

Reply 4: The value of CD4⁺ T cells in the Sevo-group in Fig. 2A is higher than normal value, as you said. The exact reason is uncertain, although we explored the cause except for lung cancer which increase the lymphocyte such as leukemia and lymphoma.

Comment 5. Figure 3 gives the percentage of Treg as 0.01%. This seems a very low percentage, since Treg has been shown to be 5% of CD4⁺ T cells. Could the authors compare their CD127-CD25⁺Treg values to Treg values from a control group and from literature. Why Tregs are not shown as percentage of CD4⁺ T cells?

Reply 5: Unfortunately, we did not measure and compare their CD127-CD25+Treg values from a control group or from literature since these were not measured, however, we will plan to add these parameters in the next study.

Treg (%) is shown additionally in Fig 3 as you suggested.

Comment 6: Line 145-149: Vacutainers with sodium citrate were used, this means, plasma and not serum was prepared.

Reply 6: Thank you so much for your comment. According to your helpful comment, we correct it as plasma, not serum.

Change in the text: (PBMC) and plasma.

Comment 7: Table “Patient characteristics”: The units of “Surgical duration” and anaesthetic duration are not given. Furthermore, the authors should complement the leukocyte numbers and lymphocyte numbers in the three groups in the table. Typo: Histrogical type

Reply 7: Thank you for your valuable comments and pointing out mistakes.

“Histrogical” was corrected to “Histlogical” in Table.

Surgical duration and anesthetic duration were already shown in Table 1 in the fourth column, leukocyte numbers and lymphocyte numbers were also put in the table.

Also, we added description about leukocyte and lymphocyte in result part. (Page 6 Line 178)

Change in the text. Pre-and post-operative numbers of leukocyte and lymphocyte were also shown in Table 1. Leukocyte values among three groups were significantly different at both points, however the values of lymphocyte were not different among three groups at either point.

Comment 8: The first blood sample was collected directly from the arterial catheter, the second blood sample was collected after stopping anaesthetics. How diluted is the blood and does the dilution has an effect on the results of lymphocyte numbers?

Reply 8: Thank you for your valuable comments, slightly blood dilution may be seen. However, we restrict the fluid volume according to the thoracic anesthesia protocol of our department which we usually use, hence, we think the difference of blood dilution can be permissive in the cases of similar anesthetic duration. In further study, the

parameter which indicates blood dilution such as hematocrit or hemoglobin should be presented as you suggested.