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Frontiers in Immunology
Manuscript ID: 1570717

April 8, 2025

Dear Academic Editor and Reviewers,

Please find enclosed our revised manuscript entitled "**Development of caninized anti-CTLA-4 antibody as salvage combination therapy for anti-PD-L1 refractory tumors in dogs**".

We would like to express our sincere gratitude to all the reviewers and the editor for their consideration. We are grateful to reviewers # 5 and #6 for their positive feedback. We have revised our manuscript according to additional input from reviewer #1. We believe that the modifications have significantly improved the clarity of our manuscript and that the revised manuscript is now suitable for publication in *Frontiers in Immunology*.

Our responses are outlined in red letters, and the Manuscript with the tracked changes is provided as a supplemental item.

Reviewers' comments:

Reviewer 1

Thank you for your response. I believe you have appropriately addressed the comments from the reviewer. However, I would like to provide the following additional comments. Further revisions are necessary for publication, and I ask for your reconsideration of these points.

We sincerely appreciate Reviewer #1 for the thorough review and additional input on this manuscript. We have further revised our manuscript based on your valuable comments.

Regarding the objective response rate: Even if non-measurable disease cases are excluded, presenting the objective response rate as 16.7% without including six non-measurable cases (half of the enrolled subjects) is misleading. If the efficacy and safety study cohorts were pre-separated, this would not be an issue. Still, in your study design, they were not, and it would be more appropriate to calculate the ORR, including those six non-evaluable (NE) cases.

Thank you so much for this comment. We agree with your comment that the response rate reported in the current manuscript may be misleading. In addition, as you clearly pointed out in the following comment, dogs with a variety of tumor types and treatment histories were included in the clinical study, and the response rate calculation would not add meaningful insights to the interpretation of the results. Given the exploratory (proof-of-concept) nature of the clinical study, we would like to omit the

response rate calculation from the manuscript. Instead, we would like to focus on describing the treatment outcome of individual cases. We have revised the Results, Discussion, and Methods accordingly, as follows.

(Line 291 in the revised manuscript with tracked changes)
The response evaluation eligibility statement has been removed.

(Line 312)
We have revised the sentences in the Results to describe the treatment outcome in a case-by-case basis. We have omitted the response rate calculation and deleted Table 4 from the manuscript.
"Among dogs with non-target disease (n = 6), one dog (Dog #3) died a day after the first combination treatment. The remaining five dogs experienced unequivocal disease progression within 8 weeks (Supplementary Table 4). Among dogs with target disease (n = 6), five dogs experienced PD as their best overall response. Notably, one dog with recurrent OMM (Dog #9) achieved a partial response (PR)."

(Line 352)
The sentence has been revised to reflect the above changes.

(Line 393)
The sentence has been revised to reflect the above changes and to explicitly discuss that the response rate calculation will require further studies.
"While the ORR should be calculated in further clinical studies involving a larger number of dogs with a uniform tumor type, …"

(Line 723)
The sentence has been revised to reflect the above changes.

We have recently published 2 papers with clinical study data evaluating tumor response using the same rationale (npj Precision Oncology (2021) 5:10; PLoS ONE (2023) 18: e0291727). In the review process of the former manuscript (npj Precision Oncology (2021) 5:10), anonymous reviewer(s) have argued that the response evaluation should include only dogs with measurable disease as defined by cRECIST (Vet Comp Oncol (2015) 13:176-183), and we reported the response rate as such. The study design was considered to be acceptable due to the explicit statement that the study was exploratory rather than conclusive.

24. Maekawa, N. et al. PD-L1 immunohistochemistry for canine cancers and clinical benefit of anti-PD-L1 antibody in dogs with pulmonary metastatic oral malignant melanoma. NPJ Precis. Oncol. 5, 10 (2021).
25. Maekawa, N. et al. Safety and clinical efficacy of an anti-PD-L1 antibody (c4G12) in dogs with advanced malignant tumours. PLoS One 18, e0291727 (2023).
29. Nguyen, S. M., Thamm, D. H., Vail, D. M. & London, C. A. Response evaluation criteria for solid tumours in dogs (v1.0): a Veterinary Cooperative Oncology Group (VCOG) consensus document. Vet. Comp. Oncol. 13, 176–183 (2015).

In response to lines 270-271: Although the inclusion criteria specify “dogs with tumors that are not expected to be cured by existing therapies,” there is a lack of consistency in your results, as half of the enrolled cases have non-measurable disease. Adhering to inclusion and exclusion criteria is crucial in clinical trials, and a more appropriate response must be provided for the manuscript submission. Additionally, the progression rates of tumors can vary depending on the tumor type, so it is advisable to avoid evaluating PFS and OS for all 12 cases together.

The inclusion criteria “(2) dogs with detectable tumors that can be monitored repeatedly during the study” and “(3) dogs with tumors that are not expected to be cured by existing therapies” do not necessarily exclude the enrolment of dogs that have only non-measurable lesions. The word “detectable” in inclusion criteria (2) is used to express the situation that there are detectable/visible (regardless of the “measurability”; please see the definition explained below) tumors present in the body as evidenced by clinical examination or diagnostic imaging. In clinics, tumors of approximately more than 5 mm are detectable/visible by clinical examination/CT, and those >10 mm can clearly be depicted by chest X-ray/ultrasound. However, to allow accurate measurement of the longest diameter, tumors less than 10 mm on clinical examination/CT and those <20 mm on X-ray/ultrasound are considered “non-measurable” based on the definition by cRECIST (Vet Comp Oncol (2015) 13:176-183). Nonetheless, we apologize the confusing statement in the inclusion criteria (2), which is originally written in non-English language and is insufficiently translated in the R1 version of the manuscript. We have amended the sentence in Methods as follows to improve the clarity of the inclusion criteria.

(Line 679 in the revised manuscript with tracked changes)
“The inclusion criteria for study enrollment were as follows: (1) dogs with a histopathologic or cytopathologic diagnosis of malignant tumor, (2) dogs with clinically detectable tumors that can be monitored repeatedly during the study, (3) dogs with tumors that are not expected to be cured by

existing therapies, and (4) dogs with written informed consent obtained from their owners. Given the exploratory nature of the clinical study, the presence of measurable lesions as defined by cRECIST [29] was not a prerequisite for enrollment."

As discussed above regarding the omission of response rate reporting, we agree that PFS and OS for all 12 cases may not add meaningful insight to the interpretation of the results because dogs with a variety of tumor types and treatment histories were enrolled in the clinical study. We appreciate this input and have removed the sentences describing PFS and OS in the Results section.

(Line 311 in the revised manuscript with tracked changes)
The PFS and OS reporting was deleted.

Regarding Figure 2a: The difference in CTLA-4 expression between healthy controls and melanoma cases is small. To enhance the persuasive power of the data, it would be helpful to include representative dot plot results as well.

Thank you so much for this input. We have added the gating strategy and representative dot plots as Supplementary Figure 7. We have revised the Methods accordingly to cite the added data.

(Line 636 in the revised manuscript with tracked changes)
"The gating strategy is shown in Supplementary Figure 7."

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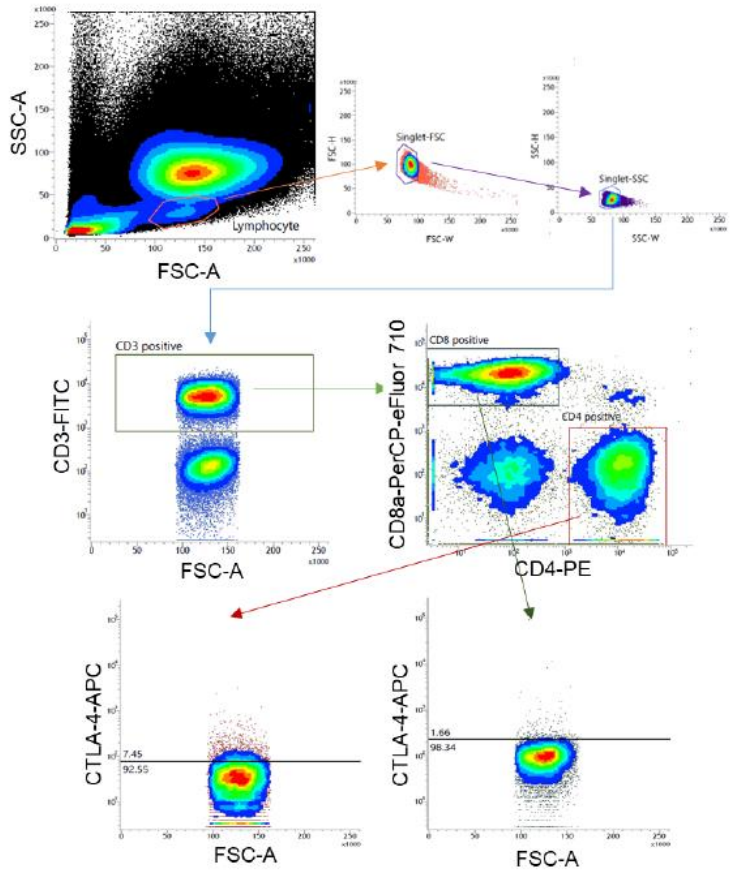

Supplementary Fig 7. Gating strategy and example plots for expression analysis of CTLA-4 on T cells.

CTLA-4 expression on $CD4^+$ or $CD8^+$ T cells ($CD3^+$ lymphocytes) was analyzed in white blood cells (WBCs) by flow cytometry.

We deeply appreciate your consideration and the opportunity that you gave us to improve our manuscript. Please let us know if we can be of any further assistance in the process of considering this article for publication in *Frontiers in Immunology*.

Sincerely yours,

The Authors