nature portfolio

Peer Review File

Tumor-targeted IL-10 synergizes with IL-2 to Enhance Antitumor Immunity while Minimizing Immune-Related Adverse Events

Corresponding Author: Dr Jian Qiao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Sun et al. studied how combined immunotherapy of tumor-targeted IL-10 (CmAb-$(IL10)_2$) and tumor-targeted IL-2 (CmAb-IL2) exerts antitumor immune responses and limits immune-related adverse events (irAEs). Using mouse tumor models, humanized mice, and ex vivo patient-derived tumor fragments, the authors demonstrated that endogenous IL-10 was essential for IL-2-mediated antitumor effects. The combination therapy preferentially expanded less exhausted intratumoral $CD8^+$ T cells and elicited stronger antitumor immune responses than IL-2 alone. Mechanistically, the authors identified the IL-10/IL-10 receptor axis as a key mediator of efficacy while IL-10 signaling in macrophages mitigated IL-2-induced toxicities. Overall, this is an interesting study demonstrating that tumor-targeted IL-10 synergizes with IL-2 to enhance antitumor immunity while reducing IL-2-associated irAEs, offering new insights into a cytokine-based strategy to improve the efficacy and safety of cancer immunotherapy. However, some conclusions appear somewhat unsubstantiated, and certain aspects of data presentation and figures should be improved.

(1) The distinction used in Figure 5 and the related supplementary figures to distinguish cDC1s is problematic. Defining DCs as CD11c+ CD11b– cells the authors risk to include B cell subsets, pDCs, and some progenitor and activated cells. Did the authors use either XCR1, CD103 or CD8a in their analysis to define cDC1 cells? This would be critical, as the main conclusions throughout the manuscript are drawn from analysis presented in Fig. 5 and onwards.

(2) Related to Fig. 3d-k, please show UMAPs and dot plots (RNA-seq / cytometry) depicting expression of PD-1 and TIM-3 in the supplementary materials.
Additionally, the concatenated dot plot in Fig. 3k is rather confusing and overclustered, especially since it used TCF-1 as one of the markers whereas the authors did not mention it in the manuscript. The authors should reconsider their clustering strategy considering biological relevance. Additionally, they should show exemplary gating strategy that Fig. 3i is based on, since it is difficult to relate the depicted frequencies of antigen-specific CD8+ T cells to selected gates on concatenated dot plots in Fig. 3l.

(3) Did the authors measure the proliferation of antigen-specific intratumoral CD8+ T cells after treatments?

(4) Related to Fig. 3e-g and Supplementary Fig. 2, please elaborate on the observed therapeutic effect on tumor growth for CmAb-(IL10)2 in comparison to the previously published work on this molecule (ref. 17). Notably, in their previous work (ref. 17), the authors observed near-curative responses for B16-cEGFR tumors and TC-1-cEGFR tumors by using CmAb-(IL10)2 treatment starting 7-11 days after tumor inoculation. Please describe in detail how the prior results compare to the ones presented in the current manuscript, including the dosing, administration routes, treatment time as well as the distinction that authors took on specifying early-stage and advanced tumors for tumor cell lines used in the study.

(5) Did authors quantify changes in T cell counts in peripheral lymphoid organs and the tumor for treatments depicted in Fig. 2e? Related to this, were there any changes in proliferation of T cell subset, including CD8+, conventional CD4+ and Treg cells, after administration of treatments, comparing tumor and peripheral lymphoid organs?

(6) Could the authors elaborate in more detail on the origin of the observed less-exhausted phenotype of CD8+ TILs after

combination treatment? Should higher costimulatory molecule levels on antigen-presenting cells and overall higher APC cell counts not translate to more potent antigen presentation in the combination treatment setting?

(7) How is the T cell metabolism and mitochondrial fitness affected in single/combination treatment settings? Please discuss the functional consequences of CmAb-(IL10)2 treatment for progenitor exhausted vs terminally exhausted CD8+ TILs as well as for regulation of T cell exhaustion, compared to results shown in ref. 38.

Minor comments:

(a) Please double check the manuscript for typing errors, such as on page 6, line 150 (sing-cell RNA sequencing), line 153 (exhaustion-associate genes), including the figures and figure legends: Supplementary Fig. 6b (CD11c1 instead of CD11c), Fig. 5f graph title (cluster, instead of currently existing labels).

(b) If available, please provide the quantification in Supplementary Fig. 6b as frequencies of live CD45+ immune cells for accuracy, not tumor cells.

(c) Please verify the clustering labels in Fig. 5e, supplementary Fig. 7a and corresponding quantification in S7b.

(d) Please expand the single-cell RNA sequencing and analysis method section to include more details on how exactly the analysis was performed, including the subsetting process for DC analysis.

Reviewer #2

(Remarks to the Author)
The authors present a bispecific cytokine approach that combines a cetuximab-based IL-10 fusion protein, CmAb (IL-10)2, with a cetuximab-based tumor targeted IL-2, CmAb IL-2, to enhance antitumor immunity while limiting IL-2 associated systemic inflammation. The combination shows improved tumor control compared with either monotherapy across multiple murine tumor models. Mechanistically, the authors propose that antitumor efficacy requires IL-10R signaling in dendritic cells, particularly cDC1s, where IL-10 preserves mitochondrial integrity and survival through reduced mitochondrial reactive oxygen species (ROS) and increased Mcl-1, thereby sustaining antigen presentation and expansion of less-exhausted PD1+Tim3- tumor specific CD8+ T cells. In parallel, they proposed that the combination therapy mitigates high-dose IL-2–driven systemic inflammation through an IL-10R-dependent mechanism in macrophages, suggesting that therapeutic efficacy and systemic protection are mediated by distinct cellular programs.
This is a technically strong and conceptually interesting study supported by extensive conditional genetics and multi-modal immune profiling. The dendritic cell-centered IL-10 axis is plausible and potentially differentiating, and the macrophage-dependent protection is supported by genetic necessity. The main limitations relate to mechanistic resolution and framing. In particular, the manuscript does not adequately reconcile its conclusion that direct IL-10 signaling in CD8+ T cells is dispensable with prior literature supporting direct IL-10-driven functional rescue of exhausted CD8+ TILs. In addition, the downstream mechanism by which macrophages mediate systemic protection is insufficiently defined, and the Mcl 1-centered dendritic cell survival model is not tested with direct perturbation. Finally, characterization of the CmAb IL-2 construct is limited. Specific comments are listed below.
1. The manuscript concludes that IL-10 primarily acts through dendritic cells rather than directly on CD8 positive T cells, based on conditional loss of IL-10r in CD8+ cells showing preserved combination efficacy and preserved expansion of tumor antigen specific CD8 positive T cells. This interpretation requires clearer reconciliation with prior evidence that IL-10 can directly enhance terminally exhausted CD8+ TILs via metabolic reprogramming (Nat Immunol 2021;22:746-56). The two models may reflect different boundary conditions, such as cytokine format and exposure kinetics, disease context, or the presence of IL-2 shifting the limiting step toward antigen presentation rather than intrinsic T cell metabolism. The discussion should explicitly address these possibilities. A focused analysis of the CD8+ compartment would further strengthen the manuscript, for example testing whether terminal exhaustion programs and metabolic signatures are present and whether they depend on dendritic cell IL-10R signaling rather than T cell IL-10R signaling.
2. The macrophage axis is supported by genetic necessity but remains mechanistically underdefined. However, the manuscript does not define the key macrophage population involved, the dominant tissue source of systemic cytokines, or downstream pathways mediating protection. Serum cytokines and AST are supportive endpoints, but they do not establish mechanisms. The manuscript would be strengthened by identifying the relevant myeloid compartment and tissue context, for example liver, spleen, or circulating myeloid cells, and by adding pathway level evidence consistent with an IL-10 anti-inflammatory program in macrophages or reduced inflammatory cytokine production at the cellular source.
3. The proposed dendritic cell mechanism places Mcl-1 as a key mediator linking IL-10R signaling to dendritic cell survival and mitochondrial integrity. The association is supported by the genetic requirement for dendritic cell IL-10R signaling and by accompanying mitochondrial readouts and Mcl-1 changes. However, direct causal testing of the Mcl-1 axis is limited. Since Mcl-1 is central to the mechanistic model, at least one perturbation of the Mcl-1 pathway would strengthen the conclusions.
4. The CmAb IL-2 construct requires more complete characterization. The Methods describe production and purification and basic quality assessment, but the manuscript does not provide sufficient biochemical and functional definition of CmAb IL-2 as a standalone agent, including binding parameters to EGFR and IL-2 receptor, stability, and receptor engagement potency relative to recombinant IL-2. Given that the therapeutic rationale depends on altered exposure and toxicity, these data would improve interpretability and reproducibility.
5. The dosing strategy and translational framing of IL-2 require clarification. The manuscript employs lower IL-2 doses in efficacy studies and higher doses in toxicity assessments, while the discussion intermixes concepts of tolerated-dose and high-dose IL-2. However, the rationale underlying dose selection and the operational definitions of “low-dose” and “high-

dose" IL-2 are not clearly articulated. Importantly, the authors do not sufficiently address how the high-dose IL-2 regimen used in mice relates to clinically relevant high-dose IL-2 toxicity in humans, which is historically defined by capillary leak syndrome and systemic inflammatory sequelae. The authors should more explicitly define the intended clinical context for each dosing regimen and justify the translational relevance of the murine high-dose model. Moreover, they should avoid implying that systemic protection alone enables clinically meaningful high-dose IL-2 use, unless superior antitumor efficacy under high-dose conditions is directly demonstrated through appropriate comparative experiments.

Reviewer #3

(Remarks to the Author)
Sun et al. investigate the therapeutic combination of tumor-targeted IL-2 and IL-10 delivered as antibody–cytokine conjugates for the immunotherapy of solid tumors. The authors employ a tumor-targeted IL-10 construct (CmAb-(IL-10)\₂) and a tumor-targeted IL-2 construct (CmAb-IL-2), administered either as monotherapies or in combination.
The manuscript advances several important claims:
(i) endogenous IL-10 plays a critical role in mediating the antitumor effects of IL-2;
(ii) combined CmAb-(IL-10)\₂ and CmAb-IL-2 therapy preferentially expands less-exhausted intratumoral $CD8^+$ T cells;
(iii) $CD8^+$ T cells are essential for the therapeutic efficacy of tumor-targeted cytokine antibodies;
(iv) IL-10 receptor signaling in dendritic cells is required for the effect of CmAb-(IL-10)\₂ on T cell responses, as shown by conditional IL-10R deletion in DCs using Zbtb46^Cre, with a proposed mechanism involving altered mitochondrial metabolism; and
(v) IL-10 receptor signaling in macrophages limits IL-2–driven immune-related adverse events (irAEs), particularly liver toxicity, as demonstrated using LysM^Cre-mediated IL-10R inactivation.
Overall, the study is well performed, and the data are clearly presented. The work is potentially important, as it proposes a novel strategy to mitigate IL-2–associated immune-related adverse events while preserving antitumor efficacy. The demonstration that dendritic cells respond directly to IL-10 and that this signaling pathway modulates $CD8^+$ T cell–dependent immunotherapy responses is particularly interesting and conceptually novel.
However, several minor concerns currently limit the impact of the study.

Major and Minor Comments
1. Requirement for tumor targeting
The manuscript does not clearly establish whether tumor targeting is required for IL-10–mediated modulation of IL-2 therapy. Additional evidence demonstrating that tumor localization of IL-10 is essential for the observed effects would strengthen this conclusion.
2. Comparison with non-targeted cytokines
It remains unclear whether non-targeted IL-2 and IL-10 exhibit similar immunological and therapeutic profiles. Direct comparison with untargeted cytokines would help clarify the added value of the targeting strategy.
3. Activity in non-EGFR–expressing tumors
The authors should clarify whether the targeted cytokines retain activity in EGFR-negative tumor models (e.g., non-EGFR–expressing B16 variants or other tumor lines). This would address the generalizability of the approach.
4. Depth of $CD8^+$ T cell functional characterization
The characterization of $CD8^+$ T cell responses in treated wild-type mice remains limited. Inclusion of polyfunctionality assays—ideally in antigen-specific settings—would substantially strengthen the conclusions. Ex vivo assays assessing tumor-infiltrating lymphocyte function following TCR engagement would be particularly informative.
5. Assessment of T cell exhaustion markers
Additional markers such as CD39 and TOX would allow a more precise assessment of $CD8^+$ T cell exhaustion status and would complement the current phenotypic analysis.
6. DC subset-specific metabolic analysis
The Mitotracker analysis presented in Figure 5A would be more informative if performed at the level of dendritic cell subsets, specifically comparing $XCR1^+$ (cDC1) and $SIRP\alpha^+$ (cDC2) populations.
7. Antigen presentation and cross-presentation
Ex vivo analysis of antigen presentation and cross-presentation of tumor-associated antigens by myeloid cells from control and treated mice would further support the proposed mechanism involving dendritic cells.
8. Specificity of LysM^Cre-mediated IL-10R deletion
Given that LysM^Cre also affects subsets of dendritic cells, it would be valuable to directly compare Zbtb46^Cre and LysM^Cre models using the same readouts, including:
o $CD8^+$ T cell–mediated antitumor immunity (Figure 5), and
o IL-2–mediated liver toxicity (Figure 6).
In addition, evidence of IL-10–driven anti-inflammatory programs in liver macrophages (Kupffer cells) or liver dendritic cells would strengthen the conclusions regarding mitigation of irAEs.
9. Biodistribution of the IL-10 antibody conjugate
Visualization of the biodistribution of the IL-10 antibody–cytokine conjugate would be valuable. In particular, demonstrating whether IL-10–responsive myeloid cells localize near tumor cells decorated with the targeting conjugate would provide important mechanistic insight.
Summary
In summary, this is a well-executed and conceptually interesting study that addresses an important challenge in cytokine-based cancer immunotherapy. Addressing the points above would significantly strengthen the mechanistic depth and translational relevance of the manuscript.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Sun et al. present a revised version of their study on how combined immunotherapy of tumor-targeted IL-10 (CmAb-(IL10)$_2$) and tumor-targeted IL-2 (CmAb-IL2) exerts antitumor immune responses and limits immune-related adverse events (irAEs). The authors have addressed most of our previous concerns, which has significantly improved the manuscript. Below are a few remaining points.

(1) Please elaborate on the differences in frequencies of PD1+ Tim3- tetramer+ CD8+ T cells between results obtained in WT mice after combination therapy presented in Fig. 4i (average for combination therapy approx. 17%) and in Fig. R3c (average for combination therapy approx. 65%) following the same treatment settings.
(2) Related to Figure R6, please provide absolute cell counts of cDC1 and cDC2 subsets in the tumor.
(3) Performing a pseudobulk analysis of the data presented in Fig. 3e-g would be better than a Wilcoxon test on single cell data with large cell numbers as currently performed.
(4) The relevance and basis of clustering and analysis presented in Fig. 5d-e and Fig. S8a-b and is confusing.
• If the data indeed reflect tumor-infiltrating CD45+ CD3- cells that were clustered in the initial UMAP shown in Fig. S8a, the overall clustering pattern and following DC cluster annotation is unusual. Three distinct clusters currently annotated as DC clusters 1-3 are spatially separated and belong to larger, currently unannotated clusters (cells connected to current DC clusters 1 and 3). Please provide the basis for annotating the selected clusters as dendritic cells (by providing lineage and functional marker expression). Additionally, please provide cell type annotation and validation in the form of marker expression for all major (currently unannotated) clusters in Fig. S8a, as it appears that there are marked differences in the overall non-T cell (CD45+ CD3- cells) composition between the treatments.
• After providing the above-mentioned validation, please elaborate on the unusual spatial distribution of currently existing DC clusters. What are the cells currently neighbouring the DC clusters 1 and 3? Why are those cells (belonging to currently unannotated cell lineage) spatially closer based on UMAP coordinates than other dendritic cell subclusters?
• Were the cell numbers evenly downsampled between treatments during generation of UMAP in Fig. S8a?

Reviewer #2

(Remarks to the Author)
The revised manuscript is substantially improved. The additional clarifications regarding the dendritic cell centered IL-10R mechanism, macrophage dependent attenuation of systemic inflammation, and characterization of the CmAb-IL2 construct are helpful. My only remaining concern relates to the newly added experiment shown in Fig. S8d. I appreciate the authors' effort to interrogate the MCL-1 pathway using an MCL-1 inhibitor and to assess mitochondrial integrity by JC-1 staining. However, the increase in the JC-1 red/green ratio induced by CmAb-(IL10)2 alone appears modest and does not seem to reach statistical significance. Thus, while the inhibitor result is directionally consistent with the proposed model, the current data do not provide strong evidence that CmAb-(IL10)2 significantly enhances mitochondrial integrity through MCL-1 in this assay. I therefore suggest that the authors temper the wording related to this point. A more balanced interpretation would be that MCL-1 inhibition attenuates a trend toward IL-10 mediated improvement in mitochondrial integrity, thereby supporting, but not definitively proving, the involvement of the MCL-1 pathway.

Reviewer #3

(Remarks to the Author)
The authors have successfully adressed my concerns, in most cases by bringing additional experimental materials that clarify the mechanisms of action of tumor targeted IL10.
In particular, it is now clearer that IL10 or IL2 requires tumor targeting for efficiency.
The point if further made clear by the implementation of EGFR negative tumors.
The CD8+ T cell response is now characterized with state-of-the-art polyfunctionality assay, thereby establishing the relevance of observed changes awarded by immunotherapy. Exhaustion markers have been included like CD39.
Also the role of IL10R on mitochondrial function in tumor-infiltrating DC subsets is now better established with a distinction of DC1 and DC2s.
Adoptive transfer of DCs in the context of IL10R deficiency now demonstrates that IL10R signaling promotes antigen presentation.
Finally specificity issue raised by the implementation of Lyz2Cre+Il10rFl/Fl are aleviated by the fact that these mice showed no impairment in tumor antigen-specific CD8+ T cell responses or tumor control in CmAb-(IL10)2 plus CmAb-IL2 treated

mice, thereby contrasting with DC-specific depletion.
ALtogether the study is much improved and highlight a novel mechanism that can be activated to improve anti-tumor immunity.

Reviewer #4

(Remarks to the Author)
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Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors revised the manuscript appropriately.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #1 (Remarks to the Author):

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Overall, this is an interesting study demonstrating that tumor-targeted IL-10 synergizes with IL-2 to enhance antitumor immunity while reducing IL-2-associated irAEs, offering new insights into a cytokine-based strategy to improve the efficacy and safety of cancer immunotherapy. However, some conclusions appear somewhat unsubstantiated, and certain aspects of data presentation and figures should be improved.

We thank the reviewer for the constructive comments and for recognizing the potential significance of our study.

(1) The distinction used in Figure 5 and the related supplementary figures to distinguish cDC1s is problematic. Defining DCs as $CD11c^+$ $CD11b^-$ cells the authors risk to include B cell subsets, pDCs, and some progenitor and activated cells. Did the authors use either XCR1, CD103 or CD8a in their analysis to define cDC1 cells? This would be critical, as the main conclusions throughout the manuscript are drawn from analysis presented in Fig. 5 and onwards.

We appreciate the reviewer's thoughtful comments. We agree with the concerns raised regarding DC subsets. As the reviewer suggested, we performed further analysis of both cDC1 ($XCR1^+$ $CD11c^+$ $MHC\text{-}II^+$ and cDC2 ($Sirpa^+$ $CD11c^+$ $MHC\text{-}II^+$) subsets following the combination therapy with CmAb-$(IL10)_2$ and CmAb-IL2. Consistent with our previous analysis, we found that combination therapy increases both cDC1 and cDC2 subsets within the tumors (**Fig. R1a, b**). Furthermore, MCL-1 expression was upregulated in both intratumoral cDC1 and cDC2 populations (**Fig. R2c, d**), suggesting that CmAb-$(IL10)_2$ and CmAb-IL2 combination therapy broadly regulates cDC1 and cDC2 in this context. In conjunction with our results derived from *Zbtb46*$^{Cre+}$*Il10r*$^{Fl/Fl}$ mice (which lack IL-10R in both cDC1 and cDC2), these data support our conclusion that IL-10/IL-10R axis in DCs plays a critical role in mediating the efficacy of the combination therapy by promoting intratumoral DC function and maintaining mitochondrial health and function. To reflect these findings more accurately, we have updated the manuscript to focus on the overall conventional DCs (cDCs) population, rather than attributing effects exclusively to a single subset. Accordingly, from Fig.4 onward, we refer to this population collectively as "DCs" throughout the text.

Additionally, we observed that the therapeutic efficacy of the combination treatment was significantly reduced in *Batf3*$^{-/-}$ mice lacking cDC1 cells and in mice with cDC1-specific IL-10R knockout (*Xcr1*$^{cre+}$*Il10r*$^{Fl/Fl}$), suggesting an important role of IL-10/IL-10R axis in cDC1 cells. However, these results may not exclude a contribution from cDC2 population to the observed therapeutic effects. Given that both cDC1 and cDC2 subsets showed treatment-associated changes (e.g. **Fig. R1**), and to improve

the logical coherence of the manuscript, we have relocated these data to the supplementary materials in the revised manuscript to maintain focus and clarity in the main figures.

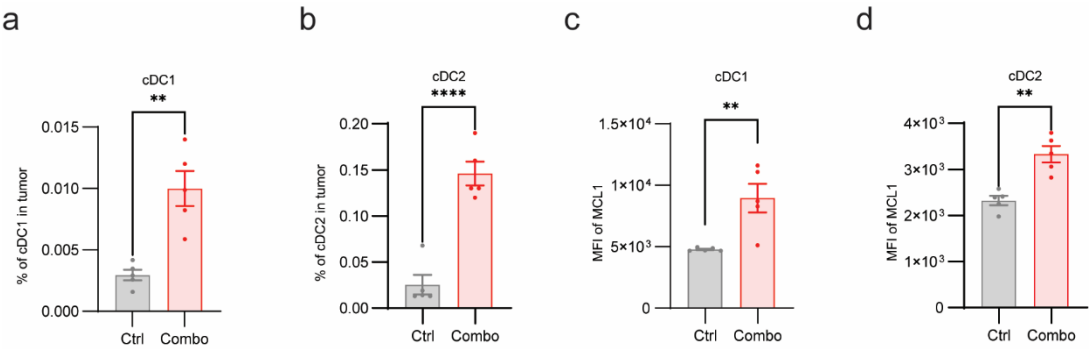

Figure R1. WT C57/BL6 mice (n=5/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA cells. Nine days after tumor cell inoculation, mice were i.p. treated by 80 μg of CmAb-$(IL10)_2$ with 5 μg of CmAb-IL2. Tumor tissues were collected on day 12, and the frequency of cDC1 (a) and cDC2 (b) in tumor; the expression levels of MCL-1 on cDC1 (c) and cDC2 (d) were analyzed by flow cytometry.

(2) Related to Fig. 3d-k, please show UMAPs and dot plots (RNA-seq / cytometry) depicting expression of PD-1 and TIM-3 in the supplementary materials.

We have added UMAP of $CD8^+$ T cells subsets and shown the expression of PD-1 and Tim-3 (related to Fig.3d-g) (**Fig. R2**), which has been added into the supplementary materials (Fig. S3e-3g).

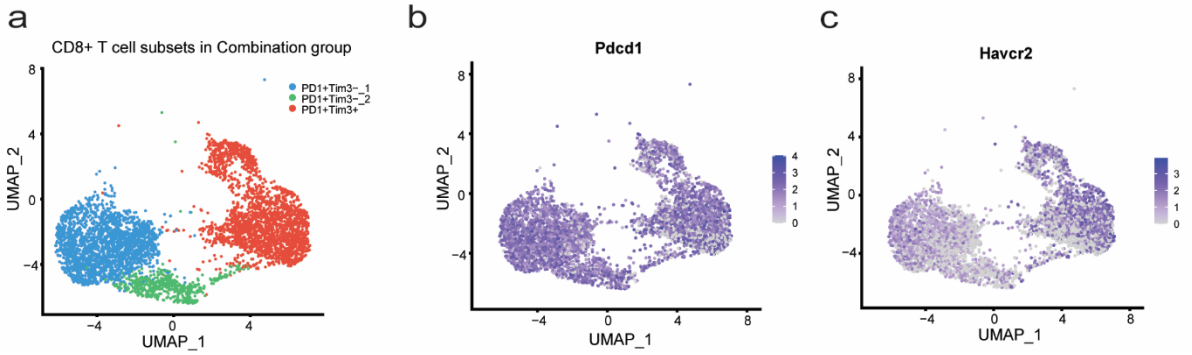

Figure R2. (a) The UMAP analysis of intratumoral $CD8^+$ T cells in the combination treatment group, termed as $PD1^+$ $Tim3^+$, $PD1^+$ $Tim3^-$-1 and $PD1^+$ $Tim3^-$-2. UMAP plots showing the expression of PD-1 (b) and Tim-3 (c).

Additionally, the concatenated dot plot in Fig. 3k is rather confusing and overclustered, especially since it used TCF-1 as one of the markers whereas the authors did not mention it in the manuscript. The authors should reconsider their clustering strategy considering biological relevance. Additionally, they should show exemplary gating strategy that Fig. 3i is based on, since it is difficult to relate the depicted frequencies of antigen-specific CD8+ T cells to selected gates on concatenated dot plots in Fig. 3l.

We thank the reviewer for pointing out this confusion. To avoid over clustering and improve clarity, we have removed Fig. 3k-l. As suggested, we replaced the frequency data of antigen-specific

CD8⁺ T cells with a dot plot (Fig. S4 / **Fig. R3a-b**) and a bar graph (**Fig R3c**), and representative gating strategy has been shown in supplementary materials (Fig. S4).

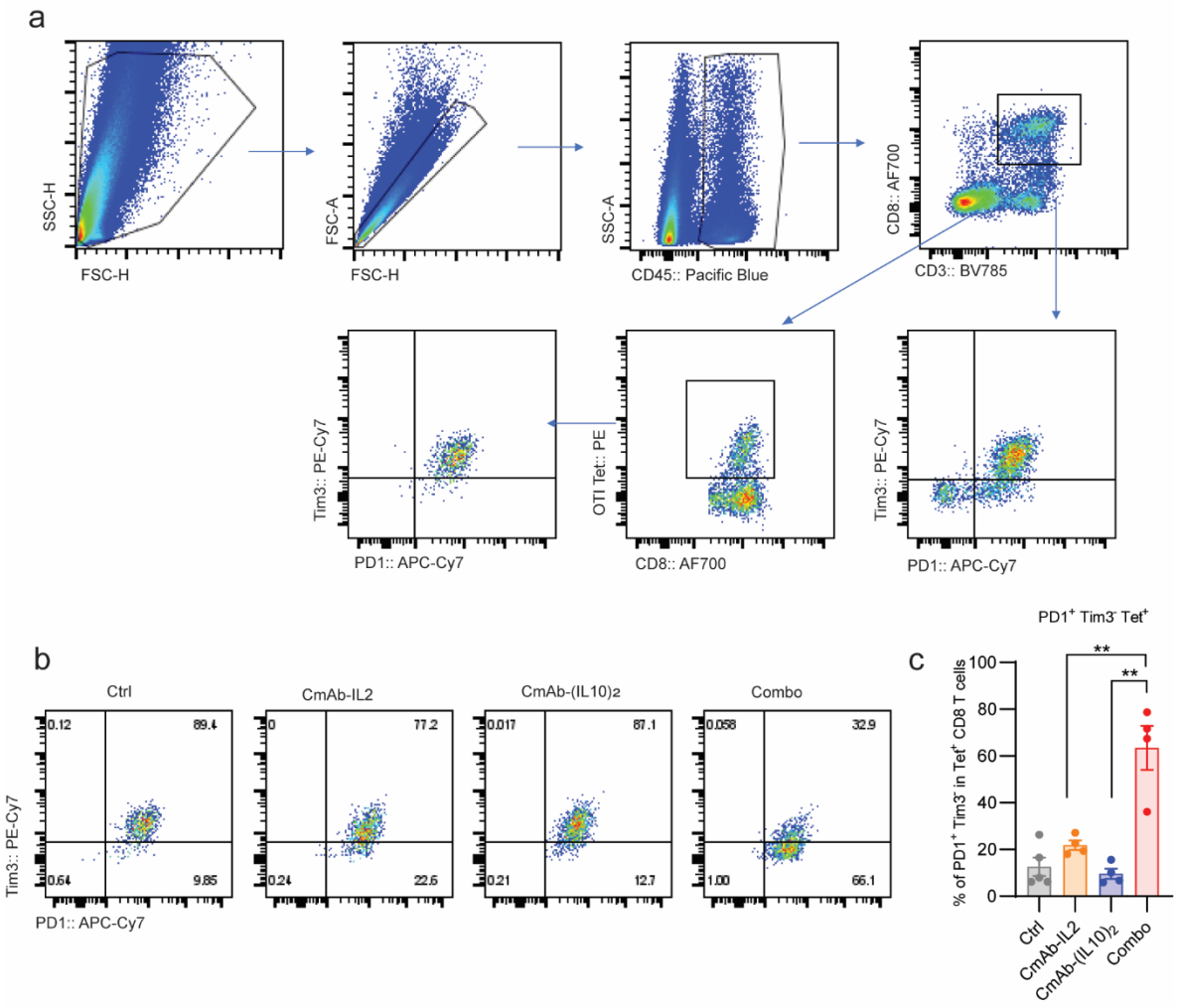

Figure R3. WT C57BL/6J mice (n=4-5/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA and i.p. treated by 80 μg of CmAb-$(IL10)_2$, 5 μg of CmAb-IL2 or the combination on days 9 and 12. Tumor tissues were harvested on day 14, and the frequency of PD1⁺ Tim3⁻ in Tetramer⁺ OVA-specific CD8⁺ T cells in each group was analyzed by flow cytometry. Representative gating strategy and expression pattern of PD-1 and Tim-3 on Tetramer⁺ CD8⁺ T cells in each group (a, b), with corresponding quantification results shown in (c).

(3) Did the authors measure the proliferation of antigen-specific intratumoral CD8+ T cells after treatments?

We assessed the proliferation of intratumoral antigen-specific CD8⁺ T cells by performing Ki67 staining. As shown in **Fig. R4**, treatment with the combination of CmAb-$(IL10)_2$ and CmAb-IL2 significantly increased both the expression level and the frequency of Ki67⁺ cells among antigen-specific intratumoral CD8⁺ T cells, indicating enhanced proliferative activity in these cells.

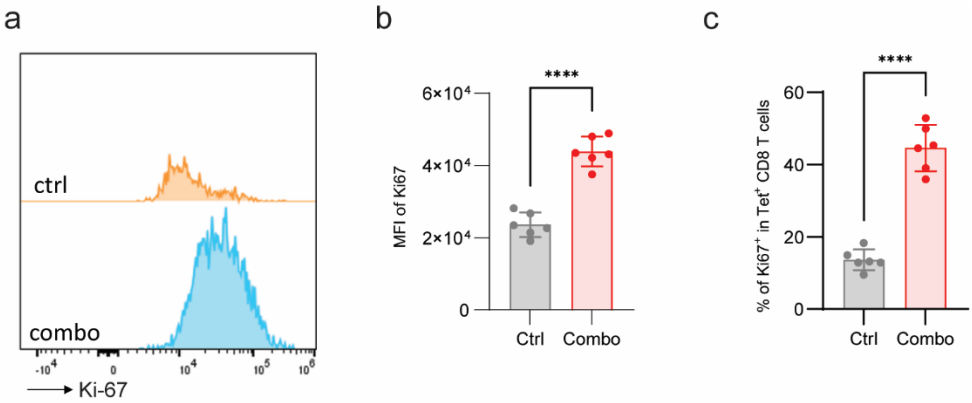

Figure R4. WT C57BL/6J mice (n=6/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA and i.p. treated by 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 days 9 and 12. Tumor tissues were harvested on day 14, and the expression of Ki67 in tetramer$^+$ antigen specific CD8$^+$ T cells (a, b) and the frequency of PD1$^+$ Tim3$^-$ Tetramer$^+$ OVA-specific CD8$^+$ T cells (c) were analyzed by flow cytometry.

(4) Related to Fig. 3e-g and Supplementary Fig. 2, please elaborate on the observed therapeutic effect on tumor growth for CmAb-(IL10)2 in comparison to the previously published work on this molecule (ref. 17). Notably, in their previous work (ref. 17), the authors observed near-curative responses for B16-cEGFR tumors and TC-1-cEGFR tumors by using CmAb-$(IL10)_2$ treatment starting 7-11 days after tumor inoculation. Please describe in detail how the prior results compare to the ones presented in the current manuscript, including the dosing, administration routes, treatment time as well as the distinction that authors took on specifying early-stage and advanced tumors for tumor cell lines used in the study.

Thank you very much for the reviewer's insightful comment. In our previous study (ref17, PMID: 31185213), CmAb-$(IL10)_2$ monotherapy induced near-curative responses in B16-cEGFR and TC-1-cEGFR tumor models when treatment was initiated at an early-stage, with a tumor size cutoff of ~ 50mm^3. Here, our objective was to evaluate whether therapeutic efficacy can be further enhanced by combining CmAb-$(IL10)_2$ with CmAb-IL2. Therefore, the dosing regimen and route of administration of CmAb-$(IL10)_2$ were kept consistent with our previous work (ref17), while to better model a more clinically relevant and challenging setting, we expanded the treatment window to include tumors up to ~ 150 mm^3. Under these conditions, CmAb-$(IL10)_2$ monotherapy showed reduced efficacy relative to our prior report, consistent with the known impact of increased tumor burden on immunotherapy responsiveness. By maintaining dosing while increasing tumor burden, this study allows direct comparison with prior work and highlights the added benefit of combination therapy in a more challenging disease setting.

(5) Did authors quantify changes in T cell counts in peripheral lymphoid organs and the tumor for treatments depicted in Fig. 2e? Related to this, were there any changes in proliferation of T cell subset, including CD8+, conventional CD4+ and Treg cells, after administration of treatments, comparing tumor and peripheral lymphoid organs?

We quantified T cell frequency and proliferation in tumors and peripheral lymphoid organs following CmAb-$(IL10)_2$ plus CmAb-IL2 therapy, with or without FTY720. In tumors, combination therapy increased CD8$^+$ T cells and decreased CD4$^+$ T cells, independent of FTY720. It also enhanced proliferation, as indicated by increased Ki67 expression and frequency of Ki67$^+$ cells among CD8$^+$ T

cells, with similar increases in Ki67 expression in CD4$^+$ and Treg cells and a trend toward increased frequency of Ki67$^+$ cells (**Fig. R5 a-h**). In contrast, analysis of draining lymph nodes (dLNs) showed that T cell subset frequencies were unchanged after combination therapy, and Ki67 expression and the frequency of Ki67$^+$ T cells in dLNs remained substantially lower compared to those observed in tumors. These data further support the conclusion that intratumoral CD8$^+$ T cells are sufficient to mediate tumor control following combination therapy (**Fig. R5 i-p**).

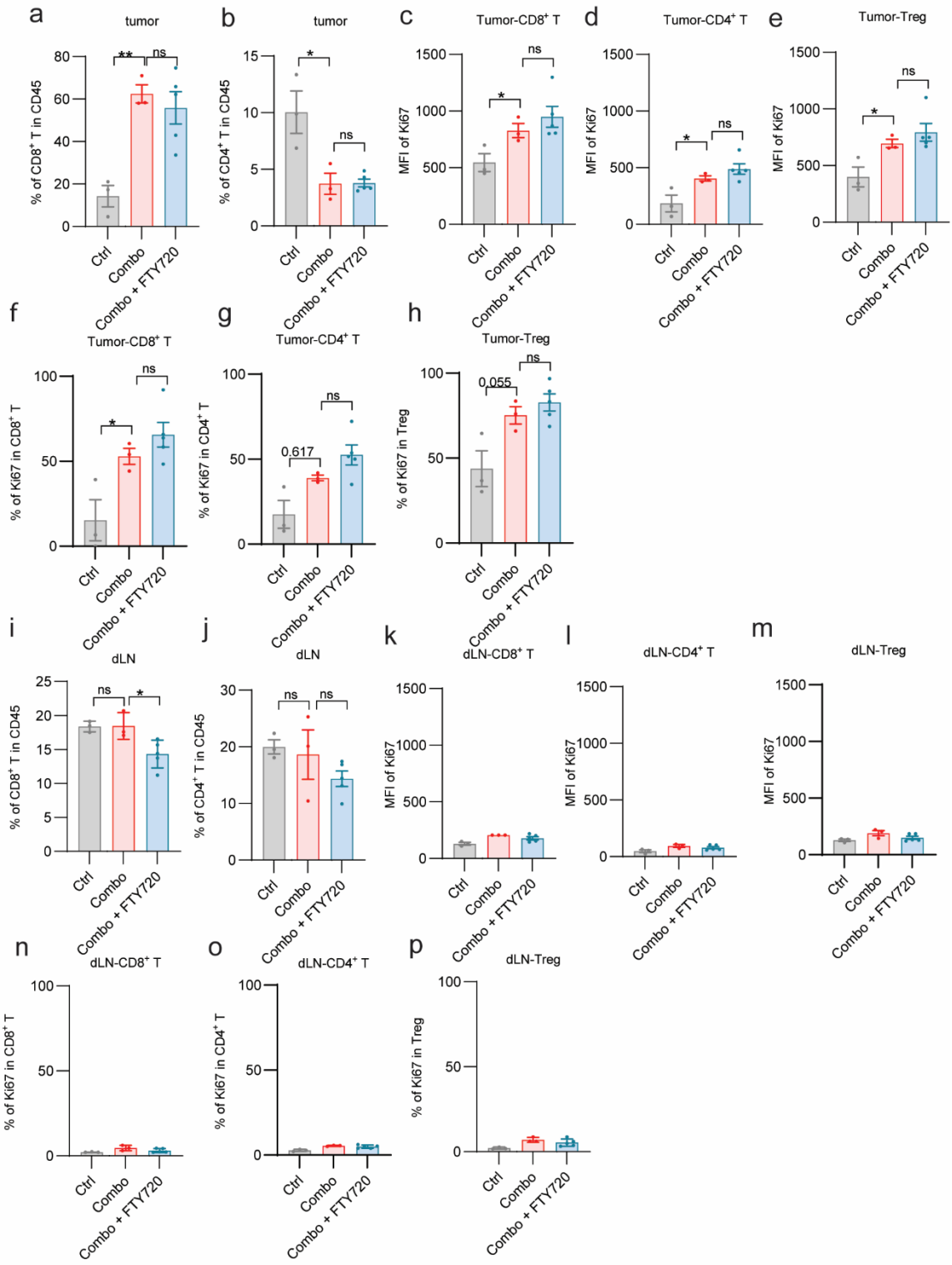

Figure R5. WT C57BL/6J mice (n=3-5/group) were injected subcutaneously with 4×10^5 B16-cEGFR and treated i.p. with 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 on days 9 and 12. To block T cell trafficking, FTY720 was administrated i.p. every other day starting on day 9 for a total of three doses. Tumor tissues and tumor draining lymph node were harvested on day 14. Flow cytometry was used to analyze the frequency of $CD8^+$ T (a) and $CD4^+$ T (b) in tumors; the expression levels of Ki67 in $CD8^+$ T (c), $CD4^+$ T (d) and Treg cells (e) in tumors; the frequency of $Ki67^+$ among $CD8^+$ T (f), $CD4^+$ T (g) and Treg cells (h) in tumors; the frequency of $CD8^+$ T (i) and $CD4^+$ T (j) in dLNs; the expression levels of Ki67 in $CD8^+$ T (k), $CD4^+$ T (l) and Treg cells (m) in dLNs; the frequency of $Ki67^+$ among $CD8^+$ T (n), $CD4^+$ T (o) and Treg cells (p) in dLNs. Data are presented as mean ±SEM. Unpaired t-tests were used for the other data. ns. not significant, $*P < 0.05$ and $**P < 0.01$.

(6) Could the authors elaborate in more detail on the origin of the observed less-exhausted phenotype of CD8+ TILs after combination treatment? Should higher costimulatory molecule levels on antigen-presenting cells and overall higher APC cell counts not translate to more potent antigen presentation in the combination treatment setting?

We thank reviewer's comment. We observed that intratumoral DCs exhibited increased MHC-I and CD40 expressions (Fig. 5j, 5k), and DC IL10R-dependent increase in tumor-specific CD8+T cells within tumors (Fig.4a) following combination therapy. We further show that CmAb-$(IL10)_2$ plus CmAb-IL2 combination therapy expands less-exhausted tumor antigen-specific $CD8^+$ TILs in an IL-10/IL-10R/DC - dependent manner (Fig. 4h-k), indicating that IL-10 signaling in DCs is required for the expansion of this less-exhausted phenotypic $CD8^+$TILs. We therefore propose that the combination therapy preserves DC function, including maintenance of DC mitochondrial fitness (Fig. 5d-i) – rather than simply enhancing classical costimulatory molecule levels. Improved APC quality may sustain antigen presentation in a manner that favors the expansion and maintenance of less-exhausted, tumor- specific $CD8^+$ T cells. This is consistent with prior reports demonstrating that intratumoral APCs play a key role in sustaining stem-like $CD8^+$ T cells during immunotherapy (PMCID: PMC7108171). While intrinsic IL-10/IL-10R signaling in T cells has been reported to limit terminal exhaustion and preserving progenitor-like, less exhausted $CD8^+$ T cells through maintenance of NFAT: AP-1 cooperativity (PMID: 34879221). We cannot exclude the possibility that this direct effect on T cells may also contribute to the less-exhausted phenotype observed with our combination therapy, warranting further investigation in this setting (please also see Reviewer #2 - Point 1).

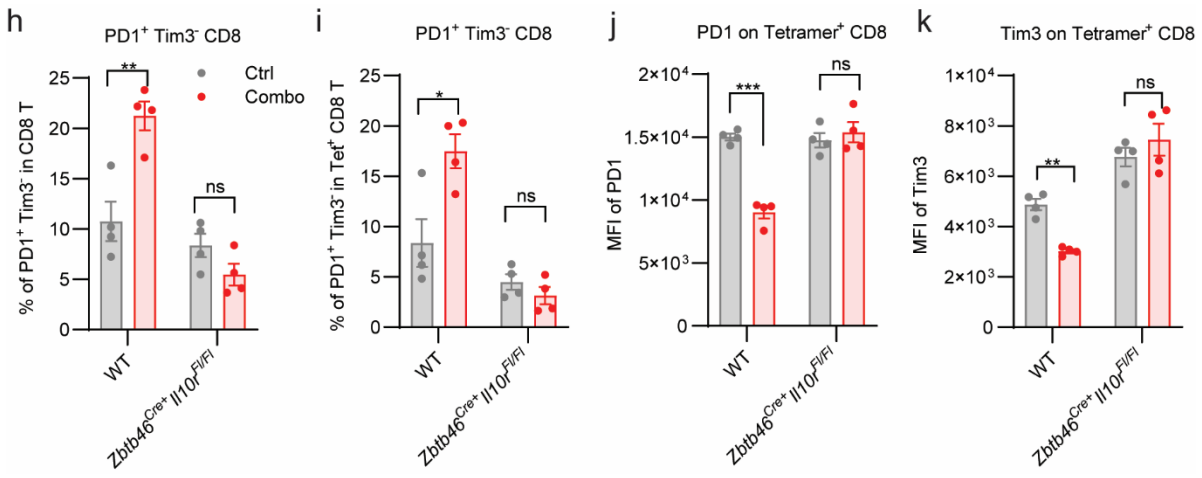

Figure 4 (h-k) WT C57BL/6J and IL-10R knockout specially in DCs (*Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$) mice (n=4/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA cells and i.p. treated with 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 on days 9 and 12 after tumor cell inoculation. Tumor tissues were collected on day 14. The frequency of PD1$^+$ Tim3$^-$ population among CD8$^+$ T cells (h) and tetramer$^+$ CD8$^+$ T cells (i) in the tumor were analyzed. Additionally, the expression levels of PD-1 (j) and Tim-3 (k) on tetramer$^+$ CD8$^+$ T cells were assessed by flow cytometry. The results are shown as mean ±SEM.

(7) How is the T cell metabolism and mitochondrial fitness affected in single/combination treatment settings? Please discuss the functional consequences of CmAb-(IL10)2 treatment for progenitor exhausted vs terminally exhausted CD8+ TILs as well as for regulation of T cell exhaustion, compared to results shown in ref. 38.

We agree that T cell metabolism and mitochondrial fitness are important considerations in the context of our therapeutic modality; however, these respects were not directly assessed and are beyond the scope of the current study. As the reviewer suggested, we have expanded the discussion (Lines 411-424) regarding the distinction between the less-exhausted CD8$^+$ TILs phenotype observed in our study and terminally exhausted CD8$^+$ TILs reported in ref 38. These differences are most likely attributable to distinct therapeutic context and formats. Ref 38 employed the peritumoral administration of IL-10-Fc in adoptively transferred T cells therapy settings, with effects linked to metabolism and mitochondrial regulation. However, in contrast, IL-10/IL-10R signaling also has a cell-intrinsic role in preventing terminal exhaustion of CD8$^+$ T cells while preserving a progenitor-like, less exhausted CD8+T cell population. This effect has been linked to maintenance of NFAT: AP-1 cooperativity (PMID: 34879221, also see the response to Point 6 above and Reviewer #2-Point 1).

Minor comments:

(a) Please double check the manuscript for typing errors, such as on page 6, line 150 (sing-cell RNA sequencing), line 153 (exhaustion-associate genes), including the figures and figure legends: Supplementary Fig. 6b (CD11c1 instead of CD11c), Fig. 5f graph title (cluster, instead of currently existing labels).

Thank you for pointing out. We have checked the typing errors and corrected them.

(b) If available, please provide the quantification in Supplementary Fig. 6b as frequencies of live

CD45+ immune cells for accuracy, not tumor cells.

We have revised Suppl Fig. S7 to include analysis of cDC1 and cDC2 populations as suggested. We performed quantification in both tumors and live CD45$^+$ immune cells. Quantification was performed both within total tumors (cells) and within CD45$^+$ immune cells. Consistent with our original observations, combination therapy increased the abundance of DCs within tumors. However, this increase was not reflected as a frequency of CD45$^+$ immune cells. (**Fig. R6**). We attribute this discrepancy to a marked expansion of T cells within the CD45$^+$ compartment following combination therapy, which reduces the relative frequency of DCs despite their increased absolute presence.

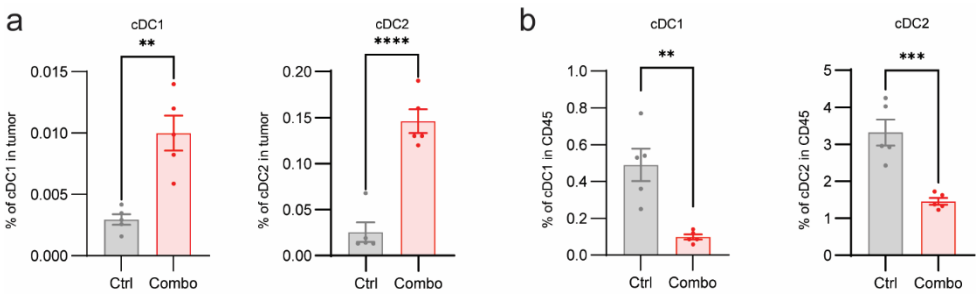

Figure R6. WT C57BL/6J mice (n=5/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA cells and treated i.p. with 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 on day 9 post tumor cell inoculation. Tumor tissues were harvested on day 12, and the frequencies of cDC1 and cDC2 within the tumor (a) or $CD45^+$ TILs (b) were quantified by flow cytometry. Data are presented as mean ±SEM. Unpaired t-tests were used for the other data. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

(c) Please verify the clustering labels in Fig. 5e, supplementary Fig. 7a and corresponding quantification in S7b.

Thank you for pointing out. We apologize for the mislabeled clusters and any confusion caused. We have verified the clustering labels and corrected the figures with right labels.

(d) Please expand the single-cell RNA sequencing and analysis method section to include more details on how exactly the analysis was performed, including the subsetting process for DC analysis.

We have provided more information on scRNA sequencing methods and analysis including for DC analysis, which now are included in Method section (Lines 582-603).

Reviewer # 2 (Remarks to the Author):

The authors present a bispecific cytokine approach that combines a cetuximab-based IL-10 fusion protein, CmAb-$(IL-10)_2$, with a cetuximab-based tumor targeted IL-2, CmAb IL-2, to enhance antitumor immunity while limiting IL-2 associated systemic inflammation. The combination shows improved tumor control compared with either monotherapy across multiple murine tumor models. Mechanistically, the authors propose that antitumor efficacy requires IL-10R signaling in dendritic cells, particularly cDC1s, where IL-10 preserves mitochondrial integrity and survival through reduced mitochondrial reactive oxygen species (ROS) and increased Mcl-1, thereby sustaining antigen presentation and expansion of less-exhaustedPD1$^+$Tim3$^-$ tumor specific $CD8^+$ T cells. In parallel, they proposed that the combination therapy mitigates high-dose IL-2–driven systemic inflammation through an IL-10R-dependent mechanism in macrophages, suggesting that therapeutic efficacy and systemic protection are mediated by distinct cellular programs.

This is a technically strong and conceptually interesting study supported by extensive conditional genetics and multi-modal immune profiling. The dendritic cell-centered IL-10 axis is plausible and potentially differentiating, and the macrophage-dependent protection is supported by genetic necessity. The main limitations relate to mechanistic resolution and framing. In particular, the manuscript does not adequately reconcile its conclusion that directIL-10 signaling in CD8+ T cells is dispensable with prior literature supporting direct IL-10-driven functional rescue of exhausted CD8+ TILs. In addition, the downstream mechanism by which macrophages mediate systemic protection is insufficiently defined, and the Mcl 1-centered dendritic cell survival model is not tested with direct perturbation. Finally, characterization of the CmAb IL-2 construct is limited. Specific comments are listed below.

We sincerely thank the reviewer for the thoughtful evaluation of our work. We truly appreciate the reviewer for the recognition of our genetic approach, multi-modal immune profiling, and dendritic cell-centered IL-10 axis conceptually compelling. We address each specific comment/concern in detail below.

1. The manuscript concludes that IL-10 primarily acts through dendritic cells rather than directly on CD8 positive T cells, based on conditional loss of IL-10r in CD8+ cells showing preserved combination efficacy and preserved expansion of tumor antigen specific CD8 positive T cells. This interpretation requires clearer reconciliation with prior evidence that IL-10 can directly enhance terminally exhausted CD8+ TILs via metabolic reprogramming (NatImmunol 2021;22:746-56). The two models may reflect different boundary conditions, such as cytokine format and exposure kinetics, disease context, or the presence of IL-2 shifting the limiting step toward antigen presentation rather than intrinsic T cell metabolism. The discussion should explicitly address these possibilities. A focused analysis of the CD8+compartment would further strengthen the manuscript, for example testing whether terminal exhaustion programs and metabolic signatures are present and whether they depend on dendritic cell IL-10R signaling rather than T cell IL-10R signaling.

We thank the reviewer for this insightful comment and agree that our findings should be more explicitly discussed with prior evidence demonstrating a direct role of IL-10 in $CD8^+$ T cells. In our study, we do observe evidence supporting a cell-intrinsic contribution of IL-10 signaling in $CD8^+$ T cells. As shown, $CD8^+$ T cell–specific IL-10R deficiency reduces total and tumor antigen–specific $CD8^+$ T cell frequencies (Fig. S6 a-b) and impairs antitumor efficacy relative to WT controls (Fig. S6c), whereas combination therapy in these mice still leads to better tumor control than CmAb-IL2 monotherapy (Fig. S6c). Our work represents a key finding that is complementary but not disrupting the previous findings where IL-10 can directly act on $CD8^+$ T cells.

As the reviewer suggested, to address the direct effects on $CD8^+$ T cells explicitly including enhancing terminally exhausted $CD8^+$ TILs, we have revised the discussion to highlight potential context-dependent effects. Differences in cytokine format, exposure kinetics, and therapeutic setting likely influence the relative contribution of IL-10 signaling across cell type. For example, IL-10-Fc administration peritumorally in combination with adoptive T cell transfer therapy, can directly enhance terminally exhausted $CD8^+$ TILs through metabolic and mitochondrial regulation (PMID: 34031618). In contrast, IL-10/IL-10R signaling in $CD8^+$ T cells has also been reported to preserve progenitor-like, less exhausted $CD8^+$ T cells by maintaining NFAT:AP-1 cooperativity (PMID: 34879221). In our system, the combination therapy of IL-10 and IL-2 may shift toward/amplify DC-mediated $CD8^+$ TILs phenotype and responses. While at the same time, we cannot exclude those direct effects of IL-10 on $CD8^+$ T cells, including modulation of exhaustion states, also contribute to the phenotype observed with the combination therapy of CmAb-$(IL10)_2$ plus CmAb-IL2. We have revised the manuscript to incorporate these considerations and to better position our findings by refining, rather than contradict, the current understanding of IL-10 biology (Lines 411-424).

We performed a more focused analysis of total and tumor antigen specific $CD8^+$ T cells, directly comparing WT and *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice following combination therapy. CmAb-$(IL10)_2$ plus CmAb-IL2 significantly increased the frequency of PD-1^+ Tim-3^- $CD8^+$ T cells in tumors from WT mice, but not in *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice (Fig. 4h). Consistently, analysis of tumor antigen–specific $CD8^+$ T cells revealed that the combination treatment similarly elevates the proportion of PD-1^+ Tim-3^- tetramer$^+$ $CD8^+$ T cells in WT mice but not in *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice (Fig. 4i). In addition, combination therapy

reduced PD-1 and Tim-3 expression on tetramer$^+$ CD8$^+$ T cells (Fig. 4j, k), consistent with a less exhausted phenotype. These data support a requirement for IL-10/IL-10R signaling in dendritic cells in promoting less-exhausted CD8$^+$ TILs following combination therapy. We agree that defining metabolic programs would provide further insight; however, this is beyond the scope of the current study and will be addressed in future work (see also Reviewer #1 - Point 7).

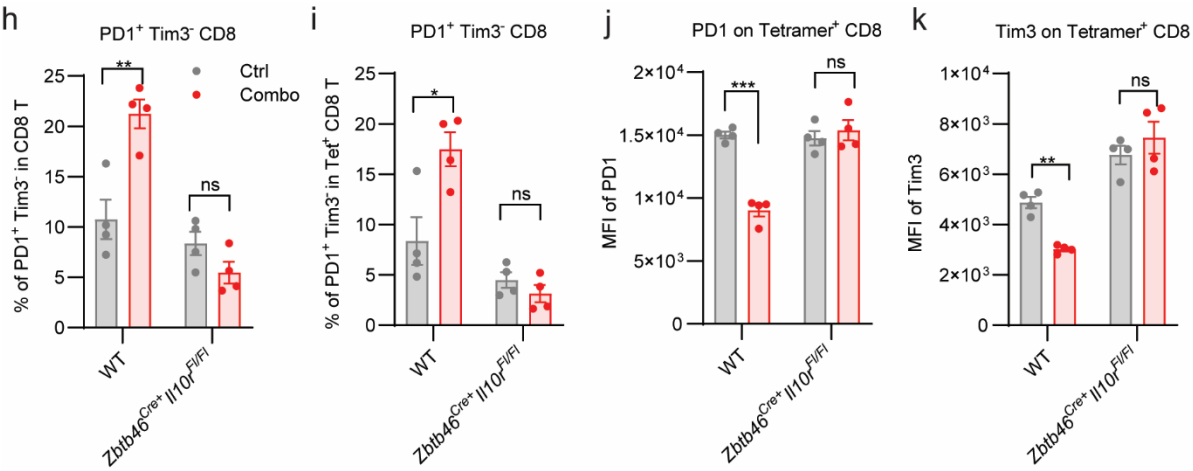

Figure 4 (h-k) WT C57BL/6J and IL-10R knockout specially in DCs (*Zbtb46^{cre+}Il10r$^{Fl/Fl}$*) mice (n=4/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA cells and i.p. treated with 80 μg of CmAb-(IL10)$_2$ and 5 μg of CmAb-IL2 on days 9 and 12 after tumor cell inoculation. Tumor tissues were collected on day 14. The frequency of PD1$^+$ Tim3$^-$ population among CD8$^+$ T cells (h) and tetramer$^+$ CD8$^+$ T cells (i) in the tumor were analyzed. Additionally, the expression levels of PD-1 (j) and Tim-3 (k) on tetramer$^+$ CD8$^+$ T cells were assessed by flow cytometry. The results are shown as mean ±SEM.

2. The macrophage axis is supported by genetic necessity but remains mechanistically underdefined. However, the manuscript does not define the key macrophage population involved, the dominant tissue source of systemic cytokines, or downstream pathways mediating protection. Serum cytokines and AST are supportive endpoints, but they do not establish mechanisms. The manuscript would be strengthened by identifying the relevant myeloid compartment and tissue context, for example liver, spleen, or circulating myeloid cells, and by adding pathway level evidence consistent with an IL-10 anti-inflammatory program in macrophages or reduced inflammatory cytokine production at the cellular source.

We thank the reviewer for these thoughtful comments. We agree that, although our genetic data support a requirement for the IL-10/IL-10R/macrophage axis, the precise cellular and tissue context, as well as downstream pathway would further extend the mechanistic depth of the study. However, we respectfully note that the primary objective of this work is to establish the functional requirement of this axis in controlling IL-2 induced toxicity, rather than to provide a comprehensive dissection of macrophage subsets and tissue context. To strengthen the mechanistic framework within this scope and based on our current results along with the reports implicating TNF-α as a one of the key drivers in IL-2 associated toxicity and the role of IL-10 in anti-inflammatory program in macrophages. we performed additional experiments targeting this pathway. Systemic administration of anti-TNF-α

antibody significantly reduced HD-CmAb-IL2 induced inflammatory cytokines and serum AST, providing direct evidence that TNF-α is a key downstream mediator in this setting.

'We also note that the IL-10/IL-10R/Macrophage axis is unlikely to be explained by a single, well-defined cellular source or linear pathway. Instead, it likely integrates both direct and indirect mechanisms. In addition to suppressing the production of inflammatory mediators such as TNF-α and IL-6, this axis may also regulate a broader immune network, potentially through intermediates like TNF-α (Fig. S10), involving multiple inflammatory cell types. Through these combined effects, it may contribute to the reduction of IL-2- induced toxicity. A definitive dissection of macrophage subsets, tissue- context dependency, and downstream pathways remain to be further investigated".

This discussion (Lines 434-441) along with the new data (**Fig. R7**), has been included in the revised manuscript (Fig. S10). A definitive dissection of macrophage subsets, tissue context dependency, and associated downstream pathways will require dedicated studies in order to fully elucidate this system - level regulatory mechanism and therefore lies beyond the scope of the present work. Nevertheless, it represents an important direction for future investigation.

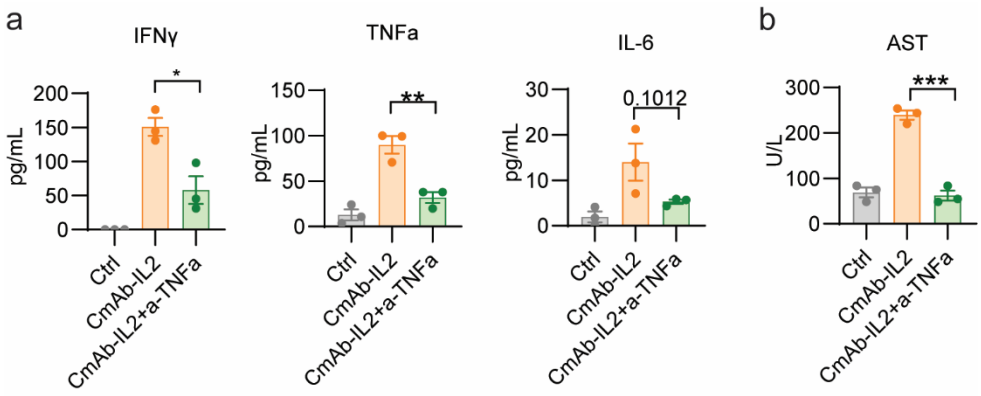

Figure R7. WT C57BL/6 mice were i.p. treated with PBS, or 20 μg of CmAb-IL2 with or without i.p. administrated with a TNFa blocking antibody on days 0 and 2. Cytokines level (a) and AST (b) were measured on day 4. Data are presented as mean ±SEM. Unpaired t-tests were used for the other data. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

3. The proposed dendritic cell mechanism places Mcl-1 as a key mediator linking IL-10R signaling to dendritic cell survival and mitochondrial integrity. The association is supported by the genetic requirement for dendritic cell IL-10R signaling and by accompanying mitochondrial readouts and Mcl-1 changes. However, direct causal testing of the Mcl-1 axis is limited. SinceMcl-1 is central to the mechanistic model, at least one perturbation of the Mcl-1 pathway would strengthen the conclusions.

We thank the reviewer for this important suggestion. We show that the combination therapy upregulates MCL-1 expression in DCs (Fig. 5g. h). As suggested, to directly test the MCL-1 axis, we treated JAWSII cells, a murine DC cell line, with CmAb-$(IL10)_2$ in the presence or absence of an MCL-1 inhibitor and assessed mitochondrial integrity using JC-1 fluorescence. Consistent with our model, IL-10 treatment increased the JC-1 red/green ratio, indicating improved mitochondrial integrity. Importantly, this effect was attenuated upon addition of the MCL-1 inhibitor, demonstrating that perturbation of the MCL-1 pathway compromises IL-10–mediated mitochondrial protection (**Fig. R8**). The quantification data (Fig. R8b) has been included in revised manuscript (Fig. S8d). These findings provide functional evidence supporting a causal role for MCL-1 in linking IL-10R signaling to

mitochondrial integrity in DCs, supporting our genetic findings that DC-intrinsic IL-10R signaling is required to maintain mitochondrial health.

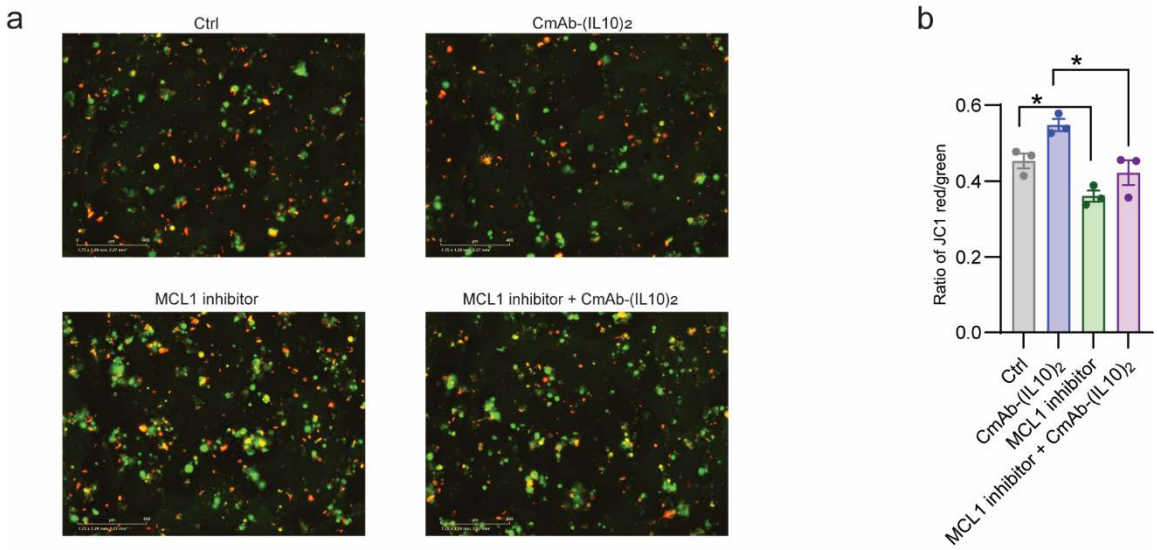

Figure R8. DC cell line (JAWSII) was treated with the indicated reagents and stained with JC-1 (5 µM) for 3 hours (a). The ratio of intracellular red to green fluorescence of JC-1 (invitrogen™) was assessed using Incucyte™ (b). Data are presented as mean ±SEM. Unpaired t-tests were used for the other data. *P < 0.05.

4. The CmAb IL-2 construct requires more complete characterization. The Methods describe production and purification and basic quality assessment, but the manuscript does not provide sufficient biochemical and functional definition of CmAb IL-2 as a standalone agent, including binding parameters to EGFR and IL-2 receptor, stability, and receptor engagement potency relative to recombinant IL-2. Given that the therapeutic rationale depends on altered exposure and toxicity, these data would improve interpretability and reproducibility.

We thank the reviewer for this insightful suggestion. Indeed, CmAb-IL2 has been previously evaluated by our group related to toxicity and functional studies (PMID: 31185213, PMID: 31462678). To further address this point, we performed additional experiments with biochemical and functional analyses. We confirmed that CmAb-IL2 binds EGFR with similar specificity to Cetuximab and engages IL-2 receptor comparably to the IL-2-Fc fusion protein. Functionally, CmAb-IL2 stimulates STAT5 signaling at levels comparable to or modestly higher than recombinant IL-2 (**Fig. R9**).

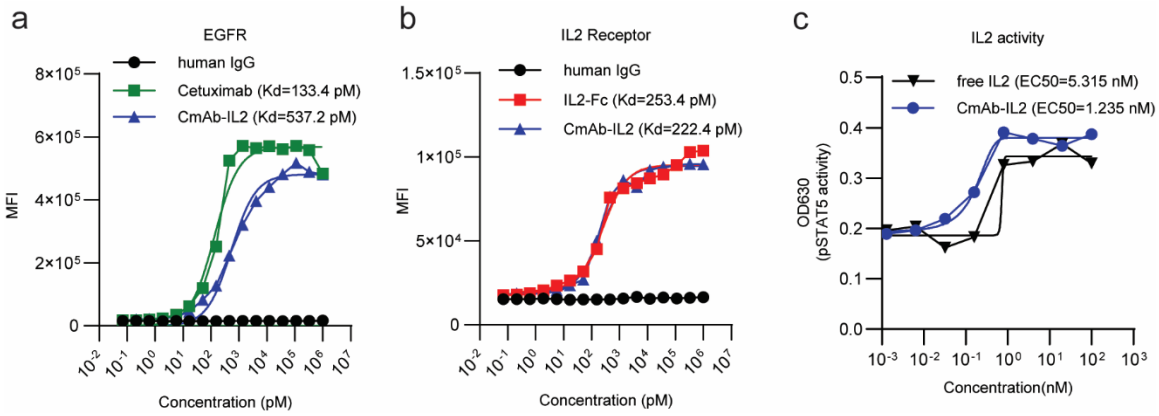

Figure R9. (a), B16-cEGFR cells were incubated with Cetuximab or CmAb-IL2 fusion protein, followed by anti-human IgG-PE staining. (b), HEKBlue™ IL-2 reporter cells were incubated with IL2-Fc or CmAb-IL2 fusion protein, followed by anti-human IgG-PE staining. (c), HEKBlue™ IL-2 reporter cell assay was used to determine the functional activity of CmAb-IL2 and recombinant IL-2 (free IL-2) protein.

5. The dosing strategy and translational framing of IL-2 require clarification. The manuscript employs lower IL-2 doses in efficacy studies and higher doses in toxicity assessments, while the discussion intermixes concepts of tolerated-dose and high-dose IL-2. However, the rationale underlying dose selection and the operational definitions of “low-dose” and “high-dose” IL-2 are not clearly articulated. Importantly, the authors do not sufficiently address how the high-dose IL-2 regimen used in mice relates to clinically relevant high-dose IL-2 toxicity in humans, which is historically defined by capillary leak syndrome and systemic inflammatory sequelae. The authors should more explicitly define the intended clinical context for each dosing regimen and justify the translational relevance of the murine high-dose model. Moreover, they should avoid implying that systemic protection alone enables clinically meaningful high-dose IL-2 use, unless superior antitumor efficacy under high-dose conditions is directly demonstrated through appropriate comparative experiments.

We thank the reviewer for raising this point regarding dosing rationale and translational interpretation. In this study, we define a “low dose” of CmAb-IL2 (LD-CmAb-IL2) as a well – tolerated dose and a “high dose” (HD-CmAb-IL2) as one that induces measurable systemic inflammatory responses. These designations are based on the assessment of both antitumor activity and induction of inflammatory cytokines, as shown in Fig. S1. LD-CmAb-IL2 is associated with lower efficacy and minimal systemic inflammation, whereas HD-CmAb-IL2 produces greater antitumor activity but is accompanied by elevated inflammatory cytokines. Importantly, combining CmAb-$(IL\text{-}10)_2$ with either dosing regimen improves efficacy compared to monotherapy (Fig.1g and Fig.S9).

We agree that the current model does not directly replicate clinically defined low- or high-dose IL-2 regimens in humans, and that mitigation of systemic toxicity alone does not justify clinical use of high-dose IL-2. Accordingly, we have removed language implying direct equivalence to clinical high-dose IL-2 therapy. The discussion has been revised to clarify that our intent is to model two biological IL-2 exposure states: a low, well-tolerated condition versus a condition associated with systemic inflammatory toxicity- rather than to quantitatively recapitulate clinical dosing (Lines 442-456: “For the proof of concept… to further determine whether this strategy can enhance efficacy while reducing toxicity in patients”).

Reviewer #3 (Remarks to the Author):

Sun et al. investigate the therapeutic combination of tumor-targeted IL-2 and IL-10 delivered as antibody–cytokine conjugates for the immunotherapy of solid tumors. The authors employ a tumor-targeted IL-10 construct (CmAb-(IL-10)\$_2$) and a tumor-targeted IL-2 construct (CmAb-IL-2), administered either as monotherapies or in combination.

The manuscript advances several important claims:

(i) endogenous IL-10 plays a critical role in mediating the antitumor effects of IL-2;

(ii) combined CmAb-(IL-10)\$_2$ and CmAb-IL-2 therapy preferentially expands less-exhausted intratumoral CD8$^+$ T cells;

(iii) CD8$^+$ T cells are essential for the therapeutic efficacy of tumor-targeted cytokine antibodies;

(iv) IL-10 receptor signaling in dendritic cells is required for the effect of CmAb-(IL-10)\$_2$ on T cell responses, as shown by conditional IL-10R deletion in DCs using Zbtb46^Cre, with a proposed mechanism involving altered mitochondrial metabolism; and

(v) IL-10 receptor signaling in macrophages limits IL-2–driven immune-related adverse events(irAEs), particularly liver toxicity, as demonstrated using LysM^Cre-mediated IL-10R inactivation.

Overall, the study is well performed, and the data are clearly presented. The work is potentially important, as it proposes a novel strategy to mitigate IL-2–associated immune-related adverse events while preserving antitumor efficacy. The demonstration that dendritic cells respond directly to IL-10 and that this signaling pathway modulates CD8$^+$ T cell–dependent immunotherapy responses is particularly interesting and conceptually novel. However, several minor concerns currently limit the impact of the study.

We sincerely thank the reviewer for the constructive evaluation of our manuscript. We are particularly grateful for the recognition that our work proposes a novel strategy to mitigate IL-2 - associated toxicity while preserving antitumor efficacy, and that the demonstration of direct IL-10 signaling in dendritic cells and its impact on CD8$^+$ T cell-dependent immunotherapy responses is considered conceptually novel and potentially important. We appreciated the reviewer's assessment of the overall study design and data presentation. In response to the reviewer's minor concerns, we have carefully revised the manuscript to further strengthen clarity and impact.

Major and Minor Comments

1. Requirement for tumor targeting

The manuscript does not clearly establish whether tumor targeting is required for IL-10–mediated modulation of IL-2 therapy. Additional evidence demonstrating that tumor localization of IL-10 is essential for the observed effects would strengthen this conclusion.

We thank the reviewer for this comment. Our prior studies demonstrated that tumor targeted delivery of IL-10 (PMID: 31185213) or IL-2 (PMID: 31462678) is required for effective antitumor efficacy. To directly test whether IL-10 targeting is necessary for modulation of IL-2 therapy, we performed additional experiments comparing tumor-targeted IL-10 (CmAb-(IL10)$_2$) with non-targeted

IL-10 (TmAb-$(IL10)_2$). As shown in Fig. R10, tumor-targeted IL-10 significantly enhanced tumor-targeted IL-2 mediated antitumor efficacy, whereas equivalent doses of non-targeted IL-10 failed to recapitulate this effect when combined with either tumor-targeted IL2 (**Fig.R10**, orange line) or non-tumor targeted IL-2 (**Fig. R10**, blue line). These data demonstrated that tumor-targeted delivery of IL-10 is required for its modulation of IL-2 therapy.

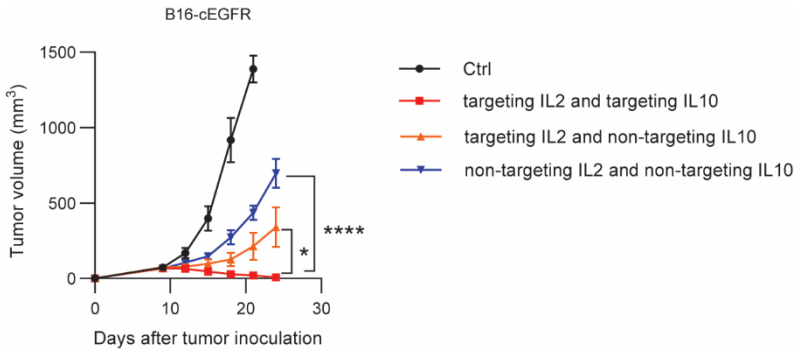

Figure R10. B16-cEGFR tumor-bearing C57BL/6 mice (n=5/group) were treated i.p. with PBS, targeting IL-2 and targeting IL-10 (CmAb-IL2 and CmAb-$(IL10)_2$), targeting IL-2 and non-targeting IL-10 (CmAb-IL2 and TmAb-$(IL10)_2$), or non-targeting IL-2 and non-targeting IL-10 (IL2-Fc and TmAb-$(IL10)_2$) on days 9, 12, and 15. Tumor volumes were measured, and the data are presented as means ± SEM. Two-way ANOVA tests were used to analyze the tumor growth data. *$P < 0.05$ and **** $P < 0.0001$.

2. Comparison with non-targeted cytokines

It remains unclear whether non-targeted IL-2 and IL-10 exhibit similar immunological and therapeutic profiles. Direct comparison with untargeted cytokines would help clarify the added value of the targeting strategy.

We agree with the reviewer. As detailed above (Point 1), we performed direct comparisons between targeted and non-targeted cytokines. Non-targeted cytokine therapy did not reproduce the therapeutic effects observed with tumor-targeted cytokine (**Fig. R10**, blue line vs. red line), supporting the importance of the targeting strategy.

3. Activity in non-EGFR–expressing tumors

The authors should clarify whether the targeted cytokines retain activity in EGFR-negative tumor models (e.g., non-EGFR–expressing B16 variants or other tumor lines). This would address the generalizability of the approach.

We thank the reviewers for this point. To assess dependence on EGFR expression, we evaluated efficacy in EGFR-negative B16 parental tumors. As shown in **Fig. R11**, the combination of CmAb-$(IL10)_2$ and CmAb-IL2 showed substantially reduced activity in EGFR-negative tumors compared to the pronounced tumor regression observed in EGFR-expressing B16-cEGFR tumors (see the results above in response to Point -1). These findings indicate that therapeutic efficacy depends on tumor targeting, highlighting the potential importance of tumor-targeted delivery in cytokine therapy.

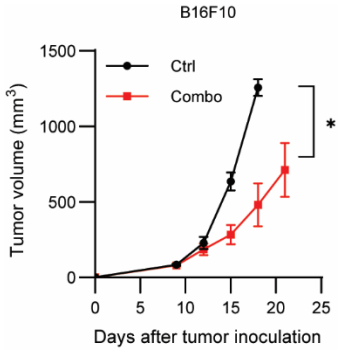

Figure R11. B16F10 tumor-bearing C57BL/6 mice (n=5/group) were treated i.p. with PBS, 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 combination on days 9, 12, and 15. Tumor volumes were measured, and the data are presented as means ± SEM. Two-way ANOVA tests were used to analyze the tumor growth data. *$P < 0.05$.

4. Depth of $CD8^+$ T cell functional characterization

The characterization of $CD8^+$ T cell responses in treated wild-type mice remains limited. Inclusion of polyfunctionality assays—ideally in antigen-specific settings—would substantially strengthen the conclusions. Ex vivo assays assessing tumor-infiltrating lymphocyte function following TCR engagement would be particularly informative.

We thank the reviewer for this insightful suggestion. In response, we have expanded the functional characterization of $CD8^+$ T cell responses by incorporating polyfunctionality analyses. Treated wild-type mice exhibited a significant increase in polyfunctional tumor antigen–specific (tetramer$^+$) $CD8^+$ tumor-infiltrating lymphocytes (TILs), as evidenced by enhanced production of IFNγ, TNFα, and granzyme B (**Fig. R12**). These data have been added into the revised manuscript (Fig. S5). In addition to the directly assessing tumor antigen specific $CD8^+$ TILs function, we performed *ex vivo* restimulation assays to further evaluate their effector capability. TILs isolated from treated mice showed enhanced antigen specific $CD8^+$ T cell effector function upon restimulation, whereas no such increase was observed following stimulation with an irrelevant antigen (**Fig. R13**). This data has been added into the revised manuscript (Fig. 3n).

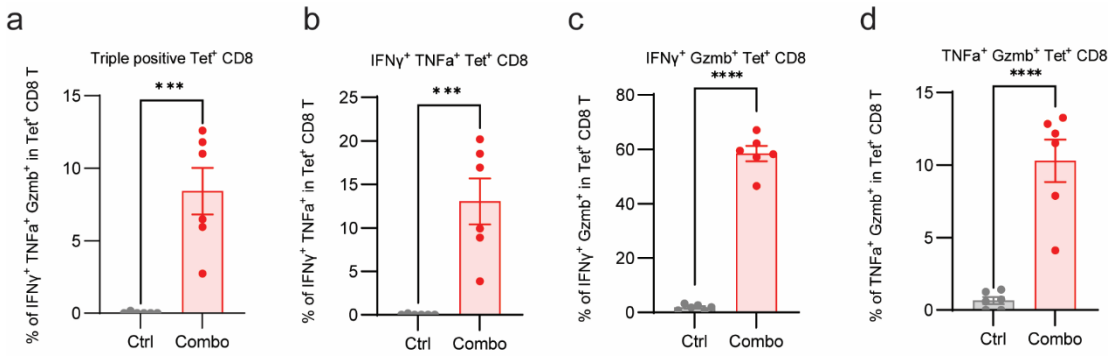

Figure R12. B16-cEGFR-OVA tumor-bearing C57BL/6 mice (n=6/group) were i.p. treated with 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 on days 9 and 12 after tumor cell inoculation. Tumor tissues were collected on day 14, and single-cell suspensions of tumor tissue were stimulated with PMA and Ionomycin in the presence of BFA (Brefeldin A) at 37°C for 4 hours. The frequencies of $IFN\gamma^+$ $TNFa^+$

GzmB$^+$ (a), IFNγ$^+$ TNFa$^+$ (b), IFNγ$^+$ GzmB$^+$ (c), TNFa$^+$ GzmB$^+$ (d) among OVA-specific Tetramer$^+$ CD8$^+$ T cells in the tumor tissue were analyzed by flow cytometry. Data are presented as mean ±SEM. Unpaired t-tests were used to analyze the data. ***P < 0.001 and ****P < 0.0001.

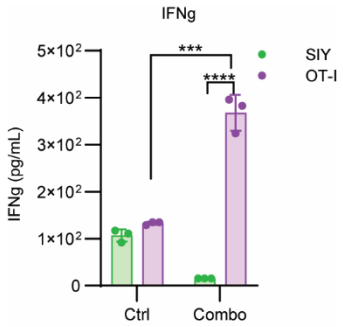

Figure R13. B16-cEGFR-OVA tumor-bearing C57BL/6 mice (n=6/group) were i.p. treated with 80 μg of CmAb-(IL10)$_2$ and 5 μg of CmAb-IL2 on days 9 and 12 after tumor cell inoculation. Tumor tissues were collected on day 14, and the CD45$^+$ TILs were isolated and pooled. Subsequently, 2×10^5 CD45$^+$ TILs were stimulated with either control peptides (SIY) or specific antigen peptides (OT-I) for 3 days. IFNγ secretion in the culture supernatant was measured by BD CBA assay. Each dot represents an individual experimental replicate. Data are presented as mean ±SEM. Unpaired t-tests were used to analyze the data. ***P<0.001 and ****P<0.0001.

5. Assessment of T cell exhaustion markers

Additional markers such as CD39 and TOX would allow a more precise assessment of CD8$^+$ T cell exhaustion status and would complement the current phenotypic analysis.

In response, we have incorporated CD39 as an additional marker to better evaluate the exhaustion status of CD8$^+$ T cells within tumor. Our results show that treatment with CmAb-(IL10)$_2$ in combination with CmAb-IL2 increases the frequency of less-exhausted CD8$^+$ T cells, characterized as PD-1$^+$ Tim-3$^-$ CD39$^-$ (**Fig. R14a**). In addition, this combination therapy increases the proportion of functional CD8$^+$ T cells, defined as PD-1$^+$ Tim-3$^-$ CD39$^-$ IFN-γ$^+$ cells (**Fig. R14b**). These data have been incorporated into the revised manuscript (Fig. 3i. m).

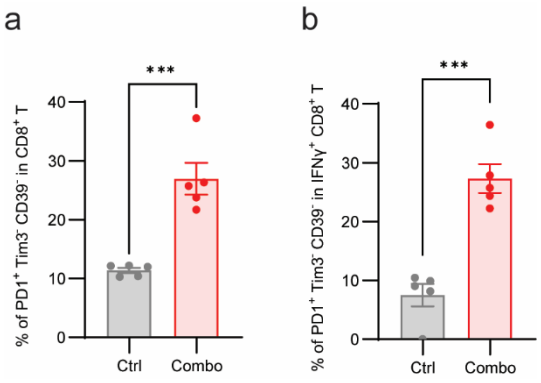

Figure R14. B16-cEGFR tumor-bearing C57BL/6 IFNγ-YFP mice (n=5/group) were i.p. treated with 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 on days 9 and 12 after tumor cell inoculation. Tumor tissues were harvesteded on day 14; the frequency of $PD1^+$ $Tim3^-$ $CD39^-$ among either total $CD8^+$ T cells (a) or $IFN\gamma^+$ $CD8^+$ T cells (b) in the tumor tissue were analyzed by flow cytometry. Data are presented as mean ±SEM. Unpaired t- tests were used to analyze the data. ***$P < 0.001$.

6. DC subset-specific metabolic analysis

The Mitotracker analysis presented in Figure 5A would be more informative if performed at the level of dendritic cell subsets, specifically comparing $XCR1^+$ (cDC1) and $SIRP\alpha^+$ (cDC2) populations.

We thank the reviewer for this suggestion. We have now analyzed mitochondrial function in tumor-infiltrating DC subsets. Both $XCR1^+$ (cDC1) and $SIRP\alpha^+$ (cDC2) from *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice exhibited increased JC-1 green fluorescence compared to WT controls, indicating elevated mitochondrial dysfunction (**Fig. R15**). These data support a role for DC-intrinsic IL-10R signaling in maintaining mitochondrial health (Please also see the response to Reviewer #1-Point 1).

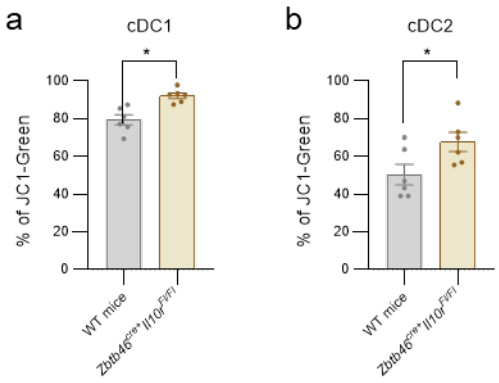

Figure R15. WT C57BL/6 mice and *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice (n=6/group) were injected subcutaneously with 4×10^5 of B16-cEGFR cells. Tumor tissues were collected on day 9 post-tumor cell inoculation. Mitochondrial dysfunction in cDC1 (a) and cDC2 (b) cells was stained by JC-1 and assessed by flow cytometry. Data are presented as mean ±SEM. Unpaired t-tests were used to analyze the data. *$P < 0.05$.

7. Antigen presentation and cross-presentation

Ex vivo analysis of antigen presentation and cross-presentation of tumor-associated antigens by myeloid cells from control and treated mice would further support the proposed mechanism involving dendritic cells.

We thank reviewer for this comment. Our study is primarily aimed at defining a mechanism by which IL-10/IL-10R signaling in DCs enhances intratumoral cDC function to promote tumor antigen-specific $CD8^+$ T cell responses. We attempted to isolate DCs from tumors for *ex vivo* antigen

presentation and cross-presentation assays; however, we were unable to obtain sufficient numbers of viable DCs to perform these experiments reliably.

To address this, we employed an in vivo approach. We administrated exogenous DCs derived from wild-type (WT) or IL-10R-deficient mice intratumorally into *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice. This strategy enabled direct assessment of whether WT DCs could restore or enhance tumor-specific CD8$^+$ T cell responses in a setting where endogenous DCs lack IL-10R signaling. Our results show that WT DC transfer significantly increased tumor antigen–specific CD8$^+$ T cells compared to *Il10r*$^{-/-}$ DCs (Fig. 4c-e). Co-administration experiments by transferring a mixture of WT and *Il10r*$^{-/-}$ DCs intratumorally further showed higher MHC-I and sustained CD80/CD86 expression in WT DCs (Fig. 5i-n). Consistently, tumor antigen-specific CD8$^+$ T cell responses depend on DC-intrinsic IL-10R signaling (Fig. 4a,4i). Together, although *ex vivo* cross-presentation assays were not feasible, these in vivo functional and phenotypic data support the conclusion that IL-10/IL-10R signaling enhances DC antigen presentation capacity, thereby promoting tumor-specific CD8$^+$ T cell immunity.

8. Specificity of LysM^Cre-mediated IL-10R deletion

Given that LysM^Cre also affects subsets of dendritic cells, it would be valuable to directly compare Zbtb46^Cre and LysM^Cre models using the same readouts, including:

o CD8$^+$ T cell–mediated antitumor immunity (Figure 5), and

o IL-2–mediated liver toxicity (Figure 6)

In addition, evidence of IL-10–driven anti-inflammatory programs in liver macrophages (Kupffer cells) or liver dendritic cells would strengthen the conclusions regarding mitigation of irAEs.

We thank the reviewer for highlighting the specificity of *LysM-Cre* (*Lyz2-Cre*) mediated deletion. Although *LysM-Cre* targets a minor subset of DCs, its activity is largely restricted to myeloid populations. Consistent with this, *Lyz2*$^{Cre+}$*Il10r*$^{Fl/Fl}$ mice showed no impairment in tumor antigen-specific CD8$^+$ T cell responses or tumor control in CmAb-(IL10)$_2$ plus CmAb-IL2 treated mice (Fig. S6d–f), indicating that major DC populations driving CD8$^+$ T cell responses are not substantially affected.

As suggested, we further analyzed CD8$^+$ T cell responses. Combination therapy with CmAb-(IL10)$_2$ and CmAb-IL2 increased tumor antigen–specific CD8$^+$ T cells, including PD1$^+$Tim3$^-$ tetramer$^+$ cells, and reduced PD-1 and Tim-3 expression on tetramer$^+$ CD8$^+$ T cells within tumors (Fig. R16). These data indicate that, in contrast to *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice, CD8$^+$ T cell – mediated antitumor immune responses remain effective in *Lyz2*$^{Cre+}$*Il10r*$^{Fl/Fl}$ mice. (**Fig. R16**).

To assess IL-2 mediated liver toxicity and the potential contribution of liver-resident Kupffer cells and DCs to IL-10 driven anti-inflammatory programs and mitigation of irAEs, we performed additional experiments using the limited number of available *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ mice. We observed a trend toward reduced AST levels in mice treated with high-dose CmAb-IL2 in combination with CmAb-(IL10)$_2$, although this difference did not reach statistical significance, likely due to limited sample size (**Fig. R17**). While these data suggest a possible contribution that liver dendritic cells would be involved in reduction of liver toxicity; our overall findings support a more dominant role for macrophages in this process. This point has been addressed in greater details in response to Reviewer #2 (Point 2) and incorporated into the revised manuscript (Lines 434-441).

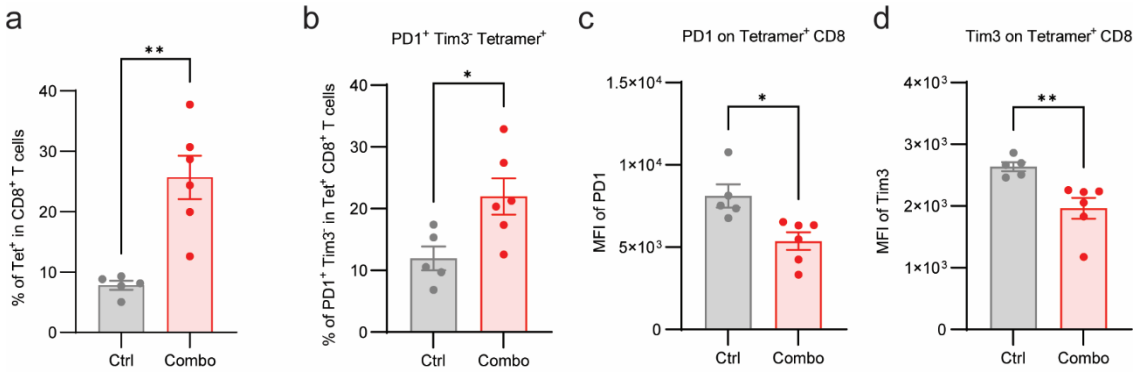

Figure R16. *Lyz2*$^{cre+}$*Il10r*$^{Fl/Fl}$ C57BL/6 mice (n=5-6/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA cells, and i.p. treated with PBS, 80 μg of CmAb-(IL10)$_2$ and 5 μg of CmAb-IL2 on days 9 and 12. Tumor tissues were collected on day 14. The frequency of OVA antigen- specific CD8$^+$ T cells (a); the frequency of PD1$^+$ Tim3$^-$ among Tetramer$^+$ CD8$^+$ T cells (b); the expression levels of PD-1 (c) and Tim-3 (d) on Tetramer$^+$ CD8$^+$ T cells in the tumor tissue were analyzed by flow cytometry. Data are presented as mean ±SEM. Unpaired t-tests were used to analyze the data. *P<0.05 and **P<0.01.

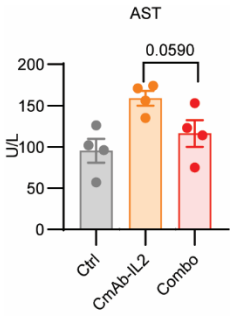

Figure R17. *Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$ C57BL/6 mice (n=4/group) were i.p. treated with PBS, 20μg of CmAb-IL2, 20 μg of CmAb-IL2 with 80 μg of CmAb-(IL10)$_2$ on days 0 and 3. The levels of AST in serum were measured on day 4. Data are shown as mean ±SEM. Unpaired t-tests were used to analyze the data.

9. Biodistribution of the IL-10 antibody conjugate

Visualization of the biodistribution of the IL-10 antibody–cytokine conjugate would be valuable. In particular, demonstrating whether IL-10–responsive myeloid cells localize near tumor cells decorated with the targeting conjugate would provide important mechanistic insight.

We appreciate the reviewer for this comment. The design of this IL-10 antibody-cytokine fusion protein is specifically intended to enable tumor-targeted delivery of IL-10. Our previous work (PMID: 31185213) together the current data highlight the importance of tumor-targeted delivery for achieving effective IL-10 mediated antitumor tumor immune responses, either as monotherapy or in combination with IL-2 (please also see responses to Points 1-3 above). Following delivery and retention within tumors, IL-10 is expected to act on multiple responsive populations, including myeloid and T cells (see also Reviewer #2 - Point 1). Accordingly, physical clustering of IL-10–responsive myeloid cells specifically around tumor cells decorated with the targeting conjugate may not be essential for achieving therapeutic efficacy; however, this still requires specific validation. We agree with the reviewer that

direct visualization of biodistribution, as well as high-resolution spatial analysis of tumor - immune cell would provide valuable mechanistic insight, however, these studies are beyond the scope of the current work, and such studies, including spatial and dynamic colocalization analyses will be pursued in future investigations.

Summary

In summary, this is a well-executed and conceptually interesting study that addresses an important challenge in cytokine-based cancer immunotherapy. Addressing the points

We thank the reviewer for the insightful evaluation of our work. In response, we have strengthened the mechanistic framework with additional data and analyses and clarified the translational relevance in the context of cytokine-based cancer immunotherapy.

Reviewer #1 (Remarks to the Author):

Sun et al. present a revised version of their study on how combined immunotherapy of tumor-targeted IL-10 (CmAb-$(IL10)_2$) and tumor-targeted IL-2 (CmAb-IL2) exerts antitumor immune responses and limits immune-related adverse events (irAEs). The authors have addressed most of our previous concerns, which has significantly improved the manuscript. Below are a few remaining points.

(1) Please elaborate on the differences in frequencies of PD1+ Tim3- tetramer+ CD8+ T cells between results obtained in WT mice after combination therapy presented in Fig. 4i (average for combination therapy approx. 17%) and in Fig. R3c (average for combination therapy approx. 65%) following the same treatment settings.

We thank the reviewer for highlighting this discrepancy. The data in Fig. 4i and Fig. R3c were obtained from independent experiments performed under the same treatment conditions, and the difference likely reflects inter-experimental variability, including staining assay for flow cytometry acquisition and biological variation. To address this concern, we pooled data from another independent experiment and replaced the original Fig. 4i data with the combined dataset (Fig R1). Importantly, the pooled analysis supports the same conclusion that the frequency of PD1+ Tim3- tetramer+ CD8+ T cells is increased in WT mice after combination therapy, and the overall interpretation remains unchanged.

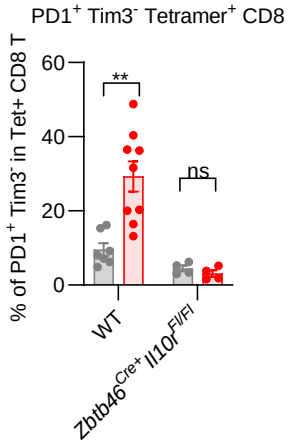

Fig R1. WT C57BL/6J (n=7-9/group) and IL-10R knockout specially in DCs (*Zbtb46*$^{cre+}$*Il10r*$^{Fl/Fl}$) mice (n=4/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA cells and i.p. treated with 80 μg of CmAb-$(IL10)_2$ and 5 μg of CmAb-IL2 on days 9 and 12 after tumor cell inoculation. Tumor tissues were collected on day 14. The frequency of PD1$^+$ Tim3$^-$ population among tetramer$^+$ CD8$^+$ T cells in the tumor were analyzed. The results are shown as mean ±SEM. ns, not significant, **P < 0.01.

(2) Related to Figure R6, please provide absolute cell counts of cDC1 and cDC2 subsets in the tumor.

We thank the reviewer for this suggestion. In the original Fig. R6, we presented the frequencies of cDC1 and cDC2 subsets relative to tumor cells, which demonstrated their increased abundance following combination therapy. To further address the reviewer's comment, we have now included the absolute numbers of cDC1 and cDC2 cells normalized to tumor mass (Fig. R2). These additional data confirm that combination therapy significantly increases the intratumoral abundance of both cDC1 and cDC2 subsets, further supporting our original findings.

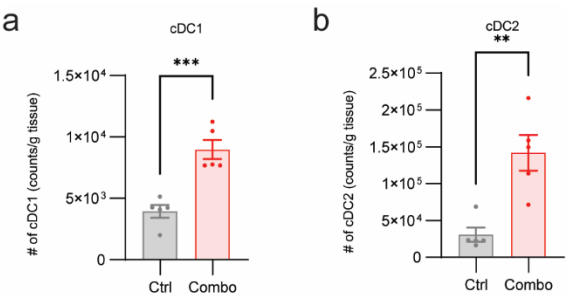

Fig R2. WT C57/BL6 mice (n=5/group) were injected subcutaneously with 4×10^5 B16-cEGFR-OVA cells. Nine days after tumor cell inoculation, mice were i.p. treated by 80 μg of CmAb-$(IL10)_2$ with 5 μg of CmAb-IL2. Tumor tissues were collected on day 12, and the number of cDC1 (a) and cDC2 (b) in tumors were quantified by flow cytometry and normalized to tumor mass.

(3) Performing a pseudobulk analysis of the data presented in Fig. 3e-g would be better than a Wilcoxon test on single cell data with large cell numbers as currently performed.

We thank the reviewer for this suggestion. In Fig. 3e-g, our objective was not to perform a comprehensive differential gene expression analysis, but rather to assess the expression of key exhaustion - and effector- associated genes (e.g., *Tox*, *Ifng*, and *Gzmb*) across the indicted CD8+T cell subsets. These analyses were included to support our conclusion that the combination therapy promotes a less-exhausted and more functional state within $PD1^+$ $Tim3^-$ $CD8^+$T cell subset. We agree that pseudobulk analysis can provide a more informative and rigorous framework for differential expressing testing. However, in our experimental design, the scRNA-seq libraries for each group were generated from pooled tumor tissues (n = 6 mice per group) and sequenced as a single sample without individual mouse-specific barcoding. Consequently, biological replicates cannot be deconvolved from the dataset, which precludes the application of standard

pseudobulk differential expression methods (e.g., DESeq2) that require replicate-level variance estimation. Importantly, together with the independent flow cytometry data, these findings corroborate our interpretation, and the absence of pseudobulk analysis does not affect the conclusions of the study.

(4) The relevance and basis of clustering and analysis presented in Fig. 5d-e and Fig. S8a-b and is confusing.

• If the data indeed reflect tumor-infiltrating CD45+ CD3- cells that were clustered in the initial UMAP shown in Fig. S8a, the overall clustering pattern and following DC cluster annotation is unusual. Three distinct clusters currently annotated as DC clusters 1-3 are spatially separated and belong to larger, currently unannotated clusters (cells connected to current DC clusters 1 and 3). Please provide the basis for annotating the selected clusters as dendritic cells (by providing lineage and functional marker expression). Additionally, please provide cell type annotation and validation in the form of marker expression for all major (currently unannotated) clusters in Fig. S8a, as it appears that there are marked differences in the overall non-T cell (CD45+ CD3- cells) composition between the treatments.

We thank the reviewer for this important comment. To clarify the basis for cell-type annotation including DCs, we have now provided the marker genes used to define all clusters identified in the $CD45^{+}CD3^{-}$ cell dataset in Supplementary Table S1. In addition, annotations for major clusters have been added to the revised UMAP shown in Fig. R3. The UMAP visualization and cell cluster annotation were performed using standard Seurat workflows. Samples from control and combination treatment groups were merged and processed together to enable direct comparison of cluster distributions across conditions. As noted by the reviewer, the $CD45^{+}$ $CD3^{-}$ compartment comprises diverse immune populations and the updated annotations reveal differences in cluster enrichment between the groups. We agree that a detailed characterization of these broader compositional changes would be informative. However, because the objective of these analyses was to investigate the DC populations underlying the mechanism described in this study, we have therefore restricted our analysis to the DC clusters directly relevant to the study conclusions. We believe that the additional annotations and marker-gene information provided in the revised manuscript adequately address the reviewer's concern and clarify the basis for the analyses presented in Fig. 5d-e and Fig. S8a-c.

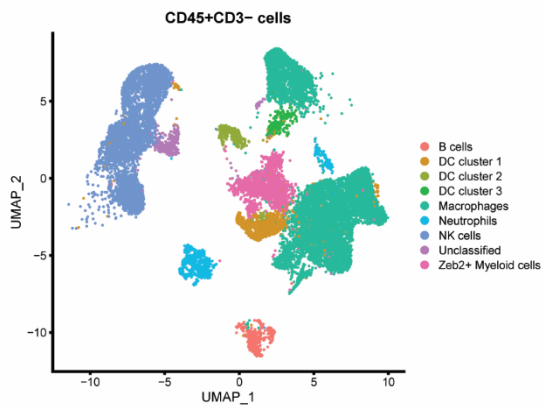

Fig R3. UMAP visualization of scRNA-seq data of intratumoral CD45^{+} CD3^{-} cells from control and the combination treatment group.

• After providing the above-mentioned validation, please elaborate on the unusual spatial distribution of currently existing DC clusters. What are the cells currently neighbouring the DC clusters 1 and 3? Why are those cells (belonging to currently unannotated cell lineage) spatially closer based on UMAP coordinates than other dendritic cell subclusters?

We thank the reviewer for this question. Based on the comprehensive cluster annotation provided above, the cell populations adjacent to DC clusters 1 and 3 are predominantly macrophages and other myeloid cell populations (Fig. R3). Importantly, UMAP proximity is driven by overall transcriptional states and does not necessarily reflect lineage relationships. Accordingly, the spatial separation of DC clusters reflects distinct transcriptional states, suggesting the transcriptomic remodeling of intratumoral DCs following combination therapy. Likewise, the adjacent macrophages and myeloid populations neighboring the DC clusters may also reflect broader treatment-associated transcriptional changes within the tumor myeloid compartment. While a detailed characterization of these additional myeloid populations would be of interest, it is beyond the primary focus of the current study. Further investigation of these treatment-induced changes across the broader intratumoral myeloid compartment will be pursued in future studies.

• Were the cell numbers evenly downsampled between treatments during generation of UMAPin Fig. S8a?

In the original analysis, cell numbers were not downsampled between the groups for UMAP generation. The control group contained 12,903 cells and the combination treatment group contained 6,235 cells. While the cell numbers differ between groups, the UMAP embedding was computed on the merged dataset to enable direct comparison of cluster distributions across treatment conditions, which is a standard approach in single-cell analyses.

Reviewer #2 (Remarks to the Author):

The revised manuscript is substantially improved. The additional clarifications regarding the dendritic cell centered IL-10R mechanism, macrophage dependent attenuation of systemic inflammation, and characterization of the CmAb-IL2 construct are helpful. My only remaining concern relates to the newly added experiment shown in Fig. S8d. I appreciate the authors'effort to interrogate the MCL-1 pathway using an MCL-1 inhibitor and to assess mitochondrial integrity by JC-1 staining. However, the increase in the JC-1 red/green ratio induced by CmAb-(IL10)2 alone appears modest and does not seem to reach statistical significance. Thus, while the inhibitor result is directionally consistent with the proposed model, the current data do not provide strong evidence that CmAb-(IL10)2 significantly enhances mitochondrial integrity through MCL-1 in this assay. I therefore suggest that the authors temper the wording related to this point. A more balanced interpretation would be that MCL-1 inhibition attenuates a trend toward IL-10 mediated improvement in mitochondrial integrity, thereby supporting, but not definitively proving, the involvement of the MCL-1 pathway.

We thank the reviewer for this thoughtful comment and suggestion. In response, we have revised the manuscript to provide a more cautious interpretation (Lines 283-285).

Reviewer #3 (Remarks to the Author):

The authors have successfully adressed my concerns, in most cases by bringing additional experimental materials that clarify the mechanisms of action of tumor targeted IL10. In particular, it is now clearer that IL10 or IL2 requires tumor targeting for efficiency. The point if further made clear by the implementation of EGFR negative tumors.

The CD8+ T cell response is now characterized with state-of-the-art polyfunctionality assay, thereby establishing the relevance of observed changes awarded by immunotherapy.

Exhaustion markers have been included like CD39.

Also the role of IL10R on mitochondrial function in tumor-infiltrating DC subsets is now better established with a distinction of DC1 and DC2s.

Adoptive transfer of DCs in the context of IL10R deficiency now demonstrates that IL10R signaling promotes antigen presentation.

Finally specificity issue raised by the implementation of Lyz2Cre+Il10rFl/Fl are aleviated by the fact that these mice showed no impairment in tumor antigen-specific CD8+ T cell responses or tumor control in CmAb-(IL10)2 plus CmAb-IL2 treated mice, thereby contrasting with DC-specific depletion.

Altogether the study is much improved and highlight a novel mechanism that can be activated to improve anti-tumor immunity.

We sincerely thank the reviewer for the thoughtful and insightful evaluation of our work.

We greatly appreciate the careful assessment of our revisions and the recognition that the additional experiments and analyses have addressed the reviewer's concerns and strengthened the manuscript.

We are also grateful for the reviewer's recognition of the significance of our findings and the mechanistic insights advanced by this study.

Reviewer #4 (Remarks to the Author): I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.