

CX3CR1⁺ CD8⁺ Cytotoxic T Cells Drive Tumor Killing in Esophageal Squamous Cell Carcinoma During Neoadjuvant Therapy

Corresponding Author: Professor Zhihua Liu

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)

Esophageal squamous cell carcinoma (ESCC) is one of the leading cancers with high mortality in East Asia, causing a significant health burden in this region of the world. ESCC generally does not respond well to immune checkpoint inhibitor (ICI) monotherapy. However, a combination of ICI with chemotherapy as neoadjuvant therapy is emerging as a promising treatment for this disease. The underlying mechanisms for clinical responses to chemoimmunotherapy remain unclear, and this lack of knowledge is one of the hurdles in the rational design of this regimen for treating ESCC. This report takes a comprehensive approach using omics techniques to dissect tumor biology and immune responses in the context of chemoimmunotherapy leveraging on their current clinical trials. The knowledge base built by this report is fundamental for clinical implications. However, some details of clinical data and relevant issues from previous studies in this field have not been thoroughly considered and addressed, which may compromise the novelty of this report.

The details of clinical features of ESCC patients are missing for what chemotherapy (platinum or taxane) used in this study? What kind of immunotherapy (anti-PD-1 or PD-L1 antibody, etc.) used in this study? Patient information about their age, sex, etc. Importantly, when the biopsy were collected during the chemoimmunotherapy. What is the exact definition or timing of “pre-therapy”, i.e. were these patients are treatment naïve or after prior immunotherapy.

The identification of Gp1 and Gp2 tumor types regarding their sensitivity to chemoimmunotherapy is interesting but lacks further consideration in the context of immunogenicity. The authors did not show whether the expression of genes encoding antigen presentation in ESCC tumor cells differs between Gp1 and Gp2 tumors based on their RNA-seq data. Are there any differences between Gp1 and Gp2 tumors in their expression of HLA I or II molecules, PD-L1, and Ki67? MUC1 is mainly identified in Gp2, but as one of the outstanding tumor antigens expressed by tumor cells, MUC1 should be validated for their RNA-seq data with IHC or IF to confirm the differential expression of MUC1 in Gp1 and Gp2 tumor tissues. The immunoediting score is an attractive marker for selecting potential therapy-sensitive tumors after therapy, but the authors did not clarify whether chemotherapy or immunotherapy would be involved in immunoediting that would eliminate tumors with nonsynonymous mutations. Additionally, the decrease of T-NK cells in the post-therapy group has not been clearly explained, particularly in these well responders (Fig. 1d). Of note, the method of calculating “cell percent in sample” should be added in the Methods section with sufficient details.

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As reflected by the title of this report, one major finding in immune responses to chemoimmunotherapy for ESCC is the increase of CX3CR1⁺ CD8⁺ T cells in these patients. This finding aligns with the discovery reported by Yan et al. in JCI Insight (2018) in patients with metastatic melanoma after chemoimmunotherapy. Yan et al. reported that CX3CR1

expression increased in peripheral blood CD8 T cells in responders to chemoimmunotherapy and defined a unique feature of CX3CR1+ CD8 T cells that can withstand the toxicity of chemotherapy while remaining responsive to ICI therapy. They also identified that CX3CR1+ CD8 T cells are effective cytotoxic cells with granzyme B expression (both by flow cytometry and IHC in melanoma tissues) and high degranulation capability (CD107a assay). While this report confirmed the high cytotoxic capacity of CX3CR1+ CD8 T cells in patients with ESCC, but authors need to validate whether the CX3CR1+ CD8 T cells isolated from well responders with ESCC would also express granzyme B and mobilize CD107a in degranulation assay, as this is missing from their RNA-seq data set and in vitro study (Fig. 5a, 5d, and 5e).

The tumor-killing ability of freshly isolated CX3CR1+ CD8 T cells was tested in comparison with other subsets of CD8 T cells. However, the authors did not show whether the freshly isolated CX3CR1+ CD8 T cells are tumor-antigen specific or not. If they are not, are they able to kill tumor cells due to alloantigen expression? Additionally, it is unclear whether CX3CR1+ CD8 T cells need to be preactivated before being used in the 4-hour tumor-killing assay. Details are missing from their 2-day culture of CX3CR1+ CD8 T cells in the Methods section, specifically whether these cells were cultured with or without tumor antigen. Notably, normal human T cells would lose their viability and functionality over 2 days of culture without any antigen stimulation or cytokine supplements. If CX3CR1+ CD8 T cells can overcome these limitations, it would be another notable feature of this type of T cell and should be highlighted in this report.

Similar to what Yan et al. reported, CX3CR1+ OT-1 CD8 T cells have superior antitumor activity compared to CX3CR1- OT-1 CD8 T cells in adoptive transfer therapy for B16-OVA tumors. The authors confirmed the expansion of CX3CR1+ OT-1 CD8 T cells in their ESCC-OVA model. However, the authors should test whether the transfer of CX3CR1+ OT-1 CD8 T cells can suppress the growth of ESCC-OVA tumors in vivo. Of note, gating of CD8 T cells for CX3CR1 expression should be added with CD3 or TCR beta marker to exclude mouse dendritic cells or NKT cells that may also express CD8 (extended Fig. 9). Lastly, it is suggested that the authors discuss to what degree this work presents distinguished novelty from Yan et al.'s study.

Reviewer #2

(Remarks to the Author)

Dear Authors, I read your manuscript "CX3CR1+ CD8+ Cytotoxic T Cells Drive Tumor Killing in Esophageal Squamous Cell Carcinoma During Neoadjuvant Therapy" with interest.

This is a study of the TME in surgical patients, treated with either chemo and ICB or chemo alone. Tumors were studied for scRNAseq, TCR, WES. confirmatory studies were done in vitro and in an animal model . scRNAseq suggested that there is a signature for pts who displayed a good response that was in contrast to (Gp2) pts who exhibited higher expression of chemo resistance and immune evasion.

study points to two specific T-cell phenotypes that are upregulated and exhausted respectively, in pts who respond well to therapy.

The manuscript is well written but editing would make it stronger. ICI (check point inhibition) is a more common abbreviation than ICB for example.

I have the following comments that I hope will be helpful to the authors:

- 1) did the time from therapy to collection differ in the group of patients? Is it possible that the selective expansion/exhaustion of a specific cell phenotype can also be time dependant? For instance, did all pts have similar ability to show a good response, or are there intermediate sequences? Was the phenotype time dependant in any way?
- 2) Was chemo or ICB same in the whole cohort of pts? Would it be reasonable to add this information to methods as well as stage of disease (range) of treated pts? There are chemo groups that are known to produce a hot tumor, and variability in the chemo within your study would be of interest.
- 3) indicating homogeneity in the sample would be of interest. Were there pts that are HPV+, or MSI-High, and was PDL-1 or CPS score available on the sample set? correlating the highly responsive pts with known molecular markers of prognosis and therapy response would be of interest.
- 4) was there a difference in the CD8+ subgroup in pts treated with Immune checkpoint inhibition compared to those w chemo only, and was there a correlation to CPS/PDL-1 score?

Reviewer #3

(Remarks to the Author)

Authors present here the analysis of an interesting dataset obtained by sc-RNAseq, TCRseq and WES performed on samples coming from 52 ESCC patients recruited in a phase III to evaluate the efficacy of a combination of anti-PD1 antibody and chemotherapy in a neoadjuvant setting. In particular, they observe that a population of non-exhausted cytotoxic CD8+ expressing CX3CR1 seems to expand in patient experiencing a tumor regression. On the opposite, the exhausted CXLC13+ CD8+T cells population, generally associated with response to immune checkpoint blocker in other tumor types is observed contracting upon treatment in responders. This finding is reminiscent of the results published by Cappuyns et al.,

Nature Communication 2023, where clonally expanded CD8 Temra (CX3CR1+) and not exhausted CD8 T cells were found to be associated with response to an anti-PD-L1 + anti-VEGF therapy in advanced hepatocellular carcinoma, suggesting a similar characteristic of action of PD1 blockers therapy in both ESCC and HCC. If confirmed, this could be particularly valuable to orient the development of specific therapies and response biomarkers.

Sadly, this manuscript seems suffers from several discrepancies in the way the results have been generated when comparing the main text, the figures and the methods, questioning the robustness of the observations made. The bioinformatic analysis seem to have been performed according to standard practice in the field but the link to the analysis code leads to the home page of Github which makes difficult any check on this. The absence of direct access to the data although available from the corresponding author upon reasonable request, limit the scientific community benefit from this study. At least an online data exploratory interface would have been appreciated. This manuscript also contains one in-vitro and one in-vivo mouse experiment supposed to support the findings. Unfortunately, the setup, results and analysis are not convincing.

Given the initial potential of this study, I suggest correcting the manuscript to better support the findings since they could be of great interest.

See below points to clarify and improve:

Authors states that 41 patients received anti-PD1 blockade combined with chemotherapy, while 11 were treated with chemotherapy alone. However, in Extended fig.1 only 36 patients are indicated to have received chemo+immune. 5 are indicated as NA. Why?

11 patients don't have a Mandard grade (NA), which is the same number of chemo only patient but Extended fig.1 reveals that only 1 chemo only patient doesn't have a Mandard grade. Then this implies that the authors have included in PR and WR groups patients that don't have received immunotherapy. This is very confusing in a text block entitled "Characterization of the TME in ESCC patients undergoing chemo-Immunotherapy" Can authors clarify this point? A table summarizing at a patient level characteristic such age, gender, tumor stage, treatment, response would be welcomed.

Authors use "well" and "poor responder" groups which could be led the reader to assume the existence of a positive response present in all patients with variable intensity. Responder vs non-responder groups would be more expected in such study.

Authors state that "none of these genomic alterations, including tumor mutational burden (TMB), were associated with immunotherapy efficacy (Extended Data Fig. 2b). However Extended Data Fig. 2b, shows a graph indicating immune editing score correlation with response.

Authors state that "mutations with a high CCF (CCF > 50%) were significantly more prevalent in well responders compared to poor responders (Fig. 2a)". In Fig 2a, 14 dots are visible for Poor responders whereas Fig1 shows 16 responder patient among which 13 have paired data. Can authors explain the inclusion of 14 PR patients in this analysis ?

In the same scope, why showing only 8 matched pre post for Fig 2b and d whereas the data is supposed to be available for 16WR and 13PR.

Authors state that "exhausted T cell clusters, including CD8_CXCL13 and CD8_TYMS, showed a marked decrease in TCR expansion in well responders (Extended Data Fig. 5f)" and define expansion as "Any clonotype composed of at least 2 cells and at least 1% all T cells". Therefore high number of "expanded TCR according to this definition fit more to the description of an oligoclonal TCR repertoire distribution than a proper measurement of T cell expansion since the pre and post samples links have not been taken into account (eg. A TCR start at 20% in pre and ending at 2% in post in clear not an expander but would count in authors expansion definition). Here, more accuracy in the analysis is critical since it will have impact on all downstream analysis. The illustration of repertoires changes during the different time point at a patient level and according the phenotypes would be welcomed.

Authors state that they examined the efficacy of CX3CR1+ CD8+ T cells using patient peripheral blood mononuclear cells (PBMCs) sorted and co-culture with patient-derived ESCC tumor cells. However the Method Co-culture and cytotoxicity assays part states that

KYSE150 cells and PBMC from healthy donors were used instead. Authors have to clarify since the relevance of a tumor killing assay using patient unmatched tumor and T cells is likely to rely more on an allogenic reaction than a proper tumor specific reactivity.

Fig5g is supposed to be " FACS showing CX3CR1+ OT1+ CD8+ T cell enrichment in OVA- vs WT-tumors whereas the graph shows arrows of different size and color without any legend. Extended fig9 shows high variability in CX3CR1 and tetramer signals among the 4 mice analysed. Moreover, it is unclear why having a small fraction of OT-I T cells being CX3CR1+ would support that the CD8_CX3CR1 found in human tumors are tumor-specific.

Related to the introduction:

Authors state that "Esophageal squamous cell carcinoma (ESCC), a significant health burden in China, accounts for approximately half of the global incidence and mortality rates of this

disease^{3,4}". Supporting reference 3 (from 2016) states that "Approximate, 70% of global esophageal cancer cases occur in China" and reference 4 (from 2023) states "Approximately 50% of cases occur in East and Central Asia, especially in China". Accurate numbers for a given date and a reference to the primary source of data would be preferred. A comment about why China is more affected than other country would also be welcomed.

Authors develop a comparison of pCR between one phase 1b and two phase II clinical trial (ref 10,11,12). Since this kind of studies are not powered to assess a reliable pCR expected for a whole population, their comparison and associated conclusion seems inappropriate.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Authors have appropriately and comprehensively addressed my comments and suggestions with new and solid data.

Reviewer #3

(Remarks to the Author)

The authors present a multi-omics analysis (scRNA-seq, scTCR-seq and WES) of tumor samples from a neoadjuvant ESCC clinical trial evaluating chemotherapy with or without anti-PD-1 therapy. The central finding is that treatment response is associated with an expansion and clonal enrichment of non-exhausted, cytotoxic CD8 T cells expressing CX3CR1, whereas exhausted CXCL13 CD8 T cells contract in responders. This contrasts with observations in several other tumor types and suggests a distinct immune mechanism of response in ESCC.

The revised manuscript has clearly improved in terms of transparency, data availability, and internal consistency compared with the original submission. In particular, public deposition of scRNA-seq/scTCR-seq data and release of the analysis code represent important steps forward.

If fully supported, the identification of a CX3CR1 cytotoxic CD8 T-cell axis associated with neoadjuvant immunotherapy response in ESCC would be of interest to the cancer immunology and translational oncology communities. The work aligns with, and extends, recent observations in other tumor types (e.g. hepatocellular carcinoma) suggesting that terminally differentiated CD8 T cells, rather than exhausted populations, can underpin responses to immune checkpoint blockade in specific therapeutic contexts.

The study therefore has potential conceptual significance. However, its impact is currently limited by ambiguities in cohort definition, overinterpretation of functional data, and insufficient rigor in some analyses linking CX3CR1 cells to tumor-specific immunity.

While the correlative single-cell and TCR-seq data suggest an association between CX3CR1 CD8 T cells and response, several key claims are not fully supported:

1. Clinical stratification and treatment heterogeneity

Responders and non-responders include patients treated with chemotherapy alone as well as chemo-anti-PD-1. This substantially weakens conclusions regarding immunotherapy-specific mechanisms. Several analyses (e.g. genomic correlates, immune phenotypes) would need to be stratified by treatment arm or restricted to anti-PD-1-treated patients to justify claims about ICI response.

2. Clonal enrichment versus phenotypic frequency

Despite improvements in terminology, there remains confusion between changes in cell-type frequency, TCR clonality, and true longitudinal clonal expansion. Some figures and supplementary panels still conflate these concepts or lack sufficient methodological clarity.

3. Functional validation of CX3CR1 CD8 T cells

The in vitro cytotoxicity assay relies on an allogeneic co-culture system with a large excess of T cells and an ATP-based viability readout, which cannot reliably distinguish tumor cell killing from T-cell death. Given the poor viability of sorted CX3CR1 cells, the conclusions drawn from this assay are not convincing.

4. Tumor specificity claims

The mouse OT-I/OVA model demonstrates antigen-driven differentiation but does not establish tumor specificity in a clinically meaningful sense, as OVA is an artificial antigen. Statements suggesting direct relevance to tumor-specific immunity in human ESCC remain overstated.

5. Limited human validation

Conclusions based on flow-cytometric analyses from one responder and one non-responder are anecdotal. Such data should be framed as illustrative rather than supportive evidence unless expanded.

Despite improvements, several issues persist:

- Discrepancies remain between figures, text, and supplementary tables (e.g. patient numbers, WES pairs, responder counts).
- Some clusters (e.g. CD8_ANXA1, CD8_GZMK) show stronger associations with response than CD8_CX3CR1 in certain analyses, yet the narrative selectively emphasizes CX3CR1 without sufficient justification.
- Overinterpretation of mouse data and limited human validation undermine the translational claims.

The revised manuscript shows significant improvement in terms of reproducibility and transparency of the methods. Code

availability and public deposition of sequencing data are commendable. However, full reproducibility will only be possible once datasets are released (The data under accession HRA011005 will be available on 2027-04-07).

Overall assessment

This revised manuscript represents a meaningful improvement over the original submission and addresses several major concerns. The dataset is potentially valuable, and the central hypothesis is interesting and timely. However, important conceptual, analytical, and interpretative issues remain—particularly regarding treatment heterogeneity, functional validation, and overstatement of tumor specificity.

I believe the work requires further substantial revision before it can be considered for publication in Nature Communications. With more rigorous stratification, clearer analysis of clonality, and a more cautious interpretation of functional experiments, the study could make a solid contribution to the field.

Details remarks

The Introduction would benefit from editing for clarity and readability:

Heavy to read:

- Line 49: “Esophageal squamous cell carcinoma (ESCC) imposes a disproportionate health burden in China, which contributes a major share of the global esophageal cancer incidence and mortality, and in which ESCC is the predominant histologic subtype”

- Line 72: “has proven invaluable” ; Line 75 “has proven invaluable”

Terminology inconsistency

- Line 83: ICB instead of ICI

Cohort definition, treatment assignment, and response stratification:

- Line 106: “41 received neoadjuvant chemotherapy ($\pm$ anti-PD-1 antibody)” is ambiguous and misleading. The “ $\pm$ ” notation suggests heterogeneity within the 41 patients, whereas the authors likely mean that 41 patients received the combination therapy, in contrast to 11 who received chemotherapy alone. This should be clarified explicitly.

- In Supplementary Table 1, among scRNA-seq-characterized patients, 11 are listed as chemotherapy only, 36 as camrelizumab plus chemotherapy, and 5 patients (P02, P31, P35, P37, P52) have no treatment annotation. The treatment status of these five patients must be clarified, or they should be excluded from analyses.

- Line 109: Response categories include patients treated with and without anti-PD-1. Since the total number of patients with evaluable response equals the number treated with combination therapy, this is potentially misleading. The text should clearly state that patients receiving chemotherapy alone were also included in responder/non-responder categories.

- Figure 1b indicates 34 responders, whereas Supplementary Table 1 reports 32. This discrepancy must be resolved.

- Line 116: The text states that WES was performed on 53 tumor-normal pairs (45 patients), including 35 overlapping with scRNA-seq samples, whereas Supplementary Table 1 indicates 34 overlapping pairs. This inconsistency requires correction.

Clarity and consistency between text, figures, and labels:

- Line 132: “cell clusters that were unique to individual patients (Fig. 1e, Extended Data Fig. 1c).” None of these figures present data at patient level.

- Line 212 contains an incomplete sentence (“Using scRNA-seq. A significant portion, we identified viral RNA sequences...”), which should be corrected.

- Figure 6h typo: “the volumn of tumor”

- Supplementary Figure 5f: The figure is described as showing the “Proportion of TCR clones,” while the y-axis is labelled “Cell percent in cluster,” and the main text refers to TCR clonality assessment. The authors should clarify how these values were generated and what exactly is being represented. It is unclear whether this corresponds to the percentage of T cells sharing identical TCR sequences (i.e., clonally expanded T cells).

I do not understand why cells from the CD8_ANXA1 cluster are associated with a strong increase in responders in Figure 3c and Supplementary Figure 5f, yet show no change in Figure 5a. Conversely, CD8_CX3CR1 cells show no apparent change in Supplementary Figure 5e, but display the largest change in Figure 5a. The authors should clarify the reasons for these apparent inconsistencies and explain how these analyses relate to one another.

In vitro cytotoxicity assays:

- Figure 6 reports “tumor killing efficiency,” but this metric is not defined.

- Extended Data Fig. 9c shows poor viability of sorted cells, but it is unclear whether this reflects post-sort viability, pre-assay viability, or viability after co-culture.

- The CellTiter-Glo assay measures total ATP and cannot distinguish tumor cells from T cells. Given the 20:1 excess of T cells over tumor cells and the poor viability of CX3CR1 T cells, changes in signal may largely reflect T-cell death rather than tumor killing. The authors should clarify how tumor-specific killing was inferred and address this major technical limitation.

Mouse models and tumor specificity claims:

- Statements such as “to directly address tumor-antigen specificity” are overstated. The OVA model relies on an artificial antigen and demonstrates antigen-driven differentiation rather than tumor specificity per se.

- In AKR-OVA tumors, OT-I cells are expected to differentiate into effector cells, whereas no such differentiation occurs in WT tumors lacking antigen. This observation is expected and does not uniquely implicate CX3CR1.

- According to Fig. 6c, more than 50% of OT-I tumor-infiltrating cells in AKR-OVA tumors are CX3CR1⁺, while CX3CR1⁺ cells represent <20%. The emphasis on CX3CR1⁺ cells therefore requires stronger justification.

- In Fig. 6f–h, a critical control group is missing: anti-PD-1 treated mice receiving CX3CR1⁻ OT-I cells. Without this control, the claimed synergy cannot be attributed specifically to CX3CR1 expression.

Human validation:

- Fig. 6i shows increased CX3CR1⁺GZMB⁺ CD8⁺ T cells in the blood of one responder but not in one non-responder. While interesting, this is anecdotal and insufficient to support general conclusions. Inclusion of additional patients (at least 2–3 per group) is necessary to lend credibility to this observation.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The revised submission has addressed all of my previous concerns. The manuscript, figures, and supplementary table are now very clear, and all inconsistencies have been corrected. The authors now present a more appropriately tempered interpretation of the data.

I still have a minor reservation regarding the reliability of the short-term tumor killing assay shown in Fig. 6a. In particular, I wonder whether the removal of non-adherent T cells fully excludes the possibility that the measured ATP signal largely reflects activated T cells bound to adherent tumor cells, rather than tumor cell viability alone. However, this remains a technical concern that does not affect the overall quality of the work and the broad biological conclusions presented.

I therefore support publication of this manuscript in its current form.

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Point-by-point response

Reviewer #1 (Remarks to the Author):

Comment 1#

Esophageal squamous cell carcinoma (ESCC) is one of the leading cancers with high mortality in East Asia, causing a significant health burden in this region of the world. ESCC generally does not respond well to immune checkpoint inhibitor (ICI) monotherapy. However, a combination of ICI with chemotherapy as neoadjuvant therapy is emerging as a promising treatment for this disease. The underlying mechanisms for clinical responses to chemoimmunotherapy remain unclear, and this lack of knowledge is one of the hurdles in the rational design of this regimen for treating ESCC. This report takes a comprehensive approach using omics techniques to dissect tumor biology and immune responses in the context of chemoimmunotherapy leveraging on their current clinical trials. The knowledge base built by this report is fundamental for clinical implications. However, some details of clinical data and relevant issues from previous studies in this field have not been thoroughly considered and addressed, which may compromise the novelty of this report.

The details of clinical features of ESCC patients are missing for what chemotherapy (platinum or taxane) used in this study? What kind of immunotherapy (anti-PD-1 or PD-L1 antibody, etc.) used in this study? Patient information about their age, sex, etc. Importantly, when the biopsy were collected during the chemoimmunotherapy. What is the exact definition or timing of “pre-therapy”, i.e. were these patients are treatment naïve or after prior immunotherapy.

Response: We thank the reviewer for these constructive comments and fully agree that clearer clinical and treatment information is essential for interpreting our findings.

In the revised manuscript, we have substantially expanded the description of the clinical cohort and now provide a detailed per-patient summary in Supplementary Table 1. This table lists baseline demographic and clinical characteristics, as well as the

neoadjuvant regimen actually received by each patient. We now clarify in the *Human ESCC tissue samples* section that all patients received platinum-based doublet chemotherapy as neoadjuvant treatment. In the chemo-immunotherapy arm this backbone was combined with a fixed-dose anti-PD-1 antibody, whereas patients in the comparator arm received chemotherapy alone. No anti-PD-L1 or CTLA-4-targeting agents were used in this cohort. We also explicitly define the timing of tissue sampling. “Pre-treatment” refers to diagnostic endoscopic tumor biopsies obtained immediately before initiation of neoadjuvant treatment; all patients were systemic-therapy-naïve for ESCC and had not received prior immune-checkpoint inhibition. “Post-treatment” refers to the esophagectomy specimen collected at surgery after completion of neoadjuvant therapy. These time points are now consistently labelled in the Methods, main text and figure legends.

Overall, 63 ESCC patients were enrolled. Among them, 52 patients contributed at least one sample for scRNA-seq/scTCR-seq, and 41 patients treated with neoadjuvant treatment had evaluable clinical response (Responder vs Non-responder). In total, we profiled 81 tumor samples by scRNA-seq and scTCR-seq (49 pre-treatment and 32 post-treatment) and 53 tumor samples by WES (45 pre-treatment and 8 post-treatment). These cohort- and sample-level statistics are summarized in **Figure R1-1** and **Supplementary Table 1**.

We believe these additions and clarifications address the reviewer’s concerns regarding chemotherapy and immunotherapy regimens, baseline patient characteristics and the exact definition of the “pre-treatment” biopsy.

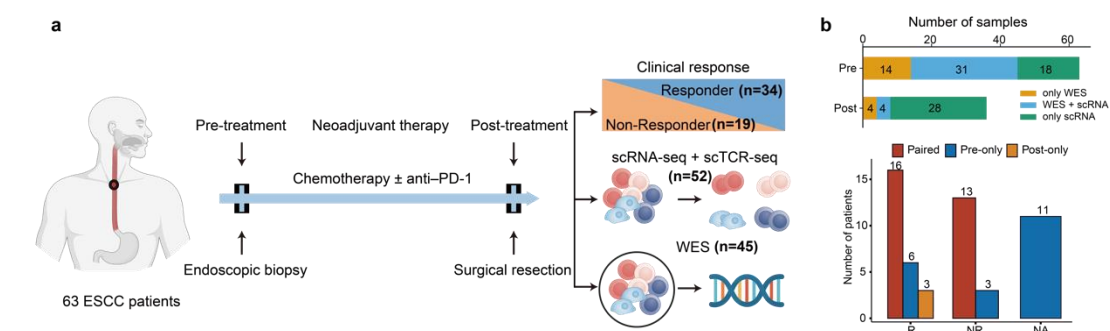


Figure R1-1. Study design and sample overview. **a**, Schematic of the neoadjuvant treatment and sequencing strategy in the ESCC cohort, indicating pre-treatment endoscopic biopsies, post-treatment surgical resections and the allocation of samples to scRNA-seq/scTCR-seq and WES. **b**, Sample statistics. Top, numbers of pre- and post-treatment tumor samples with WES only, scRNA-seq only, or paired WES and scRNA-seq data. Bottom, numbers of patients stratified by clinical response (Responder, Non-responder, Not assessed) and by availability of longitudinal scRNA-seq samples (paired pre- and post-treatment, pre-only, or post-only).

Comment 2#

The identification of Gp1 and Gp2 tumor types regarding their sensitivity to chemoimmunotherapy is interesting but lacks further consideration in the context of immunogenicity. The authors did not show whether the expression of genes encoding antigen presentation in ESCC tumor cells differs between Gp1 and Gp2 tumors based on their RNA-seq data. Are there any differences between Gp1 and Gp2 tumors in their expression of HLA I or II molecules, PD-L1, and Ki67? MUC1 is mainly identified in Gp2, but as one of the outstanding tumor antigens expressed by tumor cells, MUC1 should be validated for their RNA-seq data with IHC or IF to confirm the differential expression of MUC1 in Gp1 and Gp2 tumor tissues. The immunoediting score is an attractive marker for selecting potential therapy-sensitive tumors after therapy, but the authors did not clarify whether chemotherapy or immunotherapy would be involved in immunoediting that would eliminate tumors with nonsynonymous mutations. Additionally, the decrease of T-NK cells in the post-therapy group has not been clearly explained, particularly in these well responders (Fig. 1d). Of note, the method of calculating “cell percent in sample” should be added in the Methods section with sufficient details.

Response: Thank you for the suggestion. We have performed additional analyses to address this question. Our results demonstrate that the expression levels of both HLA class I and class II molecules are significantly higher in Gp2 tumors compared to Gp1

tumors (**Figure R1-2a, b**), suggesting a potentially enhanced antigen presentation capacity in the Gp2 subtype. Regarding Ki67 and PD-L1, although minor differences might be detected by statistical tests, the overall expression levels of both genes were relatively low in both groups. Consequently, their expression at the RNA level may not serve as a robust distinguishing feature between the two subtypes (**Figure R1-2c, d**).

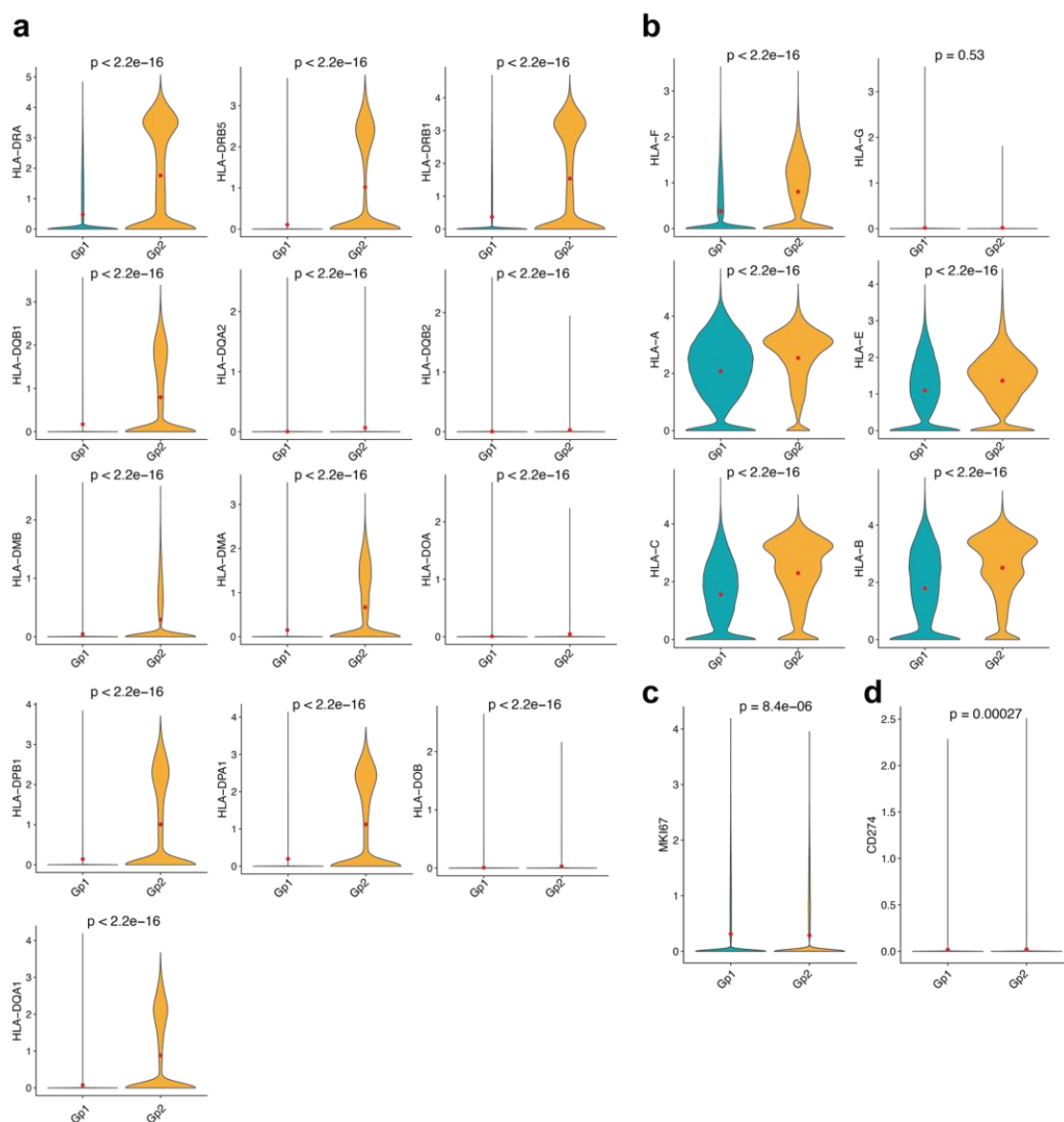


Figure R1-2. Tumor cell expression of HLA-II (a), HLA-I (b), MKI67 (c) and CD274 (d) in Gp1 versus Gp2.

Our results also demonstrate that the expression levels of MUC1 is significantly higher in Gp2 tumors compared to Gp1 tumors (**Figure R1-3a**). We further performed

IHC/IF on representative tumors to confirm increased MUC1 protein signal in non-responders, together with reduced CX3CR1⁺GZMB⁺ and CX3CR1⁺CD107a⁺ CD8⁺ T-cell signals (**Figure R1-3b–e**). We now discuss that high MUC1 can coexist with apparently intact antigen presentation yet still associate with inferior response, consistent with tumor-intrinsic program in which MUC1 has been linked to tumor survival and cisplatin resistance and can contribute to immune-evasion phenotypes (e.g., PD-L1 induction), rather than necessarily reflecting favorable immunogenicity^{1,2}.

To address whether chemotherapy or immunotherapy is involved in the immunoediting process that eliminates tumors with nonsynonymous mutations, we compared immunoediting scores separately in patients receiving chemotherapy alone and those receiving chemo-immunotherapy combination (**Figure R1-3f**). While the limited sample size in the chemotherapy-alone group may have precluded the detection of a significant treatment effect, we observed a statistically significant decrease in the immunoediting score after treatment in responders within the chemo-immunotherapy group.

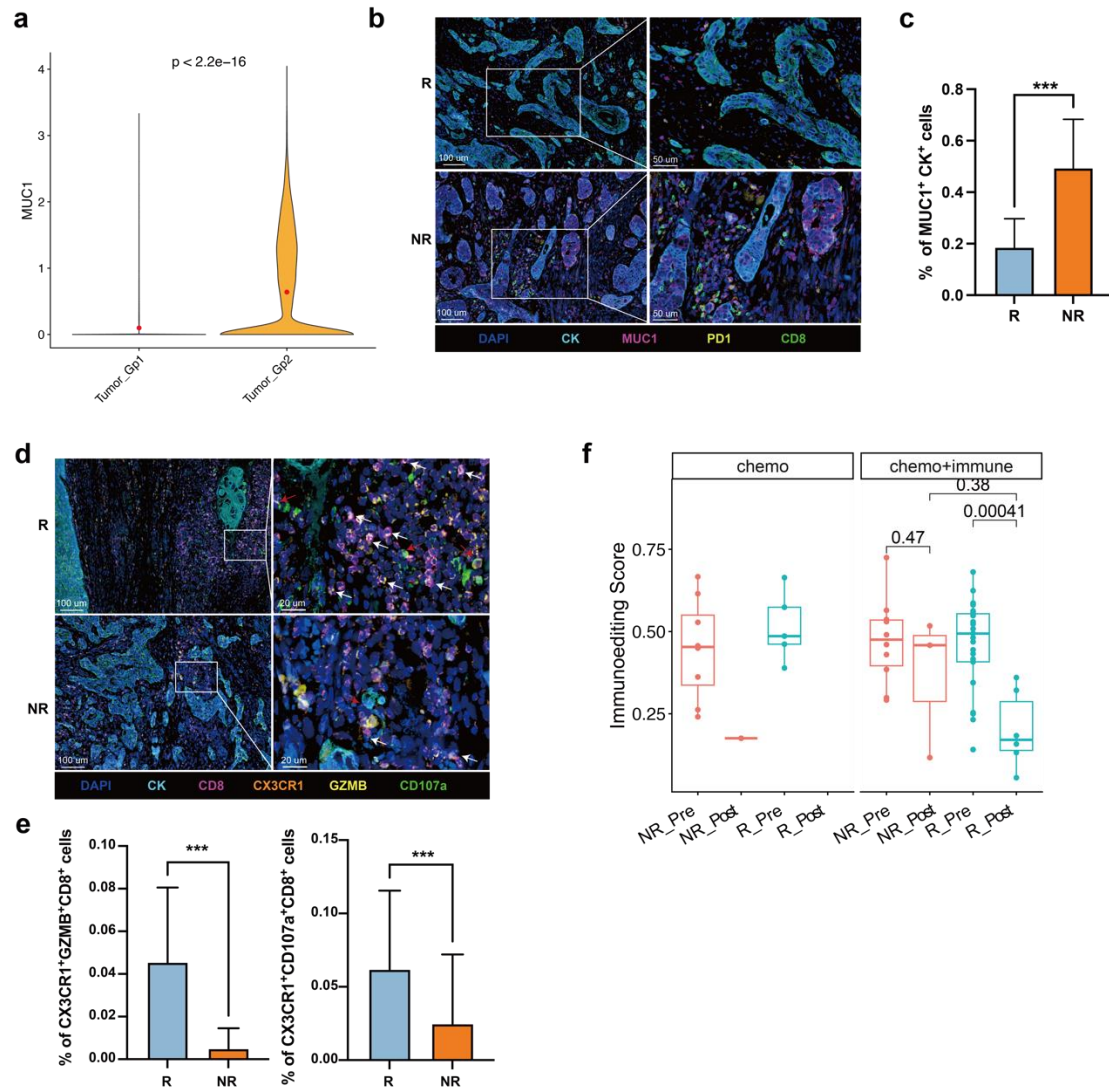


Figure R1-3. MUC1 expression (a), protein-level validation and associated cytotoxic CD8⁺ T-cell markers (b–e), and stratified immunoediting analysis by treatment arm (f).

We agree with the reviewer that the post-treatment decrease in T and NK cell percentages is more pronounced in responders. Importantly, this signal primarily reflects a compositional effect in our single-cell dataset: in responders, effective tumor clearance and subsequent wound-healing–like remodeling are accompanied by an expansion of stromal (fibroblast) and endothelial compartments, which increases the denominator and thereby dilutes the relative proportion of lymphocytes when abundance is reported as “cell percent in sample” (revised Fig. 1d). Consistent with this interpretation, despite the global compositional shift, cytotoxic T-cell programs are

strengthened within the T-cell lineage, including increased intra-lineage frequency of cytotoxic CD8⁺ T-cell subsets and higher cytotoxic signatures (revised Fig. 3c).

We also provide mechanistic context for this response-associated remodeling. In responders, the accumulation of cytotoxic T cells is paired with a shift in recruitment mechanisms: most chemokine axes linked to canonical effector T-cell trafficking (including *CXCL9–CXCR3*) decrease during treatment (revised Extended Data Fig. 6a, b), whereas *CXCL12–CXCR4* emerges as the dominant axis that increases in both ligand and receptor expression (revised Extended Data Fig. 6a, b). Fibroblasts and endothelial cells continue to show positive regulation of T cells post-treatment in responders, whereas this regulation is attenuated in non-responders (revised Fig. 2j). *CXCL12* is mainly expressed by fibroblasts and endothelial cells (revised Fig. 3e) and is significantly elevated in responders after therapy (revised Fig. 3f; Extended Data Fig. 6c); *CXCR4* is broadly expressed but enriched in cytotoxic T cells (revised Fig. 3e) and shows a greater increase in responders (revised Fig. 3g). We have incorporated these results into the revised Results/Discussion to clarify that reduced percentages of T/NK cells do not contradict enhanced cytotoxic immune activity, but rather occur alongside stromal remodeling and altered recruitment cues in responders.

Finally, following the reviewer's request, we have added an explicit definition of "cell percent in sample" in the Methods: for each sample, it is calculated as (QC-passed cells assigned to a given cluster) / (total QC-passed cells in that sample), and this definition is now referenced in the relevant figure legends.

Comment 3#

As reflected by the title of this report, one major finding in immune responses to chemoimmunotherapy for ESCC is the increase of CX3CR1⁺ CD8 T cells in these patients. This finding aligns with the discovery reported by Yan et al. in JCI Insight (2018) in patients with metastatic melanoma after chemoimmunotherapy. Yan et al. reported that CX3CR1 expression increased in peripheral blood CD8 T cells in responders to chemoimmunotherapy and defined a unique feature of CX3CR1⁺ CD8 T

cells that can withstand the toxicity of chemotherapy while remaining responsive to ICI therapy. They also identified that CX3CR1⁺ CD8 T cells are effective cytotoxic cells with granzyme B expression (both by flow cytometry and IHC in melanoma tissues) and high degranulation capability (CD107a assay). While this report confirmed the high cytotoxic capacity of CX3CR1⁺ CD8 T cells in patients with ESCC, but authors need to validate whether the CX3CR1⁺ CD8 T cells isolated from well responders with ESCC would also express granzyme B and mobilize CD107a in degranulation assay, as this is missing from their RNA-seq data set and in vitro study (Fig. 5a, 5d, and 5e).

Response: We thank the reviewer for highlighting the important prior work by Yan et al. (JCI Insight, 2018)³ and for the request to validate granzyme B and degranulation-related features of CX3CR1⁺ CD8⁺ T cells in our ESCC cohort.

In our scRNA-seq dataset, the CX3CR1⁺ CD8⁺ T-cell cluster exhibits a strong cytotoxic effector program, with elevated expression of PRF1, NKG7, IFNG and multiple granzymes (e.g., GZMH/GZMM) (revised Fig. 3a). To provide protein-level validation aligned with the reviewer's suggestion, we have added multiplex IHC analyses in representative ESCC tumors showing that CX3CR1⁺ CD8⁺ T cells in responders more frequently co-express GZMB and the degranulation marker CD107a compared with non-responders (**Figure R1-4a, b**). In addition, we included flow-cytometric analyses from two ESCC patients with paired pre-/post-treatment samples, showing a marked increase of CX3CR1⁺GZMB⁺ CD8⁺ T cells after treatment in the responder but not in the non-responder (**Figure R1-4c**). Together, these data support that CX3CR1 marks a cytotoxic CD8⁺ T-cell population with granzyme B expression and degranulation-associated features in our ESCC cohort, consistent with the observations reported by Yan et al³.

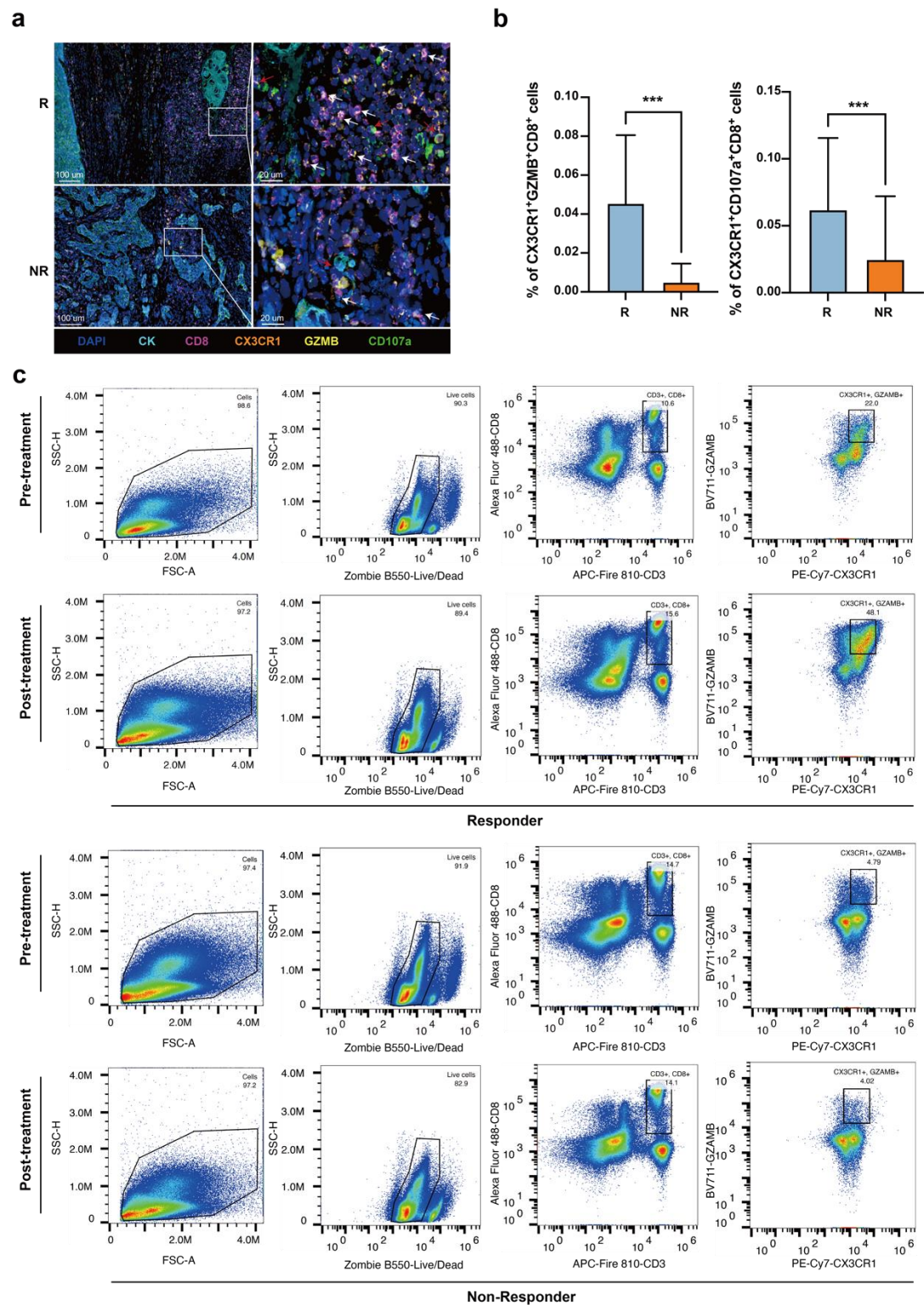


Figure R1-4. Protein-level validation of cytotoxic features of CX3CR1⁺ CD8⁺ T cells in ESCC. a and b, Multiplex IHC showing CX3CR1, CD8, GZMB and CD107a and quantification in responder vs non-responder tumors. c, Flow cytometry of paired

pre-/post-treatment samples showing CX3CR1⁺GZMB⁺ CD8⁺ T-cell frequencies in a responder and a non-responder.

Comment 4#

The tumor-killing ability of freshly isolated CX3CR1⁺ CD8 T cells was tested in comparison with other subsets of CD8 T cells. However, the authors did not show whether the freshly isolated CX3CR1⁺ CD8 T cells are tumor-antigen specific or not. If they are not, are they able to kill tumor cells due to alloantigen expression? Additionally, it is unclear whether CX3CR1⁺ CD8 T cells need to be preactivated before being used in the 4-hour tumor-killing assay. Details are missing from their 2-day culture of CX3CR1⁺ CD8 T cells in the Methods section, specifically whether these cells were cultured with or without tumor antigen. Notably, normal human T cells would lose their viability and functionality over 2 days of culture without any antigen stimulation or cytokine supplements. If CX3CR1⁺ CD8 T cells can overcome these limitations, it would be another notable feature of this type of T cell and should be highlighted in this report.

Response: We appreciate the reviewer's thoughtful comments regarding the specificity and culture conditions of the CX3CR1⁺ CD8⁺ T-cell killing assay. The original 4-h in vitro killing assay was performed in an allogeneic setting, using CX3CR1⁺ and CX3CR1⁻ CD8⁺ T-cell subsets from healthy donor PBMCs co-cultured with an unrelated ESCC cell line. We have now revised the Methods and Results to explicitly describe this assay as an allogeneic model that was intended to compare the relative cytotoxic potential of CX3CR1⁺ versus CX3CR1⁻ CD8⁺ T cells under standardized conditions, rather than to demonstrate tumor-antigen specificity.

To directly address the question of antigen specificity, we have performed additional experiments in an ESCC-OVA model using CX3CR1⁺ OT-1 CD8⁺ T cells. In this model, CX3CR1⁺ OT-1 cells accumulate within AKR-OVA tumors and mediate robust, OVA-specific cytotoxicity against tumor cells, as shown in the figures (revised

Fig. 6a-h and Extended Data Fig. 9). These data complement the allogeneic assay and demonstrate that antigen-experienced, tumor-reactive CX3CR1⁺ CD8⁺ T cells can exert potent, antigen-specific killing in an ESCC-relevant context.

Regarding the 2-day culture conditions, we now specify in the Methods that CX3CR1⁺ and CX3CR1⁻ CD8⁺ T-cell subsets were cultured for 48 h with IL-2 supplementation but without cognate tumor antigen before the killing assay. As shown in revised Extended Data Fig. 9c, CX3CR1⁺ CD8⁺ T cells actually exhibited the highest proportion of cell death after this 2-day culture, indicating that they do not overcome the usual limitations of human T cells maintained without antigenic stimulation. Consistent with this, we have stated in the main text that, despite expressing minimal exhaustion markers, CX3CR1⁺ T cells are highly short-lived and undergo rapid cell death under in vitro activation (revised Extended Data Fig. 9b, c). Accordingly, we interpret this assay as probing short-term effector function rather than enhanced long-term survival in antigen-free culture.

We believe these clarifications and additional data address the reviewer's concerns regarding alloantigen versus tumor-antigen specificity and the interpretation of the 2-day culture conditions.

Comment 5#

Similar to what Yan et al. reported, CX3CR1⁺ OT-1 CD8 T cells have superior antitumor activity compared to CX3CR1⁻ OT-1 CD8 T cells in adoptive transfer therapy for B16-OVA tumors. The authors confirmed the expansion of CX3CR1⁺ OT-1 CD8 T cells in their ESCC-OVA model. However, the authors should test whether the transfer of CX3CR1⁺ OT-1 CD8 T cells can suppress the growth of ESCC-OVA tumors in vivo. Of note, gating of CD8 T cells for CX3CR1 expression should be added with CD3 or TCR beta marker to exclude mouse dendritic cells or NKT cells that may also express CD8 (extended Fig. 9). Lastly, it is suggested that the authors discuss to what degree this work presents distinguished novelty from Yan et al.'s study.

Response : We thank the reviewer for this important suggestion and have now

performed additional in vivo experiments to directly address these points and to better clarify the novelty of our work relative to Yan et al.

Following the reviewer's recommendation, we refined our gating strategy and sorted CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells for the adoptive transfer experiments. By restricting CX3CR1 analysis to the CD3⁺CD8⁺ compartment, this gating efficiently excludes CD8⁺ dendritic cells and NK/NKT cells, which are CD3⁻, thereby enriching for bona fide CD8⁺ T cells. The full gating scheme is now shown in the revised Extended Data Fig. 9.

We adoptively transferred the purified CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells into mice bearing subcutaneous AKR-OVA ESCC tumors and evaluated their antitumor efficacy alone and in combination with chemotherapy or chemo-immunotherapy (chemotherapy plus anti-PD-1). CX3CR1⁺ OT-1 T cells alone significantly delayed AKR-OVA tumor growth, and their effect was further enhanced when combined with chemotherapy, with the strongest tumor control observed in the chemo-immunotherapy plus CX3CR1⁺ OT-1 group. These new data are presented in **Figure R1-5** and described in the Results section.

Together, these experiments extend the findings of Yan et al.³ by demonstrating that stringently defined CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells are directly capable of controlling ESCC-OVA tumor growth in vivo, and by showing that CX3CR1⁺ OT-1 T cells can further potentiate the efficacy of chemotherapy and chemo-immunotherapy in an ESCC-relevant model that closely mirrors our clinical regimen. We have revised the Discussion to explicitly contrast our ESCC-OVA chemo-immunotherapy model and mechanistic integration with the melanoma B16-OVA system used by Yan et al., and to clarify the additional mechanistic and translational insights provided by our study.

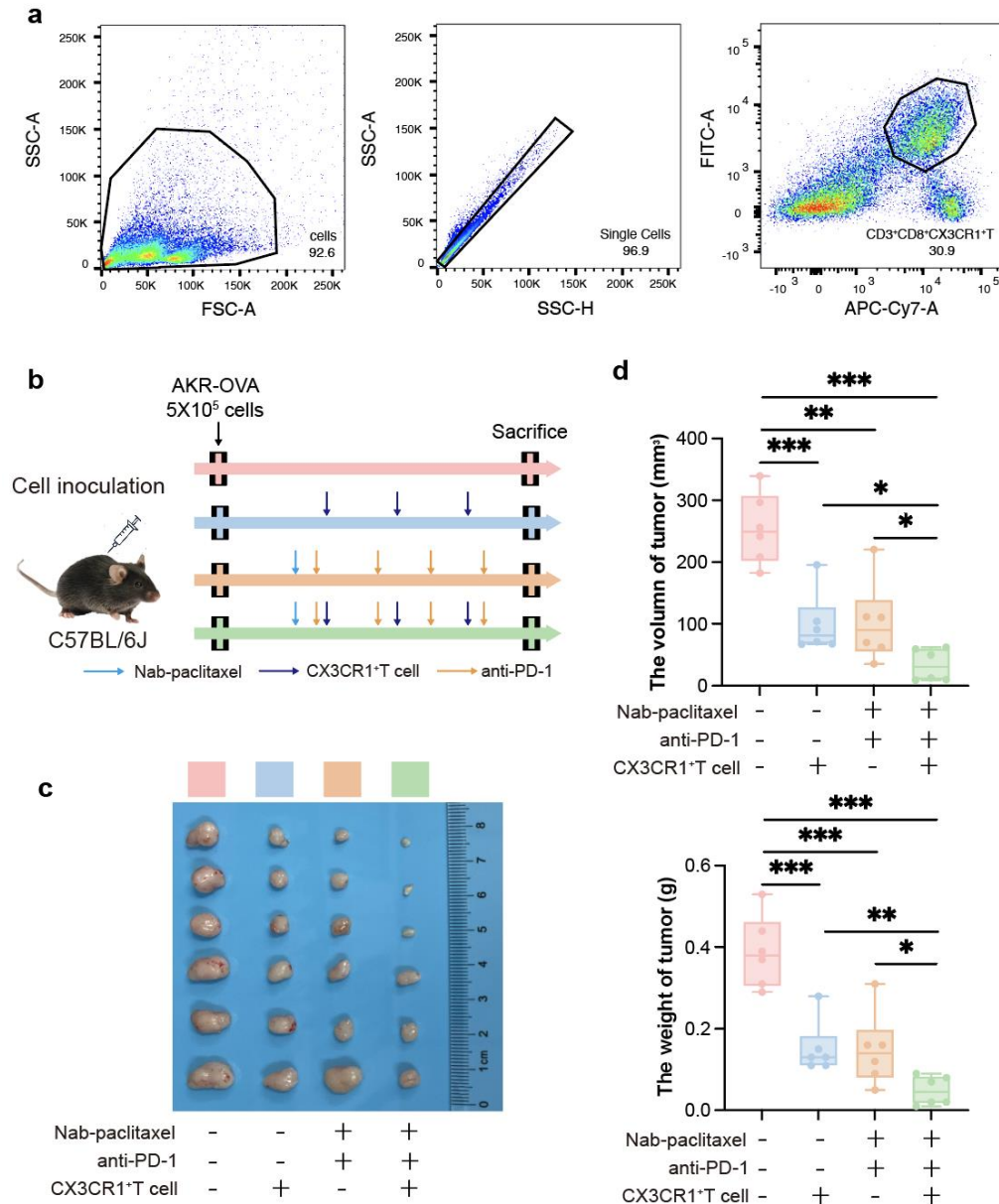


Figure R1-5. CX3CR1⁺ OT-1 T cells potentiate chemo-immunotherapy in an ESCC-OVA model. **a**, Flow-cytometric gating strategy used to identify and sort CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells for the adoptive transfer experiments. **b**, Schematic diagram of the treatment schedule in mice bearing subcutaneous AKR-OVA ESCC tumors, showing administration of CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells alone and in combination with chemotherapy or chemo-immunotherapy (chemotherapy plus anti-PD-1). **c** and **d**, Representative macroscopic images of tumors (**c**) and quantification of tumor volume and tumor weight in mice (**d**).

References

1. Zhao, Y.-Q. *et al.* Targeting MUC1-C reverses the cisplatin resistance of esophageal squamous cell carcinoma in vitro and in vivo. *Transl Cancer Res TCR* **10**, 645–655 (2021).
2. Grewal, U. S. & Kurzrock, R. Mucin-1: A promising pan-cancer therapeutic target. *NPJ Precis Oncol* **9**, 218 (2025).
3. Yan, Y. *et al.* CX3CR1 identifies PD-1 therapy-responsive CD8⁺ T cells that withstand chemotherapy during cancer chemoimmunotherapy. *JCI Insight* **3**, e97828, 97828 (2018).

Reviewer #2 (Remarks to the Author):

Dear Authors, I read your manuscript "CX3CR1⁺ CD8⁺ Cytotoxic T Cells Drive Tumor Killing in Esophageal Squamous Cell Carcinoma During Neoadjuvant Therapy" with interest.

This is a study of the TME in surgical patients, treated with either chemo and ICB or chemo alone. Tumors were studied for scRNAseq, TCR, WES. confirmatory studies were done in vitro and in an animal model . scRNAseq suggested that there is a signature for pts who displayed a good response that was in contrast to (Gp2) pts who exhibited higher expression of chemo resistance and immune evasion.

study points to two specific T-cell phenotypes that are upregulated and exhausted respectively, in pts who respond well to therapy.

The manuscript is well written but editing would make it stronger. ICI (check point inhibition) is a more common abbreviation than ICB for example.

Response: We thank the reviewer for the positive overall assessment of our study and for the helpful suggestion regarding terminology and language editing. In the revised manuscript, we have carefully edited the text for clarity and consistency. In particular, we now use the more common abbreviation “ICI” (immune checkpoint inhibitor) instead of “ICB” throughout, and we explicitly state that the only ICI used in this cohort is the anti-PD-1 antibody camrelizumab. Where appropriate, we refer to the treatment arm as “chemotherapy + anti-PD-1 (camrelizumab)” rather than a generic ICI. We have also undertaken an additional round of language editing to improve readability and to ensure consistent terminology when describing the tumor microenvironment, the treatment arms (chemo versus chemo + anti-PD-1) and the distinct T-cell phenotypes associated with treatment response.

I have the following comments that I hope will be helpful to the authors:

Comment 1#

1) did the time from therapy to collection differ in the group of patients? Is it possible that the selective expansion/exhaustion of a specific cell phenotype can also be time

dependant? For instance, did all pts have similar ability to show a good response, or are there intermediate sequences? Was the phenotype time dependant in any way?

Response: We thank the reviewer for raising this important point about the potential impact of sampling time on the observed T-cell phenotypes.

Our cohort was enrolled from the randomized phase 3 ESCORT NEO/NCCES01 trial, in which all patients received two cycles of neoadjuvant therapy (chemotherapy ± anti-PD-1) followed by esophagectomy according to a predefined schedule. In the revised *Human ESCC tissue samples* section we now clarify that: all patients were treated with platinum-based doublet chemotherapy; in the chemo-immunotherapy arm this backbone was combined with camrelizumab, whereas the comparator arm received chemotherapy alone; and tissue sampling was anchored to fixed protocol-defined time points. Specifically, “pre-treatment” samples correspond to diagnostic endoscopic tumor biopsies obtained immediately before initiation of neoadjuvant therapy, and “post-treatment” samples correspond to esophagectomy specimens collected after completion of two cycles of neoadjuvant therapy. All patients were systemic-therapy-naïve for ESCC and had not received prior immune-checkpoint inhibition. These details, together with per-patient information on treatment and sampling, are now summarized in Supplementary Table 1.

Within this trial framework, the interval from the last cycle of therapy to surgery was constrained to a narrow, protocol-defined window, and we did not observe systematic differences in this interval between responders and non-responders. Thus, while we cannot fully exclude subtle timing effects within this restricted window, our data do not support a major confounding effect of variable sampling time on the selective expansion or exhaustion of specific T-cell phenotypes. Instead, we interpret the observed differences—such as enrichment of CX3CR1⁺ cytotoxic CD8⁺ T cells versus exhausted CD8⁺ states—as primarily reflecting underlying biological response patterns to a uniform two-cycle neoadjuvant regimen rather than heterogeneity in sampling schedules.

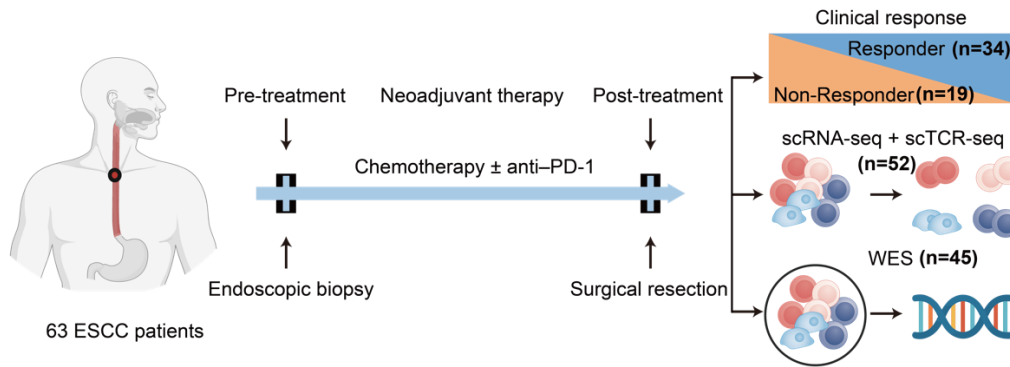


Figure R2-1. Schematic of the neoadjuvant treatment and sequencing strategy in the ESCC cohort, indicating pre-treatment endoscopic biopsies, post-treatment surgical resections and the allocation of samples to scRNA-seq/scTCR-seq and WES.

Comment 2#

2) Was chemo or ICB same in the whole cohort of pts? Would it be reasonable to add this information to methods as well as stage of disease (range) of treated pts? There are chemo groups that are known to produce a hot tumor, and variability in the chemo within your study would be of interest.

Response : We thank the reviewer for highlighting the importance of treatment homogeneity and disease stage in interpreting tumor immune profiles.

In the revised *Human ESCC tissue samples* section and revised Supplementary Table 1, we now provide detailed information on the neoadjuvant regimens and clinical stage for each patient. All patients received platinum–taxane as neoadjuvant chemotherapy. In the chemo-immunotherapy arm, this backbone was combined with the anti-PD-1 antibody camrelizumab; in the chemotherapy-alone arm, the same platinum–taxane regimens were administered without immunotherapy. Specifically, patients received paclitaxel or nab-paclitaxel plus platinum, and in our stratified analyses both solvent-based paclitaxel and nab-paclitaxel were considered taxane–platinum backbones, as they share the same mechanism of action despite differences in formulation. We agree that certain chemotherapy backbones can differentially

modulate tumor inflammation. However, within this trial the variability in taxane–platinum regimens was limited. We additionally summarize the range of clinical stages at enrolment (resectable, non-metastatic ESCC, predominantly stage I–III) in the revised Methods and Supplementary Table 1, so that readers can appreciate the stage distribution of the treated cohort.

Comment 3#

3) indicating homogeneity in the sample would be of interest. Were there pts that are HPV+, or MSI-High, and was PDL-1 or CPS score available on the sample set? correlating the highly responsive pts with known molecular markers of prognosis and therapy response would be of interest.

Response : We thank the reviewer for this helpful suggestion regarding cohort homogeneity and established biomarkers. HPV status and MSI status were not routinely assessed in this trial-based ESCC cohort and therefore were not available for systematic analysis. PD-L1 expression was evaluated as combined positive score (CPS) where sufficient tumor tissue was available. CPS values are now included in the updated Supplementary Table 1; however, PD-L1 CPS data were available for only 22 patients. Accordingly, any analyses relating CPS to response-associated immune features are presented as exploratory, and we explicitly report the sample size in the corresponding Results/figure legends to avoid over-interpretation.

Comment 4#

4) was there a difference in the CD8+ subgroup in pts treated with Immune checkpoint inhibition compared to those w chemo only, and was there a correlation to CPS/PDL-1 score?

Response: We thank the reviewer for this thoughtful question regarding treatment-specific effects on CD8⁺ T-cell subsets and their relationship to PD-L1 expression.

To address this, we performed exploratory analyses of tumor PD-L1 expression (CPS score) and the abundance of CD8⁺ T-cell clusters stratified by treatment arm

(**Figure R2-2**). In the chemotherapy-only group ($n = 4$ patients), higher CPS showed a positive association with the cytotoxic CD8_GZMK cluster and a negative association with the exhausted CD8_TYMS cluster. In contrast, in the chemo + anti-PD-1 group, CPS tended to correlate positively with the cytotoxic CD8_CCL5 cluster, although this trend did not reach statistical significance ($P = 0.072$). We did not observe a consistent CPS-linked enrichment of exhausted clusters in either arm.

Given the very small number of chemotherapy-only cases with available CPS ($n = 4$), and the limited overall sample size for these analyses, we now explicitly present these findings as hypothesis-generating and refrain from over-interpreting CPS as a definitive biomarker of specific CD8⁺ phenotypes or treatment arm-specific effects.

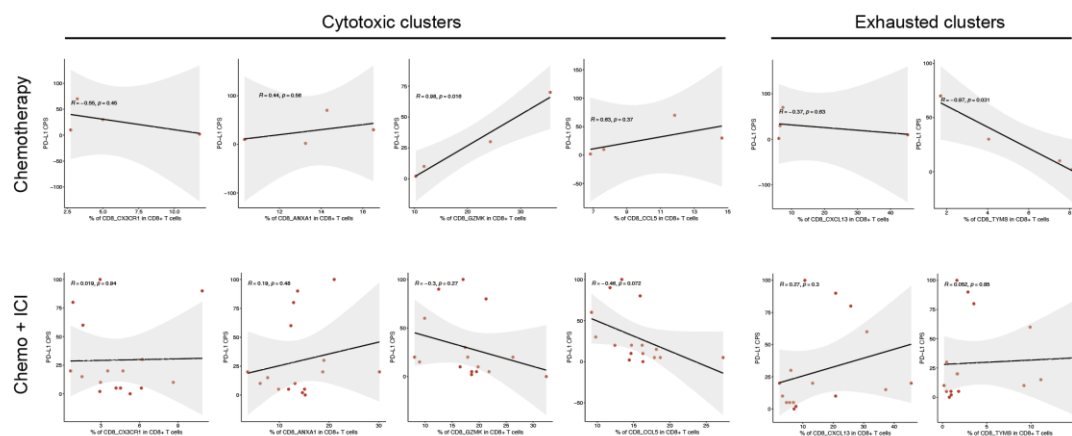


Figure R2-2. Correlation between tumor PD-L1 CPS and CD8⁺ T-cell subsets in different treatment arms. Linear regression plots showing the relationship between PD-L1 CPS and the relative abundance of cytotoxic and exhausted CD8⁺ T-cell clusters in tumors from patients treated with chemotherapy alone (top row) or chemotherapy plus anti-PD-1 (bottom row). Each point represents one patient.

Reviewer #3 (Remarks to the Author):

Authors present here the analysis of an interesting dataset obtained by scRNA-seq, TCR-seq and WES performed on samples coming from 52 ESCC patients recruited in a phase III to evaluate the efficacy of a combination of anti-PD1 antibody and chemotherapy in a neoadjuvant setting. In particular, they observe that a population of non-exhausted cytotoxic CD8⁺ expressing CX3CR1 seems to expand in patient experiencing a tumor regression. On the opposite, the exhausted CXCL13⁺ CD8⁺T cells population, generally associated with response to immune checkpoint blocker in other tumor types is observed contracting upon treatment in responders. This finding is reminiscent of the results published by Cappuyns et al., Nature Communication 2023, where clonally expanded CD8 Temra (CX3CR1⁺) and not exhausted CD8 T cells were found to be associated with response to an anti-PD-L1 + anti-VEGF therapy in advanced hepatocellular carcinoma, suggesting a similar characteristic of action of PD1 blockers therapy in both ESCC and HCC. If confirmed, this could be particularly valuable to orient the development of specific therapies and response biomarkers.

Sadly, this manuscript seems suffers from several discrepancies in the way the results have been generated when comparing the main text, the figures and the methods, questioning the robustness of the observations made. The bioinformatic analysis seem to have been performed according to standard practice in the field but the link to the analysis code leads to the home page of Github which makes difficult any check on this. The absence of direct access to the data although available from the corresponding author upon reasonable request, limit the scientific community benefit from this study. At least an online data exploratory interface would have been appreciated. This manuscript also contains one in-vitro and one in-vivo mouse experiment supposed to support the findings. Unfortunately, the setup, results and analysis are not convincing. Given the initial potential of this study, I suggest correcting the manuscript to better support the findings since they could be of great interest.

Response: We thank the reviewer for the thoughtful evaluation of our study and for pointing out the conceptual link with Cappuyns et al.¹; we now explicitly discuss these parallels in the Discussion.

To address concerns about robustness and traceability, we have re-checked the manuscript for internal consistency between the main text, figures, Extended Data and Methods, particularly cohort and sample numbers; set up a dedicated public GitHub repository containing the full analysis pipeline and updated the Code availability section with the direct link (<https://github.com/songmingl/escc-codes>); and deposited raw and processed scRNA-seq, scTCR-seq data in a public repository (<https://ngdc.cncb.ac.cn/gsa-human/browse/HRA011005>). We believe these steps substantially improve the transparency and reproducibility of our work.

See below points to clarify and improve:

Comment 1#

Authors states that 41 patients received anti-PD1 blockade combined with chemotherapy, while 11 were treated with chemotherapy alone. However, in Extended fig.1 only 36 patients are indicated to have received chemo+immune. 5 are indicated as NA. Why? 11 patients don't have a Mandard grade (NA), which is the same number of chemo only patient but Extended fig.1 reveals that only 1 chemo only patient doesn't have a Mandard grade. Then this implies that the authors have included in PR and WR groups patients that don't have received immunotherapy. This is very confusing in a text block entitled "Characterization of the TME in ESCC patients undergoing chemo-Immunotherapy" Can authors clarify this point? A table summarizing at a patient level characteristic such age, gender, tumor stage, treatment, response would be welcomed.

Response: We are sorry for the confusion caused by the inconsistent patient numbers in the original version. In the revised manuscript we now clearly distinguish between the underlying clinical trial and the multi-omics cohort. Overall, 63 ESCC patients were enrolled in the trial. Among these, 52 patients contributed at least one tumor sample for scRNA-seq/scTCR-seq and/or WES and constitute the cohort used for our mechanistic analyses. A detailed per-patient summary (age, sex, stage, treatment, Mandard grade/response and sequencing modality) is now provided in Supplementary Table 1.

Within these 52 patients: 41 patients completed neoadjuvant therapy and esophagectomy and therefore have an evaluable Mandard grade (used to define responder vs non-responder groups); 11 patients have no Mandard grade (NA)—4 withdrew after the baseline biopsy and did not receive protocol neoadjuvant therapy, and 7 initiated neoadjuvant chemotherapy ($\pm$ anti-PD-1) but did not proceed to surgery.

The apparent discrepancy noted by the reviewer stems from us mixing trial-level and sequencing-cohort numbers in the original text. We have now corrected the relevant sentences and **Figure R3-1** so that treatment arms and response categories are always reported with respect to the 52-patient multi-omics cohort, and we explicitly state that the R/NR analyses in the section *Characterization of the TME in ESCC patients undergoing neoadjuvant therapy* include only patients with an evaluable Mandard grade.

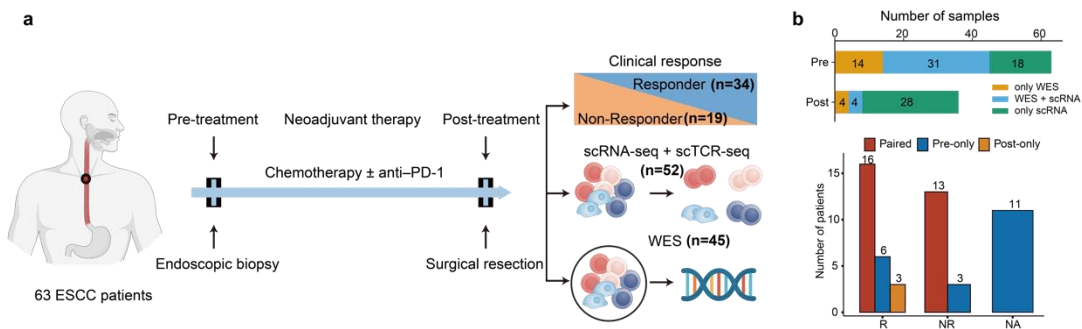


Figure R3-1. Study design and sample overview. **a**, Schematic of the neoadjuvant treatment and sequencing strategy in the ESCC cohort, indicating pre-treatment endoscopic biopsies, post-treatment surgical resections and the allocation of samples to scRNA-seq/scTCR-seq and WES. **b**, Sample statistics. Top, numbers of pre- and post-treatment tumor samples with WES only, scRNA-seq only, or paired WES and scRNA-seq data. Bottom, numbers of patients stratified by clinical response (Responder, Non-responder, Not assessed) and by availability of longitudinal scRNA-seq samples (paired pre- and post-treatment, pre-only, or post-only).

Comment 2#

Authors use “well” and “poor responder” groups which could be led the reader to assume the existence of a positive response present in all patients with variable intensity. Responder vs non-responder groups would be more expected in such study.

Response: We thank the reviewer for this helpful suggestion. We agree that the terms “well responder” and “poor responder” could be misinterpreted as implying that all patients derived some degree of benefit. In the revised manuscript, we have therefore replaced these terms with the more standard “Responder (R)” and “Non-responder (NR)” throughout the text, figures and tables. We now explicitly define these categories in the Results section (“Characterization of the TME in ESCC patients undergoing chemo-immunotherapy”) based on pathological response:

"Patients were categorized based on surgical evaluations into two groups: non-responders (NR, n = 16), who were characterized post-treatment with Mandard grades 3, 4, and 5, and responders (R, n = 25), who achieved Mandard grades 1 and 2 (Fig. 1b and Extended Data Fig. 1a)."

Comment 3#

Authors state that “none of these genomic alterations, including tumor mutational burden (TMB), were associated with immunotherapy efficacy (Extended Data Fig. 2b). However Extended Data Fig. 2b, shows a graph indicating immune editing score correlation with response.

Response: We thank the reviewer for carefully checking Extended Data Fig. 2b and apologize for the confusion. The panel shown in the original submission was incorrectly placed: it displayed the immunoediting score, whereas the corresponding statement in the text referred to baseline genomic features, including tumor mutational burden (TMB) (**Figure R3-2**). In the revised manuscript, we have corrected this error and updated Extended Data Fig. 2b to present the intended analysis, showing that TMB is not significantly associated with treatment efficacy in our cohort. The immunoediting score plot has been moved to the appropriate figure and is now described consistently in the Results and figure legend.

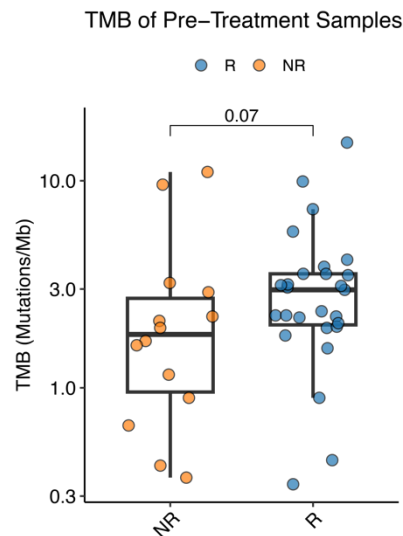


Figure R3-2. Tumor mutational burden in pre-treatment samples by response. *P* values were calculated by unpaired two-sample Wilcoxon test.

Comment 4#

Authors state that “mutations with a high CCF (CCF > 50%) were significantly more prevalent in well responders compared to poor responders (Fig. 2a)”. In Fig 2a, 14 dots are visible for Poor responders whereas Fig1 shows 16 responder patient among which 13 have paired data. Can authors explain the inclusion of 14 PR patients in this analysis ? In the same scope, why showing only 8 matched pre post for Fig 2b and d whereas the data is supposed to be available for 16WR and 13PR.

Response: We thank the reviewer for carefully checking the patient numbers. Based on the revised Supplementary Table 1, the WES cohort includes 45 patients with pre-treatment WES, of whom 14 are non-responders (NR). Fig. 2a therefore depicts 27 R and 14 NR patients with pre-treatment WES data. By contrast, the numbers 16 WR and 13 PR mentioned in revised Fig. 1 refer to the subset of patients with paired pre- and post-treatment scRNA-seq, and not to the WES cohort.

For Fig. 2b and 2d, only 8 patients had both pre- and post-treatment WES, and these 8 matched pairs are the ones shown in the longitudinal analyses. We have revised the text and figure legends to clearly distinguish between the scRNA-seq and WES

cohorts, explicitly report the exact n for each panel, and avoid any ambiguity in the correspondence between the numbers in Fig. 1 and Fig. 2.

Comment 5#

Authors state that “exhausted T cell clusters, including CD8_CXCL13 and CD8_TYMS, showed a marked decrease in TCR expansion in well responders (Extended Data Fig. 5f)” and define expansion as “Any clonotype composed of at least 2 cells and at least 1% all T cells”. Therefore high number of “expanded TCR according to this definition fit more to the description of an oligoclonal TCR repertoire distribution than a proper measurement of T cell expansion since the pre and post samples links have not been taken into account (eg. A TCR start at 20% in pre and ending at 2% in post in clear not an expander but would count in authors expansion definition). Here, more accuracy in the analysis is critical since it will have impact on all downstream analysis. The illustration of repertoires changes during the different time point at a patient level and according the phenotypes would be welcomed.

Response: We thank the reviewer for this important comment and for pointing out the potential confusion in our use of the term “expanded”.

For analyses that explicitly compare pre- versus post-treatment dynamics, we indeed used a paired, time-resolved definition. Specifically, in revised Fig. 4d–h and Extended Data Fig. 7b, we included only T cells from the 29 patients with paired pre- and post-treatment samples. For each clonotype, we compared its cell counts between the two time points using a χ^2 test; clonotypes with a significantly higher proportion post-treatment ($P \leq 0.05$) were classified as “expanded”, and those with a significantly lower proportion as “contracted”.

By contrast, in the original manuscript, when analyzing clonotype-associated phenotypes within a single sample (revised Fig. 2m and Fig. 5a,b), we applied a descriptive threshold (≥ 2 cells and $\geq 1\%$ of total T cells in that sample) and referred to such clonotypes as “expanded”. We agree that this usage is potentially misleading,

because it does not capture longitudinal changes but simply indicates that a clonotype is present at relatively high frequency at one time point.

To address this issue, we have revised the manuscript to remove the term “expanded” from all single-sample analyses. In these panels we now report only the percentage of T cells belonging to each clonotype, without attributing this to clonal “expansion”. The corresponding text, figure labels and figure legends have been updated accordingly.

This change is purely terminological and does not alter the underlying analyses or the conclusions drawn from these data. We believe that this revision improves conceptual clarity and ensures a more rigorous and transparent use of terminology throughout the manuscript.

Comment 6#

Authors state that they examined the efficacy of CX3CR1⁺ CD8⁺ T cells using patient peripheral blood mononuclear cells (PBMCs) sorted and co-culture with patient-derived ESCC tumor cells. However the Method Co-culture and cytotoxicity assays part states that KYSE150 cells and PBMC from healthy donors were used instead. Authors have to clarify since the relevance of a tumor killing assay using patient unmatched tumor and T cells is likely to rely more on an allogenic reaction than a proper tumor specific reactivity.

Response: We thank the reviewer for pointing out this inconsistency and for raising the important issue of tumor specificity in our killing assay. We apologize for the misleading wording in the original text, where we referred to “patient PBMCs co-cultured with patient-derived ESCC tumor cells”. As correctly noted by the reviewer, the assay described in the Methods used KYSE150 ESCC cells co-cultured with CX3CR1⁺ and CX3CR1⁻ CD8⁺ T-cell subsets sorted from healthy-donor PBMCs. In the revised Results and Methods, we now explicitly describe this experiment as an allogeneic co-culture model, whose purpose was to compare the relative cytotoxic capacity of CX3CR1⁺ versus CX3CR1⁻ CD8⁺ T cells under standardized conditions,

rather than to assess patient-matched, tumor-antigen-specific reactivity. We have removed the inaccurate reference to patient-derived tumor cells and added a brief caveat in the Discussion regarding this limitation.

To directly address tumor-antigen specificity and in vivo relevance, we performed additional experiments in an ESCC-OVA model using CX3CR1⁺ OT-1 CD8⁺ T cells. In this system, CX3CR1⁺ OT-1 cells accumulate within AKR-OVA tumors and mediate robust, OVA-specific cytotoxicity against tumor cells. Moreover, adoptive transfer of stringently gated CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells into AKR-OVA-bearing mice significantly delayed tumor growth, and this effect was further enhanced when combined with chemotherapy, with the strongest tumor control observed in the chemo-immunotherapy plus CX3CR1⁺ OT-1 group (**Figure R3-3a-d**). These data complement the allogeneic in-vitro assay and demonstrate that antigen-experienced, tumor-reactive CX3CR1⁺ CD8⁺ T cells can exert potent, antigen-specific killing and cooperate with chemo-immunotherapy in an ESCC-relevant setting. We have revised the Discussion to highlight these additional mechanistic and translational insights.

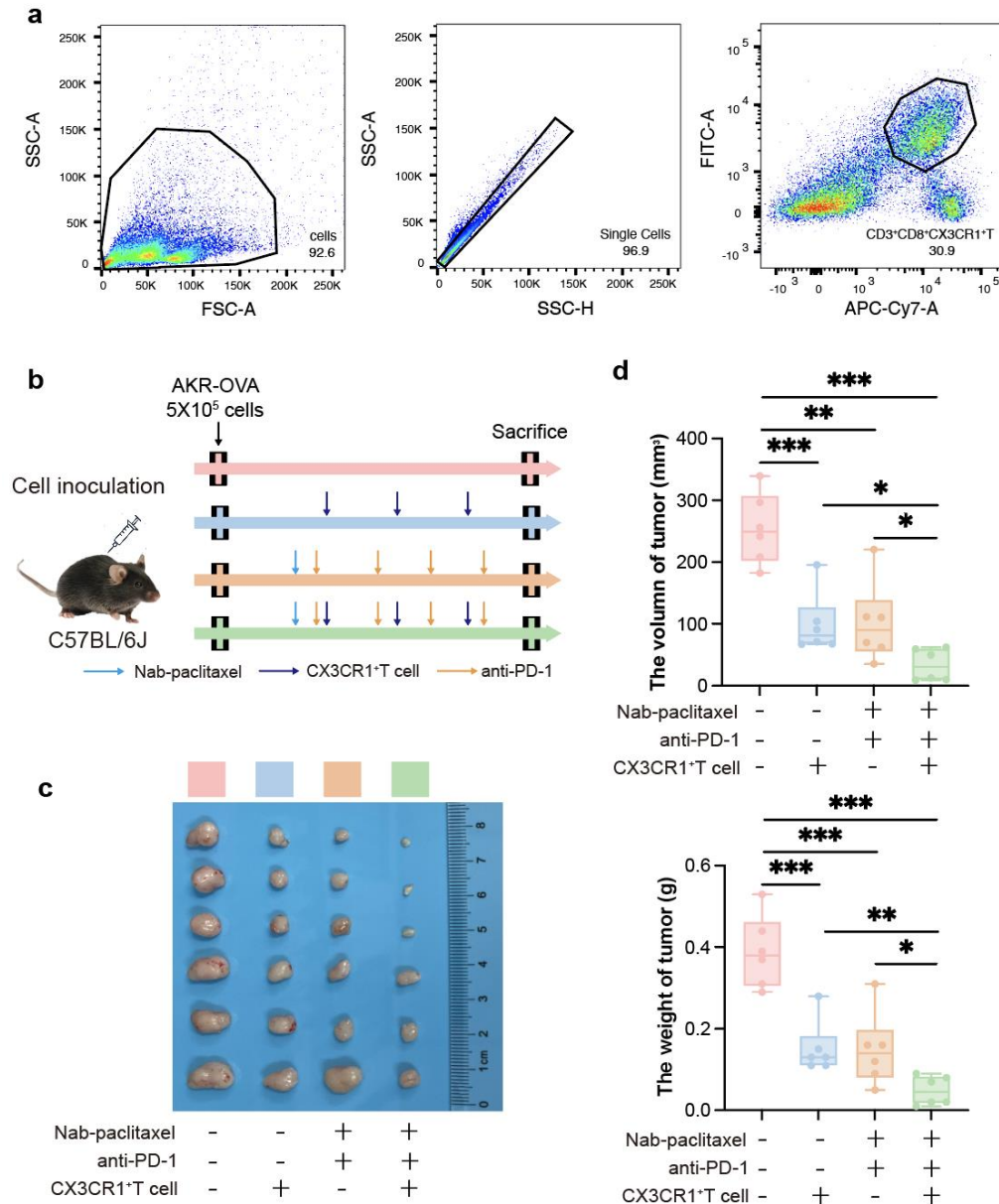


Figure R3-3. CX3CR1⁺ OT-1 T cells potentiate chemo-immunotherapy in an ESCC-OVA model. **a**, Flow-cytometric gating strategy used to identify and sort CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells for the adoptive transfer experiments. **b**, Schematic diagram of the treatment schedule in mice bearing subcutaneous AKR-OVA ESCC tumors, showing administration of CD3⁺CD8⁺CX3CR1⁺ OT-1 T cells alone and in combination with chemotherapy or chemo-immunotherapy (chemotherapy plus anti-PD-1). **c** and **d**, Representative macroscopic images of tumors (**c**) and quantification of tumor volume and tumor weight in mice (**d**).

Comment 7#

Fig5g is supposed to be “FACS showing CX3CR1⁺ OT1⁺ CD8⁺ T cell enrichment in OVA- vs WT-tumors whereas the graph shows arrows of different size and color without any legend. Extended fig9 shows high variability in CX3CR1 and tetramer signals among the 4 mice analysed. Moreover, it is unclear why having a small fraction of OT-I T cells being CX3CR1⁺ would support that the CD8_CX3CR1 found in human tumors are tumor-specific.

Response: We thank the reviewer for this helpful comment and agree that our original presentation of Fig. 5g was unclear. In the revised version, we have re-analyzed and re-plotted the frequency of CX3CR1⁺OT-1⁺, CX3CR1⁺OT-1⁻, CX3CR1⁻OT-1⁻, and CX3CR1⁺OT-1⁻ CD8⁺ T cells among tumor-infiltrating CD8⁺ T cells in OVA- versus WT-expressing AKR tumors. The updated data are now shown in **Figure R3-1b** with standard box/point plots and full legends. As illustrated in these panels, OVA expression leads to a clear enrichment of CX3CR1⁺ OT-1⁺ CD8⁺ T cells in AKR-OVA tumors compared with WT tumors (fold change = 9.4), as shown in **Figure R3-4a-c**. CX3CR1⁺ OT-1⁺ cells in this system are by definition OVA-specific, and, together with our functional data showing that CX3CR1⁺ CD8⁺ T cells display the highest cytotoxic activity, support the interpretation that CX3CR1 marks a population of highly antigen-experienced effector CD8⁺ T cells.

We fully agree that mouse data alone cannot prove tumor specificity in human ESCC. To provide additional translational support, we included flow-cytometric analyses from two ESCC patients who underwent neoadjuvant therapy (**Figure R3-4d**). In the responder, the proportion of CX3CR1⁺GZMB⁺ CD8⁺ T cells increased markedly in post-treatment blood, whereas no such increase was observed in the non-responder. Together, these mouse and human data suggest that CX3CR1⁺ cytotoxic CD8⁺ T cells are preferentially enriched in antigen-driven responses.

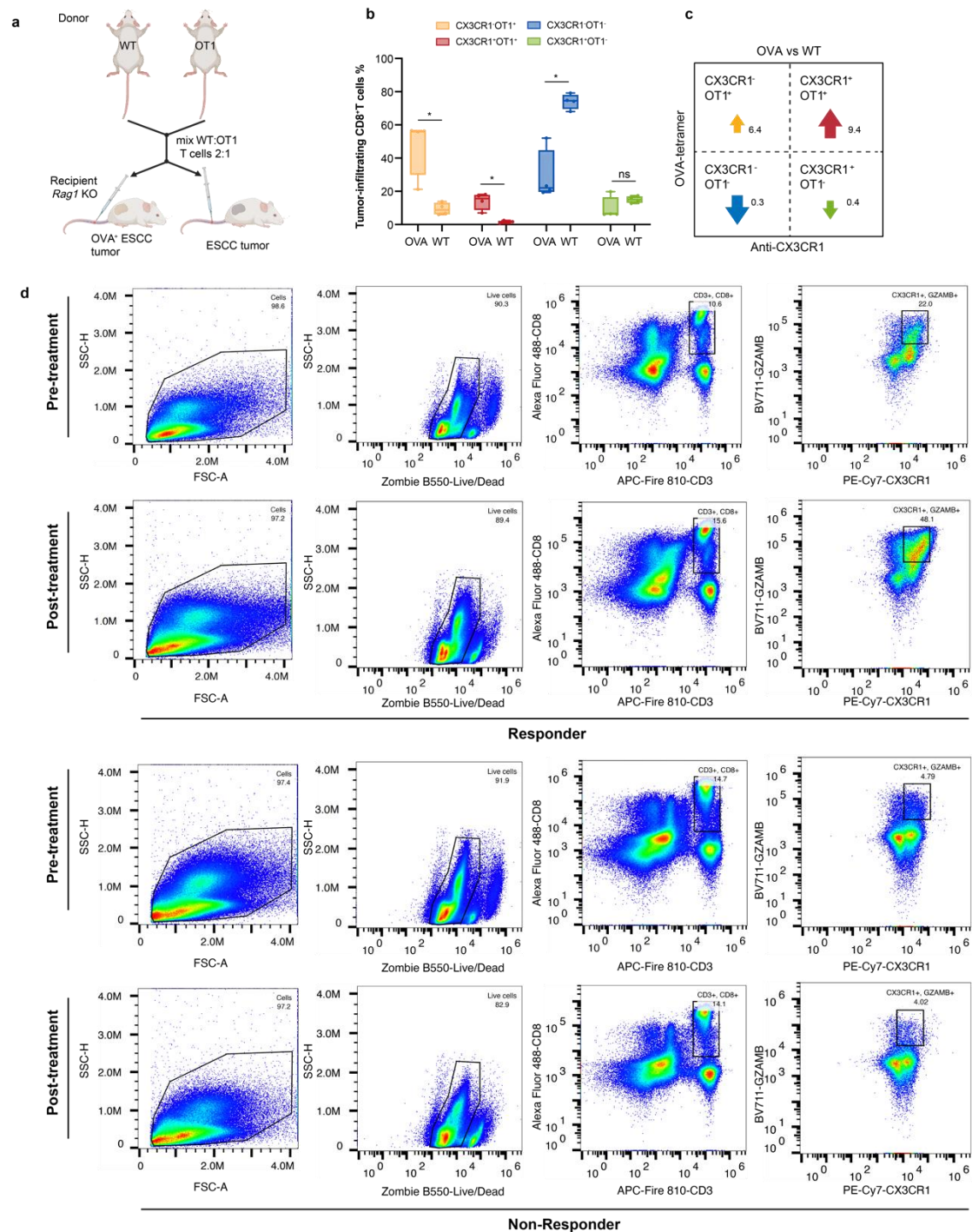


Figure R3-4. CX3CR1⁺ OT-1 and human CX3CR1⁺GZMB⁺ CD8⁺ T cells in antigen-driven responses. **a**, Schematic of AKR-WT and AKR-OVA tumor models and OT-1 transfer. **b** and **c**, Quantification of CX3CR1⁺OT-1⁺, CX3CR1⁻OT-1⁺, CX3CR1⁺OT-1⁻, and CX3CR1⁻OT-1⁻ CD8⁺ T cells among tumor-infiltrating CD8⁺ T cells in OVA versus WT tumors. **d**, Representative flow cytometry and quantification

of CX3CR1⁺GZMB⁺ CD8⁺ T cells in pre- and post-treatment ESCC samples from a responder and a non-responder.

Comment 8#

Related to the introduction:

Authors state that “Esophageal squamous cell carcinoma (ESCC), a significant health burden in China, accounts for approximately half of the global incidence and mortality rates of this disease^{3,4}”. Supporting reference 3 (from 2016) states that “Approximate, 70% of global esophageal cancer cases occur in China” and reference 4 (from 2023) states “Approximately 50% of cases occur in East and Central Asia, especially in China”. Accurate numbers for a given date and a reference to the primary source of data would be preferred. A comment about why China is more affected than other country would also be welcomed.

Response : We thank the reviewer for pointing out the inconsistency in our epidemiological statements and for requesting clearer sourcing.

In the revised Introduction, we now base our numbers on recent GLOBOCAN-2020–derived analyses. In 2020, there were ~604,000 new esophageal cancer cases and ~544,000 deaths worldwide, making esophageal cancer the seventh most common cancer and the sixth leading cause of cancer death globally. China contributed roughly half of all new cases and deaths, with an estimated ~320,000 incident cases in 2020. We have therefore rephrased the sentence to state that “*ESCC represents a major health burden in China, which accounts for about half of the global esophageal cancer burden*” and we now cite high-impact epidemiologic studies and reviews (Morgan *et al.* Gastroenterology 2022; Zhu *et al.* Int J Cancer 2023)^{2,3}. The earlier, potentially conflicting 70% versus 50% figures have been removed to avoid mixing estimates from different years and regions.

As requested, we also added a brief explanation of why China and neighbouring regions are disproportionately affected. ESCC is the predominant histologic subtype in

esophageal cancer, and high incidence in China has been linked to a combination of environmental and lifestyle factors, including tobacco and alcohol use, consumption of very hot beverages and foods, nitrosamine-rich or pickled foods, low intake of fresh fruits and vegetables, poor nutritional status, and region-specific gene–environment interactions^{2,4}. These points are now briefly summarized in the Introduction with appropriate references.

Comment 9#

Authors develop a comparison of pCR between one phase 1b and two phase II clinical trial (ref 10,11,12). Since this kind of studies are not powered to assess a reliable pCR expected for a whole population, their comparison and associated conclusion seems inappropriate.

Response: We appreciate the reviewer’s point that formal comparison of pCR rates across individual phase 1b and phase II trials is statistically under-powered and can be misleading. In the revised manuscript, we have therefore removed the direct numerical comparison of pCR rates between these early-phase studies (previous refs. 10–12) and no longer draw conclusions based on cross-trial contrasts.

Instead, we now frame the clinical context using higher-level evidence and prospective randomized data, emphasizing both the progress and the remaining unmet need. A systematic review and meta-analysis of neoadjuvant immunotherapy trials in resectable esophageal cancer reported a pooled pCR rate of ~30%, indicating meaningful activity while also showing that most patients do not achieve pCR⁵. Consistently, the phase 3 ESCORT-NEO/NCCES01 trial showed that neoadjuvant camrelizumab plus chemotherapy achieved pCR rates of 15.4–28.0%, versus 4.7% with chemotherapy alone⁶, again underscoring that a substantial proportion of patients have residual disease despite contemporary chemo-immunotherapy.

We have revised the Introduction/Discussion accordingly to emphasize that, while neoadjuvant chemo-immunotherapy has improved pathological responses in resectable ESCC, heterogeneous and incomplete responses remain common, motivating the need

for single-cell, TCR-seq and WES to delineate the intratumoral immune programs and resistance mechanisms (including the balance between CX3CR1⁺ cytotoxic versus exhausted CD8⁺ T-cell states) that may underlie differential sensitivity to treatment.

References

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2. Morgan, E. *et al.* The global landscape of esophageal squamous cell carcinoma and esophageal adenocarcinoma incidence and mortality in 2020 and projections to 2040: New estimates from GLOBOCAN 2020. *Gastroenterology* **163**, 649-658.e2 (2022).
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4. Abnet, C. C., Arnold, M. & Wei, W.-Q. Epidemiology of esophageal squamous cell carcinoma. *Gastroenterology* **154**, 360–373 (2018).
5. Ge, F. *et al.* Evaluation of clinical and safety outcomes of neoadjuvant immunotherapy combined with chemotherapy for patients with resectable esophageal cancer: A systematic review and meta-analysis. *JAMA Netw Open* **5**, e2239778 (2022).
6. Qin, J. *et al.* Neoadjuvant chemotherapy with or without camrelizumab in resectable esophageal squamous cell carcinoma: The randomized phase 3 ESCORT-NEO/NCCES01 trial. *Nat Med* **30**, 2549–2557 (2024).

Reviewer #3 (Remarks to the Author):

The authors present a multi-omics analysis (scRNA-seq, scTCR-seq and WES) of tumor samples from a neoadjuvant ESCC clinical trial evaluating chemotherapy with or without anti-PD-1 therapy. The central finding is that treatment response is associated with an expansion and clonal enrichment of non-exhausted, cytotoxic CD8 T cells expressing CX3CR1, whereas exhausted CXCL13 CD8 T cells contract in responders. This contrasts with observations in several other tumor types and suggests a distinct immune mechanism of response in ESCC.

The revised manuscript has clearly improved in terms of transparency, data availability, and internal consistency compared with the original submission. In particular, public deposition of scRNA-seq/scTCR-seq data and release of the analysis code represent important steps forward.

If fully supported, the identification of a CX3CR1 cytotoxic CD8 T-cell axis associated with neoadjuvant immunotherapy response in ESCC would be of interest to the cancer immunology and translational oncology communities. The work aligns with, and extends, recent observations in other tumor types (e.g. hepatocellular carcinoma) suggesting that terminally differentiated CD8 T cells, rather than exhausted populations, can underpin responses to immune checkpoint blockade in specific therapeutic contexts.

The study therefore has potential conceptual significance. However, its impact is currently limited by ambiguities in cohort definition, overinterpretation of functional data, and insufficient rigor in some analyses linking CX3CR1 cells to tumor-specific immunity.

While the correlative single-cell and TCR-seq data suggest an association between CX3CR1 CD8 T cells and response, several key claims are not fully supported:

1. [Clinical stratification and treatment heterogeneity](#)

Responders and non-responders include patients treated with chemotherapy alone as well as chemo–anti–PD-1. This substantially weakens conclusions regarding immunotherapy-specific mechanisms. Several analyses (e.g. genomic correlates, immune phenotypes) would need to be stratified by treatment arm or restricted to anti–PD-1–treated patients to justify claims about ICI response.

Response: We thank the reviewer for raising this important point. We agree that the treatment cohort was not described with sufficient clarity in the previous version.

In the revised manuscript, we have corrected and optimized the cohort description in the Results section. Specifically, all 52 scRNA-seq-characterized patients received neoadjuvant therapy: 41 patients received neoadjuvant chemotherapy plus camrelizumab, an anti–PD-1 antibody, whereas 11 patients received neoadjuvant chemotherapy alone. The previous wording, “41 received neoadjuvant chemotherapy (± anti–PD-1 antibody), while 11 were treated with chemotherapy alone,” was imprecise and could have caused ambiguity regarding treatment assignment. We have revised this sentence to more accurately reflect the cohort structure.

Revised manuscript text:

“To establish an overview of the ESCC tumor microenvironment under neoadjuvant therapy, 63 treatment-naïve ESCC patients were enrolled, and scRNA-seq was ultimately performed on cells isolated from 81 samples collected from 52 patients (Fig. 1a, Extended Data Fig. 1a; Supplementary Table 1). Among these 52 scRNA-seq-characterized patients, 41 received neoadjuvant chemotherapy plus camrelizumab, an anti–PD-1 antibody, whereas 11 received neoadjuvant chemotherapy alone.”

We have also reviewed the relevant Results and figure legends to ensure that the treatment groups and analytical cohorts are described more precisely. This textual clarification does not affect the underlying analyses or conclusions.

2. Clonal enrichment versus phenotypic frequency

Despite improvements in terminology, there remains confusion between changes in cell-type frequency, TCR clonality, and true longitudinal clonal expansion. Some figures and supplementary panels still conflate these concepts or lack sufficient methodological clarity.

Response: We thank the reviewer for pointing out this important conceptual issue. We agree that the previous version did not sufficiently distinguish between changes in cell-state frequency, TCR clonal enrichment, and longitudinal clonal expansion. In fact, Fig. 3 and Fig. 5 address different biological layers. Fig. 3 is based on tumor-tissue scRNA-seq data and primarily describes the abundance and frequency changes of transcriptionally defined T-cell states. By contrast, Fig. 5 is based on paired scTCR-seq data and evaluates clonotype enrichment, clonal dominance, and treatment-associated clonal dynamics within these T-cell states. Therefore, an increase in the cellular abundance of a cluster, such as CD8_ANXA1 in Fig. 3, does not necessarily imply the strongest TCR clonal enrichment. Conversely, CD8_CX3CR1 shows a relatively modest change in overall abundance but more pronounced clonotype enrichment in Fig. 5, suggesting that it represents a numerically smaller but more clonally selected cytotoxic effector subset. We have now clarified the analytical focus, denominators, and figure legends for Fig. 3 and Fig. 5 to avoid conflating cell frequency, TCR clonality, and longitudinal clonal expansion. We thank the reviewer for this important comment. We agree that cell-state frequency, TCR clonal enrichment, and paired pre-to-post clonal expansion/contraction are distinct measurements. We have revised the Results, Methods, and figure legends to clarify these differences.

First, we clarified that Fig. 3c and Extended Data Fig. 5e describe relative abundance of T-cell states, not TCR clonal expansion. We added the following sentence to the Results:

“Collectively, these relative-abundance analyses describe changes in T-cell state composition and do not directly measure TCR clonal enrichment, which we examined separately using scTCR-seq data.”

Second, we revised Extended Data Fig. 5f to clarify that it measures cluster-level TCR clonal enrichment. The y-axis now indicates the percentage of cells within each cluster that belonged to clonally expanded TCR clonotypes.

Third, we reserved the terms clonal expansion, contraction, and replacement for paired pre- and post-treatment scTCR-seq analyses. Accordingly, we revised the Fig. 4 section title to:

“Paired pre- and post-treatment TCR analysis reveals clonal replacement toward non-exhausted cytotoxic CD8⁺ T-cell states in responders.”

We also revised the Fig. 4 text to describe pre-to-post changes in clonotype abundance rather than expansion of whole T-cell states.

Fourth, we revised the Fig. 5 section to clarify that Extended Data Fig. 5f and Fig. 5a measure related but distinct aspects of clonality. Extended Data Fig. 5f shows cluster-level clonal enrichment across selected cytotoxic and exhausted T-cell states, whereas Fig. 5a compares responder-associated changes in clonotype abundance across clusters. We also revised the section title to:

“CX3CR1⁺ CD8⁺ T cells represent a clonally enriched, cytotoxic effector state associated with response.”

Finally, we added methodological clarification:

“Clonal enrichment or clonotype abundance was used to describe the representation of TCR clonotypes within a given sample or cell cluster. Pre-to-post treatment clonal expansion or contraction was assessed only in patients with paired pre- and post-treatment scTCR-seq data. Single-time-point clonal enrichment was therefore not interpreted as evidence of longitudinal clonal expansion.”

Together, these revisions distinguish phenotypic cell-state abundance, cluster-level TCR clonal enrichment, and paired pre-to-post clonotype dynamics, avoiding conflation of these related but distinct analyses.

3. Functional validation of CX3CR1 CD8 T cells

The in vitro cytotoxicity assay relies on an allogeneic co-culture system with a large excess of T cells and an ATP-based viability readout, which cannot reliably distinguish tumor cell killing from T-cell death. Given the poor viability of sorted CX3CR1 cells, the conclusions drawn from this assay are not convincing.

Response: We agree that the previous description of the in vitro cytotoxicity assay did not sufficiently define “tumor killing efficiency” or clearly distinguish the tumor-killing assay from the T-cell viability assay.

First, we have revised the Methods and figure legends to clarify that Fig. 6a and Extended Data Fig. 9a assess short-term tumor-killing efficacy after 4 h of co-culture between sorted CD8⁺ T-cell subsets and adherent tumor cells. After co-culture, non-adherent T cells were gently removed before CellTiter-Glo measurement, and luminescence was used as a readout of residual adherent tumor-cell viability. Tumor killing efficiency was calculated as:

$$\text{Tumor killing efficiency (\%)} = \left(1 - \frac{\text{RLU}_{\text{co-culture}}}{\text{RLU}_{\text{tumor only}}}\right) \times 100\%$$

Second, we clarified that Extended Data Fig. 9b,c represent a separate in vitro activation/viability assay of sorted T-cell subsets, rather than post-co-culture tumor-cell viability. These data show that CX3CR1⁺ CD8⁺ T cells are relatively short-lived under in vitro activation conditions, which we now explicitly note when interpreting the short-term CellTiter-Glo cytotoxicity assay.

4. Tumor specificity claims

The mouse OT-I/OVA model demonstrates antigen-driven differentiation but does not establish tumor specificity in a clinically meaningful sense, as OVA is

an artificial antigen. Statements suggesting direct relevance to tumor-specific immunity in human ESCC remain overstated.

Response: We thank the reviewer for this important comment. We agree that the OT-I/OVA model uses an artificial model antigen and therefore does not establish endogenous tumor-antigen specificity in human ESCC. We have revised the manuscript to avoid overstating the clinical implication of this model. In the revised manuscript, we no longer describe the OT-I/OVA experiment as directly addressing tumor-antigen specificity. Instead, we present it as an antigen-driven CD8⁺ T-cell differentiation model used to assess whether antigen recognition can promote CX3CR1 expression in cytotoxic CD8⁺ T cells. We also toned down statements linking this model to human tumor-specific immunity and now interpret the data as supportive of antigen-driven induction of a CX3CR1⁺ cytotoxic effector state, rather than as proof of endogenous ESCC tumor specificity.

Revised Results text:

“To assess whether antigen recognition can promote CX3CR1 expression in cytotoxic CD8⁺ T cells, we used the OT-I/OVA model. This model does not establish endogenous ESCC tumor-antigen specificity, but provides an antigen-driven system to evaluate CD8⁺ T-cell differentiation.”

We also revised the Discussion to clarify the limitation of this model:

Revised Discussion text:

“CX3CR1⁺ effector-memory CD8⁺ T cells showed strong short-term tumor-killing activity in vitro, and the OT-I/OVA ESCC model supported the induction of CX3CR1 expression in an antigen-driven context. Adoptive transfer of CD3⁺CD8⁺CX3CR1⁺ OT-I⁺ T cells delayed AKR-OVA tumor growth, with enhanced tumor control when combined with chemo-immunotherapy. These findings support the functional relevance of CX3CR1⁺ CD8⁺ T cells as a cytotoxic effector state, while further studies, including direct comparison with CX3CR1⁻ antigen-specific CD8⁺ T cells, will be required to determine whether

CX3CR1 expression itself is mechanistically required for enhanced antitumor activity.”

These revisions clarify that the OT-I/OVA model supports antigen-driven differentiation, but does not directly prove clinically meaningful tumor specificity in human ESCC.

5. Limited human validation

Conclusions based on flow-cytometric analyses from one responder and one non-responder are anecdotal. Such data should be framed as illustrative rather than supportive evidence unless expanded.

Response: We thank the reviewer for this important comment. We agree that the flow-cytometric analysis in the previous version, which included only one responder and one non-responder, was limited in sample size and should be considered illustrative rather than sufficient to support a general conclusion.

To address this concern, we expanded the human peripheral blood validation cohort by collecting paired pre- and post-treatment blood samples from six ESCC patients receiving neoadjuvant therapy, including three responders and three non-responders (Figure R1A). We performed flow-cytometric analysis to quantify circulating CX3CR1⁺GZMB⁺ CD8⁺ T cells before and after treatment in each patient (Figure R1B).

In the revised manuscript, we have updated Fig. 6i, 6j and Extended Data Fig. 9e to include representative flow-cytometry plots and quantitative analysis of the expanded paired cohort. The expanded analysis showed an increase in circulating CX3CR1⁺GZMB⁺ CD8⁺ T cells after treatment in responders, whereas no comparable increase was observed in non-responders. These data provide additional exploratory human validation consistent with the tumor-tissue scRNA-seq, scTCR-seq and mIHC findings.

We have also revised the wording throughout the manuscript to avoid overinterpreting the peripheral blood flow-cytometry data. Rather than presenting these data as definitive evidence, we now describe them as

exploratory human translational validation supporting the association between CX3CR1⁺ cytotoxic CD8⁺ T cells and treatment response. We have further added a limitation statement noting that larger prospectively collected paired blood cohorts will be required to validate circulating CX3CR1⁺CD8⁺ T cells as a response-associated biomarker in ESCC.

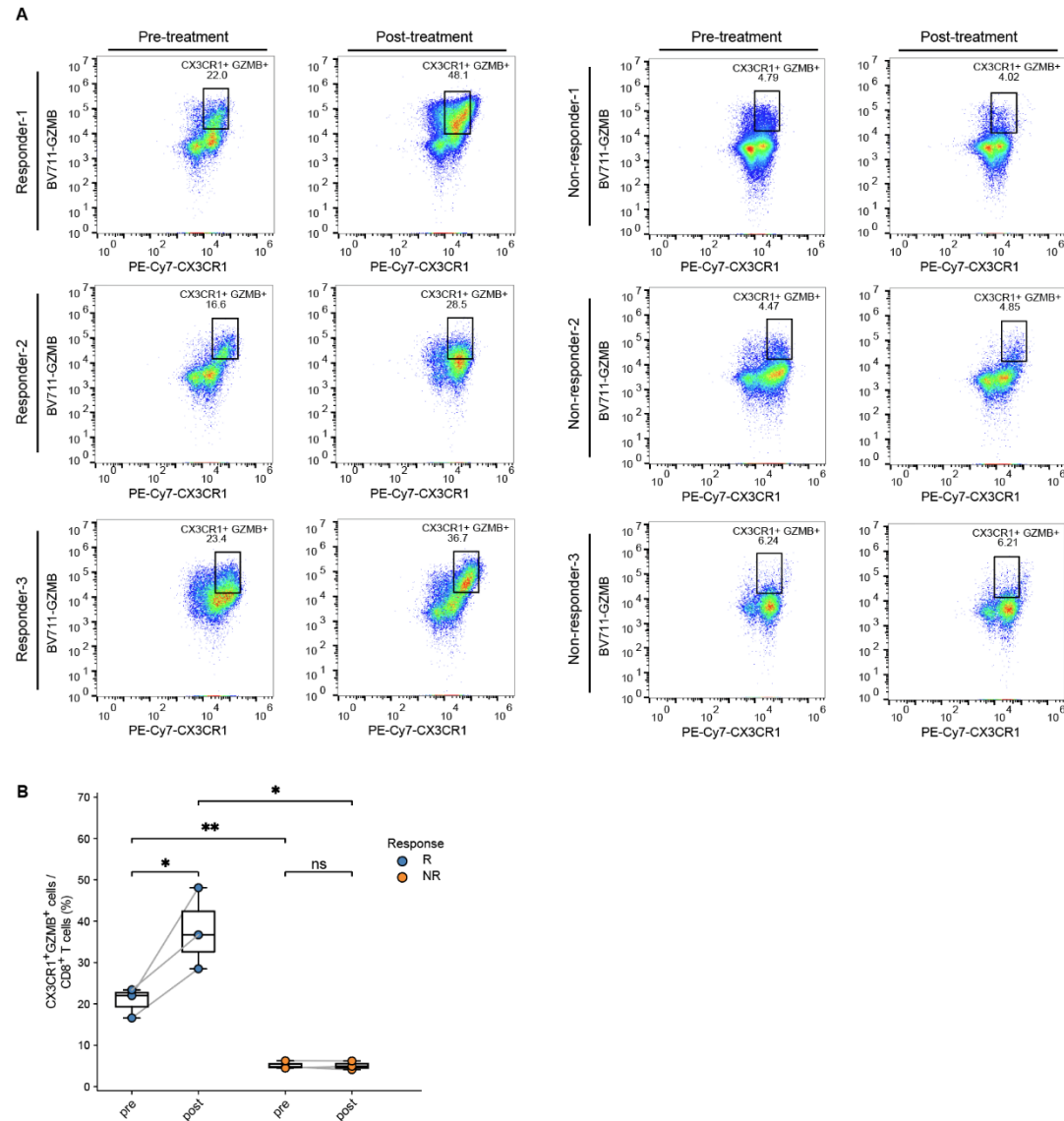


Figure R1 Peripheral blood flow-cytometric validation of CX3CR1⁺GZMB⁺ CD8⁺ T cells in ESCC patients receiving neoadjuvant therapy. A, Flow-cytometry plots showing CX3CR1 and GZMB expression in peripheral blood CD8⁺ T cells from paired pre- and post-treatment samples of three responders and three non-responders. Numbers indicate the percentage of CX3CR1⁺ GZMB⁺ cells among CD8⁺ T cells. **B,** Quantification of CX3CR1⁺GZMB⁺ cells

among CD8⁺ T cells in paired pre- and post-treatment peripheral blood samples from responders and non-responders. Each dot represents one patient, and lines connect paired samples from the same patient. Box plots show the median, interquartile range and whiskers extending to 1.5× the interquartile range. *P < 0.05; **P < 0.01; ns, not significant.

6. Despite improvements, several issues persist:

- Discrepancies remain between figures, text, and supplementary tables (e.g. patient numbers, WES pairs, responder counts).
- Some clusters (e.g. CD8_ANXA1, CD8_GZMK) show stronger associations with response than CD8_CX3CR1 in certain analyses, yet the narrative selectively emphasizes CX3CR1 without sufficient justification.
- Overinterpretation of mouse data and limited human validation undermine the translational claims.

Response: We thank the reviewer for this constructive comment. We have revised the manuscript, figures, figure legends, and Supplementary Table 1 to address these concerns.

First, we rechecked all clinical annotations and sequencing information and corrected inconsistencies among the main text, Fig. 1b, and Supplementary Table 1. The revised manuscript now consistently reports 63 enrolled patients, 52 patients with scRNA-seq data, and WES performed on 53 tumor–normal pairs (pre- and post-treatment tumor sample) from 45 patients. Among these, 34 patients also had scRNA-seq data, contributing 39 WES tumor–normal pairs. We also clarified response status: among all 63 patients, 32 were responders, 19 were non-responders, and 12 were not evaluable; within the scRNA-seq cohort, 25 were responders, 16 were non-responders, and 11 were baseline-only or response-unevaluable cases.

Second, we revised Fig. 1b to show patient-level sequencing data availability rather than sample-level WES counts. The figure legend and Supplementary Table 1 were updated accordingly.

Third, we agree that CD8_ANXA1 and CD8_GZMK show strong response-associated changes. We have therefore revised the narrative to clarify that therapeutic response is associated with broader cytotoxic CD8⁺ T-cell remodeling, rather than CX3CR1⁺ CD8⁺ T cells alone. We now present CX3CR1⁺ CD8⁺ T cells as a clonally enriched, cytotoxic effector state within this broader program. The focus on CX3CR1 is based on its responder-associated clonotype abundance, reduced TCR diversity, cytotoxic non-exhausted phenotype, surface-marker accessibility for functional assays, and association with the CX3CL1–CX3CR1 axis.

Fourth, we toned down the interpretation of the functional and mouse experiments. We no longer state that CX3CR1⁺ CD8⁺ T cells alone “drive” response, but instead describe them as a cytotoxic effector CD8⁺ T-cell state that may contribute to tumor control.

Finally, we expanded the human peripheral blood validation to six ESCC patients, including three responders and three non-responders. Fig. 6i and Extended Data Fig. 9e shows representative flow-cytometry plots, and Fig. 6j shows paired quantification in all six patients. These data are now presented as supportive translational evidence rather than definitive proof.

7. The revised manuscript shows significant improvement in terms of reproducibility and transparency of the methods. Code availability and public deposition of sequencing data are commendable. However, full reproducibility will only be possible once datasets are released (The data under accession HRA011005 will be available on 2027-04-07).

Response: We thank the reviewer for recognizing the improvements in method transparency, code availability, and sequencing-data deposition. We agree that full independent reproducibility requires public access to the raw sequencing datasets.

The sequencing data have been deposited in the Genome Sequence Archive for Human under accession code HRA011005. To address the

reviewer's concern, we have revised the Data and code availability statement to clarify that the deposited dataset will be made publicly available immediately upon publication in accordance with journal policy and repository requirements.

Revised Data availability statement:

“Raw transcriptomic sequencing data have been deposited in the Genome Sequence Archive for Human (GSA-Human) under accession HRA011005 (<https://ngdc.cncb.ac.cn/gsa-human/browse/HRA011005>). The deposited dataset will be made publicly available immediately upon publication in accordance with journal policy and repository requirements.”

In addition, we have provided detailed Methods descriptions and code availability information to support reproducibility before full dataset release.

8. Overall assessment

This revised manuscript represents a meaningful improvement over the original submission and addresses several major concerns. The dataset is potentially valuable, and the central hypothesis is interesting and timely. However, important conceptual, analytical, and interpretative issues remain—particularly regarding treatment heterogeneity, functional validation, and overstatement of tumor specificity.

I believe the work requires further substantial revision before it can be considered for publication in Nature Communications. With more rigorous stratification, clearer analysis of clonality, and a more cautious interpretation of functional experiments, the study could make a solid contribution to the field.

Response: We thank the reviewer for the overall positive assessment and for recognizing the value of the dataset and the relevance of the central hypothesis. We also appreciate the reviewer's constructive comments regarding the remaining conceptual, analytical, and interpretative issues.

In response, we have substantially revised the manuscript in three major aspects. First, we clarified the cohort definition, treatment assignment, and response stratification. We corrected the ambiguous treatment description,

updated Fig. 1b and Supplementary Table 1, and clearly annotated baseline-only or response-unevaluable cases. These revisions address the concerns regarding treatment heterogeneity and cohort consistency.

Second, we improved the clarity of the clonality analyses. We now explicitly distinguish cell-state frequency, cluster-level TCR clonal enrichment, and paired pre-to-post clonal expansion or contraction. The Results, Methods, and figure legends have been revised accordingly, including clarification of Extended Data Fig. 5f and the relationship between Extended Data Fig. 5f and Fig. 5a.

Third, we revised the interpretation of the functional experiments and tumor-specificity analyses to better align the conclusions with the experimental design and available evidence. The OT-I/OVA model is now described as an antigen-driven CD8⁺ T-cell differentiation model rather than direct evidence of endogenous ESCC tumor-antigen specificity. We also clarified the limitations of the in vitro cytotoxicity assay and now present it as a supportive short-term functional readout. In addition, we describe CX3CR1⁺ CD8⁺ T cells as a functionally relevant cytotoxic effector state within a broader response-associated cytotoxic CD8⁺ T-cell program, rather than as the only response-associated subset.

Together, these revisions provide clearer stratification, more rigorous interpretation of TCR clonality, and a more cautious presentation of functional and tumor-specificity data. We believe these changes substantially improve the clarity, reproducibility, and interpretative balance of the manuscript.

Details remarks

9. The Introduction would benefit from editing for clarity and readability:

Heavy to read:

- Line 49: "Esophageal squamous cell carcinoma (ESCC) imposes a disproportionate health burden in China, which contributes a major share of the global esophageal cancer incidence and mortality, and in which ESCC is the

predominant histologic subtype”

- Line 72: “has proven invaluable” ; Line 75 “has proven invaluable”

Terminology inconsistency

- Line 83: ICB instead of ICI

Response: We thank the reviewer for these helpful suggestions. We have edited the Introduction to improve clarity, readability, and terminology consistency.

First, we simplified the sentence describing the disease burden of ESCC in China.

Revised text:

“Esophageal squamous cell carcinoma (ESCC) is the predominant histologic subtype of esophageal cancer in China, where the disease accounts for a substantial proportion of global esophageal cancer incidence and mortality^{3,4}.”

Second, we revised repeated wording in the Introduction. Specifically, we replaced the repeated phrase “has proven invaluable” with more concise and varied expressions.

Revised text examples:

“In this context, integrated single-cell RNA sequencing (scRNA-seq) and T-cell receptor sequencing (scTCR-seq) can further reveal treatment-associated immune remodeling and clonal dynamics^{13,14}.”

“scRNA-seq has enabled high-resolution characterization of the tumor microenvironment (TME) and dissection of the heterogeneity and dynamics of T-cell responses after ICI treatment.”

Third, we corrected the terminology inconsistency by using ICI consistently throughout the Introduction instead of switching between ICB and ICI.

These revisions improve the readability of the Introduction and ensure consistent terminology throughout the manuscript.

10. Cohort definition, treatment assignment, and response stratification:

- Line 106: “41 received neoadjuvant chemotherapy ($\pm$ anti-PD-1 antibody)” is

ambiguous and misleading. The “±” notation suggests heterogeneity within the 41 patients, whereas the authors likely mean that 41 patients received the combination therapy, in contrast to 11 who received chemotherapy alone. This should be clarified explicitly.

- In Supplementary Table 1, among scRNA-seq-characterized patients, 11 are listed as chemotherapy only, 36 as camrelizumab plus chemotherapy, and 5 patients (P02, P31, P35, P37, P52) have no treatment annotation. The treatment status of these five patients must be clarified, or they should be excluded from analyses.

- Line 109: Response categories include patients treated with and without anti-PD-1. Since the total number of patients with evaluable response equals the number treated with combination therapy, this is potentially misleading. The text should clearly state that patients receiving chemotherapy alone were also included in responder/non-responder categories.

- Figure 1b indicates 34 responders, whereas Supplementary Table 1 reports 32. This discrepancy must be resolved.

- Line 116: The text states that WES was performed on 53 tumor-normal pairs (45 patients), including 35 overlapping with scRNA-seq samples, whereas Supplementary Table 1 indicates 34 overlapping pairs. This inconsistency requires correction.

Response: We thank the reviewer for carefully pointing out these inconsistencies. We agree that the cohort definition, treatment assignment, and response stratification were not described with sufficient clarity in the previous version. We have now rechecked the clinical annotation, Supplementary Table 1, Fig. 1b, and the corresponding text, and have revised them to ensure consistency.

First, we corrected the ambiguous wording in the Results section. The previous phrase “41 received neoadjuvant chemotherapy (± anti-PD-1 antibody)” was indeed misleading, as it could imply heterogeneity within these 41 patients. We have revised the sentence to explicitly state that, among the

52 scRNA-seq-characterized patients, 41 received neoadjuvant chemotherapy plus camrelizumab, an anti-PD-1 antibody, whereas 11 received neoadjuvant chemotherapy alone.

Revised text:

“Among these 52 scRNA-seq-characterized patients, 41 received neoadjuvant chemotherapy plus camrelizumab, an anti-PD-1 antibody, whereas 11 received neoadjuvant chemotherapy alone.”

Second, we clarified the status of patients with incomplete annotation in Supplementary Table 1. These patients had pre-treatment endoscopic biopsy samples collected for scRNA-seq, but did not subsequently undergo surgery within the study workflow; therefore, post-treatment surgical specimens, Mandard grades, and complete pathological response information were not available. These cases are now annotated as baseline-only / response-unevaluable cases where applicable. They were retained only for the global scRNA-seq landscape analysis and were excluded from paired analyses or analyses requiring evaluable pathological response.

Third, we revised the response-stratification text to clarify that pathological response was assigned according to Mandard tumor regression grades in patients with available post-treatment surgical specimens, regardless of whether they received chemotherapy alone or chemotherapy plus anti-PD-1 therapy. Patients without surgical specimens or evaluable Mandard grades were annotated as not evaluable for pathological response.

Fourth, we corrected the responder-count discrepancy between Fig. 1b and Supplementary Table 1. After rechecking the source data, the revised manuscript consistently reports 32 responders, 19 non-responders, and 12 response-unevaluable cases among all 63 enrolled patients. Within the 52 scRNA-seq-characterized patients, there were 25 responders, 16 non-responders, and 11 baseline-only or response-unevaluable cases. Fig. 1b and its legend have been updated to clearly specify the denominator.

Finally, we corrected the WES overlap description. WES was performed on 53 tumor–normal pairs from 45 patients, including 45 pre-treatment and 8 post-treatment tumor samples. Of these 45 patients, 34 also had scRNA-seq data, contributing 39 WES tumor–normal pairs. We revised the text to distinguish patient number from sample-pair number and updated Supplementary Table 1 accordingly.

Together, these revisions clarify the cohort structure, treatment assignment, response evaluability, and WES/scRNA-seq overlap. They represent annotation and textual corrections and do not alter the underlying analyses or conclusions.

11. Clarity and consistency between text, figures, and labels:

- Line 132: “cell clusters that were unique to individual patients (Fig. 1e, Extended Data Fig. 1c).” None of these figures present data at patient level.
- Line 212 contains an incomplete sentence (“Using scRNA-seq. A significant portion, we identified viral RNA sequences...”), which should be corrected.
- Figure 6h typo: “the volumn of tumor”
- Supplementary Figure 5f: The figure is described as showing the “Proportion of TCR clones,” while the y-axis is labelled “Cell percent in cluster,” and the main text refers to TCR clonality assessment. The authors should clarify how these values were generated and what exactly is being represented. It is unclear whether this corresponds to the percentage of T cells sharing identical TCR sequences (i.e., clonally expanded T cells).

Response: We thank the reviewer for carefully pointing out these issues. We have revised the relevant text, figure legends, labels, and Methods to improve clarity and consistency.

First, we revised the statement that some cell clusters were “unique to individual patients.” Because Fig. 1e and Extended Data Fig. 1c mainly show subcluster annotation and distribution rather than patient-level specificity, we

removed this wording and replaced it with a more accurate description of transcriptionally distinct subclusters across the scRNA-seq cohort.

Second, we corrected the incomplete sentence describing viral RNA analysis. The revised text now clearly states that viral RNA sequences were identified using scRNA-seq, and that the distribution of viral-read-associated cells was examined across major cell types.

Third, we corrected the typo in the Fig. 6 legend from “the volumn of tumor” to “Tumor volume.”

Finally, we clarified Extended Data Fig. 5f. This panel was intended to show cluster-level TCR clonal enrichment, not overall cell-type frequency. We revised the legend and axis label to indicate that the y-axis represents the percentage of cells within each cluster that belonged to clonally expanded TCR clonotypes.

We also added a Methods clarification defining clonal enrichment and distinguishing it from paired pre-to-post clonal expansion. These revisions improve consistency between the main text, figures, supplementary figures, and Methods.

12. I do not understand why cells from the CD8_ANXA1 cluster are associated with a strong increase in responders in Figure 3c and Supplementary Figure 5f, yet show no change in Figure 5a. Conversely, CD8_CX3CR1 cells show no apparent change in Supplementary Figure 5e, but display the largest change in Figure 5a. The authors should clarify the reasons for these apparent inconsistencies and explain how these analyses relate to one another.

Response: We thank the reviewer for pointing out this apparent inconsistency. We agree that the relationship among these panels was not sufficiently clear in the previous version. We have now revised the Results, Methods, and figure legends to clarify that these analyses measure different aspects of the T-cell response.

Fig. 3c and Extended Data Fig. 5e quantify cell-state frequency, namely the relative abundance of each T-cell cluster within the T/NK-cell compartment. In

these analyses, CD8_ANXA1 and CD8_GZMK showed strong increases in relative abundance in responders, indicating broad expansion of cytotoxic CD8⁺ T-cell states.

Extended Data Fig. 5f measures cluster-level TCR clonal enrichment, not overall cell frequency. Specifically, it shows the percentage of cells within each cluster that belonged to clonally expanded TCR clonotypes. Thus, CD8_ANXA1 and CD8_GZMK showing strong signals in this panel indicates that these cytotoxic clusters contain a high proportion of clonally expanded cells.

By contrast, Fig. 5a compares responder-associated treatment-related changes in clonotype abundance across T-cell clusters. In this analysis, CD8_CX3CR1 showed the strongest responder-associated increase in clonotype abundance, even though its overall cell frequency did not markedly change in Extended Data Fig. 5e. This indicates that CD8_CX3CR1 did not necessarily expand as a whole cell population, but its internal TCR clonal structure became more focused in responders, as further supported by reduced TCR diversity in Fig. 5b.

Therefore, these findings are not contradictory. CD8_ANXA1 and CD8_GZMK primarily reflect broader cytotoxic CD8⁺ T-cell abundance and cluster-level clonal enrichment, whereas CD8_CX3CR1 reflects a responder-associated change in clonotype abundance and TCR diversity. We have added explanatory text to the Results and revised the relevant figure legends to make this distinction clear.

13. In vitro cytotoxicity assays:

- Figure 6 reports “tumor killing efficiency,” but this metric is not defined.
- Extended Data Fig. 9c shows poor viability of sorted cells, but it is unclear whether this reflects post-sort viability, pre-assay viability, or viability after co-culture.
- The CellTiter-Glo assay measures total ATP and cannot distinguish tumor cells from T cells. Given the 20:1 excess of T cells over tumor cells and the

poor viability of CX3CR1⁺ T cells, changes in signal may largely reflect T-cell death rather than tumor killing. The authors should clarify how tumor-specific killing was inferred and address this major technical limitation.

Response: We agree that the previous description of the in vitro cytotoxicity assay did not sufficiently define “tumor killing efficiency” or clearly distinguish the tumor-killing assay from the T-cell viability assay.

We have revised the Methods and figure legends to clarify that Fig. 6a and Extended Data Fig. 9a assess short-term tumor-killing efficacy after 4 h of co-culture between sorted CD8⁺ T-cell subsets and adherent tumor cells. After co-culture, non-adherent T cells were gently removed before CellTiter-Glo measurement, and luminescence was used as a readout of residual adherent tumor-cell viability. Tumor killing efficiency was calculated as:

$$\text{Tumor killing efficiency (\%)} = \left(1 - \frac{\text{RLU}_{\text{co-culture}}}{\text{RLU}_{\text{tumor only}}} \right) \times 100\%$$

We also clarified that Extended Data Fig. 9b,c represent a separate in vitro viability assay of sorted T-cell subsets, rather than post-co-culture tumor-cell viability. These data show that CX3CR1⁺ CD8⁺ T cells, despite low exhaustion-marker expression, are relatively short-lived and undergo rapid cell death under in vitro activation.

To address the concern regarding the 20:1 effector-to-target ratio, we provide a pilot E:T ratio titration assay in Figure R2. This pilot experiment was performed before the formal cytotoxicity assay to guide assay-condition selection. Tumor-cell survival gradually decreased with increasing E:T ratios in CD8⁺ T cells derived from PBMCs of two different patients, while tumor-cell-only controls maintained high viability. Based on this titration, an E:T ratio of 20:1 was selected for the formal assay as a condition that produced a measurable but not saturated reduction in adherent tumor-cell viability.

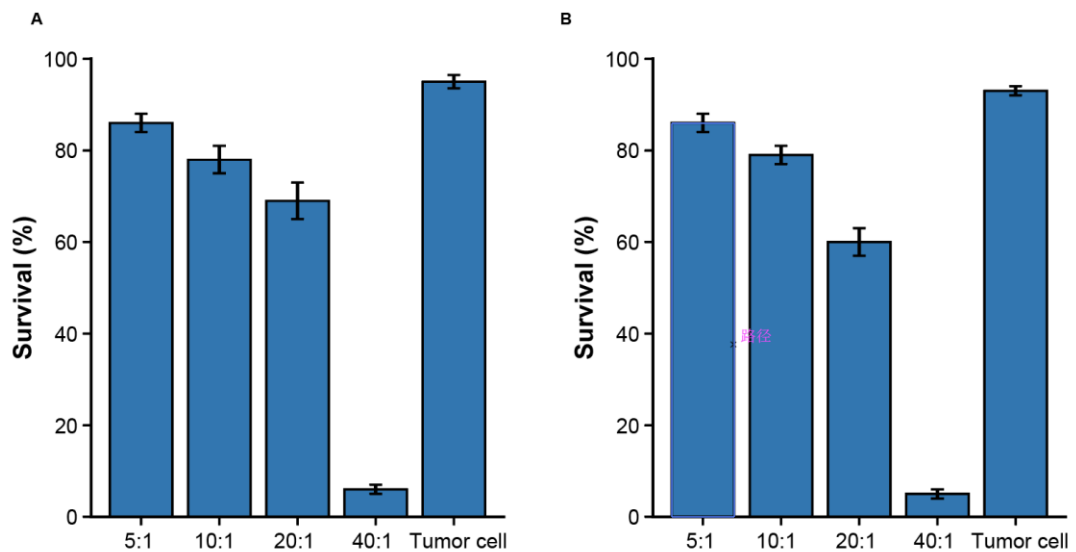


Figure R2. Pilot effector-to-target ratio titration before the formal in vitro cytotoxicity assay. A, B, Survival of adherent tumor cells after 4 h of co-culture with sorted PBMC-derived CD8⁺ T cells from two different patients at the indicated effector-to-target ratios. Non-adherent T cells were removed before CellTiter-Glo measurement.

14. Mouse models and tumor specificity claims:

- Statements such as “to directly address tumor-antigen specificity” are overstated. The OVA model relies on an artificial antigen and demonstrates antigen-driven differentiation rather than tumor specificity per se.
- In AKR-OVA tumors, OT-I cells are expected to differentiate into effector cells, whereas no such differentiation occurs in WT tumors lacking antigen. This observation is expected and does not uniquely implicate CX3CR1.
- According to Fig. 6c, more than 50% of OT-I tumor-infiltrating cells in AKR-OVA tumors are CX3CR1⁻, while CX3CR1⁺ cells represent <20%. The emphasis on CX3CR1⁺ cells therefore requires stronger justification.

Response: We thank the reviewer for this important comment. We agree that the previous wording overstated the conclusion from the OT-I/OVA model. Because OVA is an artificial model antigen, this system does not directly demonstrate endogenous ESCC tumor-antigen specificity. We have therefore

removed statements such as “to directly address tumor-antigen specificity” and now describe this experiment more accurately as an antigen-driven CD8⁺ T-cell differentiation model.

We also agree that OT-I cells are expected to show antigen-dependent effector differentiation in AKR-OVA tumors but not in WT tumors lacking OVA. Accordingly, we have toned down the interpretation of these data. We now state that antigen recognition can induce an effector program that includes a subset of CX3CR1⁺ CD8⁺ T cells, rather than claiming that this model uniquely implicates CX3CR1 or proves endogenous tumor specificity.

We further clarified that CX3CR1⁺ cells represent a minority of OT-I tumor-infiltrating cells in AKR-OVA tumors. Therefore, our focus on CX3CR1 is not based solely on its frequency in this mouse model. Instead, we position CX3CR1⁺ CD8⁺ T cells as a functionally tractable cytotoxic effector state within a broader antigen-driven CD8⁺ T-cell response, supported by combined evidence from human scRNA-seq/scTCR-seq, mIHC, in vitro cytotoxicity assays, adoptive transfer experiments, and peripheral blood validation.

Revised Results text:

“To assess whether antigen recognition can promote CX3CR1 expression in cytotoxic CD8⁺ T cells, we used the OT-I/OVA model. This model does not establish endogenous ESCC tumor-antigen specificity, but provides an antigen-driven system to evaluate CD8⁺ T-cell differentiation.”

Revised Discussion text:

“Our functional and translational analyses therefore position CX3CR1⁺ CD8⁺ T cells as a tractable effector population within the broader response-associated cytotoxic CD8⁺ T-cell program, rather than as the only response-associated subset. CX3CR1⁺ effector-memory CD8⁺ T cells showed short-term tumor-killing activity in vitro, and the OT-I/OVA ESCC model supported the induction of CX3CR1 expression in an antigen-driven context. Adoptive transfer of CD3⁺CD8⁺CX3CR1⁺ OT-I⁺ T cells delayed AKR-OVA tumor growth, with

stronger tumor growth suppression observed when combined with chemo-immunotherapy.”

These revisions clarify the purpose and limitations of the OT-I/OVA model, reduce overinterpretation of tumor specificity, and more appropriately justify the focus on CX3CR1⁺ CD8⁺ T cells.

15. - In Fig. 6f–h, a critical control group is missing: anti-PD-1 treated mice receiving CX3CR1⁻ OT-I cells. Without this control, the claimed synergy cannot be attributed specifically to CX3CR1 expression.

Response: We thank the reviewer for this important point. We agree that the absence of an anti-PD-1-treated group receiving CX3CR1⁻ OT-I cells limits the ability to attribute the enhanced antitumor effect specifically to CX3CR1 expression itself.

In the revised manuscript, we have clarified the scope of the adoptive transfer experiment and adjusted the wording to avoid overinterpretation. The current design supports the functional activity of CX3CR1⁺ OT-I cells in the AKR-OVA model and shows stronger tumor growth suppression when these cells were combined with chemo-immunotherapy. However, because a corresponding CX3CR1⁻ OT-I control group under anti-PD-1 or chemo-immunotherapy treatment was not included, we now interpret CX3CR1 as a marker of a cytotoxic effector CD8⁺ T-cell state in this model, rather than as direct evidence that CX3CR1 expression is mechanistically required for the therapeutic effect.

We have also acknowledged that additional experiments, including direct comparison with CX3CR1⁻ OT-I cells under anti-PD-1 or chemo-immunotherapy treatment, would be needed to determine whether CX3CR1 expression itself is functionally required for enhanced antitumor activity.

Revised Results text:

“Adoptive transfer of CD3⁺CD8⁺CX3CR1⁺ OT-I⁺ T cells delayed AKR-OVA

tumor growth, with stronger tumor growth suppression observed when combined with chemo-immunotherapy (Fig. 6f–h)."

Revised Discussion text:

"These findings support the functional relevance of CX3CR1⁺ CD8⁺ T cells as a cytotoxic effector state, while further studies, including direct comparison with CX3CR1⁻ antigen-specific CD8⁺ T cells, will be required to determine whether CX3CR1 expression itself is mechanistically required for enhanced antitumor activity."

These revisions avoid attributing the combined antitumor effect specifically to CX3CR1 expression in the absence of the corresponding CX3CR1⁻ OT-I control group.

16. Human validation:

- Fig. 6i shows increased CX3CR1⁺GZMB⁺ CD8⁺ T cells in the blood of one responder but not in one non-responder. While interesting, this is anecdotal and insufficient to support general conclusions. Inclusion of additional patients (at least 2–3 per group) is necessary to lend credibility to this observation.

Response: We thank the reviewer for this important comment. We agree that the flow-cytometric analysis in the previous version, which included only one responder and one non-responder, was limited in sample size and should be considered illustrative rather than sufficient to support a general conclusion.

To address this concern, we expanded the human peripheral blood validation cohort by collecting paired pre- and post-treatment blood samples from six ESCC patients receiving neoadjuvant therapy, including three responders and three non-responders (Figure R1A). We performed flow-cytometric analysis to quantify circulating CX3CR1⁺GZMB⁺ CD8⁺ T cells before and after treatment in each patient (Figure R1B).

In the revised manuscript, we have updated Fig. 6i, 6j and Extended Data Fig. 9e to include representative flow-cytometry plots and quantitative analysis of the expanded paired cohort. The expanded analysis showed an increase in

circulating CX3CR1⁺ GZMB⁺ CD8⁺ T cells after treatment in responders, whereas no comparable increase was observed in non-responders. These data provide additional exploratory human validation consistent with the tumor-tissue scRNA-seq, scTCR-seq and mIHC findings.

We have also revised the wording throughout the manuscript to avoid overinterpreting the peripheral blood flow-cytometry data. Rather than presenting these data as definitive evidence, we now describe them as exploratory human translational validation supporting the association between CX3CR1⁺ cytotoxic CD8⁺ T cells and treatment response. We have further added a limitation statement noting that larger prospectively collected paired blood cohorts will be required to validate circulating CX3CR1⁺CD8⁺ T cells as a response-associated biomarker in ESCC.

Reviewer #3:

The revised submission has addressed all of my previous concerns. The manuscript, figures, and supplementary table are now very clear, and all inconsistencies have been corrected. The authors now present a more appropriately tempered interpretation of the data.

I still have a minor reservation regarding the reliability of the short-term tumor killing assay shown in Fig. 6a. In particular, I wonder whether the removal of non-adherent T cells fully excludes the possibility that the measured ATP signal largely reflects activated T cells bound to adherent tumor cells, rather than tumor cell viability alone. However, this remains a technical concern that does not affect the overall quality of the work and the broad biological conclusions presented.

I therefore support publication of this manuscript in its current form.

Response: We sincerely thank the reviewer for the positive assessment of the revised manuscript and for supporting publication. We also appreciate the reviewer's remaining technical concern regarding the short-term CellTiter-Glo assay in Fig. 6a.

We agree that, although non-adherent T cells were removed before measurement, the ATP-based readout cannot fully exclude a contribution from activated T cells that remained associated with adherent tumor cells. We also acknowledge that this assay alone cannot determine the exact proportion of luminescence derived from tumor cells versus residual tumor-associated T cells. We have therefore further tempered our interpretation and describe the assay as a short-term ATP-based readout of residual adherent-cell viability after co-culture, rather than definitive evidence of tumor-cell-specific killing.

We have revised the Methods section (page 28, lines 808–812) as follows:

“To minimize the contribution of T-cell-derived ATP to the CellTiter-Glo signal, non-adherent T cells were gently removed before measurement. Under this condition, luminescence was used as a short-term readout of residual adherent-cell viability after co-culture. The ATP-based readout may include a residual contribution from T cells remaining associated with adherent tumor cells.”

The reported killing metric was calculated from the reduction in residual luminescence relative to tumor-cell-only control wells. We now present this experiment as supportive, rather than definitive, functional evidence and interpret it together with the scRNA-seq/scTCR-seq analyses, mIHC validation, OT-I/OVA model, adoptive-transfer experiment and peripheral-blood flow-cytometry analysis.