nature portfolio

Peer Review File

Tumor immune dynamics and long-term clinical outcome of stage IIIA NSCLC patients treated with neoadjuvant chemoimmunotherapy

Corresponding Author: Dr Dominic Schmid

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)
Thank you for the opportunity to review this interesting manuscript, wherein the authors report on multi-omic translational analysis of specimens obtained during the SAKK 16/14 trial. This study enrolled patients with Stage IIIA NSCLC (N2 involvement) treated with neoadjuvant platinum-based chemotherapy followed 3 cycles followed by 2 cycles of durvalumab and resection. Patients then underwent 1 year of adjuvant durvalumab treatment. Translational studies included tissue and blood interrogation at baseline (prior to chemoimmunotherapy; TP1), prior to neoadjuvant durvalumab (TP2), prior to surgery (TP3) and post 4 cycles of adjuvant durvalumab (TP4). This study reports the association of improved EFS with baseline tumor characteristics, including immune inflamed phenotype, tumor infiltrating CD8 T cell density and TLS size. The study also notes increased intra-tumoral T cell diversity and its association with improved EFS. Interrogation of the systemic immune system as measured through mass cytometry and multispectral flow cytometry on PMBC samples revealed post-treatment circulating Ki-67-expressing CD39+ PD-1+ CD8+ T cells that associated with improved EFS. Serum CCL15 elevations were noted in 3 patients with durable benefit to therapy for which CCL15 and its receptors CCR1 and CCR3 were explored in a pre-existing single-cell RNA dataset.

Major Comments/questions:
- Overall, the manuscript highlights previously known biomarkers of immune checkpoint blockade response with the potential to fundamentally understand the impact of chemotherapy on the modulation of key immune cells. The association of immune phenotype as it correlates to sequential chemotherapy and immunotherapy offers an incredibly opportunity to answer questions re: the role of these agents in neoadjuvant therapy for NSCLC. Additionally, this data set offers an opportunity to understand histology specific differences in immune profile and how that correlates with potential resistance mechanisms to neoadjuvant chemoimmunotherapy.
- In its current form, the manuscript does not take full advantage of this cohort's clinical impact. TP2 is a critically important time point, as it offers the opportunity to distinguish the impact of chemotherapy induction from that of immunotherapy. Recently published translational work in an LCMV model with sequential chemotherapy --> immunotherapy (https://doi.org/10.1158/1078-0432.CCR-23-1316) could be readily compared to these findings from human specimens.
- In addition, no mention is made of differential findings with respect to lung cancer histology, though it is well-recognized that the immune composition of lung cancer may vary significantly with respect to histology
- Do mutations described as having a potential deleterious impact on immunotherapy and chemotherapy response (i.e. STK11, KEAP1) confound the authors' findings at all?
- What is the significance of EFS relative to MPR/pCR and OS in this cohort? How does this differ from the other neoadjuvant IO +/- chemo studies?
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correlation with response to treatment including MPR.
Pg 7, Figure 1; helpful to have median EFS and OS (NR) in the graph. Notable that there was a good amount of attrition for digital pathology analysis (only 21/67 patients) - why? Was NGS done on up-front tumor specimens? Change in mutations with treatment could be of interest (Ricciuti, JCO 2024)
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Pg 13, Lines 221-222; it would be interesting to evaluate the change in TCR diversity in the peripheral blood across various time points from baseline through post treatment given the non-significant findings at the post-treatment time period. Numerous studies, including Yost (cited in this paper) report on the clonal expansion that is seen with ICB. It would be interesting to see the effect of TCR diversity from chemotherapy and immunotherapy, respectively, in the neoadjuvant setting.
Pg 13; what is the relationship between elevated intratumoral T cell clonal richness and TLS size? PD-L1?
Pg 14, Fig 4. The distribution of patients appears somewhat skewed with 15 patients within the EFS <1 year and 34 patients in the EFS >1 year timeframe. Is there a sufficient sample size to create 3 cohorts of extremely poor responders, average responders and exceptional responders for further stratification of the data? Does TCR richness change with treatment?
Pg 15; was WES performed? Or just targeted NGS + RNAseq?
Pg 16, Lines 274 – 275 describes the expansion of PD1+ CD8+ T cells in the peripheral blood post-chemoimmunotherapy that was primarily seen in responders. A recent paper by Mariniello A et al. Clin Cancer Res 2024, describes the influence of platinum-based chemotherapy in reducing proliferative PD1+ CD8+ T cell expansion compared to anti-PD1 therapy alone in a LCMV mouse model. It would be interesting to see if the effects of chemotherapy are detrimental to the later use of immunotherapy based on the longitudinal assessment of proliferating PD1+ CD8+ T cells in this translational study across all the pre-surgical time points.
Pg 16, Lines 276 – 277, the presence of CD57+ CD4+ T cells has been described in several studies in the infectious disease field as marker of cell senescence with potential long-term memory components. It would be interesting to understand if the expansion of this cell subset was coupled with markers of memory (CD27, CD127, TCF1, CCR7/CD45RO/CD45RA) versus a more terminally differentiated T cell subset marked increases in checkpoint markers such as CTLA4, TIM-3, LAG-3, etc.
Pg 23; authors should comment more specifically on the impact of chemotherapy before immunotherapy - this is what is unique compared to the well-described translational datasets on neoadjuvant immunotherapy alone and combination chemoimmunotherapy. For the digital path analysis, a significant limitation is that markers were identified on serial sections - much weaker than simultaneous measurement with a multiplex.
Pg 24, Line 444, ipilimumab is misspelled.

Reviewer #2

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

Reviewer #4

(Remarks to the Author)
This manuscript profiles the immune response within a cohort of NSCLC patients that were treated as part of a phase II trial with neoadjuvant chemo and immunotherapy and a subsequent adjuvant immunotherapy regiment. These immune parameters, which are sourced from tissue, serum, and PBMCs at various time during the trial, are further correlated to 5-year survival outcomes. Major findings for patients with better outcomes include evidence of tumor inflammation, superior infiltration of CD8 T cells, TLS formation, TCR clonal diversity, and activated markers for infiltrating T cells, all of which have been accepted as signs of productive anti-tumor responses. Interestingly, tumor mutation burden did not correlate to outcomes. Novel findings include TIM-3+ cDC1 among non-responders and signs of CCL15-CCR1 signaling among some responders. The dataset is extensive and impressive, and methods are sound. Enthusiasm is dampened somewhat in that the conclusions have already been highlighted in the field, and the patients are end up being responders seem to have more CD8 T cell infiltration on the onset. It is not clear if neoadjuvant therapy had any impact on turning more patients into responders. While the study seeks to elucidate the spatial dynamics of the tumor microenvironment, the study is rather descriptive. The Introduction states that the goal is to “evaluate whether emerging biomarkers are associated with sustained clinical benefit” but it is not clear if this had been accomplished by this retrospective analysis. In summary, the unique and deep dataset and its study is to be commended, but it is not clear if any hypotheses have been supported or even generated from the work. It is not clear whether this is suitable innovation to merit appearance in Nat Comm.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily addressed our comments.

Reviewer #2

(Remarks to the Author)
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Reviewer #4

(Remarks to the Author)
Thank you for the thoughtful revision of the manuscript, which has incorporated my request for overall significance as well as the more detailed comments from other reviewers. I greatly appreciate the inclusion of a preliminary experiment in the rebuttal, which indicates that the authors have been considering fundamental aspects of the project and how this research can lead to innovation in tumor immunology. I am satisfied that the paper has addressed my concerns and recommend for acceptance.

Tumor immune dynamics and long-term clinical outcome of stage IIIA NSCLC patients treated with neoadjuvant chemoimmunotherapy

Schmid et al.

Point by point reply

Reviewer #1 (Remarks to the Author)

Thank you for the opportunity to review this interesting manuscript, wherein the authors report on multi-omic translational analysis of specimens obtained during the SAKK 16/14 trial. This study enrolled patients with Stage IIIA NSCLC (N2 involvement) treated with neoadjuvant platinum-based chemotherapy followed 3 cycles followed by 2 cycles of durvalumab and resection. Patients then underwent 1 year of adjuvant durvalumab treatment. Translational studies included tissue and blood interrogation at baseline (prior to chemoimmunotherapy; TP1), prior to neoadjuvant durvalumab (TP2), prior to surgery (TP3) and post 4 cycles of adjuvant durvalumab (TP4). This study reports the association of improved EFS with baseline tumor characteristics, including immune inflamed phenotype, tumor infiltrating CD8 T cell density and TLS size. The study also notes increased intra-tumoral T cell diversity and its association with improved EFS. Interrogation of the systemic immune system as measured through mass cytometry and multispectral flow cytometry on PMBC samples revealed post-treatment circulating Ki-67-expressing CD39+ PD-1+ CD8+ T cells that associated with improved EFS. Serum CCL15 elevations were noted in 3 patients with durable benefit to therapy for which CCL15 and its receptors CCR1 and CCR3 were explored in a pre-existing single-cell RNA dataset.

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- Overall, the manuscript highlights previously known biomarkers of immune checkpoint blockade response with the potential to fundamentally understand the impact of chemotherapy on the modulation of key immune cells. The association of immune phenotype as it correlates to sequential chemotherapy and immunotherapy offers an incredibly opportunity to answer questions re: the role of these agents in neoadjuvant therapy for NSCLC. Additionally, this data set offers an opportunity to understand histology specific differences in immune profile and how that correlates with potential resistance mechanisms to neoadjuvant chemoimmunotherapy.

We sincerely appreciate your thoughtful and constructive feedback. Your insights into the potential of our dataset to investigate the effects of chemotherapy on immune cell modulation and the role of these agents in neoadjuvant therapy for NSCLC are highly valuable.

- In its current form, the manuscript does not take full advantage of this cohort's clinical impact. TP2 is a critically important time point, as it offers the opportunity to distinguish the impact of chemotherapy induction from that of immunotherapy. Recently published translational work in an LCMV model with sequential chemotherapy --> immunotherapy (https://doi.org/10.1158/1078-0432.CCR-23-1316) could be readily compared to these findings from human specimens.

We acknowledge that TP2 provides a valuable perspective on how chemotherapy and immunotherapy influence the host immune system. Notably, as highlighted in the referenced paper, the treatment sequence plays a critical role, as concurrent chemotherapy may dampen the immunostimulatory effects of checkpoint blockade.

In this revision, in response to the reviewer's suggestion, we have addressed this topic by conducting additional TCR sequencing on peripheral TP2 samples. Furthermore, we have expanded our discussion on the proliferative capacity of peripheral $CD39^+$ $PD\text{-}1^+$ $CD8^+$ T cells during neoadjuvant chemoimmunotherapy. Please see our responses to the specific questions below for further details.

- In addition, no mention is made of differential findings with respect to lung cancer histology, though it is well-recognized that the immune composition of lung cancer may vary significantly with respect to histology

We appreciate this important comment concerning the separate analysis of different NSCLC histologies and the feasibility of further subtyping immune responses across different histological subtypes. Our dataset comprises a total of 37 cases of adenocarcinoma, 22 cases of squamous cell carcinoma, 1 case of large cell carcinoma, and 7 cases of NSCLC not otherwise specified (NOS). In line with clinical practice, we will categorize these cases in the revised manuscript as squamous cell carcinoma (SCC, n = 22) and non-squamous cell carcinoma (non-SCC, n = 45). Notably, our analysis reveals no significant differences in overall or event-free survival between patients with SCC and non-SCC (**Extended Data Fig. 1a, b**).
The histological response signature—including the inflamed immune phenotype, intra-tumoral $CD8^+$ T cell infiltrate infiltrates, and TLS size—is present across both subtypes and does not exhibit a statistically significant difference in frequency between SCC and non-SCC (**Extended Data Fig. 2a-c**). Furthermore, we observe comparable patterns in TCR indices (**Extended Data Fig. 3a**) and peripheral immune cell activation (**Extended Data Fig. 8c**) between SCC and non-SCC. However, the number of cases decreases when further subsetting the cohort, which may limit statistical power.
Despite these challenges, our analysis indicates that the observed immune phenotypes remain largely consistent across various histological subgroups of NSCLC. We recognize the need for further validation of these findings and a deeper investigation into distinct immune response subtypes within independent cohorts. Such validation would not only reinforce the broader applicability of our observations but also potentially identify histology-specific resistance mechanisms to neoadjuvant chemoimmunotherapy.
Further subtyping of immune responses within adenocarcinoma histological categories (lepidic, papillary, acinar, micropapillary and solid) presents considerable challenges in the neoadjuvant setting. First, such subtyping requires a comprehensive evaluation of resection specimens, whereas preoperative biopsies, though adequate for diagnosing lung adenocarcinoma, do not sufficiently capture the high degree of intratumoral heterogeneity. Second neoadjuvant treatment induces substantial histological alterations, making further subtyping or tumor grading unsuitable according to established WHO criteria in these samples. These limitations currently hinder the feasibility of this level of detailed analysis. We anticipate that future studies in larger, independent cohorts will build upon these observations.

- Do mutations described as having a potential deleterious impact on immunotherapy and chemotherapy response (i.e. STK11, KEAP1) confound the authors' findings at all?

In the SAKK 16/14 cohort, 17% of patients had a *STK11* mutation (n = 8) and 15% had a *KEAP1* mutation (n = 7). All patients with *STK11* mutations were diagnosed with adenocarcinoma and were current or former smokers while one patient with a *KEAP1* mutation was a never smoker, and n = 2 patients with *KEAP1* mutations did not have adenocarcinoma (n = 1 with large cell carcinoma, n = 1 NSCLC NOS).
Furthermore, when checking for *KRAS* mutation status, only n = 2 patients with *STK11* mutations had concurrent *KRAS* mutations, while n = 1 patient had concurrent *KRAS* and *KEAP1* mutations. This information is displayed in **Extended Data Fig. 4f**.
STK11 and KEAP1 have been identified as negative predictors of immunotherapy response in patients with *KRAS*-mutated adenocarcinoma who have a positive smoking history, but not in *KRAS* wild-type adenocarcinomas[1]. The limited number of patients in our cohorts prevents the calculation of odds ratios to accurately assess the impact of these two mutations. However, we note that both patients with *KRAS/STK11* mutations exhibited a very low percentage of PD-L1$^+$ tumor cells (1%, data not shown), consistent with previous reports. Consequently, we cannot definitively determine whether the deleterious impact of these mutations may have influenced our results. We look forward to future translational trials with larger patient cohorts to further investigate these questions.

- What is the significance of EFS relative to MPR/pCR and OS in this cohort? How does this differ from the other neoadjuvant IO +/- chemo studies?

Since neoadjuvant treatment regimens are performed in earlier stage tumors, surrogate endpoints such as event-free survival and pathological response rates are commonly used for preliminary analysis. This is because assessing changes in overall survival requires a longer follow-up period compared to studies in more advanced tumor settings. Accordingly, EFS at 12 months was chosen as the primary endpoint of the SAKK 16/14 trial[2].

We would like to reference a recent review that has confirmed the predictive value of MPR/pCR and EFS for OS in neoadjuvant checkpoint inhibition[3], which notably includes the SAKK 16/14 trial. Furthermore, we now provide updated survival data in **Extended Data Fig. 1c-f**, illustrating the relationship between MPR/pCR, EFS and OS. Our analysis demonstrates that patients achieving MPR and pCR have significantly longer EFS ($p < 0.0001$ and $p = 0.0858$, respectively) and OS ($p < 0.0001$ and $p = 0.0288$, respectively).

- Clinical annotation of sites of recurrence could be of interest as well relative to tumor immune composition/response

Among the n = 67 evaluable patients, n = 30 experienced an EFS event, n = 25 of which have a recorded site of progression. Of these, n = 14 developed distant metastases, with the brain (n = 6) and bone (n = 4) being the most common metastatic sites. Meanwhile, n = 11 exhibited purely locoregional recurrence, occurring either in the ipsi- or contralateral lung or in new lymph nodes. We observed no significant differences in EFS and OS between patients with locoregional vs. distant progression ($p = 0.4758$ and $p = 0.7675$, respectively). These data are presented in **Extended Data Fig. 1g, h**.

Unfortunately, digital pathology data were available for only a subset of patients with recorded progressions, limiting our ability to associate distinct tumor immune profiles with future sites of progression. We appreciate the reviewer's suggestion, as such an association would indeed be valuable to explore. We look forward to future studies with larger cohorts to further investigate this question.

Minor comments by page:
Pg 4, Line 80; multicentric -> multicenter
Pg 4, Line 88; does -> did or do not

Both of these mistakes were corrected.

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We agree with the reviewer that in NSCLC patients being considered for perioperative immunotherapy, PD-L1 expression should be carefully assessed as a factor that may help guide the selection of the most appropriate treatment regimen. In this paragraph, our intention was to emphasize the relatively limited data on the predictive value of PD-L1 expression and TMB in the perioperative setting compared to their established role in purely systemic treatment for metastatic disease. We have revised this sentence accordingly.

Pg 7, Figure 1; helpful to have median EFS and OS (NR) in the graph. Notable that there was a good amount of attrition for digital pathology analysis (only 21/67 patients) - why?

We have added median EFS and OS to the survival curves in **Fig. 1**.

Regarding the attrition in digital pathology analysis, only a subset of the initial biopsies were tissue blocks suitable for digital pathology analysis. The remaining biopsies were cytology samples, primarily used for lung cancer diagnosis and PD-L1 assessment. The choice between these sample types was determined by the accessibility of the suspected lung tumor and local clinical protocols. Importantly, allowing both specimen types ensured that patient accrual was not hindered and enabled broad participation across multiple sites in Switzerland. We have clarified this point in the text accordingly.

Was NGS done on up-front tumor specimens? Change in mutations with treatment could be of interest (Ricciuti, JCO 2024)

Mutation profiling was conducted on mainly tumor resection samples, meaning the analysis was performed on tumors that had undergone neoadjuvant chemo-immunotherapy. The DNA

requirements for the assays used (50 ng for Foundation One CDx and 20 ng for Comprehensive Plus) were considered too high to be feasibly obtained from initial biopsies without compromising their use for other tissue-based analysis.

We agree with the reviewer that comparing mutational profiles before and after neoadjuvant immunotherapy could provide valuable mechanistic insights into the anti-tumor immune response. However, we note that in the referenced study[4], samples were collected from patients who had demonstrated a confirmed response or stable disease for at least 3 months, with a median interval of 18.9 months between biopsies. In contrast, the shorter time frame inherent to the neoadjuvant setting must be considered when interpreting the dynamics of mutational profiles.
Additionally, we have clarified the sourcing of material for DNA mutation analysis in the *Materials and Me*thods section.

Pg 8, Lines 144-145; please provide classification criteria for inflamed and excluded as there is no consensus criteria for that determination. Seems this was arbitrarily determined by reading pathologists? How many fields of view were analyzed?

We appreciated the reviewer's attention to this missing information. Immune phenotyping was performed out by consensus of two board-certified pathologists (ABSB and VHK) following the recommendations of the International Immuno-Oncology Biomarker Working Group[5,6]. The complete tumor area was assessed to determine immune status, as previously described[7]. Specifically, the spatial distribution of tumor-infiltrating $CD8^+$ T cells was categorized into three immune phenotype: (1) immune desert – characterized by very rare and isolated $CD8^+$ T cells detected in any of the assessed tumor compartments, (2) immune excluded - defined by the presence of $CD8^+$ T cells at the tumor environment, primarily at the invasive margin or within the stroma, with only rare and isolated T cells found within the intratumoral compartment, and (3) inflamed – marked by $CD8^+$ T cells infiltrating the stromal compartment, directly contacting tumor cells, and penetrating the tumor parenchmya.

This information has been added to the *Materials and Methods* section.

Pg 9, Figure 2; immune desert was not described in the results section and the distribution was not conveyed.

This is correct. **Fig. 2A** presents generic examples of the three immune phenotypes in isolated tumor regions from SAKK 16/14 samples, intended to aid the reader's conceptual understanding. As noted, final immune classifications were determined by evaluating the entire tumor, rather than isolated regions. In the SAKK dataset, the immunologically "cold" subtype was entirely represented by immune-excluded tumors. While some excluded tumors can contain regions with very little immune infiltration, we did not identify any completely immune-desert tumors in the final dataset. To prevent any misunderstanding, we have clarified that these are generic examples from representative tumor regions.

Pg 9, Figure 2; graphs c and d are very difficult to interpret statistically significant from non-significant data

In **Fig. 2c, d**, our aim was to emphasize that infiltration densities of the indicated cell populations did not differ between immune-excluded and inflamed tumors, neither in the tumor compartment nor in the stroma. This observation holds true for all four analyzed populations ($CD3^+$ T cells, $CD8^+$ T cells, $FoxP3^+$ T regulatory cells and $CD20^+$ B cells). To ensure clarity, we have revised the text accordingly and have also added previously missing information on statistical testing in the figure legend.

By contrast, as expected, all immune populations tended to be more abundant in the stroma than in the tumor compartment. For improved readability, we have opted not to display test statistics for this comparison, as well as for cross-population comparisons (e.g. $CD3^+$ T cells vs. $CD20^+$ B cells).

Pg 9, Figure 2; immune inflamed and TC are similar (unless criteria is different?)

We apologize for any confusion. TC (tumor compartment) refers to the segmented area within the tissue block where tumor cells are located. This compartment is surrounded by stroma. Immune cell densities are quantified and expressed per mm^2 within either the TC or the stroma. In contrast, the immune phenotype (desert, excluded, inflamed) is determined based on the spatial distribution of immune cells across these compartments, as assessed by pathologist consensus (as detailed in our previous response).

Pg 12, Figure 3; legend explanation of panel b and panel c are switched. Disambiguation of IB/RES should be in legend.

We thank the reviewer for pointing out the missing information. Legend explanations for panels **b** and **c** have been corrected, IB/RES has been explained.

Pg 12, Figure 3; C TLS size criteria? Versus above or below the median? What is the relationship between TLS size and PD-L1 score? Need to make sure this is not confounding.

The average (i.e. arithmetic mean) area of TLS in each initial biopsy was calculated, and the median of these values was used as a cutoff to classify samples as either “TLS size high” or “TLS size low”. This clarification has been added to the figure legend.

Furthermore, we have added **Extended Data Fig. 2d** to illustrate the relationship between PD-L1 score and average TLS size. Our analysis shows no significant difference in PD-L1 scores between samples with small and large TLS ($p = 0.3502$, Mann-Whitney test).

Pg 13, Lines 221-222; it would be interesting to evaluate the change in TCR diversity in the peripheral blood across various time points from baseline through post treatment given the non-significant findings at the post-treatment time period. Numerous studies, including Yost (cited in this paper) report on the clonal expansion that is seen with ICB. It would be interesting

to see the effect of TCR diversity from chemotherapy and immunotherapy, respectively, in the neoadjuvant setting.

We agree that investigating clonal dynamics across different phases of neoadjuvant treatment is highly relevant. To complement our data, we have now performed TCR sequencing on timepoint 2 (TP2) peripheral samples (collected after neoadjuvant chemotherapy but before neoadjuvant durvalumab). Of note, no tissue samples were available at this timepoint.

Before interpreting the results, we must highlight the technical challenges associated with preparing sequencing libraries in different batches. Although RNA quality metrics were comparable, TP2 samples had fewer sequencing reads compared to TP1 and TP3 samples (**Extended Data Fig. 3f**). Since clonal richness (the number of unique TCR clones) correlates with sequencing depth, this prevented a direct comparison of clonal richness across timepoints in patients with different event-free survival (**Extended Data Fig. 3g**). However, other TCR repertoire metrics, such as evenness, were not affected (**Extended Data Fig. 3h**).

To address this issue, we performed random down-sampling of sequencing reads (10'000, 50'000, 250'000, 500'000, 750'000, 1'000'000, 1'500'000 and 2'000'000 reads) and analyzed the impact on clonal richness (number of TCR clones) and TCR evenness. This analysis, illustrated with data from four representative patients (**Extended Data. Fig 3i, j**) revealed a near-linear increase in the number of detected TCR clones with increasing sequencing depth. Conversely, TCR evenness plateaued as sequencing reads increased. Based on these observations, we concluded that down-sampling is an appropriate method to mitigate batch effects between TP1/TP3 and TP2 sequencing runs. Consequently, all further analyses for PBMC samples were conducted using 250'000 randomly selected sequencing reads. No down-sampling was performed for TCR sequencing data from tumor resections.

We observed a trend toward a higher number of TCR clones in PBMC samples from patients achieving EFS ≥ 12 months both at baseline (TP1, $p = 0.1604$) and after neoadjuvant chemotherapy (TP2, $p = 0.0551$, **Fig. 4d**). Similarly, we detected a trend toward higher TCR evenness in patients with EFS ≥ 12 months at baseline (TP1, $p = 0.0853$, **Fig. 4e**). Furthermore, Hill-Simpson diversity showed a marked decrease from baseline (TP1) to post-chemotherapy (TP2) in all patients, potentially reflecting the deleterious effect of chemotherapy on T cell expansion (EFS ≥ 12 months, $p = 0.0234$; EFS < 12 months, $p = 0.1079$). Notably, in patients with EFS ≥ 12 months, Hill-Simpson diversity significantly increased again following neoadjuvant durvalumab ($p = 0.0301$, **Fig. 4f**), suggesting a potential recovery or expansion of T cell diversity after checkpoint blockade.

Pg 13; what is the relationship between elevated intratumoral T cell clonal richness and TLS size? PD-L1?

We found no association between intratumoral T cell clonal richness and TLS size in resections, as shown in **Extended Data Fig. 3b**. It is important to note that TCR sequencing was performed on resections, not on initial biopsies, due to sample limitations. In contrast, TLS were analyzed in both initial biopsies and resections. However, for survival analysis (**Fig. 3** and **Extended Data Fig. 2c, d**), we only used initial biopsy data. This decision was made because resections included samples with complete pathological response, making it impossible to quantify intratumoral TLS in those cases.

Similarly, we found no association between T cell clonal richness and PD-L1 score, regardless of whether patients achieved EFS ≥ 12 months or not. This is illustrated in **Extended Data Fig. 2e**.

Pg 14, Fig 4. The distribution of patients appears somewhat skewed with 15 patients within the EFS <1 year and 34 patients in the EFS >1 year timeframe. Is there a sufficient sample size to create 3 cohorts of extremely poor responders, average responders and exceptional responders for further stratification of the data? Does TCR richness change with treatment?

As discussed in the paper, we selected a 12-month EFS cutoff because it has been the primary endpoint for evaluating the efficacy of this treatment regimen. However, as the reviewer correctly noted, this approach results in an imbalance in patient numbers between groups. To address this, we conducted an exploratory analysis by stratifying patients into three groups: EFS < 12 months, EFS between 12 and 36 months, EFS > 36 months. We examined both T cell clonal richness (**Fig. 4**) and proliferation of tentatively tumor-reactive $CD39^+$ $PD\text{-}1^+$ $CD8^+$ T cells (**Fig. 5**). While there appears to be no difference in clonal richness between the EFS ≥ 12 < 36 months and EFS > 36 months groups, we observed a difference in baseline proliferation of $CD39^+$ $PD\text{-}1^+$ $CD8^+$ T T cells between these cohorts. This suggests that the proliferative capacity of tumor-reactive $CD8^+$ T cells at baseline may correlate with long-term event-free survival.

[editorial note: confidential, unpublished figures have been redacted

However, the reduction in sample size limits the statistical power of the dataset, leading to a loss of statistical significance in several comparisons. We have addressed this limitation in the *Discussion* to acknowledge its potential impact on data interpretation.

Regarding TCR richness, we refer to our previous comment, where we discussed its association (or lack thereof) with event-free survival (EFS), TLS size, and PD-L1 score, as well as the impact of batch effects on the analysis.

Pg 15; was WES performed? Or just targeted NGS + RNAseq?

Mutational analysis was performed using one of two extensively validated targeted NGS assays on DNA isolated from FFPE-embedded tumor resection samples.

The Foundation One CDx assay investigates 324 cancer-specific genes for base substitutions, insertions/deletions, copy number alterations and recombinations. Furthermore, it provides tumor mutational burden.
(https://www.foundationmedicine.de/de/our-services/cdx.html).

The Oncomine Comprehensive Assay Plus (Thermo Fisher Scientific) targets 517 genes and allows detection of base substitutions, insertions/deletions, copy number alterations, as well as tumor mutational burden estimation.

Gene expression profiling was run on FFPE-isolated RNA using the Oncomine Immune Response Research Assay (Thermo Fisher Scientific). Full RNAseq was not feasible due to poor RNA quality metrics, with a typical RNA integrity number (RIN) of ~2 and low RNA input. Importantly, the Oncomine assay captures expression levels of 395 genes that are commonly involved in immunotherapy response, providing a targeted but robust alternative to full RNAseq.

Pg 16, Lines 274 – 275 describes the expansion of PD1+ CD8+ T cells in the peripheral blood post-chemoimmunotherapy that was primarily seen in responders. A recent paper by Mariniello A et al. Clin Cancer Res 2024, describes the influence of platinum-based chemotherapy in reducing proliferative PD1+ CD8+ T cell expansion compared to anti-PD1 therapy alone in a LCMV mouse model. It would be interesting to see if the effects of chemotherapy are detrimental to the later use of immunotherapy based on the longitudinal assessment of proliferating PD1+ CD8+ T cells in this translational study across all the pre-surgical time points.

Interestingly, we observed increased proliferation in peripheral $CD39^+$ $PD\text{-}1^+$ $CD8^+$ T cells after neoadjuvant chemotherapy at TP2 compared to baseline (TP1). Furthermore, only in patients with EFS > 12 months, proliferation continued to increase from TP2 to TP3 following neoadjuvant immunotherapy (**Fig. 5g**). While the proliferative response to chemotherapy alone may seem counterintuitive, we believe several clinical factors can explain this observation.

In the study by Mariniello et al[8], mice received concomitant chemo- and immunotherapy every three days, with samples collected three days after the last administration. In this preclinical model, chemotherapy was shown to reduce expansion, proliferation, and cytokine secretion of LCMV-specific CD8+ T cells. In contrast, in our clinical trial, patients received chemotherapy on the first day of a three-week cycle. Several key factors likely contribute to the observed T cell dynamics:

- Cytopenia and rebound hematopoiesis:
  Chemotherapy typically induces peripheral pancytopenia around one week after administration, followed by homeostatic hematopoiesis to restore immune cell counts.
- G-CSF administration:
  Patients also received G-CSF to stimulate hematopoiesis and mitigate the risk of infection.
- Timing of TP2 Sampling:
  TP2 samples were collected three weeks after the last chemotherapy dose, immediately before the first durvalumab dose. By this time, hematopoietic recovery

would likely be underway, contributing to the observed increase in $CD8^+$ T cell proliferation.

Thus, the dynamics of $CD8^+$ T cell proliferation appear to differ based on event-free survival (EFS). In patients with EFS ≥ 12 months, proliferation remains stable or even increases following neoadjuvant immunotherapy (TP2 to TP3), suggesting a sustained immune response. In contrast, in patients with EFS < 12 months, proliferation decreases from TP2 to TP3, potentially indicating a lack of sustained T cell activation in response to durvalumab.

For further details on the SAKK 16/14 trial, including dosing schedules and treatment regimens, we refer to the study protocol available in the initial publication presenting the clinical findings[2].

Pg 16, Lines 276 – 277, the presence of CD57+ CD4+ T cells has been described in several studies in the infectious disease field as marker of cell senescence with potential long-term memory components. It would be interesting to understand if the expansion of this cell subset was coupled with markers of memory (CD27, CD127, TCF1, CCR7/CD45RO/CD45RA) versus a more terminally differentiated T cell subset marked increases in checkpoint markers such as CTLA4, TIM-3, LAG-3, etc.

We agree that $CD4^+$ $CD57^+$ cells are an intriguing subset, particularly given the growing recognition of immunosenescence as a potential resistance mechanism to cancer immunotherapy. To further characterize this population, we analyzed CyTOF data, and the results are presented in **Extended Data. Fig. 7c-e**.
Overall, $CD4^+$ $CD57^+$ T cells exhibit distinct phenotypic features:

- they express lower levels of CCR7, CD27, and CD127 compared to $CD57^-$ cells, suggesting a more differentiated state.
- they predominantly express CD45RO rather than CD45RA, indicating a memory-like phenotype, further supported by the presence of TCF1, particularly in responders.

However, several markers also suggest terminal differentiation:

- $CD57^+$ cells express higher levels of PD-1 and TOX, both associated with exhaustion and terminal differentiation.
- they exhibit lower expression of activation markers CD25 and CD28, potentially reflecting reduced activation capacity.

Interestingly, despite their differentiated phenotype, Ki67 expression was observed only in $CD57^+$ $CD4^+$ cells from responders, and proliferation further increased after neoadjuvant chemoimmunotherapy. This was unexpected, given that the overall abundance of $CD4^+$ $CD57^+$ cells was actually higher in non-responders.

These findings raise intriguing questions about the functional role of $CD4^+$ $CD57^+$ T cells in the context of immunotherapy, and we look forward to future studies that will further elucidate the dynamics and significance of this population over the course of treatment.

Pg 23; authors should comment more specifically on the impact of chemotherapy before immunotherapy - this is what is unique compared to the well-described translational datasets on neoadjuvant immunotherapy alone and combination chemoimmunotherapy. For the digital path analysis, a significant limitation is that markers were identified on serial sections - much weaker than simultaneous measurement with a multiplex.

We have expanded the *Discussion* on chemotherapy to further clarify the distinct effects of neoadjuvant chemotherapy versus durvalumab in the SAKK 16/14 trial.

We have also included a discussion on the limitations associated with using serial sections for digital pathology analysis. The primary reason for selecting single-marker IHC assays from routine diagnostics—rather than more complex multiplex approaches—was to maintain a strong translational focus, ensuring that our findings can be readily replicated in larger clinical trials. However, we acknowledge that this method does not allow for

- co-localization studies between different cell subsets (e.g. $CD3^+$ T cells and $CD20^+$ B cells)
- Confirmation of cellular identity through co-staining (e.g. $CD3^+$ $CD8^+$ CD8 T cells or $CD3^+$ $FoxP3^+$ T reg cells).

Despite these limitations, the cellular densities presented in **Fig. 2** were measured within specific tumor compartments, which were identified using H&E staining on the same slide as the IHC stain. This approach provides a spatially informed analysis while maintaining compatibility with clinical pathology workflows.

Pg 24, Line 444, ipilimumab is misspelled.

This has been corrected.

Reviewer #2 (Remarks to the Author):

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

We sincerely appreciate the time and effort that both you and your co-reviewer have dedicated to evaluating our manuscript. We fully support initiatives like this that facilitate training in peer review and provide well-deserved recognition for Early Career Researchers. Your constructive feedback has been invaluable in refining our work, and we are grateful for your thoughtful contributions.

Reviewer #4 (Remarks to the Author):

This manuscript profiles the immune response within a cohort of NSCLC patients that were treated as part of a phase II trial with neoadjuvant chemo and immunotherapy and a

subsequent adjuvant immunotherapy regiment. These immune parameters, which are sourced from tissue, serum, and PBMCs at various time during the trial, are further correlated to 5-year survival outcomes. Major findings for patients with better outcomes include evidence of tumor inflammation, superior infiltration of CD8 T cells, TLS formation, TCR clonal diversity, and activated markers for infiltrating T cells, all of which have been accepted as signs of productive anti-tumor responses. Interestingly, tumor mutation burden did not correlate to outcomes.

Novel findings include TIM-3+ cDC1 among non-responders and signs of CCL15-CCR1 signaling among some responders. The dataset is extensive and impressive, and methods are sound. Enthusiasm is dampened somewhat in that the conclusions have already been highlighted in the field, and the patients are end up being responders seem to have more CD8 T cell infiltration on the onset. It is not clear if neoadjuvant therapy had any impact on turning more patients into responders.

While the study seeks to elucidate the spatial dynamics of the tumor microenvironment, the study is rather descriptive. The Introduction states that the goal is to "evaluate whether emerging biomarkers are associated with sustained clinical benefit" but it is not clear if this had been accomplished by this retrospective analysis.

In summary, the unique and deep dataset and its study is to be commended, but it is not clear if any hypotheses have been supported or even generated from the work. It is not clear whether this is suitable innovation to merit appearance in Nat Comm.

We thank the reviewer for raising these important conceptual questions.

Regarding the impact of neoadjuvant therapy on converting more patients into responders, we acknowledge that the single-arm design of the SAKK 16/14 study limits our ability to directly assess this question. However, previous studies have conclusively demonstrated that neoadjuvant chemoimmunotherapy increases the proportion of patients achieving major or complete pathological response and prolongs event-free and overall survival compared to neoadjuvant chemotherapy alone[9–14].

Unlike in melanoma[15], a direct comparison between neoadjuvant and purely adjuvant (chemo-)immunotherapy regimens has not yet been performed in NSCLC. However, a recent review suggests that neoadjuvant immunotherapy may outperform purely adjuvant immunotherapy in NSCLC[16].

We chose to primarily investigate pathology-based immune characteristics (such as $CD8^+$ T cell infiltration, TLS size, and immunophenotype) in initial biopsies because assessing these parameters in post-immunotherapy samples is technically more challenging. For example, in patients achieving pathological complete response (pCR), $CD8^+$ T cell densities become difficult to define due to the loss of clearly delineated tumor margins. Given that these patients have the longest EFS and OS (**Extended Data Fig. 1e-f**), these technical limitations could introduce bias when evaluating pathological correlates of response.

With regard to our stated goal in the *Introduction*, we aimed to highlight the challenges of biomarker discovery in the neoadjuvant NSCLC setting, where obtaining tumor specimens is inherently more complex than in other cancers such as melanoma. Developing and refining these methods is essential for future studies to stratify patients into treatment arms based on immune characteristics. We acknowledge that our original phrasing may have been misleading and have revised the introduction accordingly:

Finally, we would like to highlight a key hypothesis generated from our study. To our knowledge, the upregulation of TIM-3 on peripheral cDCs in non-responders following anti-PD-L1 treatment has not been previously reported. This is particularly relevant as PD-L1 blockade on cDCs is a key mechanism driving response to treatment[17].
To further investigate this mechanism, we are currently conducting reverse-translation studies in mice. In preliminary experiments, we observed that anti-PD-L1 treatment upregulates TIM-3 on intratumoral cDCs, and this effect can be partially blocked by concurrent anti-TIM-3 therapy. These experiments were performed in a murine 4T1 intramammary tumor model, designed to mimic the surgical resection and treatment scheme of the SAKK 16/14 trial.

[editorial note: confidential, unpublished figures have been redacted

Given the preliminary nature of this experiment, we have chosen not to include it in the manuscript but to present it exclusively in this point-by-point response.

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