Peer Review File

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Reviewer A

In this manuscript, authors utilize multiplex immunofluorescence to understand the spatial distribution of different immune cells in non-small cell lung cancer to better characterize tumor-immune microenvironment (TIME). Authors demonstrate intricate and interesting TIME observations seen in different stages of lung cancer. I applaud the authors on their work. I have the below comments:

Comment 1: One of the main conclusions is the change in T-cells among different cancer state/stage. Authors suggest that in higher stages, the T-cells are less functional based on the co-existence of PD-L1+ tumor cells and PD-1+CD8+ T cells. Did the authors test for markers that can tell us about the functionality of the T cells? For example, I would be interested to know among the T-cells the activated/exhausted stage. I believe there are common exhaustion markers (CTLA4, LAG3, TIGIT). Were these checked?
Reply 1: Thank you for your constructive comments. We acknowledge that exhaustion markers play an important role in regulating T-cell effector function. However, these markers are not routinely tested, and as such, were not included in our panel for this study. Our current study panel has already assessed ten biomarkers that are most relevant to clinical oncology, including CD8, CD20, PD-1/L1, etc., and has conducted preliminary exploratory analyses. In light of your valuable suggestions, we plan to include exhaustion markers such as CTLA4, LAG3, and TIGIT in our future research to gain a more comprehensively understanding of the dynamic changes and functional states of T cells within the lung tumor microenvironment.

Comment 2: Authors should include morphology (lepidic, acinar, etc) for the adenocarcinoma. Especially since the authors are making the argument to characterize TIME as the tumor progresses, this information becomes important. For example, AIS/MIA to squamous is not really a progression but rather a different disease. Same with stage progression – if the histology is different, it is not a pure disease progression since you have to take into account the different histology. Morphology is a known prognostic factor, and I would be interested in the TIME difference between lepidic/acinar/papillary vs solid/micropapillary. Similarly, more details can be included on other known pathologic prognostic factors (visceral pleural invasion, STAS, lympho-vascular invasion).
Reply 2: In our study, morphological information was only recorded for patients in stage I adenocarcinoma. We have compared the immune microenvironment of patients with lepidic/acinar/papillary adenocarcinoma (n=37) and those with solid/micropapillary/mucinous adenocarcinoma (n=8). No significant differences in the immune microenvironment were observed between the groups. However, this might be due to the limited sample size, thus warranting validation in a large cohort.

Comment 3: Do the authors have data on tumor recurrence? If there are enough numbers, it would be interested to see differences between recurrence and no recurrence cases.

Reply 3: We appreciate the helpful comments. Unfortunately, we only have follow-up information for three patients of stage II-III, and do not have mature follow-up data of those of stage I. We will perform this analysis in the future.

Comment 4: Authors mention lack of TLS in the biopsy specimen for the 3 patients receiving neoadjuvant chemo-IO. Is lack of TLS something we can accurately conclude from small biopsy samples? I would think you can only comment on this on the resected specimen. Also, it is difficult to draw any conclusions from a sample of 3 patients. Having said that, I would be interested in the details/comparison of the 2 complete responders vs the 1 other case.
Reply 4: Thank you for your comment. The clinical case highlights TLS formation and TIME change due to neoadjuvant immuno-chemotherapy, which can guide precision therapy for NSCLC. We are currently collecting more pretreatment and post-treatment paired samples.

# Pts	Age	Sex	Side	Pathology	Stage	Smoke	PD-L1	Tumor shrink	Cycles	pCR	ECOG	Methods of surgery	Extent of resection	R0
1	70	M	Right	Ade	IIIB	Yes	<1%	40%	4	No	0	VATS	Lobectomy	Yes
2	66	M	Right	Ade	IIB	No	0.4	55%	2	yes	0	VATS	Lobectomy	Yes
3	69	M	Right	SCC	IIIB	Yes	0.42	30%	5	yes	1	VATS	Bilobectomy	Yes

Pts = Patients; Ade = Adenocarcinoma; SCC = Squamous cell carcinoma; VATS = Video-assisted thoracoscopic surgery; pCR = pathologic complete response

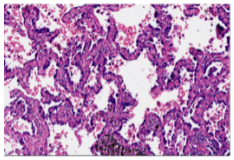
Comment 5: Of the 41 stage II-III patients, 3 received neoadjuvant chemo-IO. What was the treatment pattern for the other 38?
Reply 5: Among the 41 patients with stage II-III adenocarcinoma, 33 received neoadjuvant chemo/immuno-chemotherapy followed by surgery, while the remaining 8 patients received surgery alone.

Reviewer B

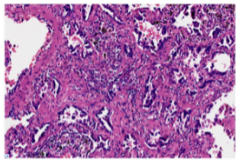
Characterization of the tumor immune microenvironment is crucial for the improvement of tumor biology knowledge and the identification of novel therapeutic targets.
The strict relationship between a specific immune landscape and a specific NSCLC subtype, stage and driver mutation is fundamental to develop a personalized therapy, as it can be very different and dynamic. Hence, it is mandatory make a distinction between subtype, stage and driver mutations. The authors' attempt in the present manuscript is to describe the TIME during NSCLC development, although they do not compare systematically a specific subtype, generalizing by stage instead.

Comment 1: Please provide Immunohistochemistry that has been used to characterized NSCLC samples.

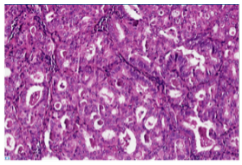
Reply 1: Here are examples of HE staining of AIS, MIA and invasive adenocarcinoma groups (200X), and HE images for all patients were provided as supplement material.



AIS 20X10



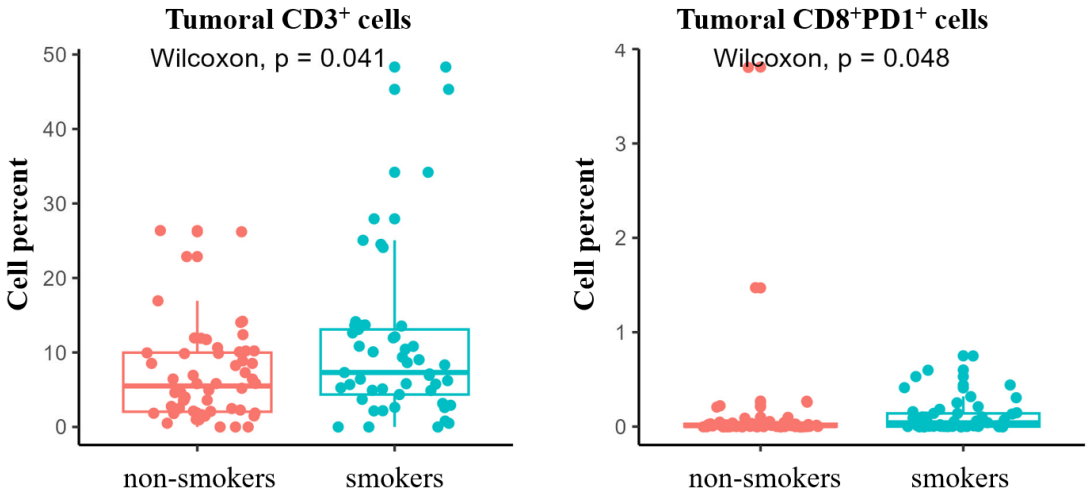
MIA 20X10



Invasive adenocarcinoma 20X10

Comment 2: In the introduction it is not clear whether the authors are focusing on Adenocarcinoma at different stages, or they are comparing different type of NSCLC. In the era on precision medicine, it is not acceptable to compare different kind of NSCLC, on the contrary it would be more important to narrow it down to specific subtypes, ADC for example, especially being the most represented in the cohort reported in the manuscript, subdividing for specific mutations. Moreover, it could be informative as a supplementary information whether smoking status affects any of the aspects mentioned in the present study.

Reply 2: Thank you for your kind suggestion. Patients who smoke showed a higher proportion of tumoral $CD3^+$ cells and CD8+PD1+ cells compared to non-smokers, and we added it as figure S6.



Comment 3: It is highly suggested to use the patients tested with NGS, in order to focus on specific ADC.

Reply 3: Thank you for your valuable comments and suggestions on our manuscript. Currently, NGS testing has been conducted only on AIS, stage I patients in our dataset. Follow-up NGS testing for stage II/III patients will continue in subsequent phases of our study. While our research was designed to provide a broad analysis of TIME changes across all subtypes of NSCLC, we recognize the particular impact of adenocarcinoma and will focus on this subtype

in greater detail in future research.

Comment 4: The authors subdivided the patient samples in AIS, MIA and stage I invasive NSCLC in the first place, then they merge AIS and MIA in the subsequent analysis, with no biological explanation. Please subdivide AIS and MIA and Stage I, focusing on ADC.
Reply 4: Thank you for your kind suggestion. We fully acknowledge the importance of distinguishing between different stages of early lung cancer, particularly the nuances between AIS and MIA. Our initial approach to combine AIS and MIA was to analyze the differences between non-invasive and invasive early-stage lung tumors. And the small number (n=3) precluded a statistically robust separate analysis for AIS. We recognize, however, that separating these groups in future studies will provide a more detailed understanding, especially when focusing on adenocarcinoma.

Comment 5: The present manuscript is entirely based on mIF, although there is a total lack of figures of the IF analysis at the basis of the graphs. IF figures should be provided. This is also fundamental since the authors perform spatial analysis to calculate the number of immune cells in proximity to tumor cells. This must be visualized.
Reply 5: Thank you for your valuable comments. mIF images could be found in figure 9. An example image of representative spatial analysis was provided upon request.

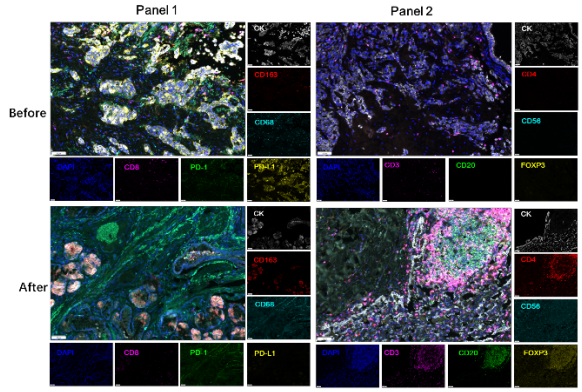
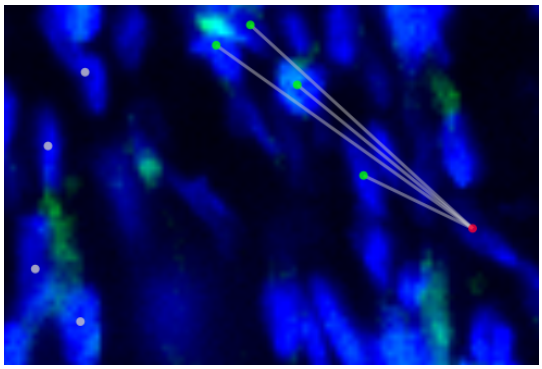


Figure 9, example image of mIF.



Example image of spatial analysis. Red dots, tumor cells; green dots, CD3+ T cells within a 30μm radius of the tumor; grey dots, CD3+ T cells outside a 30μm radius of the tumor; gray lines: nearest lines

Comment 6: Results on the counts of immune cell types between EGFR-mut Vs WT samples should be presented also subdivided according to the stage, at least in a supplementary panel since the main goal of the authors is focused on development of the NSCLC. There is no point in merging all the stage together, otherwise a clear biological explanation is required.
Reply 6: Thank you for your kind suggestion. We acknowledge the importance of analyzing the immune cell infiltration in relation to EGFR status according to stages. However, in our current study, only 64 patients with AIS or stage I NSCLC had available NGS results. We plan to expand our study in the future to include patients at different stages with NGS results, which will allow us to provide stage-specific insights into the immune cell infiltration and driver alterations.

Comment 7: Difference of LUAD and LUSC is out of focus if the authors intent is to describe the differences in immune cell types at different stages. LUAD and LUSC do not represent different stages of the same tumors, but different subtypes of NSCLC. There is a lot of confusion between NSCLC subtypes, stages and mutations through the entire manuscript.
Reply 7: Thank you for your kind suggestion. In the revised manuscript, we have deleted this part, which we agree was unnecessary.
Changes in the text: We removed S6 (see Page 10, line 330).

Comment 8: The description of the figure 5-6-7 is based on the differences between the stage I-II-III (total to 102 patients, disregarding the NSCLC subtype). Stage I according to the counts (figure 1, total of 51), this time also incorporates MIA samples (10). This means that in the previous analysis MIA samples were grouped with AIS and they showed significant differences as compared to stage I. In figure 5 they are considered together with Stage I (although they were different before) and then compared to stage II and III. This is completely confusing.
Reply 8: Thank you for your insightful thoughts. In this study, we examined the tumor microenvironment from two different perspectives. First, we analyzed the differences between non-invasive and invasive early-stage lung tumors. AIS and MIA, are early non-invasive lung tumors with no or limited histologic invasive components. These tumors have a 100% or nearly 100% probability of freedom from recurrence after complete tumor resection. Figure 3 investigate changes in the TIME during the evolution of early-stage lung cancer. Second, we also compared the immune microenvironment across clinical stages to assess how tumor progression influences immune cell infiltration. Figures 5-7 illustrate the differences among three groups according to clinical staging: stage I (n=61), stage II (n=12), and stage III (n=29). we hope these explanations would answer your doubts.

Comment 9: Explain clearly within the introduction the difference between density and percentage of cells and the biological relevance of reporting both data in the study.
Reply 9: Thank you for your kind suggestion. Immune cell density was quantified as the number of positively stained cells per mm^2, reflecting the concentration of specific cell types within the tissue. Immune cell percentage was calculated as the ratio of specific cell types to the total number of cells identified by DAPI (4',6-diamidino-2-phenylindole) staining. This approach allows us to understand both the absolute and relative abundance of immune cells in the tumor microenvironment, providing comprehensive insights into the immune landscape and its

potential impact on tumor progression. We have added this part in the introduction section.
Changes in the text: We have modified our text as advised (see Page 5, line 156-159).

Comment 10: Figure 1: in the text MIA samples accounts for 10 patients out of Stage I invasive (total of 61). In the figure 10 MIA and the remaining 51 Stage I invasive NSCLC are already subdivided. Please adjust accordingly the text or the figure to avoid confusion.
Reply 10: Thank you for your suggestion. We have modified the text accordingly.
Changes in the text: We have modified our text as advised (see Page 8, line 270-272).

Comment 11: Figure 2: please follow the order of the figure in the description within the text. AIS, MIA and Invasive stage I description is the opposite within the text. Figure legend should be more explicative.
Reply 11: Thank you for your kind suggestion. We have adjusted the description sequence in the main text according to the legend.
Changes in the text: We have modified our text as advised (see Page 9, line 281-283).

Comment 12: Numbers of percentage should be reported in the text and not assumed by the graphs.
Reply 12: Thank you for your kind suggestion. We have reported the numbers of percentage in the main text.
Changes in the text: We have modified our text as advised (see Page 9, line 283-286).

Comment 13: I would recommend using the identified cell type name within the figure on top on each corresponding graph instead of the markers and the name of the cell types within the legends.
Reply 13: Thank you for your valuable feedback. We have updated the figures accordingly, placing the cell type names directly on each corresponding graph as you suggested.
Changes in the text: We have modified our text as advised (see Figure 3-8).

Comment 14: Figure 3 reports tumoral density/percentage of specific immune cell types, i.e. CD8+ T cells, M1 and Immature NK cells. in the same paragraph the authors show in figure S2 density/percentage of other immune cell types both in the tumor and in the stroma. Explain why in the main figure 3 CD8+ T cells, M1 and Immature NK cells are described just in the tumor. Please clarify “number” on the y axis of figure 3E.
Reply 14: Thank you for your kind suggestion. In Figure 3, we have reported significant differences in the tumoral density/percentage of specific immune cell types such as CD8+ T cells, M1, and Immature NK cells. Conversely, in the stroma region, our analysis did not reveal significant differences in the density/percentage of the same immune cell types. And the results for all immune cell types in both the tumor and stroma are presented in Figure S2. This approach allows readers to concentrate on the most important findings in the main figure. The term 'number' on the y-axis of Figure 3E refers to the number of patients. We have revised the y-axis label to reflect this clarification.
Changes in the text: We have modified our text as advised (see Figure 3E).

Comment 15: Figure S3 provide average counts for different immune cell types in proximity of tumor cells and tumor cell PDL1+. There is no data showing the presence of PDL1+ cells and/or any consideration/statement whether every patient sample considered present both positive e negative tumor cells and/or the ratio of positive/negative tumor cells/sample.
Reply 15: Thank you for the valuable insights. In the Figure S3, we have reported the average counts of cells within a proximity distance of 30 μm. It is important to note that these counts are only reported when both cell types are present in the patient sample. If either type of cell is absent, we do not report the average count for that particular proximity measure. This approach ensures that the data presented is accurate and reflective of the actual cell interactions within the samples. We appreciate the opportunity to clarify this methodology and will ensure that this information is clearly stated in the method.
Changes in the text: We have modified our text as advised (see Page 8, line 251-252).

Comment 16: Figure 4 C should be "stromal" CD56bright cells, according to the legend, while title graph indicates "tumoral" instead, as the panel 4B.
Reply 16: Thank you for your comment. We are sorry for the error that occurred earlier and have modified Figure 4C.
Changes in the text: We have modified our text as advised (see Figure 4).

Comment 17: Figure S7: the figure and legend list 105 patients. In the corresponding section of the text (line 282), the authors mention 102 patients (supposing they are excluding AIS). Unclear.
Reply 17: Thank you for your comment. The correct number should be 102, and we have modified the legend.
Changes in the text: We have modified our text as advised (see Figure S7 legend).

Comment 18: Figure 7: using the same color (red for stage I in the left panel, and same color for TLS-neg in the right panel) in adjacent graph is misleading. Especially since the authors statement is "our results above showed that the occurrence rate of TLS decreased with the increasing stage.".
Reply 18: Thank you for pointing out the potential confusion regarding the color usage in Figure 7. We have changed the colors in Figure 7B, which will help readers more accurately interpret the results.
Changes in the text: We have modified the color as advised (see Figure 7B).

Reviewer C

The study characterizes the molecular and immune landscape of premalignant and early invasive lung cancer lesions using mutation analysis and RNA seq as well as a panel of multiplex staining. The study is interesting and timely and addresses questions that are difficult to answer due to limited cancer material. However, there are some major concerns and several minor aspects that should be addressed to increase the informative content, interpretability, and impact of this manuscript.

Also, there are serious incongruity that should be eliminated. The descriptions, figures and the text are sometimes negligent presented. Finally, the main aim should be in focus: what is the difference between non-invasive, early invasive, and fully invasive lesions?

Comment 1: In this lung cancer journal, the introduction does not need any general information about lung cancer. Instead, I would better describe the definition of early lesions and explain why the immune microenvironment is important in this context.

Reply 1: Thank you for your comments. We have We have removed content related to lung cancer and supplemented the role of tumor filtering lymphocytes in NSCLC.

Changes in the text: We have modified our text as advised (see Page 4, line 130-132； Page5,line 133-135).

Comment 2: I wonder why only 105 patients could be identified in the timeline 2017-2023? The proportion of MIA in this context seems too high. Is there a bias? Why so few squamous carcinomas? Please indicate clearly. Is the cohort representative of patients in the region and hospital?

Reply 2: Thank you for your valuable feedback on our manuscript. We appreciate your insightful comments regarding the patient cohort and its representativeness.

Regarding the number of identified patients from 2017 to 2023, we acknowledge your concern. The predominance of early-stage patients, particularly those willing to undergo mIF testing, reflects our department's focus on this subset for our study on minimally invasive adenocarcinoma (MIA) and invasive adenocarcinoma (IAC) of the lung. This selection bias towards adenocarcinoma patients is due to both our research interests and the natural distribution of adenocarcinoma cases in our department.

As for the apparent scarcity of squamous carcinomas in our cohort, we agree it merits clarification. The recruitment strategy prioritized adenocarcinoma patients for immunoenvironmental assessment, which naturally limited the inclusion of squamous carcinoma cases.

Comment 3: Consider excluding the squamous cell cases since they do not fit in the comparison.

Reply 3: Thank you for your kind suggestion. In the revised manuscript, we have deleted this part, which we agree was unnecessary.

Changes in the text: We removed figure S6 (see Page 10, line 330).

Comment 4: Also, the cases with neoadjuvant therapy might not be useful for the main comparison.

Reply 4: Thank you for your comment. The clinical case highlights TLS formation and TIME change due to neoadjuvant immuno-chemotherapy, which can guide precision therapy for NSCLC. Thus, we prefer retaining the result.

Comment 5: The carcinoid cases should be excluded definitely.

Reply 5: Thank you for your comments on our manuscript. Regarding the suggestion to exclude carcinoid cases from our study, we would like to address this point as follows: According to the NCCN guidelines, carcinoid tumors can be classified under the umbrella of non-small cell lung

cancer (NSCLC). Thus, we prefer retaining the case.

Comment 6: What is the other histology?
Reply 6: Thank you for your query regarding the histology of another case in our study. The specific histology of this sample is mucinous adenocarcinoma.
Changes in the text: We have modified our text as advised (see table1).

Comment 7: The study would benefit from survival and treatment data.
Reply 7: Thank you for your suggestion regarding the inclusion of survival and treatment data in our study. We are currently in the process of follow-up, and we plan to incorporate these data in our future analyses.

Comment 8: Please separate the RNA and DNA and multiplex IF analysis in the methods section.
Reply 8: Thank you for your suggestion regarding the separation of RNA and DNA and multiplex IF analysis in the methods section. We have already made the necessary revisions according to your recommendation.
Changes in the text: We have modified our text as advised (see Page 6,line 195-99;page10, line 200-228).

Comment 9: Were the samples reviewed by a pathologist with experience in lung tumors? This is important since the distinction between AIS and MIA can be difficult. Please provide HE figures with magnification inserts for all AIS and MIA as supplements, and maybe some examples as a main figure.
Reply 9: Thank you for your comments. We have provided HE images for all patients in figure S12. And representative HE images were shown in figure 1.

Comment 10: Please describe the DNA method better. It is difficult to understand that this is targeted sequencing. Same questions for the RNA analysis. Targeted or RNAseq? Only fusions? Which fusion were evaluated?
Reply 10: Thank you for your suggestion regarding the DNA method description. DNA and RNA analysis are both target sequence. We have revised the manuscript accordingly. Here is the list of genes in the 3DMed 35-gene panel: fusions were identified by RNA sequencing, and single nucleotide variations (SNVs) and insertions/deletions (Indels) were identified by DNA sequencing.

Gene list
AKT1, *ALK*, *ARAF*, *BRAF*, *EGFR*, *ERBB2*, *ESR1*, *FGFR1*, *FGFR2*, *FGFR3*, *GNA11*, *GNAQ*, *HRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *MTOR*, *NRAS*, *NRG1*, *NTRK1*, *NTRK2*, *NTRK3*, *PDGFRA*, *PIK3CA*, *RET*, *ROS1*, *CYP2C19*, *DPYD*, *TPMT*, *UGT1A1*, *BCL2L11*, *PDGFB*

Changes in the text: We have modified our text as advised (see Page 6,line 195-99;page10, line 200-228).

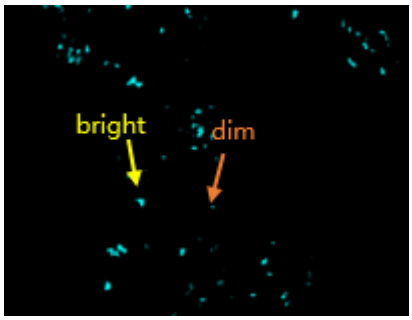
Comment 11: The multiplex staining is based on 11 markers plus DAPI. Please indicate clearly that this is done in two separate panels.
Reply 11: Thank you for your comments, we have modified the figure 9 to show our two panels.
Changes in the text: We have modified the figure 9 (see the figure 9).

Comment 12: Did you stain whole slides? How did you select the regions of interest?
Reply 12: In our study, we performed whole slide staining, and then a pathologist carefully reviewed the slides to identify and select the regions of interest (ROIs).

Comment 13: Was a pathologist involved in determining regions and excluding artifacts? Please provide representative staining with high magnification inserts as a main figure (high resolution).
Reply 13: Thank you for your comments. Tumor parenchyma and stroma were differentiated based on cytokeratin (CK) staining. We utilized image analysis software 'APTIME,' which features an integrated automated region recognition model, to facilitate this process. A pathologist reviewed the images to ensure the accuracy of the analysis and to exclude any artifacts. The mIF image was shown in figure 9.

Comment 14: How were intensity cut-offs for the markers and immune cell annotation made?
Reply 14: The multiplex IF images were scanned and then subjected to a pathologist's evaluation through our in-house image analysis software “APTIME”. The pathologist assessed images, defined phenotype parameters, and set the intensity thresholds of marker for each image. Then, the software generated and exported detailed results that included assessments of staining intensity, distribution, cell type and morphological features. These data were then utilized to classify and annotate the cells within the regions of interest (ROI).

Comment 15: What were the cut-offs for CD56 bright and dim (please show examples)? How did you address CD56 positive staining in tumor cells?
Reply 15: The threshold values for CD56 bright and dim were established for each sample by a pathologist through assessment of staining intensity, distribution pattern of the CD56 protein, and cellular context. The parameters are stored in JSON format within the image analysis software for cell annotations.
Reply 15: The pathologist has established threshold values for CD56 bright and dim expressions for each sample through a meticulous assessment of staining intensity, the distribution pattern of the CD56 protein, and the cellular context. These parameters have been meticulously stored in JSON format within our image analysis software, enabling precise cell annotations.
Subsequently, software have determined the quantities for the following immunophenotypes based on the Opal 480 fluorescence: Opal 480 Weak Positive Cytoplasms,Opal 480 Moderate Positive Cytoplasms,Opal 480 Strong Positive Cytoplasms. The count of Opal 480 Strong Positive Cytoplasms to represent CD56 bright cells. The sum of Opal 480 Weak Positive Cytoplasms and Opal 480 Moderate Positive Cytoplasms to represent CD56 dim cells.



Representative images of NK cells.

Comment 16: The presence of TLS is not clear. How many cells should be present in a cluster?
Reply 16: The tertiary lymphoid follicles were well-formed B cell germinal centers surrounded by T-cell aggregates towards the periphery. TLS were assessed on multiplex IF images and consisted of a CD3 + T cell zone and a CD20 + B cell core in our study as previous reported (PMID: 35768550; PMID: 38528050) .There was no specific cut-off for CD3+ and CD20+ number used to classify TLS positive or negative samples; rather, the presence of these cellular components in the described pattern was the basis for classification. Example image of TLS could be found in figure 9.

Comment 17: What program and code were used to calculate the distances? How were they calculated?
Reply 17: The distance could be computed with python using the squared Euclidean distance formula.
Here's the provided Python code snippet with comments:

```
# Import the pandas library
import pandas as pd
# Import the numpy library
import numpy as np
# Import the cdist function from the scipy.spatial.distance module, which computes distance between each pair of observations
from scipy.spatial.distance
import cdist
#read the data
csv_reader=pd.read_excel("../data/sample1_cell.xlsx")
# Select the rows from the DataFrame where the 'type' column is equal to ' CD8+', " CK+" and take only the 'X' and 'Y' columns
A=csv_reader.loc[csv_reader["type"] =="CD8+",['X','Y']]
B=csv_reader.loc[csv_reader["type"] =="CK+",['X','Y']]
a=cdist(A, B, metric='euclidean')
print(a)
```

Comment 18: Please show survival curves of patients. The results would be much more valuable with survival times.
Reply 18: Thank you for your suggestion regarding the inclusion of survival curves for patients

in our study. We are currently in the process of collecting patient survival data, and we plan to analyze these data in future studies.

Comment 19: In general, the figures are difficult to read and not sufficiently described in the legends. Here should some more efforts be invested.
Reply 19: Thank you for your feedback regarding the readability and description of the figures in our manuscript. We apologize for any inconvenience caused and have revised the figures and their legends according to your suggestions.
Changes in the text: We have modified revised the figures and their legends, especially figure 2 (see Figures).

Comment 20: Figure 2: Please indicate which samples were AIS and MIA. The figure is difficult to read, and the font is too small. Please improve it.
Reply 20: Thank you for your feedback regarding Figure 2. We have made the requested modifications to indicate which samples were AIS and MIA, as well as improving the readability by adjusting the font size.
Changes in the text: We have modified the image as advised (see Figure 2).

Comment 21: I have doubts that the patients had concurrent ALK and EGFR mutations; also, KIT, NTRK3, and EGFR together is not plausible. Please explain and provide evidence that this is true. Very unusual.
Reply 21: Thank you for your careful review and comments. We carefully reviewed the commutations with EGFR.

Gene	c-dot	p-dot	Variant type
NTRK3	c.1850T>G	p.F617C	Nonsynonymous
KIT	c.2484C>T	p.N828=	Synonymous
EGFR	c.2573T>G	p.L858R	Nonsynonymous

The provided table details mutations in KIT, EGFR, and NTRK3 genes from a patient. The KIT mutation, identified as a synonymous change (p.N828=), does not alter the amino acid sequence and is generally not associated with pathogenic effect. Consequently, we decided to exclude this mutation from our analysis.

Gene	c-dot	p-dot	Variant type
ALK	c.4210C>G	p.L1404V	Nonsynonymous
ALK	c.4573A>G	p.K1525E	Nonsynonymous
ALK	c.4573A>G	p.K1525E	Nonsynonymous

Among the three identified ALK mutations, two were classified as clinically benign changes (p.K1525E). As a result, we have excluded these two from our analysis, leaving one ALK mutation alongside the EGFR mutation for further consideration. Literature reviews indicate that EGFR and ALK are two critical driver genes in NSCLC. Early studies suggested they are independent molecular events that are mutually exclusive. However, there have been reports in small sample studies of cases with concurrent EGFR and ALK mutations. Concurrent EGFR and ALK mutations are rare molecular events, occurring in approximately 1% of cases. The clinical and pathological characteristics of such cases are not well-understood, and there is

currently no consensus on their treatment.
[1] Wang X, Zhong D. [Advances in Double Mutations of EGFR and ALK Gene in Non-small Cell Lung Cancer]. Zhongguo Fei Ai Za Zhi. 2018 Sep 20;21(9):686-691. Chinese.
Changes in the text: We have modified our figure 2 (see figure 2).

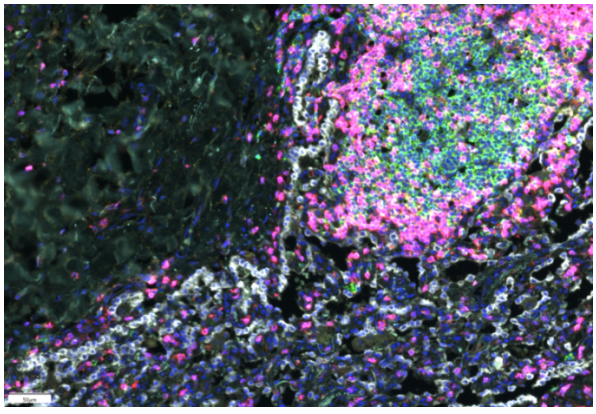
Comment 22: Please also indicate the different histologies exactly, and not only AC and non-AC, but I would exclude them anyway.
Reply 22: Thank you for your suggestion. We have addressed this concern by listing the specific subtypes of lung cancer in our table 1 to provide comprehensive information on the different histologies studied.
Changes in the text: We have modified our table as advised (see table1).

Comment 23: Please check statistically if there are differences between frequency of mutations between AIS, MIA and invasive cancer.
Reply 23: Thank you for your suggestion regarding the comparison of mutation frequencies between AIS, MIA, and invasive cancer. Due to the limited number of AIS samples (only 3 cases), they could not be analyzed as a separate group. We found no significant differences in mutation frequencies between MIA and early-stage IAC patients.

Comment 24: Please give some examples of mIF staining with annotations as figure.
Reply 24: Please find the figure 9 as example images of mIF.

Comment 25: Figure 3: Please also provide as supplementary data for AIS, MIA, and invasive AC separately. Are there differences? Please also adjust the P-value for multiple testing.
Reply 25: Due to the scarcity of AIS cases, with only three in total, they were not grouped separately. After conducting multiple tests, we did not obtain any results that were statistically significant for figure 3.

Comment 26: TLS: Please show an example of TLS as a figure and maybe also a frequency histogram (number of TLS per case). The number of cases with any TLS seems low. Please compare to the literature. Also, here please provide adjusted p-values (multiple testings).
Reply 26: Thank you for your comment. Please find the example figure of TLS below and also in figure 9.



Representative images of TLS. Green: CD20+ cells, Magenta, CD3+ cells.

In our study, 49.02% (25/51) of the patients with stage I invasive NSCLC had TLS. A previous study has reported a comparable prevalence of 44 % among the stage IB lung adenocarcinoma population (PMID: 35801360). However, it is important to note that there is a scarcity of studies on the prevalence of TLS in early stage NSCLC both domestically and internationally. Therefore, further investigation is warranted to achieve a comprehensive understanding of this phenomenon.

Comment 27: Why was the spatial analysis not performed for AIS and MIA compared to invasive? That was the aim of the study.
Reply 27: Thank you for your suggestion. Unfortunately, due to the limited number of AIS samples (only 3 cases), we were unable to perform a comparison of separate spatial results for this subgroup.

Comment 28: I do not understand figure 7. What does the average count mean? E.g., Three mean interactions of CD20 and CD3 cells+? That seems low.
Reply 28: Thank you for your comments. In Figure 7, the term "average count" refers to the mean number of CD20+ B cells found within a 30-micrometer radius of CD3+ T cells. This measurement is used to quantify the spatial interaction between these two immune cell populations. While this number may initially seem low, it is important to consider the scale of cellular interactions and the specific biological context when interpreting these results.

Comment 29: The comparison of TLS positive and negative is also confusing. Does TLS not per se show B and T-cell interaction?
Reply 29: Thank you for your comment. TLS, are indeed characterized by the interaction of B and T cells, typically with a B cell core surrounded by a T cell zone. The presence of TLS in a tissue sample generally indicates a localized immune response with the potential for antigen presentation and T cell activation. When comparing TLS positive and negative samples, the focus is often on the presence or absence of these organized structures and their potential impact on the local immune response, rather than on the absolute presence of B and T cells.

Comment 30: The same is true for figure 8. The presence of TLS might provide a higher number of B-cells or T-cells. How to extract additional information? Also, here please provide adjusted p-values (multiple testings). Maybe focus on other cell types.
Reply 30: Thank you for your comment. Regarding the spatial distribution of immune cells in TLS-positive and TLS-negative NSCLC patients, our analysis revealed that while the density of CD3+ T cells and CD20+ B cells did not differ significantly between the two groups, the spatial arrangement of these cells was notably distinct. In TLS-positive patients, these immune cells were found to be more densely clustered around tumor cells, suggesting an enhanced local immune response. This observation underscored the importance of spatial context within the tumor microenvironment and highlights the potential immunomodulatory role of TLS. We have also demonstrated that patients with TLS-positive tumors exhibited a reduced aggregation of PD-L1+ tumor cells in proximity to PD-1+CD8+ immune cells compared to TLS-negative patient. These findings indicated that the formation of TLS was associated with potential anti-

tumor activity. While we recognize the importance of a broader analysis could provide a more comprehensive understanding of the immune response and its modulation by TLS, we will incorporate additional immune cells and relevant checkpoint markers for further exploration. With conducting multiple tests, $CD4^{+}_{30\mu m}$ tumor , $CD20^{+}_{30\mu m}$ tumor, $PDL1^{+}$ tumor $_{30\mu m}$ $PD1^{+}$ $CD8^{+}$ were still statistically different between the two groups.

Comment 31: You could also compare the distance between EGFR positive and negative? And separately for invasive against MIA and AIS.
Reply 31: Thank you for your suggestion. We conducted this analysis as recommended, but unfortunately, we did not obtain significant results.

Comment 32: Figure 9: I would not include this analysis. The cases are few and are not within the scope of the study.
Reply 32: Thank you for your suggestion. While your analysis is insightful, the clinical case emphasizes TLS formation and TIME change due to neoadjuvant immuno-chemotherapy, which holds potential for guiding precision therapy in NSCLC. Therefore, we are inclined to retain this comparison.

Comment 33: Discussion: Should be updated in accordance with the updated results. The disadvantages of the study can be discussed in more detail.
Reply 33: Thank you for your suggestion. We have revised the discussion section in accordance with your advice. The updated content now reads:
"First, this study was limited by its retrospective design and small sample size. Second, only a limited number of immune markers was assessed. Nevertheless, the use of mIF allowed for the characterization of multiple biomarkers that are closely related to immune activators and suppressors, making it feasible to monitor the TIME during tumor evolution. Future studies should integrate analysis from diverse confirmatory experimental methods, including messenger RNA (mRNA) sequencing, whole-exome sequencing, and immunoproteomics."
Changes in the text: We have modified our table as advised (see Page 15, line 501-507).

Reviewer D

In this current manuscript, the authors provide novel data of the immune landscape in tumour tissue of NSCLC across development including adenocarcinoma in situ, minimally invasive adenocarcinoma and stage 1 to 3. Additionally, NGS was performed on adenocarcinoma in situ, minimally invasive adenocarcinoma and stage 1 invasive cancer.
Overall, this work contains significant novel results, with clear conclusions and improves our understanding of the TIME across NSCLC stages and its relationship with EGFR mutations.
My main concern is the lack of detail regarding the multiplex Immunofluorescence methods.

Comment 1: Could the authors clarify details of the multiplex Immunofluorescence methods and show controls to confirm true staining and lack of spectral bleedthrough, etc.? Specifically:
Reply 1: Thank you for your thoughtful comments and recommendations regarding our

manuscript. We appreciate your interest in the details of our multiplex immunofluorescence methods. The specific experimental details are as follows:

Panel development:

We determined primary antibody dilutions using chromogenic IHC, and assigned opal fluorophores to each marker, then optimized primary antibody conditions and opal dye concentration.

Staining and analysis images

Formalin-fixed paraffin-embedded (FFPE) NSCLC sections were baked at 60°C for 1 hour and dewaxed and rehydrated through gradients of Xylene and ethanol concentrations. Antigen retrieval was performed by boiling samples to 100°C for 20 minutes in either pH 6 or 9 Antigen retrieval buffer (Akoya Biosciences). Tissues were blocked for 5 minutes at room temperature before incubation for 1 hour with primary antibodies listed in the table below. Primary antibodies were detected using the OPAL Polymer HRP Ms + Rb (Akoya Biosciences) for 10 minutes, followed by a 10-minute incubation with Tyramide signal amplification and Opal Fluorophore (Akoya Biosciences). Tissue sections were then boiled at 100°C to strip antibody-HRP complexes and unbound Opal Fluorophores (Akoya Biosciences). This process was repeated until all markers have been stained (Note: Before staining with Opal780 Fluorophore (Akoya Biosciences), TSA-DIG was applied for an additional 10-minute incubation to enhance the staining intensity). Following the final marker staining, tissues were incubated with DAPI for 5 minutes. This entire process was automated using the Bond RX (Leica Biosystems) multiplex immunohistochemistry stainer. Images were captured using the Vectra polaris multispectral imaging system (Akoya Biosciences), and the unmixing process was facilitated by "inform" (Akoya Biosciences) software, and exported as .qtiff images. Images were then imported in APTIME (3Dmed) and to quantify the expression of markers on a cell by cell basis

Table: Antibodies used in the study

	Target	**Source**	**Dilution**	**Opal fluorophores**	**Color**	**Staining sequence**
Panel 1	CD163	Abcam, ab182422	1:500	Opal 620	Red	1
	CD8	Abcam, ab178089	1:200	Opal 690	Magenta	2
	CD68	Abcam, ab213363	1:1000	Opal 480	Cyan	3
	PD-1	CST, D4W2J, 86163S	1:200	Opal 520	Green	4
	PD-L1	CST, E1L3N, 13684S	1:400	Opal 570	Yellow	5
	pan-CK	Abcam, ab7753	1:100	Opal 780	White	6
Panel 2	CD20	DAKO, L26, IR604	1:1	Opal 620	Green	1
	CD3	DAKO, A0452	1:1	Opal 690	Magenta	2
	CD56	Abcam, ab75813	1:1000	Opal 480	Cyan	3
	CD4	Abcam, ab133616	1:100	Opal 520	Red	4
	FOXP3	Abcam, ab20034	1:100	Opal 570	Yellow	5
	pan-CK	Abcam, ab7753	1:100	Opal 780	White	6

Comment 2: How was the unimixing performed?

Reply 2: In our study, unstained and single stained tissue images were gathered to establish a spectral library containing each fluorophore's emission signature. After raw image acquisition by Vectra polaris multispectral imaging system (Akoya Biosciences), the unmixing process was facilitated by "inform" (Akoya Biosciences) software, and exported as .qtiff images.

Comment 3: Was an unmixing library created using the same tissue used in the study?
Reply 3: For our study, we utilized a standard spectral unmixing library built with tonsil sample. This library was specifically tailored to our laboratory's needs and the types of tissues and stains commonly used in research.

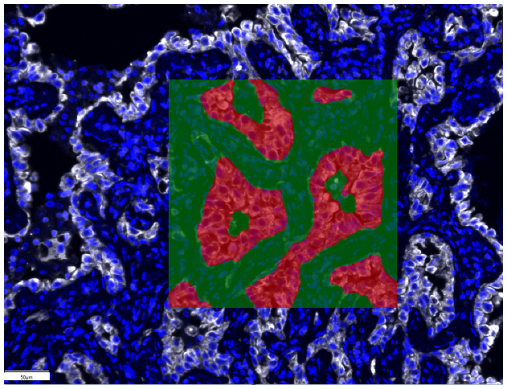
Comment 4: Could the authors provide images showing separated stains following unmixing?
Reply 4: We appreciate your suggestion and will address this by including images that demonstrate separated stains post-unmixing in our revised manuscript (Figure 9).
Changes in the text: We have modified the Figure 9 (see Figure 9).

Comment 5: Could the authors provide a stripping control to confirm the antibody complex was washed away between staining cycles?
Reply 5: Thank you for your valuable feedback on our manuscript. For our study, the clinically suitable staining pipeline was implemented using the Bond RX (Leica Biosystems) automated multiplex immunohistochemistry strainer. We did not employ a stripping control in staining cycles, as we have optimized the staining and stripping procedures to ensure minimal carryover between staining cycles during the development of our mIF method. Additionally, we included both negative and positive controls in each run to verify the accuracy and specificity of staining. In cases where the antibody complex might not have been properly washed away, potentially causing marker co-localization, such issues can be identified during the pathologist's review and analysis the images.

Comment 6: Did authors observe any spectral bleedthrough? If so, how was this handled in downstream analysis?
Reply 6: Thank you for your insightful comments. Spectral bleed-through could be an issue in the context of multispectral imaging. To address this, we employed Opal fluorophores and carefully designed our multicolor immunofluorescence assay panels to align with the expression characteristics of each marker and the fluorescence intensity of the Opal dyes. The Bond RX (Leica Biosystems) automated multiplex immunohistochemistry stainer was used to ensure consistent and controlled staining conditions. We used the Vectra Polaris multispectral imaging system (Akoya Biosciences), which employs patented multispectral imaging technology to compensate for optical spectral bleed-through among channels and to distinguish the signal from background autofluorescence. This system effectively reduces bleed-through through the use of advanced spectral unmixing techniques, thus ensuring accurate and reliable imaging results [PMID: 34150850].

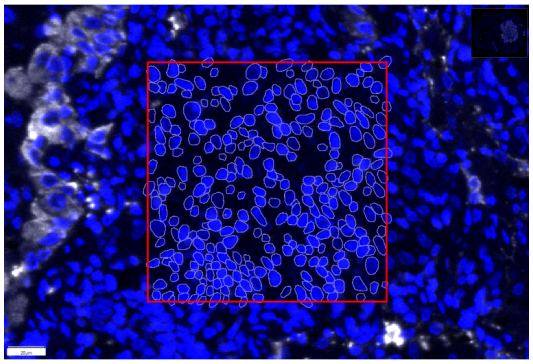
Comment 7: Could the authors elaborate on the image analysis? Specifically:
How was the tumour- and stroma area classified? (Please provide example image)
Reply 7: Tumor parenchyma and stroma were differentiated based on cytokeratin (CK) staining.

Utilizing image analysis software 'APTIME,' which features an integrated automated region recognition model, we accurately distinguished between these two tissue components.



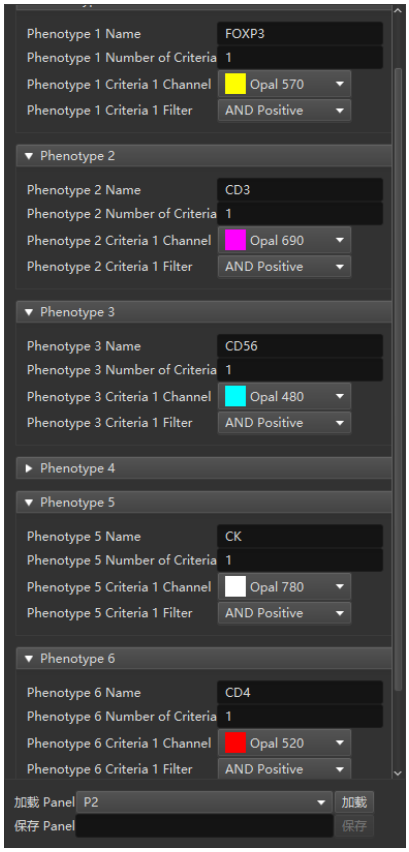
Blue, DAPI; White, CK; Green area,Tumor; Red area, Stroma

Comment 8: How was the cell detection performed? (Please provide example image)
Reply 8: A pathologist first established the threshold values for nuclear brightness, membrane radius, and nuclear/cytoplasmic & membrane thresholds for each marker. Subsequently, cell detection was achieved through a deep learning module in the 'APTIME' software. A representative image is presented below.



Blue, DAPI; Red frame, Region of cell detection

Comment 9: How were cells assigned to the respective phenotype? (Please provide example image)
Reply 9: Cells were assigned to their respective phenotypes following a pathologist's evaluation of scanned multiplex IF images with the use of image analysis software. Intensity thresholds for each marker and phenotype parameters were established and stored in the software. The software classified and annotated cells based on staining intensity, distribution, and cell type, and then exported the results. An example image was provided upon request.



Comment 10: Why were CD4+ T cells classified as CD3+ CD4+, and CD8+ T cells as CD8+ rather than CD3+CD8+?

Reply 10: Thank you for your comments. The classification of T cells into CD4+ T cells and CD8+ T cells populations is based on the presence of the CD4 and CD8 surface molecules, which serve different functions and are associated with different T cell subsets:

CD3+ CD4+: This classification refers to T helper cells.

CD8+: This denotes cytotoxic T cells, it's common to only use the "CD8+" marker because it's understood that all CD8+ cells are T cells by default.

Comment 11: How was a TLS identified? What was the cut-off for CD3+ and CD20+ to classify TLS positive or TLS negative samples? Were structure, size and maturity considered in TLS classification?

Reply 11: The tertiary lymphoid follicles were well-formed B cell germinal centers surrounded by T-cell aggregates towards the periphery. TLS were assessed by a pathologist on multiplex IF images and consisted of a CD3 + T cell zone and a CD20 + B cell core in our study as previous reported (PMID: 35768550; PMID: 38528050). There was no specific cut-off for CD3+ and CD20+ used to classify TLS positive or negative samples; rather, the presence of these cellular components in the described pattern was the basis for classification. While the structure, size, and maturity of TLS were not analyzed in the current study, these parameters are considered important for future research to further refine TLS classification.

Comment 12: Why was a cut-off of 30um proximity to tumour cell or PDL1+ cell chosen?

Reply 12: Thank you for your suggestion. This radius was pre-selected to identify cell-to-cell interactions, consistent with prior studies [PMID: 28724788, PMID: 37231145].

Comment 13: Figure legends: Abbreviations are included in each figure legend. Could the authors instead include an abbreviation section?
Reply 13: Thank you for your comments, we have added an abbreviations section in the revised manuscript.
Changes in the text: We have added the abbreviations as advised (see Page 24, line 779-794; page10,795-797).

Comment 14: Figure 2: Could authors include detailed description of what is shown in the figure legend of figure 2? Could the authors clarify the colour code? (red/ blue/ black)
Reply 14: Thank you for your comments. We have enlarged the color annotation section below the image. This enlargement ensures that the color coding more visible.
Changes in the text: We have modified the figure 2 (see Figure 2).

Comment 15: Figure 9: Could authors explain why patient 1 post-surgery (top right) and patient 2 post-surgery (bottom right) seem to be the same tissue, and the top row does not show DAPI?
Reply 15: Thank you for your feedback. The error in the placement of the pictures has been identified and rectified.
Changes in the text: We have modified our image as advised (see Figure 9).

Comment 16: Line 234: Does proportion relate to % of all cells or % of immune cells?
Reply 16: Thank you for your feedback. Cell percentage was calculated as the ratio of specific cell types to the total number of cells identified by DAPI (4',6-diamidino-2-phenylindole) staining.

Comment 17: Line 321: Why was 30μm chosen as a cut-off to assess CD20+ CD3+ aggregates?
Reply 17: Thank you for your feedback. Thank you for your suggestion. This radius was pre-selected to identify cell-to-cell interactions, consistent with prior studies [PMID: 28724788, PMID: 37231145].

Comment 18: Line 463: “At this stage, immunotherapy can effectively relieve immune suppression and yield positive therapeutic outcomes.” Could the authors provide a reference or refer to data in support of this statement?
Reply 18: Thank you for your valuable comment. We have added relevant references to support this statement in our manuscript.
Changes in the text: We have modified our text as advised (see Page 16, line 515).

Reviewer E

The authors collect a cohort of 105 NSCLCs and do an immuno-phenotyping multiplex panel,

then examine the number, fraction and spatial relationships of different immune cells in early and late stage non-small cell lung carcinomas. Their main finding is that tumours of more advanced stage have a higher number of CD8+ T cells, with a reduction in others (macrophages, B cells, NKs). They also describe TLS formation in post-NAC patients. They also relate immune phenotypes to genotypes in a sub-selection of the cohort.

Comment 1: As described in lines 217-230, they split their dataset into AIS/MIA, then of the invasive carcinomas according to stage I-III. The invasive carcinoma cohort is comprised of not just adenocarcinoma, but includes squamous cell carcinomas, carcinoid tumours and large cell carcinoma NOS. I would suggest limiting the comparison of AIS/MIA to just invasive adenocarcinomas to provide a like-for-like comparison.
Reply 1: Thank you again for your comments. We appreciate your suggestion to limit the comparison of AIS/MIA to just invasive adenocarcinomas. However, by including a broader spectrum of invasive NSCLC subtypes, we believe our analysis can offer more holistic insights into NSCLC. Therefore, we would like to retain these cases.

Comment 2: On lines 340-347, they describe TLS formation in post-NAC cases. Although they discuss some of the research in context from 429-446, I think some of the statements/conclusions eg. on lines 446-448 are a little strong, given their evidence is purely observational and has an n of 3. I would suggest revising the language used to be less definitive about the conclusions drawn regarding TLS formation, the relationship to neoadjuvant chemotherapy and the potential prognostic implication.
Reply 2: Thank you for your comment. We have updated the expression as follows:
"This finding may underpin the favorable efficacy of d the neoadjuvant chemoimmunotherapy."
Changes in the text: We have modified our text as advised (see Page 13, line 399-400).