Peer Review File

Article information: https://dx.doi.org/10.21037/tlcr-24-284

Reviewer A

In the manuscript "Stem-like exhausted CD8 T cells in pleural effusions predict improved survival in NSCLC and mesothelioma," the authors conducted a characterization of pleural effusion cells from NSCLC patients. They discovered a correlation between Texstem and OS. This correlation was validated with an analysis of mesothelioma patients. Overall, the data are reliable and their findings are significant to this area. However, some parts of the discussion could be improved.

L534: The authors posit that the absence of EGFR mutation is associated with a lower tumor mutational burden. However, there is a lack of supporting evidence for this assertion. Therefore, it is recommended that the correlation between EGFR mutation status and tumor mutational burden be validated using publicly available data.

Reply:
Thank you for the comment. In Line 534 (Line 559 in amended manuscript) we stated 'This suggests that lower levels of terminal exhaustion and the stem-like exhaustion state of CD8 T cells may be related to the **lower tumor mutational burden associated with EGFR mutant** and non-smoking related lung cancer.' This statement is supported by multiple previous publications. We have added appropriate citations to the manuscript.

Change in text: We have added citations to support our statement for the sentence in Line 559.

51. Offin M, Rizvi H, Tenet M, Ni A, Sanchez-Vega F, Li BT, et al. Tumor Mutation Burden and Efficacy of EGFR-Tyrosine Kinase Inhibitors in Patients with EGFR-Mutant Lung Cancers. Clin Cancer Res. 2019;25(3):1063-9.
52. Lin C, Shi X, Zhao J, He Q, Fan Y, Xu W, et al. Tumor Mutation Burden Correlates With Efficacy of Chemotherapy/Targeted Therapy in Advanced Non–Small Cell Lung Cancer. Frontiers in oncology. 2020;10:480-.

L568: The authors propose that Texstem cells in NSCLC and mesothelioma PE samples represent bystander T cells due to their TCR reactivity to virus antigens. Additionally,

the potential role of bystander T cells in tumor immunity is discussed. However, while the Texstem population contains bystander T cells, there is still the possibility that it also contains tumor-specific T cells. A discussion about this possibility and how tumor-specific T cells can be identified in PE T cells in future studies might be beneficial.

Reply:
Thank you for the comment. We agree that it is possible that Tex^{stem} cell population also contains tumour-specific T cells, indeed we have studied and published on tumour specific T cells in some detail already. We have added a discussion about this.

Changes in text: page 25 Line 603 – Page 26 Line 609 added

Our results however do not exclude the possibility that tumor-specific T cells are also present in the Tex^{stem} cell population. Our group and others have previously identified T cells directed against tumor neoantigens in PE by testing for IFNγ T-cell responses against bioinformatically predicted neoantigens, using methods such as IFNγ enzyme-linked immunosorbent spot (ELISpot) assay (65, 66) and neoantigen peptide-MHC tetramer staining (unpublished data). Further work is underway to delineate the exhaustion phenotype of these neoantigen-specific T cells.

Reviewer B

In the current study, the authors evaluated the composition of stem-like exhausted CD8 T cells (Texstem) and terminally exhausted CD8 T cells (Texterm) in malignant pleural effusion from non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MPM) patients, and analyzed their significances in the prognosis of these patients. The authors showed that the higher frequency of Texstem, but not Texterm, was associated with favorable overall survival (OS). Furthermore, the authors demonstrated that the phenotype of exhausted T cells from NSCLC were different from those from MPM, and Texstem cells contained “bystander” virus-specific T cells with mass cytometric analysis and single cell RNA/TCR sequences.

This manuscript is well written and reports some novel findings, such as the utility of Texstem in malignant pleural effusion as the prognostic biomarker of NSCLC and MPM patients. The reviewer recommends to address the following points to improve the quality of this manuscript.

Major comments;

Comment 1: The authors demonstrated that the lymphocytes in malignant pleural effusion can be used for the analyses of Tex cells to estimate the OS in the patients with NSCLC or MPM. The reviewer thinks it should be the strength of this manuscript, because the lymphocytes can be accessed easily by physicians or researchers, and the cells are colocalized with tumor cells. Therefore, the reviewer recommends to include one chapter in the Discussion section to discuss the advantages of the analyses using the lymphocytes in malignant pleural effusion or differences with the previous relevant analyses using PBMCs or TILs.

Reply:

Thank you for the comment. We agree that malignant pleural effusions are a valuable sample site for the study of host anti-cancer immunity and have added a discussion paragraph outlining the advantages of using pleural effusion lymphocytes and the differences between pleural effusion, PBMCs and TILs for lymphocyte analysis (specifically with regards to T-cell exhaustion).

Changes in text: added paragraph from Page 26 Line 610 to Line 624.

In advanced thoracic malignancies, patients often develop PE during the course of their disease (22, 67). PE provides a valuable sample source for translational research because it is proximal to tumor cells and is relatively easy to access compared to tumor biopsies. In addition, some patients require routine drainage of PE which allow longitudinal analysis that could reveal dynamic changes without the need for serial biopsies. Our study provides a proof of concept that PE can be used for immune analysis and that the data generated can have clinical relevance. In addition, PE is a unique immune microenvironment and its immunological characteristics do not necessarily mirror those of tumor tissue or peripheral blood, allowing greater insight into the complex landscape of the anti-tumor immune response. For example, the expression of inhibitory molecules such as PD-1, TIM3 and LAG3 are greater on PE T cells compared to peripheral blood but lower compared to TILs (31, 68-74). These data suggest that PE should not at this stage be relied upon as a surrogate for tumor or peripheral blood T-cell analysis but rather as an adjunct for studies into anti-tumor immunity.

Comment 2: Throughout the manuscript, the authors evaluated the significance of Texstem and Texterm as the prognostic biomarker for OS. The detailed information of treatments (chemotherapeutic regimens) was not included in this analysis. On the other

hand, as the authors described in the Introduction section, Texstem is reported to be associated with favorable therapeutic efficacy of immune-checkpoint inhibitor (ICI) in previous studies. Therefore, the reviewer would like to know whether the favorable OS in the patients with higher Texstem derives from favorable efficacy of ICI, because the reviewer assumes that most of the patients with NSCLC or MPM received ICI in their treatment course. The authors are encouraged to include the information of treatments and address this point.

Reply to 'The detailed information of treatments (chemotherapeutic regimens) was not included in this analysis.'

Thank you for this comment. We have now added data for treatment regimens patient received.

Changes to text:

1. Methods Page 9 Line 186 – added treatment regimens to data collected.

Clinical data including age, gender, smoking history (ex/current or never), Eastern Cooperative Oncology Group (ECOG) performance status (0-1 or 2-4), Charlson Comorbidities Index (7 – 9 or 10 – 13), histological subtype (NSCLC – squamous or non-squamous; mesothelioma – epithelioid, sarcomatoid or biphasic) and treatment (number of lines of systemic therapy received - 0, 1 or ≥2 **and treatment regimens**) were collected from hospital records.

2. Results Page 13 Line 287 – Line 292 – added information about treatment regimens for NSCLC patients.

Twenty patients received at least one line of systemic therapy. The treatment regimens used are listed in Table 1. In the first line setting, most patients received platinum doublet chemotherapy with or without maintenance pemetrexed, only five patients received ICPB (four single agent and one chemoimmunotherapy) and four received an EGFR tyrosine kinase inhibitor. In subsequent line therapies, four patient received chemotherapy and five received single agent ICPB.

3. Results Page 15 Line 348 – Line 353 – added treatment regimens for mesothelioma patients.

Twenty-nine patients received at least one line of systemic therapy. In the first line setting, 21 patients received platinum doublet chemotherapy, five patients received

chemoimmunotherapy and three patients received doublet ICPB with ipilimumab and nivolumab. Subsequent line therapies included chemotherapy (n=17), chemoimmunotherapy (n=1), doublet ICPB (n=3), single agent ICPB (n=4), FGFR inhibitor (n=2) and anti-CD80 antibody (n=1) (Table 4).

4. Added treatment regimen information to Table 1 (NSCLC) and Table 4 (mesothelioma)

Reply to 'Therefore, the reviewer would like to know whether the favorable OS in the patients with higher Texstem derives from favorable efficacy of ICI, because the reviewer assumes that most of the patients with NSCLC or MPM received ICI in their treatment course.'

We agree that it would be of interest to examine the effect of Tex cell subsets on ICPB response. To do this, it would be best to the examine the response to first line ICPB, as we only have T cell profiling data at the time of first line therapy and Tex frequencies may change with time or as a result of prior treatment. Unfortunately for both NSCLC and mesothelioma cohorts, only a small number of patients received first line ICPB (NSCLC n=5, mesothelioma n=8), so we are unable to perform any meaningful statistical analysis. For the five NSCLC patients who received first line ICPB, all of them had low Tex^{stem} cells and responses to treatment were variable (one partial response, two stable disease, one progressive disease, one not evaluable as patient died after first dose). For the eight mesothelioma patients, seven had high Tex^{stem} cells and their treatment responses were four partial response, one stable disease and two progressive disease. One patient had low frequencies of Tex^{stem} cells and derived partial response. We have not added this information to the manuscript as no conclusion can be drawn from the small number of patients.

We did however include ICPB use in univariate and multivariate OS analysis. ICPB use at any time during disease course was associated with improved OS in univariate analysis in the NSCLC cohort (HR 0.37 95% CI 0.17 – 0.83 p=0.015) and mesothelioma cohort (HR 0.46 95% CI 0.21 – 1.01 p=0.053). After adding ICPB into multivariable analysis with other clinicopathological variables, it was no longer significant for NSCLC (HR 0.49 95% CI 0.15 – 1.56 p=0.226) or mesothelioma (HR 0.38 CI 95% 0.11 – 1.34 p=0.130), while Tex^{stem} remained a significant predictor of improved OS for both NSCLC (HR 0.36 95% CI 0.16 – 0.79 p=0.011) and mesothelioma (HR 0.31 95% CI 0.10 – 0.96 p=0.041). This suggests that the prognostic value of Tex^{stem} is independent of ICPB. We have updated the results section and results tables with this information.

Changes to text:

1. Results Page 14 Line 328 to page 15 Line 332 - Changed NSCLC multivariate analysis results after adjusting for ICPB treatment.

Multivariate analysis showed that patients with higher frequency (above median) of Tex^{stem} cells had significantly higher median OS than those with a lower frequency (below median) (median 9.9 vs 3.4 months, **HR 0.36, 95% CI 0.16 – 0.79, p=0.011**) (Figure 1E, Table 3).

2. Results Page 16 Line 362 to Line 367 – Changed mesothelioma multivariate analysis results after adjusting for ICPB treatment.

In mesothelioma, as in NSCLC, increased Tex^{stem} cells was an independent predictor of improved OS irrespective of age, sex, histological subtype, smoking history, ECOG, number of lines of systemic therapy **and ICPB treatment**. Patients with a high abundance of Tex^{stem} cells had significantly longer OS compared to those with low abundance (median 32.1 vs 19.8 months, **HR 0.31, 95% CI 0.10 – 0.96, p=0.041**) (Figure 2D, Table 5).

3. Table 3 (NSCLC) and Table 5 (mesothelioma) – added univariate analysis results for ICPB treatment and updated multivariate analysis after adjusting for ICPB treatment.

Comment 3: In Table 3, the authors performed univariate and multivariate analyses to determine whether Tex^{stem} is independently associated with better OS. The authors included sex, age, smoking status, ECOG PS, comorbidity index, and number of chemotherapeutic regimens, but not included whether the patients received ICI or molecular targeted therapies, the status of driver gene alterations, the expression of PD-L1, etc. The authors should describe the rationale of the factors included in these analyses. Furthermore, the authors should describe how they adjust the significance of Tex^{stem} with these clinical factors, or should include the statistical values (HR, 95% CI, and p value) of these clinical factors in multivariate analysis in Table 3.

Reply to comment:

Thank you for the comment. We have now justified the selection of clinicopathological characteristics used in our analysis. We agree that ICPB and targeted therapy use are relevant and have included them in the analysis (ICPB use was included in the analysis for the entire NSCLC and mesothelioma cohort, targeted therapy use was included in

the analysis for the non-squamous NSCLC cohort).

With regard to driver gene alterations, EGFR and KRAS mutation status are already included in the multivariable analysis for the non-squamous carcinoma cohort (as they are only relevant to the non-squamous histology). Our results are stated on Page 15 Line 331 – Line 334 'Among the non-squamous subgroup (n=41), we also adjusted for EGFR and KRAS mutation status and targeted therapy. An association between Tex^{stem} cells and OS was observed irrespective of these characteristics (Supplemental Table 1, Supplemental Figure 3).'

With regard to PD-L1 expression – We did not include PD-L1 expression in the multivariable analysis due to missing data. Only 22 of the 43 NSCLC patients had this result available (some of the patients were treated at a time when PD-L1 testing was not routinely performed in Australia). We have added an explanation in the manuscript.

Changes to text:

1. Results page 14 Line 321 to Line 324 added

Univariate and multivariate analysis were performed using clinicopathological variables that can potentially influence clinical outcomes, including sex, age, smoking status, ECOG, comorbidities, number of lines of systemic therapy and ICPB treatment (Table 3).

2. Results page 14 Line 326 – Line 328 added

As well, we did not include PD-L1 expression levels in the multivariable analysis due to a significant number of missing data (only 22 patients had PD-L1 expression data).

3. Table 3 – added HR, 95% CI and p-values for all variables used in multivariate analysis.

Comment 4: In Figure 3B, the authors showed that the frequency of Texterm in EGFR wild type population was higher than that in EGFR mutant population. However, the authors described that "an increased proportion of Texstem cells was observed in EGFR mutant adenocarcinoma" in Discussion section (line 532). The authors should add the additional data of the correlation between with the frequency of Texstem and EGFR status in Figure 3B, as well as the correlation between the frequency of Texterm and smoking status in Figure 3A.

Reply to comment:
Thank you for the comment. The sentence "an increased proportion of Tex^{stem} cells was observed in EGFR mutant adenocarcinoma" in Discussion section (line 532) is incorrect and was an oversight. Tex^{stem} cells were not significantly different between EGFR mutant and EGFR wildtype adenocarcinomas. We have corrected this in the manuscript. We have also added the figures for Tex^{term} and smoking status, and Tex^{stem} and EGFR status in Figure 3.

Change to text

1. Results page 17 Line 385 to Line 386 added

There was no difference in Tex^{stem} when stratified by EGFR mutation status.

2. Discussion Page 24 Line 555 to Line 562 changed text to

An additional finding from our study was that in NSCLC, an increased proportion of Tex^{term} cells was observed in EGFR wildtype adenocarcinoma compared to EGFR mutant tumors and an increased proportion of Tex^{stem} cells was observed in never smokers compared to current or former smokers. This suggests that lower levels of terminal exhaustion and the stem-like exhaustion state of CD8 T cells may be related to the lower tumor mutational burden associated with EGFR mutant and non-smoking related lung cancer (51, 52).

3. Figure 3A – added data for Tex^{term} for smoker versus non-smoker.
4. Figure 3B – added data for Tex^{stem} for EGFR mutant versus EGFR wildtype.
5. Updated figure caption for Figure 3 for the additional data

Comment 5: The authors analyzed eight PE samples (three NSCLC and five MPM) and four PE samples (two NSCLC and two MPM) in CyTOF and scRNA-seq analyses, respectively. The authors should describe how they chose these samples among total samples. Furthermore, the authors described that "In comparing NSCLC with mesothelioma patient samples, we observed significant enrichment of natural killer (NK) cells among mesothelioma patients, while the frequency of myeloid cells was greater in NSCLC patients". However, the reviewer thinks it would be overstatement because the authors analyzed only three NSCLC PEs and five MPM PEs.

Reply to comment 'The authors should describe how they chose these samples among total samples.'

Thank you for the comment. The 8 samples for CyTOF were chosen based on sample availability from each cancer type. The 4 samples for scRNA-seq were chosen based on sample availability, disease representation (2 NSCLC and 2 mesothelioma) and adequate frequencies for analysis of Tex^{stem} and Tex^{term} cell populations.

Change in text: Methods Page 8 Line 170 – 173 added

For CyTOF analysis, five mesothelioma and three lung cancer samples were selected based on sample availability of each tumor type. For scRNA-seq two samples were selected for each tumor type based on sample availability and adequate frequencies of Tex cell subsets for data analysis.

Reply to comment 'However, the reviewer thinks it would be overstatement because the authors analyzed only three NSCLC PEs and five MPM PEs.'

We agree that we cannot conclusively compared NSCLC and mesothelioma patient samples in this small sample size.

Change in text: Deleted the sentence 'In comparing NSCLC with mesothelioma patient samples, we observed significant enrichment of natural killer (NK) cells among mesothelioma patients, while the frequency of myeloid cells was greater in NSCLC patients' from Page 18 and replaced with 'The distributions of key immune cell subsets in NSCLC and mesothelioma are presented in Supplemental Figure 5C.' (Page 18 Line 427 – 428)

Minor comments;

1. The font of the labels in some figures (Figure 4A and D, Figure 5B, D, and E) is too small to read. The authors should correct.

This has been corrected.

2. The authors should correct the typographs (6 to six in line 263, associate2d to associated in line 518, and Cytof to CyTOF in line 548).

These have been corrected