Editorial Commentary

Improved understanding of NSCLC immunotherapy response mechanisms from single-cell analysis

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Lung cancer is the leading cause of cancer-related deaths worldwide. In recent years, immune checkpoint blockade (ICB) has been widely used for the treatment of non-small cell lung cancer (NSCLC)—the predominant lung cancer subtype. However, ICB response rates remain limited. For example, analysis of multiple NSCLC trials using nivolumab showed this treatment increased 4-year overall survival rates from 5% to 14% but only achieved complete or partial responses in 16% of cases (1). Further understanding of how the tumour microenvironment changes in response to ICB, specifically what cell populations and/or molecular mechanisms drive response and resistance, is required to improve ICB efficacy through predictive biomarkers and novel combination therapies.

In a recent study published in *Genome Medicine* (2), Hu *et al.* used a multi-modal approach to investigate changes in the NSCLC multicellular ecosystem that underpin response to chemotherapy and ICB combination treatment in NSCLC. This study utilised bulk tissue and single cell (sc) RNA-sequencing, immunostaining and *in silico* modelling to investigate mechanisms associated with major pathological response (MPR). ScRNA-sequencing was performed on 3 treatment naive (TN) tumour biopsies; and 12 surgically resected tumour specimens following neo-adjuvant chemotherapy and ICB; a subset of these samples was also analysed by multi-marker immunostaining. Pathological response varied within the 12 post-treatment samples, with MPR achieved in 4 cases (leaving 8 exhibiting non-MPR). An independent TN cohort (n=21) was also examined with bulk RNA-sequencing, enabling the evaluation of molecular signatures' potential as predictive biomarkers. Despite this relatively small sample size, the study revealed multiple cell subpopulations and molecular signatures that varied between pathological response groups, providing valuable insight into key drivers of response to combined chemotherapy and ICB in NSCLC (*Figure 1*). Furthermore, many of these findings may also have implications for other solid cancers, as key findings were validated in cohorts of melanoma patients treated with ICBs.

The principle aim of ICB is to overcome $CD8^+$ T-cell suppression and initiate cytotoxic clearance of tumour cells (3). In NSCLC, ICB treatments most commonly target the interaction between the PD-1 receptor and ligands PD-L1/L2. Nivolumab (anti-PD-1) was the first immune checkpoint inhibitor approved for use in lung cancer, soon followed by pembrolizumab, cemiplimab, sintilimab, toripalimab and camrelizumab (the latter three were used in Hu *et al.*'s study). Anti-PD-L1 drugs atezolizumab and durvalumab have also been approved for use in NSCLC. Depending on national regulatory approvals, these drugs are used in a variety of clinical settings: including first-line neo-adjuvant therapy in combination with chemotherapy (as investigated in Hu *et al.*'s study); adjuvant treatment following surgery and chemotherapy; second-line monotherapy and as consolidation therapy in patients unable to undergo surgery (4-7).

Given that the efficacy of PD-1 blockade is predicated

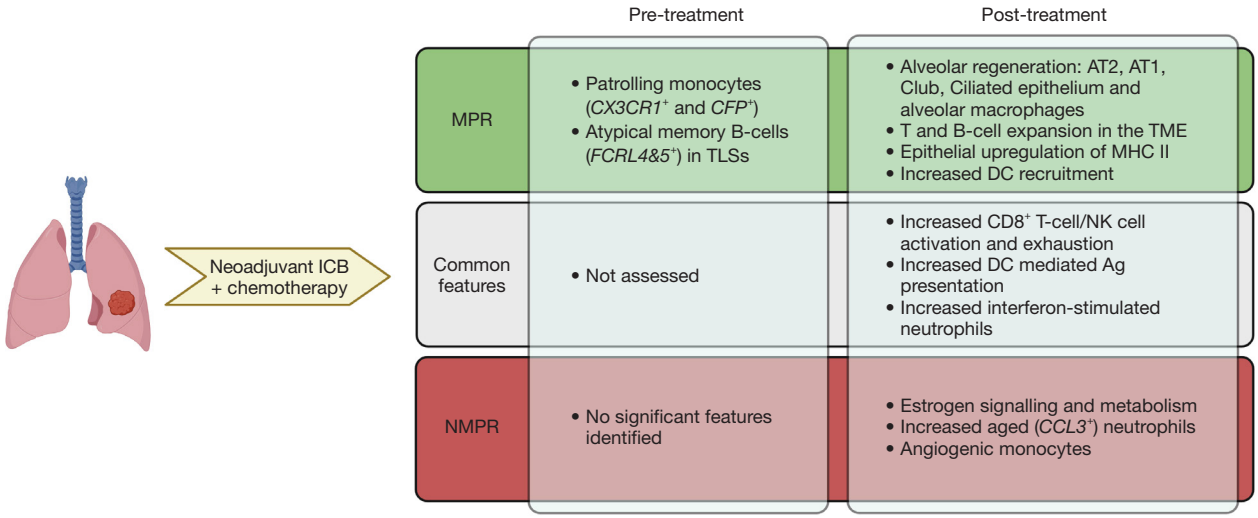

Figure 1 Graphical Summary of key findings from Hu *et al.*'s study (2), created with BioRender.com. ICB, immune checkpoint blockade; MPR, major pathological response; TLS, tertiary lymphoid structure; TME, tumour microenvironment; MHC, major histocompatibility complex; DC, dendritic cell; NK, natural killer; NMPR, non-MPR.

on establishing an adaptive immune response against the tumour, it is not surprising that Hu *et al.* found significant enrichment of T-cells and B-cells within the stromal compartment of MPR samples compared to both TN and non-MPR samples. However, closer examination of the phenotypic heterogeneity within these immune microenvironments, enabled by scRNA-seq analysis, demonstrated that specific immune cell subpopulations were variably abundant between these sample groups and also highlighted novel epithelial phenotypes associated with treatment response.

Ten clusters of epithelial cells were identified in Hu *et al.*'s scRNA-seq analysis. Non-malignant (diploid) clusters (representing AT1, AT2, club, basal and ciliated cells), were generally enriched in the MPR group. This was suggested to represent alveolar regeneration following ablation of the malignant epithelium in patients who positively responded to therapy. Notably, *CX3CL1* and genes associated with antigen presentation via major histocompatibility complex (MHC)-Class II were found to be up-regulated in the epithelial compartment of MPR samples. MHC-Class II machinery is typically expressed by professional antigen presenting cells (APCs) but can also be found expressed by other cell types, such as AT2 cells from normal lung tissue (8). Therefore, the alveolar regeneration observed in the MPR group may explain the increased MHC-Class II expression. Murine lung cancer models have also demonstrated that epithelial MHC-Class II expression can improve ICB response in lung cancer (9), providing further credence to the authors' suggestion that this pathway is actively involved in anti-tumour immunity and could be exploited to improve ICB efficacy in lung cancer.

Gene expression associated with estrogen response signalling was shown to be upregulated in epithelial cells from non-MPR patients compared to both TN and MPR samples. Hu *et al.* suggested that this pathway may represent a key mechanism of immunosuppression leading to ICB resistance. Consistent with this, gender specific response rates to ICB have been reported previously, with a meta-analysis of clinical trial data showing a reduced response rate in females across multiple cancer types (10). Differential expression analysis suggested that estrogen metabolism in the non-MPR group may be mediated by the upregulation of aldo-keto reductase family members (*AKR1C1-3*). Furthermore, metabolomic analysis of serum samples showed that beta-estradiol was upregulated in non-MPR patients' serum. Interestingly this cohort primarily consisted of men (83%) with two female participants exhibiting MPR and one non-MPR, demonstrating that the effect of estrogen mediated immune suppression may be independent of sex. Further investigation is required to elucidate the mechanism of estrogen mediated immune suppression and its impact on ICB response. However, Hu *et al.* did identify 17-AAG (an HSP90 inhibitor) as a potential means of suppressing the non-MPR epithelial signature using *in silico* analysis of data generated from

in vitro treatment of cancer cell lines.

Examination of immune cell subpopulations showed that in post-treatment samples (from both MPR and non-MPR groups) the T-cell population underwent significant changes compared to TN samples, but no significant differences in the proportion of T-cell subpopulations were found between MPR and non-MPR. A key feature of this response to treatment was shown to involve the activation of $CD8^+$ memory cells into effector phenotypes, expressing genes associated with cytotoxicity (*GZMA*, *GZMK*, *GZMB*, *NKG7* and *CCL5*) and MHC-class II antigen presentation (*CD74* and *HLA-DRA*). Given that $CD8^+$ T-cells are the main effector of ICB efficacy it is perhaps surprising that no clear differences were observed between the treatment response groups. However, these results must be carefully interpreted given that the overall abundance of $CD3^+$ T-cells was significantly higher in MPR samples compared to non-MPR samples. Additionally, the proportion and activation of Tregs was found to be lower in MPR samples, but this was not statistically significant in the small cohort analysed.

In the absence of a clear T-cell phenotype demarcating MPR and non-MPR samples, Hu *et al*. investigated alternative immune cell populations. In this analysis, a positive association with MPR was found for "atypical memory B cells" (expressing *FCRL4* and *FCRL5*); "patrolling monocytes" (PMos; expressing *CX3CR1* and *CFP*); and dendritic cells (DCs). Both the PMos and DCs were shown to express *CX3CR1*, which encodes the receptor for the ligand CX3CL1 -found to be upregulated by epithelial cells in MPR samples. Hu *et al.* went on to show that PMos found in MPR samples upregulated *CFP* [a gene known to be involved in the apoptosis of breast cancer cells (9)] and show that *CFP* significantly correlated (r=0.37) with apoptosis in lung adenocarcinoma (LUAD) samples from The Cancer Genome Atlas (TCGA) dataset. The PMos gene signature was also shown to be significantly increased in TN samples from patients that go onto achieve MPR compared to those with non-MPR, in the independent bulk RNA-sequencing cohort, suggesting PMos may be effective as a predictive biomarker. Furthermore, the PMos gene signature was also found to be significantly prognostic in a melanoma cohort. The atypical memory B cell population were similarly found to have potential as a predictive biomarker and immunostaining showed these cells were typically found in the centre of tertiary lymphoid structures (TLSs). Consistent with previous studies that have shown TLS abundance to be associated with improved ICB response rates (11,12). The mechanisms regulating TLS formation within the tumour microenvironment are yet to be fully determined. Hu *et al.* used *in silico* modelling to identify IFNα, TNF and IL27 as ligands with potential for inducing the *FCRL4/5*$^+$ atypical memory B cell phenotype, suggesting these pathways warrant further investigation to uncover the mechanisms of TLS formation.

In contrast to these MPR enriched processes, Hu *et al.* also identified myeloid cell populations associated with non-MPR and hypothesise that intercellular interactions within this cellular compartment initiate a positive feedback loop, leading to an immunosuppressive tumour microenvironment. Central to this process are "aged" neutrophils (expressing *CCL3*). *In silico* modelling identified SPP1, IFNγ and IL1β as ligands with potential to regulate this aged neutrophil phenotype. *SPP1* was found highly expressed on a population of tumour-associated macrophages (TAMs) and a highly significant correlation (r=0.77) was observed between *SPP1*$^+$ TAMs and aged (*CCL3*$^+$) neutrophils in TCGA-LUAD data. Reciprocal interactions between these two cell-types were predicted to drive an immunosuppressive microenvironment in non-MPR patients. Elevated *SPP1* expression is associated with worse survival in multiple cancers (13) and *SPP1*$^+$ TAM have been reported to be abundant in lung cancer compared to normal lung tissue (14). *SPP1*$^+$ TAM have also been associated with angiogenesis (14) and facilitating immune escape of cancer cells (15). Additionally, an angiogenic monocyte population (expressing *VEGFA*) was found to be significantly enriched in non-MPR samples compared to MPR, implicating this population as an additional contributor to the immunosuppressive microenvironment and ICB resistance.

Hu *et al.* acknowledged that the analyses performed are not without limitations, particularly regarding the limited sample size available. From this study it is not possible to disentangle whether the changes observed are due to chemotherapy, ICB or the combination. It is generally suggested that changes to the immune microenvironment are induced by ICB but whether the same alterations would be observed in patients treated with ICB alone is yet to be examined, and at present may not be clinically relevant as this is a relatively rarely used treatment modality. It is likely that some of the observed changes could be ICB independent, given that the increased abundance of epithelial subpopulations associated with the terminal respiratory unit and alveolar macrophages observed in MPR patients has also been reported in patients treated with EGFR inhibitors (16). Another area that requires further validation are the ligand-receptor pairs identified as potential

mechanisms of intercellular interaction. These findings are based on *in silico* modelling, using approaches that can be highly susceptible to false positives due to failing to account for inter-sample heterogeneity and limited concordance between transcript and protein level expression for multiple genes (16). The role of non-immune (e.g., mesenchymal) cells is also omitted from the analysis. Given the predicted role of myeloid cell populations in regulating angiogenesis it is likely that the endothelial cells profiled could provide valuable information regarding mechanisms of immune cell homing and activation within these complex multicellular ecosystems, as described previously (17). The role of cancer associated fibroblasts should also be examined given that these cells have been shown to correlate with ICB response rates in NSCLC and other cancer types (18-20).

Further work is required to expand on these findings from Hu *et al.*'s study in order to achieve clinical impact. The study presents a number of key areas that require further attention that should be addressed in larger cohorts and with experimental models. In addition to analysing larger sample sizes to validate the findings from Hu *et al.*'s study, it would be beneficial for patient matched TN and post-treatment samples to be compared, ensuring the relevant baseline material is used for inferring treatment induced phenotypic changes. To investigate intercellular interaction mechanisms further, future studies should examine the spatial distribution of cell populations and perform experimental validation using model systems to determine their functional contribution to therapy resistance. Additionally, the present study was performed across NSCLC subtypes (adenocarcinoma and squamous cell carcinoma), which vary considerably in their genetic drivers (21,22) and immune microenvironments (23). Therefore, future studies should endeavour to generate datasets sufficiently powered to enable phenotypic changes in response to therapy to be analysed within and across these major histological subtypes.

To summarise, Hu *et al.* provide valuable insight into how the immune microenvironment changes in response to chemotherapy and ICB in NSCLC, demonstrating the importance and utility of performing high resolution tissue analysis on samples from patients undergoing treatment. Due to small sample sizes, the results need to be carefully interpreted and further validation is needed to confirm the findings. Nevertheless, interesting hypotheses as to what may contribute to a better or worse response emerge from this study, including oestrogen signalling and MHC-class II mediated antigen presentation. Furthermore, novel targets for predictive biomarkers were identified that may help to stratify NSCLC patient response rates.

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Footnote

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References

1. Antonia SJ, Borghaei H, Ramalingam SS, et al. Four-year survival with nivolumab in patients with previously treated advanced non-small-cell lung cancer: a pooled analysis. Lancet Oncol 2019;20:1395-408.
2. Hu J, Zhang L, Xia H, et al. Tumor microenvironment remodeling after neoadjuvant immunotherapy in non-small cell lung cancer revealed by single-cell RNA sequencing. Genome Med 2023;15:14.
3. Chowell D, Yoo SK, Valero C, et al. Improved prediction of immune checkpoint blockade efficacy across multiple cancer types. Nat Biotechnol 2022;40:499-506.
4. Kazandjian D, Suzman DL, Blumenthal G, et al. FDA Approval Summary: Nivolumab for the Treatment of

Metastatic Non-Small Cell Lung Cancer With Progression On or After Platinum-Based Chemotherapy. Oncologist 2016;21:634-42.
5. Pai-Scherf L, Blumenthal GM, Li H, et al. FDA Approval Summary: Pembrolizumab for Treatment of Metastatic Non-Small Cell Lung Cancer: First-Line Therapy and Beyond. Oncologist 2017;22:1392-9.
6. Paik PK, Pillai RN, Lathan CS, et al. New Treatment Options in Advanced Squamous Cell Lung Cancer. Am Soc Clin Oncol Educ Book 2019;39:e198-206.
7. Reck M, Rabe KF. Precision Diagnosis and Treatment for Advanced Non-Small-Cell Lung Cancer. N Engl J Med 2017;377:849-61.
8. Cunningham AC, Milne DS, Wilkes J, et al. Constitutive expression of MHC and adhesion molecules by alveolar epithelial cells (type II pneumocytes) isolated from human lung and comparison with immunocytochemical findings. J Cell Sci 1994;107 (Pt 2):443-9.
9. Block I, Müller C, Sdogati D, et al. CFP suppresses breast cancer cell growth by TES-mediated upregulation of the transcription factor DDIT3. Oncogene 2019;38:4560-73.
10. Conforti F, Pala L, Bagnardi V, et al. Cancer immunotherapy efficacy and patients' sex: a systematic review and meta-analysis. Lancet Oncol 2018;19:737-46.
11. Schumacher TN, Thommen DS. Tertiary lymphoid structures in cancer. Science 2022;375:eabf9419.
12. Laumont CM, Banville AC, Gilardi M, et al. Tumour-infiltrating B cells: immunological mechanisms, clinical impact and therapeutic opportunities. Nat Rev Cancer 2022;22:414-30.
13. Wei T, Bi G, Bian Y, et al. The Significance of Secreted Phosphoprotein 1 in Multiple Human Cancers. Front Mol Biosci 2020;7:565383.
14. Cheng S, Li Z, Gao R, et al. A pan-cancer single-cell transcriptional atlas of tumor infiltrating myeloid cells. Cell 2021;184:792-809.e23.
15. Zhang Y, Du W, Chen Z, et al. Upregulation of PD-L1 by SPP1 mediates macrophage polarization and facilitates immune escape in lung adenocarcinoma. Exp Cell Res 2017;359:449-57.
16. Browaeys R, Gilis J, Sang-Aram C, et al. MultiNicheNet: a flexible framework for differential cell-cell communication analysis from multi-sample multi-condition single-cell transcriptomics data. bioRxiv 2023. Doi: 10.1101/2023.06.13.544751
17. Lambrechts D, Wauters E, Boeckx B, et al. Phenotype molding of stromal cells in the lung tumor microenvironment. Nat Med 2018;24:1277-89.
18. Kieffer Y, Hocine HR, Gentric G, et al. Single-Cell Analysis Reveals Fibroblast Clusters Linked to Immunotherapy Resistance in Cancer. Cancer Discov 2020;10:1330-51.
19. Dominguez CX, Müller S, Keerthivasan S, et al. Single-Cell RNA Sequencing Reveals Stromal Evolution into LRRC15(+) Myofibroblasts as a Determinant of Patient Response to Cancer Immunotherapy. Cancer Discov 2020;10:232-53.
20. Mariathasan S, Turley SJ, Nickles D, et al. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. Nature 2018;554:544-8.
21. Comprehensive genomic characterization of squamous cell lung cancers. Nature 2012;489:519-25.
22. Comprehensive molecular profiling of lung adenocarcinoma. Nature 2014;511:543-50.
23. Thorsson V, Gibbs DL, Brown SD, et al. The Immune Landscape of Cancer. Immunity 2018;48:812-830.e14.

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