Editorial Commentary

Characterization of the tumor microenvironment by single-cell RNA sequencing in non-small cell lung cancer treated with neo-adjuvant immunotherapy

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Introduction

Neo-adjuvant immunotherapy (IO) in non-small cell lung cancer (NSCLC)

Neo-adjuvant and perioperative therapies are an emerging topic in the multi-modal treatment landscape of early-stage NSCLCs (1). A growing number of clinical trials evaluates the use of either IO, or combined IO-chemotherapy (IO-CTx) or targeted therapies in this particular setting (*Table 1*). Biologically, systemic therapy before surgery (including perioperative strategies) induces profound and long-lasting changes of the tumor microenvironment (TME), encompassing multi-faceted immune cells, cancer-associated fibroblast (CAF) and tumor vasculature (1,8).

Only a proportion of patients respond to IO [i.e., immune checkpoint blockade (ICB)] comprising very few so-called super-responders, who may survive for years, even in the metastatic setting. Therefore, the identification of predictive biomarkers for response prediction to IO remains an unresolved issue (9). In early-stage NSCLC, programmed death-ligand 1 (PD-L1)-expression serves as a stratification factor for the application of ICB as (neo) adjuvant therapy (10), and besides that, no other clinically applicable predictive biomarkers have been established (9). By deciphering the TME of NSCLC, a deeper understanding of the multi-directional interactions between the clonal cancer cells, tumor-infiltrating leucocytes (TILs), tumor stroma and vasculature may aid to identify factors that are associated with response to (neo-adjuvant) IO or imprint TME-linked resistance patterns (4,6).

Currently, successful neo-adjuvant therapy is defined as major pathological response (MPR), which is a reduction of tumor viability to less than 10% (11). Together with progression-free survival (PFS), MPR serves as the main response parameter and primary endpoint in the most neo-adjuvant clinical trials (*Table 1*). Encouraging MPR rates up to 50% have been reported in several clinical trials with manageable toxicity profile and—of importance—high resection rates (2,6). Some clinical trials provided data from translational analyses as well, studying tumor immunology under the therapy pressure of IO by comparing TME composition and soluble biomarkers of patients reaching MPR *vs.* non-MPR (4-6). Importantly, an association of PD-L1-expression or TIL infiltration could not be observed consistently across these trials, again supporting the unmet clinical need to better define MPR-predictive biomarkers.

Table 1 Clinical trials evaluating neo-adjuvant/perioperative IO in NSCLC

Clinical trial (neoadjuvant IO)	Stage	Design	Treatment/intervention	Endpoint	Adjuvant treatment	Results
Checkmate 816 (2), NCT02998528	IB–IIIA	Phase 3	3 cycles CTx +/– Nivo	pCR, EFS, MPR	None	EFS+, MPR 24%
Aegean (3), NCT03800134	IIA–IIIB	Phase 3	4 cycles CTx + Durva/Plac.	pCR, EFS	Durva/Plac.	MPR/cPR+
IMpower 030, NCT03456063	II–IIIB	Phase 3	4 cycles CTx +/– Atezo	MPR, EFS	Atezo/BSC	To be published
Keynote 671, NCT03425643	IIA–IIIA	Phase 3	4 cycles CTx + Pembro/ Plac.	EFS, OS	Pembro/ Plac.	EFS+, cPR 18%, MPR 30%
NeoSTAR (4,5), NCT03158129	I–IIIA	Phase 2	Nivo + Ipi/Nivo	MPR, cPR	None	MPR 38%/22%
NeoCOAST, NCT03794544	I–IIIA	Phase 2	Durva + novel agents	MPR, translational	None	MPR 31%, cPR 12%
Nadim-II (6), NCT03794544	IIIA	Phase 2	3 cycles +/– Nivo	MPR, cPR	Nivo	MPR 52%, cPR 36%
Pacific (7), NCT02125461	$III_{unresect}$	Phase 3	Durva/Plac. after RCTx	PFS, OS	Durva/Plac.	PFS+ OS+

IO, immunotherapy; NSCLC, non-small cell lung cancer; CTx, chemotherapy; Nivo, nivolumab; pCR, pathologic complete remission; EFS, event-free survival; MPR, major pathological response; Durva, durvalumab; Plac., placebo; cPR, complete pathological response; Atezo, atezolizumab; BSC, best supportive care; Pembro, pembrolizumab; OS, overall survival; Ipi, ipilimumab; RCTx, radio-chemotherapy; PFS, progression-free survival.

The single-cell RNA sequencing (scRNAseq) landscape in NSCLC

The advent of scRNAseq technique shed light on the TME composition in NSCLC with an unprecedented precision, allowing the identification of highly diverse sub-clusters of the tumor stroma cells including immune cells in addition to the marked heterogeneity of malignant cell clones (12-15). Projects, which focused on the creation of single-cell atlases of (adjacent) lung tissue (16) and NSCLC allowed to analyse numerous TME cell subtypes with statistical power, minimizing the bias of inter-patient heterogeneity. Hereby, intra-patient and inter-patient heterogeneity as well as major differences in dependency of histology were underpinned (14,17).

Various clusters of effector T cells exhibiting a highly migratory nature and the different states of T cell exhaustion were defined by Guo *et al.* and so called pre-exhausted T cells were linked to an improved outcome. Furthermore, tumor-associated Tregs showed a substantial variability and allowed the identification of antigen-specific Tregs, which are associated with poor prognosis in lung adenocarcinoma (18). Regarding B cells, distinct subclusters, such as naïve-like and plasma-like B cells were defined by Chen *et al.* and showed evolution through different disease stages. While plasma-like B cells and their immunoglobulins inhibit cancer cell growth in early-stage NSCLC, they promote cell growth in the advanced stage disease. In contrast, naïve-like B cells suppress lung cancer growth by cell growth regulating factors (19). Moreover, the highly diverse landscape of myeloid cells in the peripheral blood as well as in the TME including macrophages, dendritic cells and neutrophils were mapped in detail. These populations seem highly conserved as they were also present in tumor-bearing mice and may also function as potential therapeutic targets (20). This also included identification of dysfunctional mature dendritic cells with a high expression of immune-regulatory molecules, resulting in impaired antigen presentation (21). Similarly, high-dimensional characterization of tumor-associated neutrophils revealed a continuum of four subtypes and finally also a gene signature of tissue-resident neutrophils, which is associated with IO failure in advanced-stage NSCLC (17). scRNAseq studies also allowed the improved annotation of a diverse sub-cluster architecture of tumor endothelial cells (TECs), such as tip-, branch- and basement-membrane-phenotypes (13) as well as various types of CAFs with a unique collagen secretion repertoire (12). Based on their gene expression signature, certain types of TEC also seem to be involved in the regulation of immune surveillance, are sensitive to vascular endothelial growth factor blockade and correlate with patient survival (13). Future scRNAseq data of patients after neo-adjuvant treatment can be compared to this large treatment-naïve reference cohort, which may ultimately help to identify therapy-induced TME changes and

potential vulnerabilities for therapeutic targeting.

scRNAseq TME characterization in tumors treated by neo-adjuvant concepts

Alterations of the TME under therapy pressure have also been intensively studied in different cancer entities by scRNAseq, both for CTx and IO. For example, Caushi *et al.* analysed reprogramming and expansion of tumor neoantigen-specific CD8$^+$ T cells in the TME and peripheral blood of NSCLC patients, however no distinct enrichment of cell populations in MPR$^+$ patients could be observed, suggesting a more complex microenvironmental pattern, including multiple TME-associated variables regulating ICB sensitivity (22). In mismatch repair-deficient and microsatellite-instability-high colorectal carcinoma patients with pathological complete remission after neo-adjuvant ICB, a decrease in mitotic CD8$^+$ T cells, CD4$^+$ Tregs, proinflammatory IL1B$^+$ monocytes and CCL2$^+$ fibroblasts and an increase of CD8$^+$ effector memory T cells, CD4$^+$ helper T cells, CD20$^+$ B cells and human leukocyte antigen-DR alpha (HLA-DRA)$^+$ endothelial cells was observed (23). A study of breast cancer patients treated with programmed cell death 1 (PD-1) inhibitors found an expansion of PD1$^+$ T cells in roughly 30% of patients. T cell expansion was associated with immunoregulatory dendritic cells (PD-L1$^+$), CCR2$^+$ or MMP9$^+$ macrophage phenotypes and major histocompatibility complex-class II (MHC-II) expression on cancer cells, but negatively correlated with TCF7$^+$, granzyme K (GZMK)$^+$ undifferentiated pre-effector/memory T cells or (CX3CR1$^+$, C3$^+$) inhibitory macrophages (24). In a study of head and neck cancer patients, neo-adjuvant nivolumab treatment resulted in substantial diversification of fibroblasts, which were predictive for ICB response, reduced exhaustion of CD8$^+$ T cells, increased resident memory phenotypes as well as enhanced the overall cytolytic profile of T cells (25).

Concerning the impact of neo-adjuvant CTx in advanced ovarian cancer, CTx induced a pronounced expansion of memory T-cell receptor (TCR) clones, central memory CD8$^+$ and classical monocytes with strong antigen presentation features (26). In oesophageal and gastric cancer patients with response to neo-adjuvant CTx, T cells (regulatory, exhausted effector) could be re-programmed towards an anti-tumor phenotype and endothelial cells as well as fibroblasts numerically expanded (27-29). Moreover, a study in oesophageal squamous carcinoma found an activation and promotion of antibody secretion of B cells under CTx (30). In neo-adjuvant radio-CTx, others found an increased CD8$^+$ T cell infiltration, with promoted exhaustion phenotype irrespective of response. T helper cell differentiation was increased but that of Treg cells was reduced in MPR$^+$ patients (31).

Comment on the paper by Hu *et al.*

With their paper "*Tumor microenvironment remodeling after neoadjuvant immunotherapy in non-small cell lung cancer revealed by single-cell RNA sequencing*" Hu *et al.* (32) presented a unique description of TME alterations induced by combined neo-adjuvant CTx and PD-1 inhibition in early-stage NSCLC. The authors collected three pre-treatment and 12 post-treatment samples from diagnostic biopsies as well as surgically resected tissue and characterized ~92,000 single cells by scRNAseq. Under therapy pressure, they depicted specific changes within the immune and stromal cell compartment, which was subsequently validated by immunohistochemistry. Stratified by the achievement of MPR, they observed major differences in the transcriptomes and metabolomes of cancer and immune cells. In our opinion the most significant findings are the following:

In cancer cells, a higher expression of *CX3CL1*, *CD74*, and *MHC-II* genes in MPR patients was found, indicating elevated immune cell recruitment and antigen presentation, as well as up-regulation of estrogen response pathways, which was negatively associated with achievement of MPR. Concerning the immune cell compartment, the expansion of cytotoxic T cells and natural killer (NK) cells in addition to the depletion of Treg cells was associated with MPR. While these effects of CTx with or without PD(L)-1 blockade have already been previously described in NSCLC (23,24,31), Hu *et al.* interestingly depict the properties of distinct T cell clusters under the combined treatment and linked these characteristics to the achievement of MPR. When compared to samples from treatment-naïve NSCLC patients, memory T cells tend to differentiate into an effector phenotype under the effect of CTx/ICB. Moreover, certain subsets of B cells (FCLR4$^+$, FCLR5$^+$) may predict MPR and are positively associated with tertiary lymphoid structure (TLS) formation, as similarly shown previously by Helmink *et al.* (33). For the myeloid compartment, patrolling CX3CR1$^+$ monocytes were expanded in patients with MPR, whereas immunosuppressive vascular endothelial growth factor A (VEGFA)$^+$ monocytes were associated with poor response to therapy. Changes in macrophage clusters indicate that

suppression of anti-inflammatory M2-macrophages may function as a response effect. Furthermore, also dendritic cells are expanded in MPR patients and may positively influence T cell activation. Interestingly, pro-inflammatory CCL3$^+$ neutrophils were downregulated in MPR patients suggesting a suppressive TME.

A strength of this study certainly is the unique scRNAseq dataset generated prior and after neo-adjuvant treatment of NSCLC, enabling to discriminate the TME between MPR positive or negative patients. Particularly, the increased antigen-presentation properties of cancer cells via MHC-II in MPR$^+$ patients, aided by ICB-activated CD8$^+$ T cells, and the subsequent activation of anti-tumor response by various cell types and down-regulation of immunosuppressive Tregs improves our understanding of the detailed mechanism of mechanism of action of ICB on a single-cell level. Moreover, the depletion of CCL3$^+$ neutrophils in MPR- patients is a finding of particular significance, as tumor neutrophils have been poorly described in the context of neo-adjuvant therapy (34).

The work of Hu *et al.* features scRNAseq data of 12 post-treatment and three pre-treatment NSCLC patients, also allowing correlation of post-therapy findings to the treatment-naïve status. Importantly, the pre- and post-treatment cohort included different patients, i.e., no patient was sampled sequentially. Hence, the observed expansion of FCRL4/5$^+$ memory B cells and CD16$^+$ and CX3CR1$^+$ monocytes in post-therapy samples, which is claimed by the authors to be a predictive biomarker, were not clearly put into context with pre-treatment samples, which is most likely due to the small pre-treatment patient cohort. Furthermore, the up-regulation of estrogen metabolism in the tumor and elevated estrogen levels in the serum of MPR patients show no clear picture in our opinion, as the authors did not contextualize their findings of the serum analysis to the sex of the study participants. Another limitation of the study is that some major results were not further validated by techniques like immunohistochemistry or covered correspondingly in an independent cohort.

Discussion

The article "*Tumor microenvironment remodeling after neoadjuvant immunotherapy in non-small cell lung cancer revealed by single-cell RNA sequencing*" by Hu *et al.* suggests that TME reprogramming by ICB mostly relies on the individual immunological and metabolic context of the tumor but not on T cell patterns. In particular, memory B cells and immune-stimulatory monocytes represent important surrogates of a pro-immunogenic TME and have the potential to serve—together with other components—as predictors for ICB response (32). Also, alterations in certain metabolic pathways (e.g., estrogen signalling) of cancer cells may be highly relevant factors for tumor immunogenicity and therefore ICB response. However, considering the great tumor cell heterogeneity observed in NSCLC, the exact role of tumor metabolism remains inconclusively addressed so far.

When relating the results on neutrophils with our previously published transcriptomic neutrophil characterization (17) the following common features but also contrarieties were observed: both studies classified mature (CXCR2^{high}, CXCR4^{low}) and aged (CXCR2^{low}, CXCR4^{high}) neutrophil clusters, and some subclusters described by Hu *et al.* were also differentiated in our analysis, e.g., an activated subcluster with high expression of the alarmin *S100A12*, a subcluster of high chemokine (*CLL3*, *CLL4*) expression as well as a pro-inflammatory subcluster with high expression of interferon-response genes (*IFIT2*, *IFIT3*). The expression of several other marker genes by distinct subclusters (e.g., *PROK2*, *PADI4*, *MMP9*, *SELL*, *VEGFA*) also overlapped. Both studies found an association of certain neutrophil populations with failure to ICB or, in the study by Hu *et al.*, achievement of MPR. While the prognostic neutrophil signatures of both studies (TRN-signature, CCL3-signature) only partly overlap, the occurrence of chemokine-signalling genes in both signatures underlines the potential role of these neutrophil pathways in IO response.

Conclusions and outlook

So-called "window of opportunity studies", most applying neo-adjuvant treatment, provide a unique opportunity to study cancer biology during therapy pressure. By treating the tumor prior to surgery, intra-patient biological changes in a real-life setting can be observed by sequential sampling. Cutting-edge techniques such as scRNAseq have massively contributed to our understanding of cancer biology by in-depth characterization the highly diverse cellular composition of cancers (12,14,15). Increasingly, studies not only describe these TME landscapes but as well evaluate the consequences of highly effective anti-cancer agents (22,27,31) and also created a longitudinal picture of cancer-immunity (23,24). Besides, combinational therapies of IO with CTx or anti-angiogenic drugs may influence the TME

in a different manner and therefore have synergistic effects (35,36). Consequently, more scRNAseq studies in NSCLC are warranted to gain a broader understanding of the TME and the utterly complex field tumor immunology, to identify predictive biomarkers, especially in the context of multimodal and combinational neo-adjuvant therapies.

With newer technologies, such as spatial transcriptomics or multiplex immunofluorescence (IF)-based technologies, we have the unique opportunity to unravel the three-dimensional composition of the TME on a single-cell level in lung cancer (37). This enables sequencing of the whole transcriptome of single cells including their regional allocation by spatially barcoded mRNA-binding oligonucleotides on fresh frozen as well as formaldehyde-fixed paraffin-embedded (FFPE) tissue (37,38). Mapping of the areal configuration of cancer and stromal cells will deepen our understanding of TME sub-compartments and indirectly also unravel distinct cellular-interactions and migration of cells within the tumor (37). Currently, there are only few studies using spatial transcriptomics in NSCLC. Previous analysis of the regional TME composition for example showed B and T cells interactions in so-called immune hotspots in squamous cell carcinoma (39), the spatial composition of brain metastasis (40) and the patterns of tumor associated macrophages (41), which could also be linked to ICB response (41,42). Further studies in this field are eagerly warranted and awaited.

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