Editorial Commentary

Single-cell insights into the dynamic tumor microenvironment changes during immunotherapy of non-small cell lung cancer

Karolina Hanna Prazanowska[1,2]^, Jiwon Hong[1,2]^, Su Bin Lim[1,2]^

[1]Department of Biochemistry & Molecular Biology, Ajou University School of Medicine, Suwon, Korea; [2]Department of Biomedical Sciences, Ajou University Graduate School of Medicine, Suwon, Korea

Correspondence to: Su Bin Lim, PhD. Department of Biochemistry & Molecular Biology, Ajou University School of Medicine, Worldcup-Ro 164, Yeongtong-Gu, Suwon 16499, Korea; Department of Biomedical Sciences, Ajou University Graduate School of Medicine, Suwon, Korea. Email: sblim@ajou.ac.kr.

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Since its introduction in 2009, single-cell RNA sequencing (scRNA-seq) has been widely used for studying transcriptomic profiles of individual cells (1). ScRNA-seq is an essential tool in various fields, including cancer biology, enabling high-resolution characterization of tumors and their microenvironment (2). Cancers of the lung are estimated to remain the leading cause of cancer-related deaths in 2023, with non-small cell lung cancer (NSCLC) being the most common type of lung cancer (3). Over the past decade, improvements in NSCLC detection and therapies, including immunotherapy, have greatly increased the 3-year relative survival rate (3). However, a significant number of patients do not respond to treatment or develop resistance during immunotherapy (4). Therefore, more effort needs to be put into understanding the mechanism of successful immunotherapy.

Cancer immunotherapy through immune checkpoint blockade (ICB), especially targeting the programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway, has emerged as a potent therapeutic regimen for resectable NSCLC as well as the advanced NSCLC (5). Neoadjuvant immunotherapy, which is applicable for resectable tumors prior to surgery, has been proved to enhance relapse-free and overall survival in operable patients by promoting systemic antitumor immunity (6). However, the underlying mechanisms assigning patients into responders or non-responders remain to be elucidated. Immunotherapy through ICB might remodel tumor microenvironment (TME), affecting the response to immunotherapy and the acquisition of resistance to the therapy. TME might influence the response to immunotherapy by multiple interactions between cancer cells and immune cells (7). The role of TME in the treatment of NSCLC through immunotherapy has been increasingly identified (8). However, which specific immune cell subtypes and interactions between the cell types affect the response to immunotherapy still remain to be elucidated during ICB treatment in NSCLC. It prompts to clarify the comprehensive landscape of TME remodeling during immunotherapy for NSCLC, which contributes to more accurate prediction of therapy response and identification of potent biomarkers that predict the patients who will benefit from neoadjuvant immunotherapy.

As currently available immunotherapy focusing on T cell anti-tumor activity is still ineffective in many patients, a deeper understanding of T cell states in the TME is of great interest. In a recently published atlas by Chu *et al.* (9), a previously undescribed subpopulation of stress response T cells (Tstr) was identified *in situ* and characterized at

^ ORCID: Karolina Hanna Prazanowska, 0000-0002-4059-1453; Jiwon Hong, 0000-0003-4224-6490; Su Bin Lim, 0000-0003-1752-7039.

single-cell level by specific expression of heat shock genes. CD4/CD8$^+$ Tstr cells were mostly identified within the tumor beds and close surroundings of non-responsive tumors, indicating they might be a potential factor of immunotherapy resistance.

In a study conducted by Hui *et al.* (10), the functional differences in T cells between patients with major pathologic response (MPR), defined as no more than 10% of viable tumor cells after therapy (11), and non-MPR patients after neoadjuvant chemoimmunotherapy against NSCLC were explained in terms of regulatory T (Treg) cells. Their data revealed that the exhausted and dysfunctional state of CD8$^+$ T cells was significantly improved in MPR tumor lesions. In these lesions, CD4$^+$CD25$^+$CD127$^-$ Treg cells and TNFRSF4$^+$ Treg cells diminished compared to non-MPR tumor lesions while FoxP3$^+$ Treg cells were more abundant in non-MPR lesions. Abundant Treg cells in non-MPR tumor lesions indicate an immunosuppressive environment, correlated with poor prognosis in non-MPR patients. Consistently, in a study by Hu *et al.* (12), activated Tregs expressing TNFRSF4 and TNFRSF9 were shown to be decreased in MPR patients, further validating their role in immunosuppression.

Another study conducted by Jia *et al.* (13) on a lung cancer patient, who nearly reached MPR with only 12.2% of cancer cells remaining after neoadjuvant anti-PD-1 immunochemotherapy, identified that cytotoxic CD8$^+$ T cells and monocyte-derived dendritic cells were the most common infiltrating cell types, indicating an activated immune microenvironment after neoadjuvant immunochemotherapy. Interestingly, a specific subtype of mitotic CD8$^+$ T cells was identified. This subtype was characterized by high expression of CD8A, GZMB, CDK1, and MKI67, indicating rapid proliferation of CD8$^+$ T cells and more production of cytotoxic CD8$^+$ T cells to execute cytotoxic functions (14). The presence of proliferating subtype with high expression of MKI67 was also observed in scRNA-seq atlas of NSCLC after neoadjuvant immunotherapy by Hu *et al.* (12), even though its relevance to clinical response between MPR and non-MPR was not fully elucidated.

Further heterogeneity within the T cell population was identified in mutation-associated neoantigen (MANA)-specific tumor-infiltrating lymphocytes (TIL). Caushi *et al.* (15) identified that T cell receptors (TCR) derived from MPR NSCLC patients were capable of strong ligand-dependent signaling after neoadjuvant anti-PD-1 treatment. This means significantly higher functional activity, while TCRs from non-MPR patients were characterized by markedly lower ligand-dependent signaling. Further, MANA-specific T cells of MPR patients were characterized by higher expression of genes associated with memory (IL7R and TCF7) and effector function (GZMK). In contrast, MANA-specific T cells from non-MPR patients highly expressed genes associated with T cell dysfunction such as TOX2, CTLA4, HAVCR2 and ENTPD1. Of note, among MANA-specific CD8$^+$ TILs of non-MPR patients, about 90% were tissue resident memory T cells (Trm) that displayed incompletely activated effector T programs, expressing high levels of HOBIT, which is involved in the development of Trm cells (16).

Heterogeneity within the T cell population in TME has been demonstrated not only between different patients (inter-patient heterogeneity), but also within single individuals (intra-patient heterogeneity) (17). In a case study by Zhang *et al.* (18), a patient with early-stage lung adenocarcinoma (LUAD) treated with pembrolizumab was found with several nodules, differentially responding to the therapy. The non-responding nodules harbored an epidermal growth factor receptor (EGFR) exon 21 L858R mutation, associated with less effective immunotherapy. However, the non-responding nodules were PD-L1 negative and carried a lower tumor mutational burden than the responding nodule, suggesting presence of an atypical immune escape mechanism. Using immunohistochemistry, the authors identified a significant enrichment of infiltrating CD8$^+$ lymphocytes and activated CD68$^+$ HLA-DR$^+$ macrophages in the responding nodule, in contrast to the nodules that did not respond to immunotherapy. At the single-cell level, clear differences were observed in the CD8$^+$ T cell population between responding and non-responding samples, which might explain lower anti-tumor T cell immunity in the latter. T cells derived from non-responding nodules were mostly subtypes specific for early stages of disease, including naïve and early activated T cells. In the responding nodule samples, resident memory T cells (Trm), beneficial in prognosis of lung cancer, accounted for the majority (~50%) of all T cells from the responding nodule and exhibited a cytotoxic phenotype, characterized by high expression of HAVCR2, TIGIT, PDCD1, GNLY, HLA-A and GZMB.

These findings are in line with the study conducted by Hu *et al.* (12), indicating an increase in the number of GZMB-expressing Trm after immunotherapy, especially in patients exhibiting MPR. Yang *et al.* (19) also describe the lack of CD8$^+$ Trm as a key factor in formation of

suppressive TME of EGFR-mutant LUAD in their paper focusing on patients' EGFR status. In this study, analysis of nine treatment-naïve samples and post treatment data from Zhang *et al.* (18), supported the finding that EGFR-mutant LUAD was deprived of $CD8^+$ Trm and indicated that recruitment of Trm may be dependent on activity of $CXCL9^+$/$CXCL10^+$ tumor-associated macrophages (TAM), which is lacking in the TME of EGFR-mutant samples. Altogether, these results show clear trends in T cell states differences between MPR and non-MPR patients, supported by several studies (*Table 1*).

The study by Hu *et al.* (12) further elucidates TAMs activity after combined therapy with a PD-1 inhibitor and chemotherapy. In MPR patients, TAMs were observed to undergo reprogramming into a neutral, anti-immunosuppressive M0 state, while the proportion of M1 and M2 TAMs decreased in these patients after therapy. In contrast, TAMs in non-responding patients showed M2 signature and expressed SPP1. Interestingly, $SPP1^+$ TAMs have been previously reported to exhibit immunosuppressive functions and contribute to immunotherapy resistance through ECM remodeling in colorectal cancer (20). Together, these findings give a novel insight into the role of TAM signature in immunotherapy response and reveal potential directions for future studies on $SPP1^+$ TAMs in NSCLC. Additionally, a subpopulation of immunosuppressive $VEGFA^+$ monocytes, an intermediate state between monocytes and macrophages, was found to be abundant in non-MPR and associated with a poor response. In contrast, signature of $CX3CR1^+$ monocytes associated with anti-tumor activity was suggested as a potential biomarker of ICB response.

Several studies note that recruitment of B cells to a tumor is crucial for tertiary lymphoid structure (TLS) formation, associated with better response to immunotherapy (19,21). The study conducted by Hui *et al.* (10) provides more evidence on this phenomenon. Through flow cytometry analysis, they identified that B cells were more abundant in tumor lesions of MPR patients and expressed a canonical surface marker CD19. Plasma cells in neoadjuvant MPR tumor lesions were characterized by significantly downregulated IGHA1, IGHA2, and JCHAIN, while IGHG1 and IGHG3 were significantly upregulated in MPR tumor lesions, indicating B cell class switching to increased IgG1 and IgG3 isotypes and diminishing IgA isotype during neoadjuvant chemoimmunotherapy. Interestingly, $CD4^+IL21^+$ T cells were significantly higher in MPR tumor tissues, which were involved in inducing B cell class switching to IgG1 and IgG3, mediating favorable anti-tumor immune response during neoadjuvant chemoimmunotherapy. Yang *et al.* (19) also observed insufficient infiltration of B cells in patients with EGFR mutations, further showing the relationship between TIME and EGFR status. Moreover, Hu *et al.* (12) identified a unique subpopulation of $FCRL4/FCRL5^+$ memory B cells, abundant in the MPR group after treatment. These atypical memory B cells were found to exhibit anti-tumor activity and located in the center of TLS, making them a potential biomarker for ICB treatment.

Presence of $CXCL17^+$ plasma and dendritic cells (DC) in the TME may further contribute to its resistant phenotype, according to Yang *et al.* (19). While in their study the population of $CD1C^+$ DCs was higher in the EGFR-positive group with resistant potential, Hu *et al.* (12) report an increase in fractions of activated conventional DCs (cDCs) in MPR patients. This might indicate existence of a specific subtype of DCs, which express some conventional cDC markers, but also exhibit immunosuppressive cell-attracting functions. Through trajectory analysis, they reported that $LAMP3^+$ DCs may be derived from cDCs, and their clinical relevance in neoadjuvant immunotherapy was implied in study by Hui *et al.* (10). The $LAMP3^+$ DCs were highly present in neoadjuvant MPR tumor lesions. Immune-related ligand-receptor pair analysis revealed $LAMP3^+$ DCs interactions with $CD4^+$ T cells, $CD8^+$ T cells and B cells, indicating their potential role in lymphocyte recruitment during neoadjuvant chemoimmunotherapy.

Neutrophils are often underrepresented in scRNAseq studies due to their short life-span and technical difficulties to capture them (19). Using BD Rhapsody technology, Yang *et al.* (19) showed insufficient infiltration of neutrophils in EGFR-positive LUAD. Neutrophils in this group highly expressed CD62L, CXCR1, and CXCR2, while CD54 and CXCL8 were downregulated, limiting the ability of neutrophils to indirectly activate T cells via IFN-γ. Some sources suggest that neutrophil function can also be regulated by mast cells, which may lead to TME remodeling and angiogenesis (22,23). Yang *et al.* noted a high proportion of $VEGFA^+$ mast cells with angiogenic characteristics in EGFR-mutant cells, supporting this conclusion. Hu *et al.* (12) identified 4 subclusters of neutrophils, showing either mature, proinflammatory or aged, immunomodulatory phenotype. A subset of aged $CCL3^+$ neutrophils was abundant in non-MPR patients and associated with poor response to therapy, through interaction with $SPP1^+$ TAMs, also previously reported to

Table 1 Distinct TME composition of MPR and non-MPR after cancer immunotherapy against NSCLC

Response	Cell type	Subtypes	Reference
MPR	T cells	High levels of infiltrating $CD8^+$ lymphocytes	Wu *et al.* (17)
		Large proportion of Trm cells	
		HAVCR2/TIGIT/PDCD1/GNLY/HLA-A/GZMB-high Trm cells	
		GZMB-expressing Trm cells	Hu *et al.* (12)
		Higher $CD4^+IL21^+$ T cells	Hui *et al.* (10)
		Decreased $CD4^+CD25^+CD127^-$ Treg cells	
		Diminished $TNFRSF4^+$ Treg cells	
		TCRs with strong ligand-dependent signaling	Caushi *et al.* (15)
		IL7R/TCF7/GZMK-high MANA-specific $CD8^+$ T cells	
		Mitotic $CD8^+$ T cells	Jia *et al.* (13); Hu *et al.* (12)
	B cells	$FCRL4^+FCRL5^+$ memory B cells	Hu *et al.* (12)
		Abundant $CD19^+$ B cells	Hui *et al.* (10)
		B cell class switch to IgG1 and IgG3 positive cells	
	Dendritic cells	Activated cDCs	Hu *et al.* (12)
		Increased $LAMP3^+$ DCs	Hu *et al.* (12); Hui *et al.* (10)
	Monocytes	Neutral $CX3CR1^+$ monocytes	Hu *et al.* (12)
		More infiltration of $CD14^+$ monocytes and $CD16^+$ monocytes	Jia *et al.* (13)
	Macrophages	Activated $CD68^+$ $HLA\text{-}DR^+$ macrophages	Wu *et al.* (17)
		TAMs reprogrammed to M0 phenotype	Hu *et al.* (12)
Non-MPR	T cells	Deficiency of $CD8^+$ T cell recruiting TAMs and CAFs	Yang *et al.* (18)*
		Presence of Tstr cells	Chu *et al.* (9)
		Abundant $FoxP3^+$ Treg cells	Hui *et al.* (10)
		TCRs with lower ligand-dependent signaling	Caushi *et al.* (15)
		TOX2/CTLA4/HAVCR/ENTPD1-high MANA-specific $CD8^+$ T cells	
		Largely confined to HOBIT-high Trm cells	
		GZMH/HLA-DRA/IFNG-high Trm cells	Wu *et al.* (17)
	B cells	Presence of $CXCL17^+$ plasma cells	Yang *et al.* (18)*
	Dendritic cells	Presence of $CXCL17^+$ DCs	Yang *et al.* (18)*
	Monocytes	Angiogenic $VEGFA^+$ monocytes	Hu *et al.* (12)
	Mast cells	Angiogenic $VEGFA^+$ mast cells	Yang *et al.* (18)*
	Macrophages	M2 signature	Hu *et al.* (12)
		Angiogenic $SPP1^+$ TAMs	
	Neutrophils	More $CCL3^+$ aged neutrophils	Hu *et al.* (12)
	Non-immune cells	$LEPR^+$ CAFs	Yang *et al.* (18)*

*, study on untreated LUAD samples with validation using post-immunotherapy data. TME, tumor microenvironment; MPR, major pathologic response; NSCLC, non-small cell lung cancer; Trm, tissue resident memory T; Treg, regulatory T; TCR, T cell receptor; MANA, mutation-associated neoantigen; cDC, conventional dendritic cell; TAM, tumor-associated macrophage; CAF, cancer-associated fibroblast; Tstr, stress response T; LUAD, lung adenocarcinoma.

facilitate angiogenesis (24). Together, these results suggest an existence of a potential crosstalk between neutrophils and other myeloid cells in tumor angiogenesis.

Changes in TME after immunotherapy have also been observed in non-immune cell populations. Yang *et al.* (19) observed that poor response to therapy was accompanied by a higher proportion of cancer-associated fibroblasts (CAF) with stem cell characteristics and recruitment of immunosuppressive cells via cytokines produced by EGFR-mutant tumor cells. Interestingly, EGFR-mutant malignant epithelial cells were shown to have similar characteristics to epithelial cells from non-responding nodules, while normal and EGFR wild-type malignant epithelial cells showed similarities with the cells from the responding nodule. Consistent with previous observations (25), cancer cells from MPR patients in Hu *et al.* (12) study could be differentiated from non-MPR by higher expression of CD74 and MHC-II genes in response to therapy, associated with a better therapy response.

Overall, the changes in TME of NSCLC after immunotherapy are highly complex, involving a variety of cell populations and dependent on additional factors, such as EGFR mutations. Studies based on scRNA-seq data contribute to a more comprehensive understanding of the differences between responding and non-responding patients (*Table 1*). However, additional studies are needed to unify the classification of the key cell types and clarify the molecular mechanisms regulating immunotherapy resistance in larger patient cohorts. The results presented by Hu *et al.* (12) complement previous reports on T and B lymphocytes and provide novel insights on other cell populations, poorly described in other studies in the context of immunotherapy, such as neutrophils and monocytes.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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