Editorial Commentary

CD66b as a prognostic and predictive biomarker in patients with non-small cell lung cancer treated with checkpoint blockade immunotherapy

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Non-small cell lung cancer (NSCLC) is the leading cause of cancer death around the world, including in the United States, where it is the leading cause of cancer-related mortality in both men and women. In recent years, the introduction of immune checkpoint inhibitors (ICI) has transformed the landscape of immunotherapy and opened up new treatment options for advanced stage (unresectable stage III and stage IV) NSCLC of both adenocarcinoma and squamous cell carcinoma histologies. Anti-PD-1/PD-L1 antibodies have demonstrated a significant benefit to a subset of patients, but a majority of patients do not respond to these antibodies or develop progressive disease after an initial clinical response. Patients who do not experience durable tumor response from ICI are exposed to potential immune-related toxicity, and the health system to financial costs, without benefit. Resistance to immunotherapy, either inherent or acquired, remains a significant challenge in improving ICI efficacy. In addition, better predictive biomarkers are needed to inform clinical care, and potentially triage patients who are unlikely to benefit from ICI to other therapies that may offer greater benefit (1).

Currently, PD-L1 expression is the most widely used biomarker for stratification of ICI treatment in NSCLC. However, PD-L1 expression is an imperfect biomarker due to inconsistencies such as variability in quantification methods and a lack of standardized cut-off points. As a result, PD-L1 immunohistochemistry may be used to determine eligibility for ICI therapies (e.g., PD-L1 TPS score >1%), but its role as a predictive marker has been observed to be limited, with an area under the receiving operating curve of <0.7 (2,3). Further optimization of this biomarker is required to improve its sensitivity and reproducibility.

One emerging area of interest in biomarker discovery is the use of neutrophils, which have been shown to be instrumental in modulating the tumor microenvironment (TME). Tumor-associated neutrophils (TANs) in the TME have been implicated in cancer progression and serve as an independent prognostic factor of unfavorable outcomes in a wide variety of cancer types (4,5). Tumor cells can alter the differentiation of TANs for their own benefit, promoting pro-tumor mechanisms, such as tumor angiogenesis, immune suppression, and neutrophil extracellular trap formation. The detection of pro-tumor TANs and TAN activity are associated with lower rates of response to ICI treatment (6). On the other hand, TANs have also been described to have some important anti-tumor functions, especially in the early stages of tumorigenesis. They can

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directly induce tumor cell apoptosis through secretion of cytotoxic molecules and target opsonized tumor cells via antibody-dependent cellular cytotoxicity (4). Thus, TAN associated markers could be promising targets for biomarker discovery for cancer prognosis and resistance to therapies. CD66b, a granulocyte activation marker typically found on neutrophils and eosinophils, may have predictive value in patients treated with immunotherapy.

In a recent study authored by Moutafi and colleagues at Yale School of Medicine, the authors sought to evaluate new candidate biomarkers for ICI response and resistance in NSCLC, using digital spatial profiling (DSP) (7). To investigate this, the authors used the recently developed NanoString GeoMX DSP technology, which combines standard immunostaining with digitally countable DNA barcodes, thereby allowing quantification of protein markers in specific regions of interest (ROIs). Leveraging this platform, Moutafi *et al.* studied the immune microenvironment of advanced NSCLC tumors from 56 patients treated at Yale with ICI. With DSP techniques, 284 protein biomarkers were analyzed. Analyses focused on 71 validated immune-oncology markers assessed in four molecular compartments—macrophage ($CD68^+$), leukocyte ($CD45^+/CD68^-$), tumor cell (panCK), and immune stroma ($panCK^-/CD45^+/CD68^+$). Of these proteins, CD66b was found to have a statistically significant association with poorer outcome; specifically, higher levels of CD66b in the immune stroma compartment were associated with shorter overall survival [OS; hazard ratio (HR) for high CD66b =1.31, 95% confidence interval (CI): 1.06–1.60] and progression-free survival (PFS; HR =1.24, 95% CI: 1.02–1.51). These associations remained significant in multivariable analyses including other key clinical covariates. Upon validation with quantitative immunofluorescence (QIF) in the same cohort, as well as in their two independent cohorts—one with ICI treatment (n=39) and one without (n=236), CD66b remained associated with worse OS but not PFS in the ICI-treated cohorts. Interestingly, in the non-ICI-treated patients, CD66b was not prognostic of OS. These findings suggest that CD66b may have prognostic value—forecasting the survival of patients with NSCLC in the context of ICI treatment—but not necessarily predictive value—forecasting the probability of tumor response to ICI therapy itself.

A particular strength of the study by Moutafi *et al.* is the inclusion of an independent validation cohort. The investigators first identified a cutoff to categorize CD66b as "high" or "low" in their discovery cohort, and then validated this cutoff in an independent validation cohort of 39 tumor samples. This is a critical step in their analysis that helps to confirm the prognostic value of this biomarker. Not all biomarkers, even those nominated with sophisticated approaches such as DSP, are necessarily able to be validated. For example, a prior study used the same GeoMX DSP platform in NSCLC patients treated with ICI, and identified the markers VISTA and CD127 as significantly associated with ICI resistance. Of note, this study did not examine CD66b, which was not on their antibody panel (8). Interestingly, in the study by Moutafi *et al.*, VISTA and CD127 markers were not found to be significantly associated with outcome, demonstrating the importance of validating candidate biomarkers in independent datasets before concluding their generalizability (7).

It is possible that CD66b has prognostic relevance only in the context of patients treated with ICI. This is suggested by the findings in Moutafi *et al.* and was also found to be the case in an analysis by Carus *et al.*, in which CD66b was studied in a cohort of 335 NSCLC tumors and found to have no prognostic associations, although CD66b was correlated with adverse prognostic inflammatory markers in the tumor and host (9). Granted, this study used immunohistochemistry to analyze CD66b in tumors, rather than isolating the immune compartment with an assay such as DSP; however, to be widely clinically available, the ideal biomarker would be measurable using widely available techniques that can be implemented in any clinical pathology laboratory.

Further suggesting nuances of how CD66b may offer the best prognostic information, Ilie *et al.* observed a significant association between CD66b and clinical outcomes in NSCLC, by using the ratio of intratumor $CD66b^+$ neutrophils to $CD8^+$ T-cells in a cohort of 632 patients (10). These findings suggest that while the presence of $CD66b^+$ TANs by themselves might not be sufficiently prognostic, the relative measure of TANs and cytotoxic T-cells could represent the balance between pro-tumor and anti-tumor contributions in the tumor immune microenvironment. This ratio was not analyzed in the current study by Moutafi *et al.* It is important to note that CD66b is also found on a subset of myeloid-derived suppressor cells (MDSCs)—the polymorphonuclear (PMN)-MDSCs—that are phenotypically similar to neutrophils but functionally distinct due to their potent immunosuppressive function (11,12). This myeloid subset could represent the pro-tumor population of TANs described previously, although this connection remains

unclear given the lack of PMN-MDSC specific markers. A recent study by Arasanz *et al.* reported an association between circulating CD66b$^+$ PMN-MDSCs and resistance to first-line anti-PD-1/PD-L1 therapy in NSCLC (13). High levels of peripheral PMN-MDSCs predicted primary resistance to ICI monotherapy but were not associated with outcome in a combined chemotherapy/ICI cohort. Interestingly, all patients with a high baseline PMN-MDSC in the chemotherapy/ICI cohort (n=9) had a significant decrease in PMN-MDSC levels between the first and second cycle. Taken together, these results suggest an active role of PMN-MDSCs in ICI resistance. Hence, chemotherapy-associated depletion of this myeloid subpopulation enhanced the effect of immunotherapy and combined treatment could overcome ICI resistance. This is consistent with the data from other studies showing similar associations between PMN-MDSC levels and ICI treatment response in NSCLC and advanced melanoma. Overall, these data suggest the role of PMN-MDSCs/TANs in promoting primary resistance and the possible use of CD66b as a predictive biomarker for ICI treatment.

We are now learning that TANs exhibit variable levels and variable directionality of prognostic impact in NSCLC, depending on the stage of the cancer, consistent with both pro- and anti-tumor functions. In some early-stage cancers, the presence of CD66b$^+$ TANs improved prognosis (4). *Ex-vivo* data of stage I to II NSCLC demonstrated enhanced T-cell proliferation and anti-tumor response mediated by TANs. In the non-ICI-treated validation cohort (7), as well as in the Carus *et al.* and Ilie *et al.* cohorts, almost all patients had early stage (stage I to III) surgically resected tumors. However, in the Moutafi *et al.* ICI-treated cohorts, the majority of the patients had advanced stage (unresectable or metastatic) disease. It is quite possible that differences in disease stage across these different studies may explain some of the divergent results. Further studies with greater statistical power are required to clearly establish an association between CD66b$^+$ status in the TME and clinical outcome in patients with NSCLC, both in the ICI and non-ICI treatment contexts.

Another important source of variability are differences in the histological subtypes of NSCLC across different studies. As reported by Rakaee *et al.*, the prognostic importance of CD66b differs between adenocarcinoma and squamous cell lung carcinomas (14). CD66b$^+$ cells were an adverse prognostic factor in patients with adenocarcinoma but may actually be a positive prognostic factor in patients with squamous cell carcinoma. Perhaps as a result of this interaction with histologic subtype, when these two NSCLC subtypes were grouped into a single NSCLC cohort, statistical significance was lost. Variable clinical outcomes observed in association with CD66b in the previous NSCLC studies are summarized along with the cohort characteristics in *Table 1*.

Given the heterogeneity of neutrophils and functional difference between pro-tumor and anti-tumor TANs in the TME, it remains to be proven whether CD66b alone can be specific enough for clinical use. While this present study by Moutafi and colleagues makes use of cutting-edge DSP technology and is to be commended for the inclusion of two independent validation cohorts, these authors do agree that additional data is needed; for example, a much larger multi-institutional dataset with sufficient power to examine possible interactions with covariates such as tumor stage, histology, prior therapy, and the site being sampled. In addition, methods to better characterize pro-tumor neutrophils are needed: these could improve the predictive value of CD66b$^+$ as a biomarker, although this is challenging given the lack of specific expression markers and the vague definitions of these populations. Ratios of CD66b$^+$ cells to CD8$^+$ T-cells or to regulatory T-cells have been described to predict ICI resistance and increase the prognostic significance when compared to CD66b$^+$ cells alone (15,16). Other cell markers involved in ICI resistance, if used in combination with CD66b, might enhance the performance and reliability of this biomarker.

Conclusions

The important work by Moutafi and colleagues, leveraging DSP technology and several independent datasets, nominates CD66b as a biomarker that is likely to have implications for prognosticating NSCLC patients being treated with ICI. Acknowledging some variability in findings across several published studies analyzing this particular marker, and TANs more broadly, in NSCLC, we can conclude that more research is needed. Nevertheless, because CD66b is a marker of TANs that can be robustly measured and appears to be associated with survival in ICI-treated patients, even if this marker alone may not be able to sufficiently move the prognostication needle, it seems very likely that this marker will add prognostic, and possibly predictive, information to more complex, multivariable models that are being developed. While the landscape of treatment options for advanced NSCLC has expanded remarkably in recent years, there remains a very strong

Table 1 Summary of study cohorts

Cohort characteristics	Cohort					
	Moutafi *et al.*—ICI Pre-treatment Cohort (N=56)	Moutafi *et al.*—Validation Cohort, ICI-treated (N=39)	Moutafi *et al.*—Validation Cohort, non-ICI-treated (N=236)	Carus *et al.*—no neoadjuvant treatment (N=335)	Ilie *et al.*—non-ICI treatment (N=632)	Rakaee *et al.*—non neoadjuvant treatment (N=509)
Tumor stage						
I	0 (0%)	0 (0%)	189 (80%)	219 (65%)	274 (44%)	160 (31%)
II	0 (0%)	0 (0%)	33 (14%)	66 (20%)	185 (29%)	251 (49%)
III	5 (9%)	1 (3%)	11 (5%)	50 (15%)	173 (27%)	92 (18%)
IV	51 (91%)	38 (97%)	3 (1%)	0 (0%)	0 (0%)	6 (2%)
Histology						
ADC	41 (73%)	28 (72%)	159 (67%)	153 (46%)	348 (55%)	188 (37%)
SCC	9 (16%)	9 (23%)	66 (28%)	153 (46%)	207 (33%)	278 (55%)
Other	6 (11%)	2 (5%)	11 (5%)	29 (8%)	77 (12%)	43 (8%)
High intratumoral CD66b$^+$	PFS HR: 1.29 (1.11–1.51; P=0.009)	Not reported	Not reported	DFS and OS were not significant	Greater CIR (P=0.002)	DSS HR: 0.92 (0.69–1.21; P=0.54)
	OS HR: 1.25 (1.04–1.50; P=0.02)				Worse OS (P=0.08)	SCC: 0.59 (0.38–0.92; P=0.02)
						ADC: 1.7 (1.1–2.65; P=0.02)
High immune stroma CD66b$^+$	PFS HR: 1.31 (1.08–1.58; P=0.02)	PFS HR: 1.49 (P=0.2)	DFS HR: 1.2 (P=0.5)	DFS and OS were not significant	Not reported	DSS was not significant
	OS HR: 1.25 (1.04–1.52; P=0.05)	OS HR: 2.08 (P=0.05)	OS HR: 1.67 (P=0.06)			

ADC, adenocarcinoma; SCC, squamous cell carcinoma; PFS, progression-free survival; HR, hazard ratio; OS, overall survival; DFS, disease-free survival; CIR, cumulative incidence of recurrence; DSS, disease-specific survival; ICI, immune checkpoint inhibitors.

clinical need for more accurate models to assist clinicians in identifying the highest value therapeutic strategies for patients presenting with new diagnoses of advanced stage NSCLC.

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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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