Editorial Commentary

Top-of-the-art cytometry as a novel tool to aid in lung cancer immunotherapy

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Comment on: Rochigneux P, Lisberg A, Garcia A, *et al.* Mass Cytometry Reveals Classical Monocytes, NK Cells, and ICOS+ CD4+ T Cells Associated with Pembrolizumab Efficacy in Patients with Lung Cancer. Clin Cancer Res 2022;28:5136-48.

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Non-small cell lung cancer (NSCLC) is perhaps the cancer type that better exemplifies the transition to precision medicine for cancer. Indeed, around 15% of patients with advanced NSCLC display genomic alterations in *EGFR*, *ALK*, *ROS1*, *BRAF* or *MET* so targeted therapies have improved their survival outcomes (1). Not in vain, immunotherapy has been one of the great advances for the treatment of advanced tumours, mostly NSCLC. The development of immune checkpoint inhibitors (ICIs) has resulted in a paradigm shift as we can now modulate the patient´s immune system to target cancer cells. Immune checkpoint molecules, such as programmed cell death protein 1 (PD-1), its ligand (PD-L1), and cytotoxic lymphocyte-associated protein 4 (CTLA-4), are proteins that contribute to the regulation of the immune system and prevent it from attacking healthy cells in the body. By targeting these proteins, ICIs can "release the brakes" on the immune system, allowing the immune system to target cancer cells more effectively. Hence, immunotherapy has significantly improved the survival outcomes of patients with advanced NSCLC, but unfortunately, not all patients benefit from this treatment approach. There is therefore a real need to discover novel biomarkers to identify the specific NSCLC patients who will respond to ICIs, namely, utilizing personalized medicine to maximize the benefits of the therapy while minimizing its side effects.

Nevertheless, and as opposed to targeted therapy, the development of biomarkers for immunotherapy success is much more complex. In addition to the alterations in tumour cells, we face a heterogeneous and dynamic scenario in which other factors, including both the tumour microenvironment and the host immune response, are involved (2). The main problem, therefore, is the absence of reliable and predictive response biomarkers that allow correct patient stratification. In this regard, the expression of PD-L1 on the surface of tumour cells is one of the

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principal biomarkers used to guide and select ICI regimens for advanced NSCLC patients in clinical practice. However, while some studies have revealed that the presence of PD-L1 expression in tumour tissue can predict a good response to ICIs (3), this association is not always observed (4), likely due to the intra- and intertumoral heterogeneity of PD-L1 expression in solid tissue tumours. Currently, the validated companion diagnostic test to detect PD-L1 expression is based on immunohistochemical (IHC) characterization of tumour tissues. Nevertheless, there are several challenges associated with the technique itself, such as the selection of the correct antibody, use of standardized protocols and consensus on the value of this biomarker (5). The tumour mutational burden (TMB) has also been reported to be a useful biomarker in the immunotherapy field. Indeed, a high TMB has been associated with an improved objective response rate (ORR) and progression-free survival (PFS) in NSCLC patients treated with an ICI (6); however, more research is needed to confirm its utility as a reliable biomarker.

Recently, microsatellite instability (MSI) has been associated with a higher likelihood of response to immunotherapy in cancer patients (7), leading to the approval of a blood-based test by the Food and Drug Administration (FDA). This blood-based test (FoundationOne Liquid CDx test, Roche, Basel, Switzerland) identifies candidates for first-line immunotherapy among patients with unresectable or metastatic solid tumours with MSI or mismatch repair deficiency (MMRD), including patients with lung cancer. Nevertheless, due to the small subset of NSCLC patients who present with an MSI-high status (8), testing for MSI is not routinely performed in clinical practice, so the value of MSI as an immunotherapy biomarker in lung cancer is poor. Hence, we lack reliable and predictive response biomarkers with real utility in the clinics to perform successful patient stratification when implementing immunotherapy.

Hence, an ideal biomarker of response to ICIs would be one that could be found in blood samples. In this regard, flow cytometry allows the characterization of the different leukocyte subsets present in a biological sample, as well as the characterization of their phenotype and function. However, flow cytometry has the technical drawback of being limited to no more than 25 markers assessed simultaneously, which makes an unbiased characterization of leukocyte subsets impossible. An alternative that circumvents this limitation is the use of mass cytometry [Cytometry Time-Of-Flight (CyTOF)], which allows the simultaneous identification of over 45 parameters in the same cell. This technique also exhibits high cost-effectiveness performance compared to other "omic" techniques, allowing characterization of the various immune subsets found in a given sample (9). CyTOF has therefore obviously revolutionized the study of the role of the immune system in various pathogenic processes (10). In fact, mass cytometry can also be applied to the study of human tissues, including the intestinal mucosa, in which it has been possible to identify more than 140 different immune subsets (11). Nevertheless, mass cytometry has important limitations, such as the slowness of the analysis (a rate of no more than 1 million cells per hour for analysis) and the low recovery of cells (more than 50% of the cells analysed are lost), a handicap that is especially relevant when working with samples that provide a very limited number of cells. One way to overcome these limitations is to use state-of-the-art spectral cytometry. This technology exploits the advantages of CyTOF equipment, such as the possibility of simultaneously evaluating complex panels of up to 40 markers while eliminating compensation and autofluorescence problems (12). In addition, spectral cytometry does not use antibodies conjugated to heavy metals but to fluorochromes, which facilitates the workflow and lowers costs. Finally, spectral cytometry allows for a high acquisition speed (up to 30,000 events per second) without the loss of cells as in CyTOF.

Having said that, CyTOF technology is still a valid and outstanding tool. Indeed, Rochigneux *et al.* performed a high-dimensional mass cytometry examination of peripheral blood mononuclear cells (PBMCs) from NSCLC patients treated with pembrolizumab prior to start the treatment (13). The authors provided evidence that the blood baseline frequencies of classical monocytes, natural killer (NK) cells and inducible T-cell costimulatory $ICOS^+CD4^+$ T-cells were associated with pembrolizumab efficacy. Despite the study limitations, mainly the small number of subjects (n=27) and the need for a validation study, the possibility of having prognostic and/or predictive non-invasive biomarkers to discriminate patients who will benefit from ICIs through deep phenotyping of immune cells prior to treatment initiation is of great interest. Hence, although the results need to be validated and the methodological approach simplified to perform these analyses with "real hospital-available cytometers", the authors provided a promising non-invasive strategy to improve the clinical management of NSCLC. Indeed, monitoring the dynamics of these immune subsets may be cost-effective and easy to implement in the routine clinic if

the analysis panel is adapted for classic cytometry.

There have been few studies on the dynamics of different circulating immune subsets before, during, and after ICI therapy to elucidate the potential roles of these subsets as predictive biomarkers of immunotherapeutic efficacy and survival in NSCLC patients (14-17). In this context, most of the current studies focus on tumour-infiltrating lymphocytes (TILs) (2). TILs are a heterogeneous population that migrated from the blood into a tumour and consists of both mononuclear and polymorphonuclear immune cells [mainly $CD4^+$ and $CD8^+$ T cells, NK cells and regulatory T cells (Tregs]]. These cells have been reported to be potential predictive biomarkers of the response to ICIs (18) and have been shown to be correlated with the efficacy of immunotherapy (19). However, their blood frequencies may not be sufficiently reflective of the tumour microenvironment conditions.

In addition, another potential field of application for this methodology might be the search for biomarkers of toxicity. Due to their mechanism of action, ICIs can cause immune-related adverse events (irAEs), as they can induce toxicity in tissues and organs that are usually protected by the immune system, resulting in autoimmune-type diseases and inflammation. The treatment of irAEs is based on the use of corticosteroids, other immunosuppressants (infliximab or mycophenolate) and temporary or definitive discontinuation of immunotherapy (20). As ICI use increases, due to the difficulty in predicting irAE rates in patients as well as new indications, a proliferation of irAEs is expected. Unfortunately, we lack a comprehensive understanding of the development of irAEs. To date, no blood-based biomarkers with the potential to accurately predict the risk of irAE development in patients undergoing ICI treatment have been identified (21).

Due to irAEs, patients exhibit relatively high diversity in $CD4^+$ and $CD8^+$ T cells, and there is an initial diversification of the T-cell repertoire after ICI treatment (22). $CD8^+$ T-cell clonal expansion has been proposed as a potential biomarker for irAEs in prostate cancer patients treated with ipilimumab (23). Das *et al.* used mass cytometry to analyse B-cell changes in patients with melanoma following ICI treatment (24), showing that early changes in B cells following combination checkpoint blockade may identify patients at an increased risk of irAEs. Indeed, pre-emptive strategies targeting B-cells may reduce toxicities in these patients. Whether these initial changes are stochastic or reflect other factors requires further investigation.

Among other potential factors, host-intrinsic polymorphisms have gained attention. The genetic predisposition to irAEs is poorly defined, and only human leukocyte antigen (HLA) profiles and a few single-nucleotide polymorphisms (SNPs) in candidate genes have been described to be associated with irAEs (2). However, recently, one SNP in the *IL-7* gene was observed to predict ICI toxicity in patients with cancer (25,26). Hence, Groha *et al.* performed a study on a cohort of 1,751 cancer patients treated with ICIs, identifying 3 significant genome-wide associations with all-grade irAEs (25). One of them, rs16906115, was located near the *IL-7* gene and colocalized with the gain of a new exon of this gene, a critical regulator of lymphocyte homeostasis. Hence, patients carrying the *IL-7* variant exhibited significantly increased lymphocyte stability after initiation of ICI treatment, which was a good indicator of potential irAEs and improved survival. In parallel, Taylor *et al.* reported the large effect size of rs16906115 as a reflection of the relevant role of *IL-7* in both B- and T-cell lymphopoiesis (26).

In summary, the biological drivers of irAEs are poorly characterized, and there is no method in standard clinical practice to identify which patients are at highest risk for developing them. A potential hypothesis states that irAEs are manifestations of autoimmunity in individuals who are genetically susceptible to autoimmune disorders and that the genetic variants underlying common autoimmune disorders could be useful predictors for irAEs, which is very interesting. In addition, whether a common immunological state precedes distinct manifestations of ICI-induced toxicity or will allow the possibility of having prognostic and/or predictive non-invasive biomarkers to discriminate patients who will benefit from ICIs is unknown. However, the findings of Rochigneux *et al.* (13) add an important approach to the identification of biomarkers for immunotherapy since pre-treatment immunological features in the peripheral blood could be associated with efficacy, toxicity and/or survival outcomes in advanced NSCLC patients. Future research should extend the observation time and monitor the changes in immune cell populations prospectively, generating high-resolution data at the single-cell level.

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Footnote

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