Multiplexed Antibody-Based Spatial Imaging Technologies in NSCLC

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Contributor: Simon Gray , Christian H. Ottensmeier

Non-small cell lung cancer (NSCLC) remains a cause of significant morbidity and mortality, despite significant advances made in its treatment using immune checkpoint inhibitors (ICIs) over the last decade; while a minority experience prolonged responses with ICIs, benefit is limited for most patients. The development of multiplexed antibody-based (MAB) spatial tissue imaging technologies has revolutionised analysis of the tumour microenvironment (TME), enabling identification of a wide range of cell types and subtypes, and analysis of the spatial relationships and interactions between them. Such study has the potential to translate into a greater understanding of treatment susceptibility and resistance, factors influencing prognosis and recurrence risk, and identification of novel therapeutic approaches and rational treatment combinations to improve patient outcomes in the clinic.

NSCLC microenvironment multiplex spatial

1. Prediction of Recurrence and Survival Following Curative-Intent Resection

Given the availability of tumour tissue following curative-intent resection, multiple studies have used MAB-based methodologies to identify signals associated with post-resection recurrence risk and OS. One relatively early study utilised a 6-plex tyramide signal amplification-based panel to assess 120 patients with resected non-small cell lung cancer (NSCLC) [1]. The cross-G function—a form of probabilistic nearest neighbour analysis—was used to demonstrate shorter OS for patients with CD4+ forkhead box P3-positive (FoxP3+) T_{reg} cells and tumour cells in close proximity (hazard ratio (HR) 1.52, 95% confidence interval [95%CI] 1.11–2.07 and $p = 0.009$). Improved OS for patients with T_{reg} cells and effector CD8+ T-cells (CD8+Ts) in close proximity (HR 0.96, 95%CI 0.92–0.99 and $p = 0.042$) was also demonstrated; the authors suggested this was related to the ability of CD8+Ts to somewhat overcome the tolerogenic effect of T_{reg} cells in the TME [1].

A further paper utilised DSP with a 52-plex panel to study a tumour microarray (TMA) comprised of 92 cases with paired histologically normal adjacent tissue (NAT) [2]. Enrichment of T-cell markers (CD3, CD4), macrophage markers (CD68, CD168), immune checkpoints (CD27 and V-domain Ig suppressor of T-cell activation [VISTA]), CD44 and CD45 were seen in the stroma relative to the tumour. Meanwhile, NAT was enriched with markers of ECM (fibronectin), indoleamine 2,3-dioxigenase 1, exhausted T-cells (lymphocyte activation gene 3 [LAG-3]), TAMs/MDSCs (arginase 1 [ARG-1]), CD34 and the tumour suppressor phosphatase and tensin homolog when compared with the TME [3][4][5][6]. Univariate analysis suggested that expression of CD34, CD3 and inducible co-

stimulatory (ICOS) were associated with favourable OS, though these signals did not persist in multivariable analysis with adjustment for age and disease stage [2].

Backman et al., studied 300 patients with resected NSCLC, and used mIF to demonstrate a positive prognostic effect for patients with high densities of tissue helper CD4+ T-cells (CD4+Ts) and CD8+Ts, M1 macrophages, B-cells, plasmacytoid dendritic cells (pDCs) and also both CD4+ and CD8+ T_{reg} cells, including when adjusted for clinical parameters; these observations were stronger when analysing tumour and stromal compartments together [7]. Similar observations were seen between lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) samples. In the spatial analysis, helper CD4+Ts and CD8+Ts, M1 macrophages and pDCs were proximal to tumour cells; other immune cell types were more evenly distributed, while mature DCs were predominantly distant from tumour cells. Co-localisation of adaptive lymphocyte subsets together was associated with longer survival, but only the CD8+T/B-cell proximity effect remained significant after multivariate analysis. The positive prognostic impact of CD8+ T_{reg} density was notably abrogated when distance to tumour cells and other immune cell types was accounted for, suggesting high CD8+ T_{reg} density was a reflection of high total immune infiltrate. In assessing relationships between distance and density, multivariable Cox regression analysis showed independently favourable prognosis for high densities of M2-like macrophages, M1 macrophages, close proximity of both CD4+ and CD8+ T_{reg} cells to B-cells, and co-localisation of effector CD8+Ts and tumour cells. Co-localisation of M2 and M1 macrophages conferred a worse prognosis [7]. This group took the commendable decision to make their entire spatial data set publicly available.

Another 2023 analysis by Sorin et al. examined 416 patients with predominantly early-stage LUAD tissue from resection or biopsy via imaging mass cytometry (IMC) of TMAs [8]. Histologically high-grade ‘solid’ tumours were enriched for myeloid cells including tumour-associated neutrophils (TANs), monocytes and CD163+ M2-like macrophages. M2-like macrophages in the ‘solid’ subtype were associated with T_{reg} cells, whereas in other histological subtypes they were strongly correlated with effector CD8+Ts. B-cell frequency was associated with improved survival, independent of a range of potentially confounding clinico-pathologic variables. Spatial analysis of direct cell–cell interaction suggested largely homotypic interactions for tumour cells, endothelial cells and CD163- macrophages; however, tumour cells interacted more with TANs and endothelial cells in higher-grade versus lower-grade histological subtypes. This is consistent with the observed ability of TANs to facilitate haematogenic metastasis [9]. Lower-grade tumours featured greater interaction between tumour cells and both CD8+ and CD4+Ts; meanwhile, though M2-like macrophages and CD8+Ts coexist across tumour grades, their degree of interaction increases as tumour grade increases. Proliferating (Ki-67+) endothelial cells, presumably implicated in hypoxia and angiogenesis, were associated with poor OS and with TAN interactions in high-grade disease.

2. Prediction of Benefit from Immunotherapy

As previously alluded to, another focus of MAB technologies has been the utilisation of both pre- and post-treatment tissue samples to identify novel biomarkers associated with response to treatment.

Several studies from the same research group utilising DSP to study NSCLC TMA tissue have reviewed multiple aspects of response to anti-PD-(L)1 therapy. One such study analysed tissue from 53 patients who received anti-PD-1 mAb monotherapy and had paired pre-treatment samples [10]. After adjustment for clinico-pathologic variables including the serum-based lung immune prognostic index (LIPI) [11], in multivariate analysis only high levels of CD4 and the natural killer cell marker CD56 measured in the immune (CD45+) compartment predicted clinical benefit (partial response or stable disease for ≥6 months), longer progression-free survival (PFS) and OS. High VISTA levels predicted lack of clinical benefit and shorter PFS [10].

A further study of 58 patients with aNSCLC and pre-anti-PD-(L)1 treatment samples studied 71 targets to determine mechanisms of treatment resistance [12]. Expression of the calcium-binding protein S100B was associated with improved OS in all four compartments. Immune stromal CD66b expression by tumour-associated neutrophils (TANs) predicted significantly shorter OS and PFS, as well as progressive disease at 12 and 24 months of therapy. A significant association between immune stromal CD66b expression and ICB resistance was seen in an ICB-treated validation cohort (HR 2.05 and p = 0.046) irrespective of pre-treatment serum neutrophil:lymphocyte ratio, but did not predict survival in a further non-ICB-treated cohort (HR 1.67 and p = 0.06) [12].

A third study focused on identifying markers of sensitivity to PD-(L)1 blockade, using pre-PD-(L)1 mAb tissue samples from a discovery cohort of 56 patients [13]. Expression of CD44, a positive regulator of PD-L1 in lung cancer, in the tumour compartment was associated with longer PFS in multivariate analysis. Intratumoural CD44 expression was significantly lower versus the immune compartment, and was higher both in patients with LUSC and without baseline liver metastasis [14]. Levels of CD44 determined using quantitative IF were associated, on multivariate analysis, with longer PFS (HR 0.31, 95%CI 0.11–0.87 and p = 0.022) and OS (HR 0.29, 95%CI 0.09–0.97 and p = 0.038), while stromal CD44 expression did not predict outcomes. In an ICB-treated validation cohort, CD44 levels in the tumour compartment predicted PFS upon multivariate analysis after adjusting for performance status, baseline liver metastasis and LIPI score (HR 0.62, 95%CI 0.40–0.96 and p = 0.035). This remained significant after adjusting for the tumour proportion score (TPS) at ≥1% and ≥50% cutoffs. A further ICB-untreated NSCLC cohort showed no prognostic association with CD44 expression. In CD44-high ROIs from both validation cohorts, upregulation of PD-L1, TIM-3, ICOS and CD40 was seen (false discovery rate (FDR)-adjusted $p < 0.05$), with other immune cell markers and co-inhibitory molecules upregulated to a lesser extent [13].

3. Study of CD8+ T-Cells in Early-Stage Resected NSCLC

Among the primary effectors of anticancer immunity are CD8+Ts, which are also potentiated using current ICB strategies [15]; they have, accordingly, been the focus of multiple studies employing MAB methods to study the TME.

One such study identified an exhausted CD8+ T-cell subset using IMC to study resected tumour and paired NAT from 25 early-stage NSCLC patients [16]. The ratio of lymphoid to non-lymphoid cells was significantly higher in tumour versus NAT; CD8+Ts in the tumour were substantially more proliferative versus those in NAT, while other

lymphoid cell types were not. Hierarchical clustering divided tumour-associated CD8+Ts into predominantly effector (CD45RA+CD45RO-) and effector memory (CD45RA-CD45RO+) subtypes. The latter was further subdivided into a conventional memory phenotype, while the other expressed high levels of CD45RO, eomesodermin, FAS, CD27, CD28, PD-1, LAG-3 and TIM-3 as well as low T-bet and granzyme B (GZMB), suggestive of a burned-out effector (E_{bo}) subset. Such E_{bo} clusters were primarily tumour-associated, while CD8+Ts in NAT showed preserved effector functions. Subsequent CD8+T whole-transcriptome RNAseq confirmed enrichment of apoptotic and dysfunctional CD8+Ts among E_{bo} cells. Anti-PD-1 therapy of NSCLC-engrafted mice demonstrated a post-treatment reduction in the E_{bo} subset while preserving effector CD8+Ts. Subsequent IMC of a mixed-stage human NSCLC cohort showed a higher proportion of CD8+Ts were E_{bo} cells in patients with late-stage, versus early-stage, disease ($p = 0.006$), suggesting expansion with time and disease progression.

A further study of tissue from 13 treatment-naïve patients with resectable NSCLC used cytometry by time-of-flight and IMC to describe a population of CD8+PD-L1+ tumour-infiltrating T-cells with low levels of expression of PD-1, CD103, GZMB and IFN-γ [17]. Cellular neighbourhood analysis demonstrated close proximity of CD8+PD-L1+ cells to activated and exhausted CD8+Ts, suggesting a regulatory role for the former subset which was subsequently corroborated with demonstration of their capacity to suppress CD8+PD-L1- cells' production of IFN-γ and TNF-α in vitro [17].

A larger study of 279 resected NSCLC cases was assessed using mIF of TMA sections, specifically seeking associations with lymph node metastasis (LNM) [18]. Density of CD8+Ts was significantly lower in both TC ($p < 0.001$) and IM ($p < 0.001$) for patients with LNM; upon CD8+T subtyping, the densities of pre-dysfunctional (odds ratio (OR) 0.51, 95%CI 0.29–0.88 and $p = 0.015$) and dysfunctional (OR 5.80, 95%CI 3.19–10.54 and $p < 0.001$) CD8+Ts were both associated with LNM, independent of a group of clinico-pathologic factors. Lower recurrence risk was predicted using total CD8+Ts in the IM (HR 0.57, 95%CI 0.35–0.92 and $p = 0.021$), pre-dysfunctional CD8+Ts in the TC (HR 0.55, 95%CI 0.34–0.89 and $p = 0.014$), while dysfunctional CD8+Ts in the IM predicted higher recurrence risk (HR 2.49, 95%CI 1.60–4.13 and $p = 0.012$).

In comparison to previously considering all CD8+Ts together, studies such as those above clearly show the value of MAB spatial dissection of this cellular compartment in detecting subsets with divergent functions [19]. This is likely to enable prognostic and predictive tools to be refined, and may enable the development of more personalised anticancer agents. While CD8+Ts have historically been synonymous with 'cytotoxic' T-cells, their roles in the TME are shown to be far less straightforward.

4. Other Multiplexed Antibody-Based Studies

An IMC-based analysis of resection specimens from 12 patients with LUSC identified a novel population of CD3-CD4+FoxP3+CD25-CD127- cells in both tumour and adjacent regions of 10 patients, which were also TNF-α-positive and IFN-γ-negative. A pro-inflammatory function, divergent from that of T_{reg} cells, was proposed in view of their TNF-α production, and negativity for CD127 also indicated they were distinct from innate lymphoid cells [20]. A CD3-CD4+CD127+ population was previously identified in autoimmune diseases such as rheumatoid arthritis and

psoriasis; despite its T-cell lineage, this was activated by innate signals such as IL-7, which can downregulate CD127 expression in CD3-CD4+ cells [21][22].

A DSP-based study of a TMA formed from 33 patients' surgically resected NSCLC tissue focused on leucocyte populations in the stroma, tumour or tertiary lymphoid structures (TLS) [23]. Versus stroma, intratumoural lymphocytes expressed higher levels of multiple molecules including PD-L2, CTLA-4 and FoxP3, indicating active immune suppression. In the stroma, fibroblast activation markers were observed, as well as significantly higher VISTA and CD27 expression versus within the tumour. Actively proliferating T- and B-cells were observed more frequently in the TLS versus stroma, with increased CD3, CD20, CD45, beta-2-microglobulin, CD11c, CD40, ICOS and Ki-67. Shorter distance from ROI to tumour was associated with increased expression of immunosuppressive molecules. Expression of co-stimulatory CD27 decreased with proximity to the tumour but was significantly expressed in stromal regions, and a CD27 agonist such as varlilumab was suggested by the authors as a potential means to exploit this. A strong correlation was observed between expression of ARG-1 and CD66b. Given prior observations of ARG-1 production by TANs and ARG-1 blockade reducing tumour growth in an animal model of NSCLC, the authors suggested that tumours with highly frequent granulocytes in either tumour or stroma could be targeted with ARG-1-blocking therapy [23][24][25].

5. Summary

As demonstrated above, a number of potential biomarkers and signatures have been identified pertaining to prognosis, risk of recurrence and prediction of benefit from ICB. While single markers could conceivably be incorporated into existing pathology workflows, other signatures may require MAB equipment for clinical application similar to that used for discovery; this may be due to a large number of included markers, or the integration of spatial information into the signature. Slow sample processing times and high cost currently represent barriers to clinical application of MAB technologies, though the opportunities and challenges of clinically implementing mIF technology have been reviewed elsewhere [26]. Prospective clinical validation of such signatures, while challenging, is likely to be crucial in evidencing the clinical benefits of MAB technologies given the potential cost of such a transition. Artificial intelligence tools have the capacity to effectively leverage the large quantities of data produced using MAB technologies [8], and are likely to feature increasingly in studies thereof. The progressive expansion and validation of larger marker panels, and the expansion of functionality within commercially available image analysis software, are also likely to expand the utility and scope of MAB-based research with time [27][28].

An inherent limitation of MAB methods, especially utilising TMAs, is tumour heterogeneity and the possibility of non-representative sampling. Where this has been studied in NSCLC, the results have been largely reassuring; in one study, 60 patients who had two TMA cores taken per patient showed 91.7% agreement in predictive outputs between their respective cores [8]. Given many MAB technologies can require the prohibitive amounts of time and expense of analysing large numbers of full biopsy sections, use of TMAs seems a reasonable compromise to allow the study of large numbers of patients. Many MAB studies within NSCLC have focused on earlier-stage, single-site disease; as such, samples are likely to be more representative of the cancer as a whole, given overall tumour burden is lower and heterogeneity between metastases does not have to be contended with. While such issues are

inherent in any analysis based on solid biopsy material, MAB technologies may be more limited in the study of metastatic disease for this reason, assuming only one site is sampled.

Multiplexed antibody-based spatial technologies continue to demonstrate their utility as a novel research tool. Moreover, techniques for analysing the data generated using these techniques continue to advance. Beyond tools for prognostication and prediction of benefit from existing standard-of-care treatments, MAB spatial technologies offer the possibility of identifying TME-based indications for additional therapeutics to overcome treatment resistance. Separation of bystander and driver events, and identifying redundancy, remain challenging but MAB technologies represent a powerful tool for deepening our understanding of the TME. In time, this may enable more rational stratification of patients into appropriate clinical trials, identifying therapies which may only benefit patients with a certain TME status.

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