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INVITED REVIEW

ADVANCES IN CELL AND GENE THERAPY WILEY

Immunological tumor heterogeneity and diagnostic profiling for advanced and immune therapies

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Abstract

Immunotherapies have changed the way how we treat cancer at all stages. The understanding of the immune system in individual tumor specimens guides the selection of immune-modulating agents such as immune checkpoint inhibitors alone or in combination with other therapeutic agents that target, modulate or unleash the patient's immune system. Despite the similar histopathological diagnosis, each tumor is unique at its primary site and site of metastasis, also depending on previous treatment regimens or genetic alterations, such as chromosomal instability or acquired mutations. The clinically well-established use of PD-1/PD-L1 inhibitors already requires the assessment of its target molecules in different cells (viable tumor cells alone or in combination with immune cells or immune cells alone) with different thresholds in various indications. Anyhow, checkpoint inhibitors show the best overall response rate when immune effector cells like tumor-infiltrating lymphocytes are in close spatial proximity without being suppressed by other humoral or cellular regulatory mechanisms. Therefore, immune cell-rich tumors ("hot tumors") are usually quite reactive to immune-modulating agents, whereas other immune-depleted or immune-excluded tumor areas are less responsive and require alternative treatment regimens such as modified immune effectors cells or immune-stimulating agents, for example, oncolytic viruses. Here, we summarize the relevance to understand the entire tumor heterogeneity and its environment, the contextual relationship and spatial quantification of all immune and tumor cells along with the genetic background of the individual cancer through the application of multiplex in-situ technologies and the application of machine learning tools.

KEYWORDS
cell therapy, immunotherapy

1 | BACKGROUND

Immunology discoveries and advancements come in waves. More than 100 years ago, different immune cells and their separate role in infectious and neoplastic diseases became obvious and some improvement in light microscopy contributed to the development of cancer immunology as a separate subject. With the advancement of analytical methods like immunohistochemistry (IHC), molecular tools, and computational solutions, immunotherapies make a greater impact in our clinical practice.[1] Today, we have advanced diagnostic tools at hand such as digital imaging for the objective and reproducible assessment of multiple markers at a time or on a single tissue slide precisely quantifying the absolute numbers of functionally distinct immune cells as well as their spatial distribution and contextual

relationships in various tissue compartments.[2] Different studies have already shown an association between immune cell infiltrates in selected tumor areas and improved outcome.[3,4]

With the integration of modern tools such as multiplexing immune phenotyping,[5,6] software solutions and machine learning into the routine work of pathologists come a deeper understanding of the communication network in tissues and reveal the existing intratumor heterogeneity that has consequences for various treatment options.[7,8]

Along with the advancement of diagnostic tools, also immune-therapeutic modalities emerged. Cytokine-stimulated tumor-infiltrating lymphocytes (TILs) were also used to enhance an anti-tumor effect as were vaccination strategies with tumor-specific antigens or immune-stimulating viruses.[9] A quantum leap was the implementation of monoclonal and eventually engineered antibodies, designed to target selected epitopes, followed by molecular engineered T cells as chimeric antigen receptor (CAR) T cells.[10,11]

Such a complex and comprehensive arsenal of advanced therapeutic modalities requires a biomarker-based diagnostic strategy. According to the understanding of the tumor heterogeneity, pathologists may guide the oncologist to select the optimal treatment for each individual cancer patient that will yield the best tumor response.

2 | IMMUNE TUMOR MICROENVIRONMENT

It has always been a core task of histopathologists to describe the resected tumor first as a gross specimen, followed by microscopic inspection of different sites of the tumor, its surrounding microenvironment, and its adjacent normal tissue. This yields a diagnosis according to the existing guidelines including a statement on the prognosis and possible predictions for the most effective treatment.

At the end of the last century, Ki67 and Her2/neu were accepted as the first predictive biomarker that required the pathologist to strictly quantify those invasive cancer cells that (over)expressed those markers in any part of the tumor area. In the meantime, a large number of other biomarkers including overexpressed immune-related proteins and molecular aberrations have been identified that guide therapeutic strategies and decisions in many tumor entities.[12] With respect to immunological properties of tumors, it was Galon's landmark paper in 2012[13] that demonstrated the combination and simultaneous presence of two markers (CD3 and CD8) with a defined spatial distribution in different compartments of the cancer tissue (tumor center versus invasive margin), which showed a better prognostic value than each single marker alone.[14] However, this requires a computer-based analysis, and it is only possible with (1) a good understanding of cancer immunology, (2) expert knowledge of the histopathologist reading the case, and (3) the implementation of an automated image analysis technology. Galon's group diligently worked out and proposed a classification of different immune stages of tumors based on the quantity and quality of immune infiltrates, ranging from "hot" to "immune-suppressed/excluded" or even "deserted" (cold).[15]

Figure 1 gives a simplified overview of those different categories and lists some potential biomarkers and morphological characteristics. Other groups followed this example and included more immune cells and immune-related biomarkers to further compartmentalize the tumor microenvironment (TME), for example, in non-small cell lung cancer.[16]

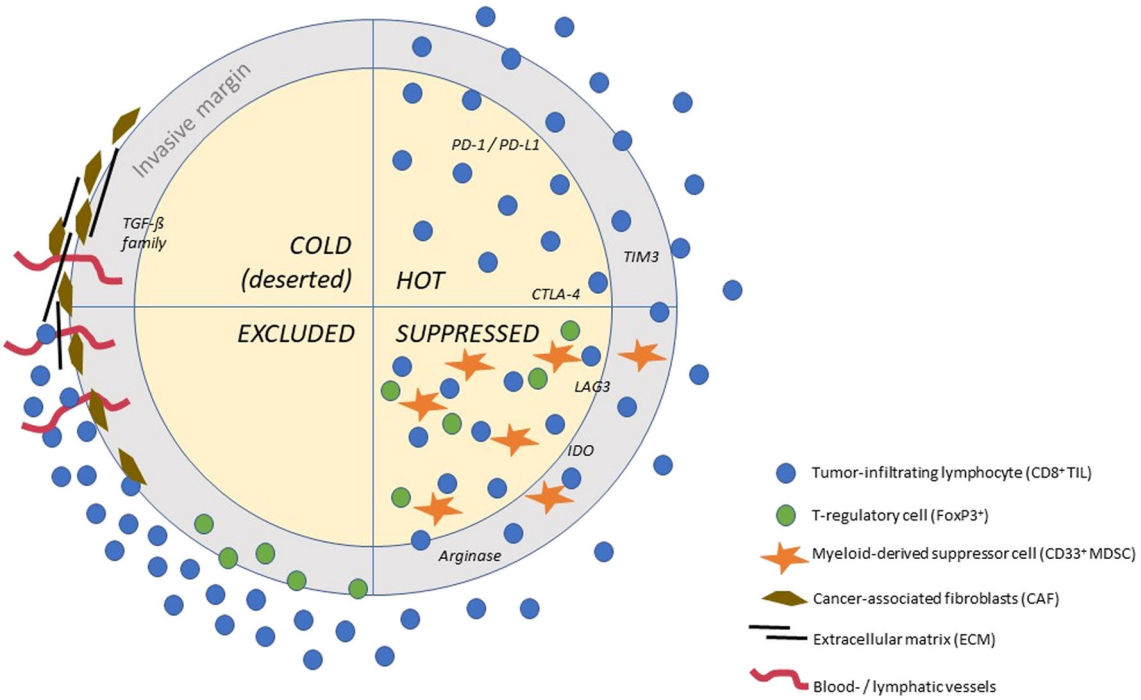

FIGURE 1 A simplified overview on the different categories of the tumor immune microenvironment and it lists some potential biomarkers (PD-1/PD-L1, TIM3, CTLA-4, LAG3, arginase, indolamin-2,3-dioxygenase = IDO) and morphological characteristics that characterizes immunological and morphological heterogeneity

3 | TUMOR HETEROGENEITY

Tumor heterogeneity is an essential part of tumor development and progression. It causes three significant problems: therapy resistance, poor reproducibility of studies, and uncertainty of histomorphological diagnoses. Tumor heterogeneity can be subdivided into intratumoral, intertumoral heterogeneity, and interpatient.[17] A different classification discriminates clonal- from non-clonal heterogeneity. Whereas the first form includes genetic aberrations (point mutations, deletions, insertions, fusions, inversions, copy number variations) and epigenetic aberrations, the second form consists of the microenvironment and stochastic plasticity.[18] Non-clonal heterogeneity can often be determined by histomorphological methods and is also correlated in part with genetics. Type, number, and homogeneity of genetic alterations, in turn, vary from tumor entity to entity and can undergo changes under therapy. Melanoma and lung cancers, for example, are caused by exogenic damage and harbor high numbers of homogeneous mutations. On the other hand, glioma starts with few genetic alterations and acquires a high number of aberrations during treatment with Temozolomide, which results in substantial heterogeneity.[19] Noteworthy, heterogeneity is not restricted to silent mutations. Driver mutations can also occur subclonal.[20] Both the detection and the failure of its identification imply therapy relevant misinterpretations. Genetic heterogeneity can be remarkable. Ling et al. identified 35 polymorphic single nucleoid variations representing 20 tumor clones in a single hepatocellular carcinoma that has been extensively sampled.[21] Tumor progression is an evolutionary process. The main topics of the evolution theory also take place in tumor biology. Such principles are selection, neutral evolution, contingency, convergence, gradualism, and punctuated evolution.[19] The detailed explanation of these principles is far beyond this review. However, all these mechanisms that partly work in the opposite direction can lead to a considerable high complexity that causes, in particular, vital challenges concerning the correct diagnosis and therapy of such malignancies. Some genetic aberrations can be identified on the protein levels by IHC, for example, EGFR, ROS and ALK TP53. Depending on the context, these markers are of diagnostic, prognostic, or predictive relevance. Besides this clonal heterogeneity of genetic or epigenetic origin, non-clonal heterogeneity represents a field, which is increasingly recognized. Stochastic plasticity belongs to this category and reflects the inherent variation of biochemical reactions resulting, for example, in different protein expression levels.[17] Although influenced in certain circumstances by genetic alterations like defect of the mismatch repair (MMR) system, the TME also belongs to the non-clonal category.

4 | TARGET ANTIGEN HETEROGENEITY

The interaction of the tumor and its microenvironment can influence the heterogeneity of the tumor by eliminating clones, which are more vulnerable by the immunosystem because of its neoantigen formation. Rooney et al. showed a gap between estimated and detected neo-epitopes in several tumor entities, indicating the elimination of neo-epitope-rich clones by the immunosystem.[22] Tumor antigen presentation is determined by number of tumor environmental and genetic factors like the overall tumor mutational burden (TMB),[23] the loss of heterozygosity (LOH),[24] or the somatic HLA class I loss.[25] Also cancer-associated fibroblasts (CAFs) play an essential role in tumor progression by providing growth factors, cytokines, metabolic support, and tissue remodeling.[26] Moreover, CAFs can be the source of therapy resistance, as shown by Hirata et al. for BRAF-inhibitor therapy in melanoma.[27] These are broad but also cancer-type specific mechanisms to evade immune surveillance but can also be used to identify the best possible and individual treatment opportunity. The understanding of the tumor-wide heterogeneity of target antigen expression is also an important information for treatment selection especially in the field of immune therapies.

5 | HETEROGENEITY OF PD-L1 EXPRESSION

The immunohistochemical evaluation of PD-L1 is currently the diagnostic backbone for the prediction for the response of a checkpoint inhibitor therapy. PD-L1 testing is way more complicated than Ki-67 or Her2/neu scoring, because of different antibodies, different testing algorithms, and constantly changing cut-offs. The reading and reporting of PD-L1 scores requires skilled and trained pathologists, also considering the substantial intratumorous heterogeneity of PD-1 and PD-L1 with prevalent expression in the invasive front. PD-L1 expression is not genetically determined but rather inducible through soluble factors provided by immune cells like TILs. Differences in intratumoral of PD-L1 expression is readily recognized in daily practice and documented in the literature.[28–30] This results in differing testing and also results between biopsies and relating surgical specimens.[31] Moreover, several authors report a considerable heterogeneity of PD-L1 expression between the primary versus lymph node metastases[32] and distant metastases.[33] Biomarkers reflecting the individual tumor immune microenvironment and tumor intrinsic factors like TMB or MMR deficiency associate with the treatment efficacy of anti-PD-1/anti-PD-L1 therapy. Microsatellite instability (MSI) seems to play a tumor agnostic role, when pembrolizumab is given in MSI-positive tumors regardless of the entity.[14] Marabelle et al. have demonstrated the association of TMB with outcomes in patients with advanced solid tumors.[34] Similar results have been obtained in hypermutated tumors[35] and TMB as an indicator for cytolytic activity and prognosis in malignant melanoma and other tumors.[36,37]

6 | TISSUE IMMUNE PROFILING

Routine histopathology to diagnose cancer still heavily relies on conventional hematoxylin and eosin (H&E) stains and an IHC usually of single marker molecules. The existing guidelines provide guidance on the composition of biomarkers that allow the diagnosis of certain subtypes along with prognosis and the possibility to predict response to different ways of treatment.

Although molecular markers are already well established in many indications and respective assays are readily available (e.g., mutational analysis of EGFR, BRAF, K-RAS, ROS, and ALK or the assessment of the overall TMB or chromosomal instability), the first immune-related biomarker has recently entered the market. PD-L1 testing is an accepted biomarker as a companion or complementary diagnostic for various immune checkpoint inhibitors (ICIs). However, recent studies have shown the relevance of other components of the immune system that have a significant prognostic value and can be included in therapeutic considerations.[38] This does not only refer to TILs but also refer to other cells of the monocyte–macrophage systems (T_{regs}, MDSC, type 1/type 2 macrophages, natural killer (NK), or dendritic cells [DCs]) and their spatial relationship to each other.[39,40] Although it could be shown that the spatial relationship, for example, proximity of TILs with FoxP3 + T_{regs} in malignant melanoma, is a predictive biomarker for the therapeutic use of Ipilimumab, similar algorithms play no role in low to intermediate prostate cancer.[41] Instead, other contextual dimensions including macrophages have a higher informative value related to prognosis and therapy selection.[42]

7 | IN SITU MULTIPLEXING

With the increasing understanding of the immunological heterogeneity of different cancer types and their TME as well as the availability of more therapeutic modalities, more information needs to be retrieved from the tissue specimens. While the number of tests requested on each sample is increasing, the size of biopsies tends to decrease (e.g., core needle biospsies), often limiting the availability of tissue sections for the pathology lab. As a result, multiplexed IHC techniques offer a solution to sample scarcity, by labeling an entire panel of biomarkers on a single section where conventional methods would require many serial sections. In situ multiplexing also enables the identification of cell populations of increasingly complexes phenotypes through colocalization of multiple markers on the same cells, which would not be possible through the use of serial sections in conventional methods. Modern multiplexing technologies allow the visualization of up to hundreds of biomarker candidates on a single slide.[43–45] As these panels enter trials for clinical validation or validation, the combination of high complexity due to the number of markers and their spatial relationship along with the large number of relevant sample require would render a standardized and robust analysis of single and multiple (molecular or protein) marker molecules implausible without the assistance of machines, such as the digitization of images, computer-based image analysis, and further data breakdown through machine learning tools. This applies in particular to the assessment of spatial relationships to evaluate the contextual information from high-dimensional functional interactions. The understanding of complex relationships through standardized tools and the use of validated algorithms allow the prediction of immune responses and the educated selection of the most appropriate treatment modality.[46] This also opens up the opportunity to identify biomarker candidates for advanced therapeutic medicinal products (ATMPs) such as engineered T cells (e.g., CAR-Ts), cancer vaccines, or oncolytic viruses.[47,48]

8 | IMAGE AND DATA ANALYSIS

The primary objective of image analysis in the context of immune profiling of tissue specimens is the ability to accurately quantify and calculate relevant spatial relationships of all immune cells and other immune-related biomarkers.[49] A second goal is the discovery of novel features and contextual information that were previously unknown or could not have been discovered otherwise. Along with a sophisticated analysis of multiplexed images, the integration of other available

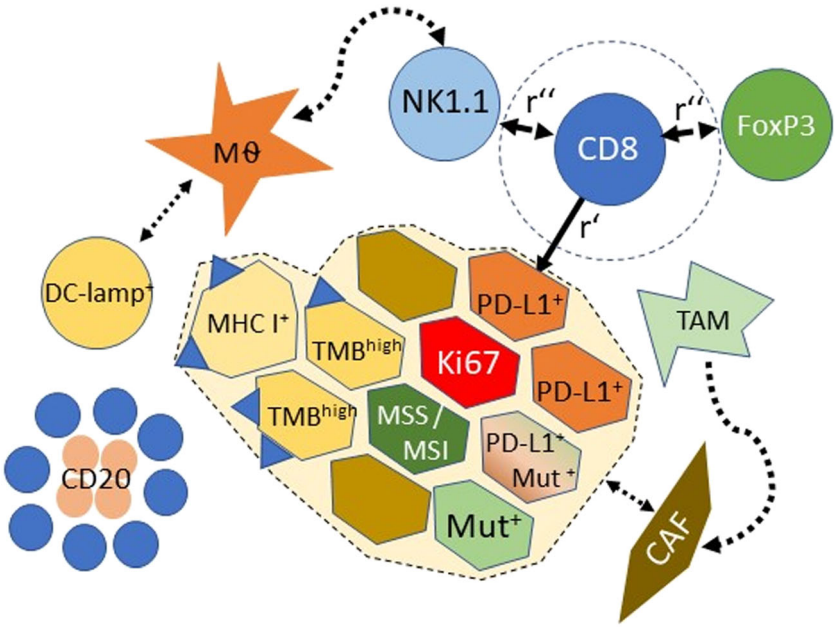

FIGURE 2 A schematic presentation of various histomorphological and molecular biomarker candidates expressed in the tumor area and the tumor microenvironment (TME) and their spatial relationship. "R" describes the spatial relationship between immune effector cells and regulatory cells (FoxP3) or NK-cells, which belong to the group of anti-tumor and antigen-presenting cells (MΘ, macrophages; DC, dendritic cells; TAM, tumor-associated macrophages; CAF, cancer-associated fibroblasts). The tumor itself is characterized by the proliferation index (Ki67), the immune checkpoint expression (PD-L1), the possible microsatellite instability (MSS/MSI), and the tumor mutational burden (TMB). In the future, it will be warranted to include all markers and available information (also from other "*omics*") into a single comprehensive analysis and report

molecular data from the cancer (epi)genome or transcriptome analysis provides an even more granular assessment of a certain cancer type. However, this may reflect the personal situation of an individual cancer patient, the stage of the disease, and/or any pretreatment conditions in times where neo-adjuvant treatment regimens are on the rise. To verify or even clinically validate any hypothesis, clinical trials are warranted. But there is one particular commonality with genomics and other *OMICS* technologies: even when applying machine learning tools that provide a hierarchical probability of relevant events, the number of potential features is usually higher than the number of patients and the statistical power of a given validation study. Therefore, the increased number of candidate biomarkers and their spatial relationships is both, a blessing and a curse. Figure 2 is a schematic presentation of various histomorphological and molecular biomarker candidates expressed in the tumor area and the TME. Depending on the spatial relationships and any other contextual information contained on the image and retrieved by computational measures, prognostic and predictive statements are possible in different indications.

The first examples were the ImmunoScore in stage 2 colorectal cancer[14] and metastatic disease.[50] Complex tissue-based signatures already need image analysis solutions. However, a widely accepted example is the expression of PD-L1 above-defined thresholds to predict treatment responses to ICI. Until now, this requires detailed and diligent training of pathologists to apply the scoring algorithm accurately.

The use of machine learning algorithms along with image analysis tools allows the discovery of immune signatures that are either too subtle or even too counterintuitive to be discovered by human experts only. But the development of a novel hypothesis, supported by the human mind or solely generated by artificial intelligence such as a neural network, generates new insights into the biology of cancer and the interaction with the immune system. By such measured, we found a possible new for lymphoid aggregates in renal and gastric cancer,[44,45] which was supported by a publication on the use of deep learning.[46] The publications even suggest a relationship between a molecular genotype and a histomorphological finding.

Figure 3 shows the intratumor heterogeneity of a colorectal cancer tissue using an 8-color immunostain kit (I/O Ultimapper, Ultivue, Boston, MA) and an adopted Visiopharm image anaylsis software solution (Visiopharm, Hoersholm, Denmark). It identifies tumor regions that might require different treatment regimens and further

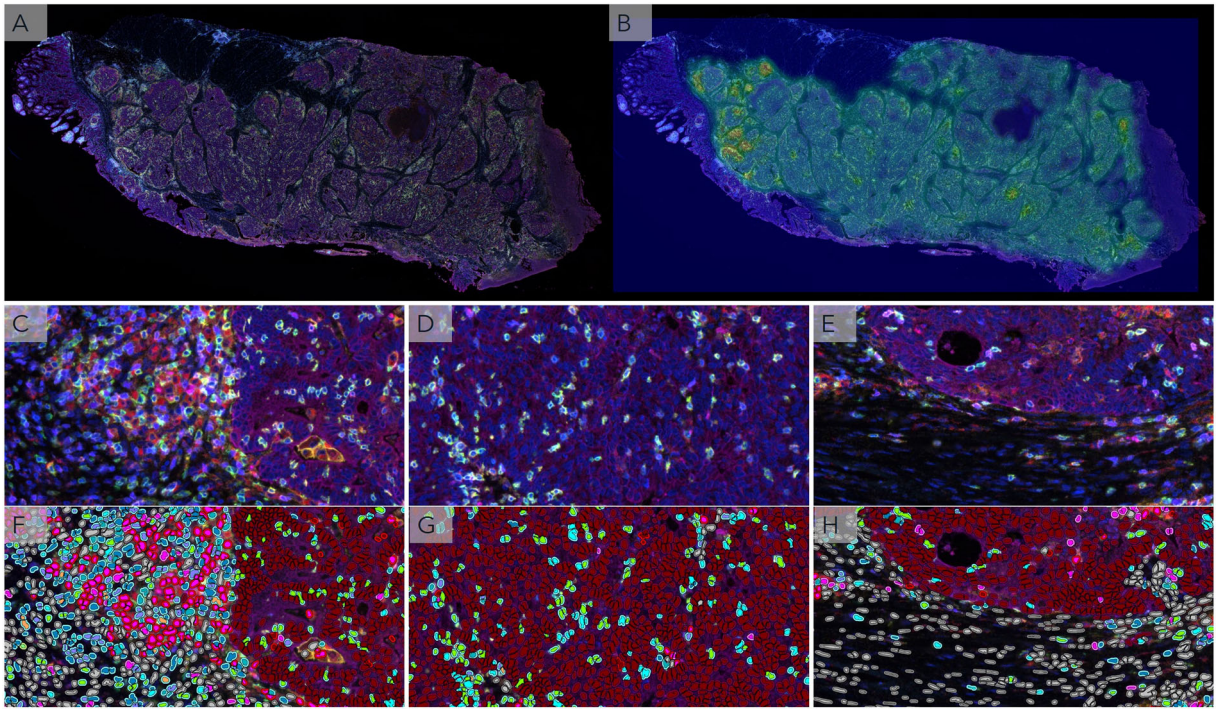

FIGURE 3 UltiMapper reagents were used to perform 8-plex immune profiling of a colorectal cancer (CRC) FFPE sample (a). Slides were stained with a cocktail of primary antibodies using a Leica biosystems BOND RX autostainer and imaged on an Olympus Slideview VS200 in the DAPI, FITC, TRITC, Cy5, and Cy7 channels. After a first round of imaging, the slides were decoverslipped, the signal was removed, and new targets were probed with a reagent incubation step on the BOND RX, termed exchange. The slides were then re-imaged on the VS200 using the same dye channels as in the first round. Finally, image pairs were automatically aligned and overlayed using a custom tool. The resulting images were exported for downstream analysis with the Visiopharm author and AI architect image analysis software. We categorized all cells into the relevant phenotypes using a deep learning-based APP and created density heatmaps of the immune landscape (B) to investigate the heterogeneity. Here, we could select "hot" regions on the border of the tumor (C and F) and inside the tumor (D and G), and "colder" regions (E and H)

research is warranted to establish a precision treatment schedule to optimize the expected outcome.

9 | DECISIONS FOR ADVANCED THERAPIES

The current dogma of immunotherapy is (1) "unleashing the immune system" or (2) "turning a cold tumor into a hot tumor."[51] The first works primarily, when enough functional (non-exhausted) T lymphocytes are present or available and the selected treatment—for example, ICI—can lift any immune suppression or allow the T cells to infiltrate the tumor.[48] However, there are a substantial number of tumors or histological tumor types that are primarily immune deserted with a relevant fibrotic component or lack the presentation of tumor antigens. Those tumors can be candidates for treatment with advanced therapeutics, such as oncolytic viruses to immunize the tumor environment alone or in combination with primed immune cells.[52–54] Therefore, different cell types are considered for advanced and cellular therapies, alone or in combination, depending on the TME and its heterogeneity.

Figure 4 shows possible treatment opportunities considering the individual tumor heterogeneity and the potential to include advanced therapeutics like cell and gene therapies including cancer vaccines and oncolytic viruses into such combination strategies.

Several studies have already shown the value of advanced treatment regimens in individual cancers which otherwise do not adequately respond to current strategies.[55–57] Those tumors are usually hard to treat exemplified in high-grade astrocytoma, hepatocellular carcinoma, pancreatic cancer, or prostate cancer.[58–60] Currently, there are hardly any or even no biomarker signatures for complex or combination therapies available, but a combination of tissue immune (and molecular) multiplexing along with advanced image analysis will offer also clinically relevant predictive tests.[61] The diagnostic challenge is to understand, visualize, and quantitatively measure the spatial relationship between an effector cell (e.g., TIL or NK cell) and the detectable target structure in the tissue or region of interest. The determination of relevant spatial relationships with multiple denominators in the therapeutic equation requires advanced tools like multiplex immunostaining on a single slide, high-resolution image acquisition, and analysis and the application of clinically validated machine learning-based algorithms to stratify patients to the best individual advanced treatment option.

10 | SUMMARY

The availability of increasingly more and advanced cancer drugs and their combinations beyond conventional radiochemotherapy, targeting antibodies or pathway-specific small molecules, adds another level of complexity to the diagnosis of malignant diseases and the understanding of the individual tumor biology. It is a challenge for "precision oncology" and expert members of the tumor boards need to integrate more (big) data from different sources (histopathology, (epi)genomics, transcriptomics, metabolomics, radiomics, liquid biopsies, etc.) into their decision-making process. The current number of available and approved biomarker assays is limited and somewhat misleading since they usually measure only a single parameter and do not

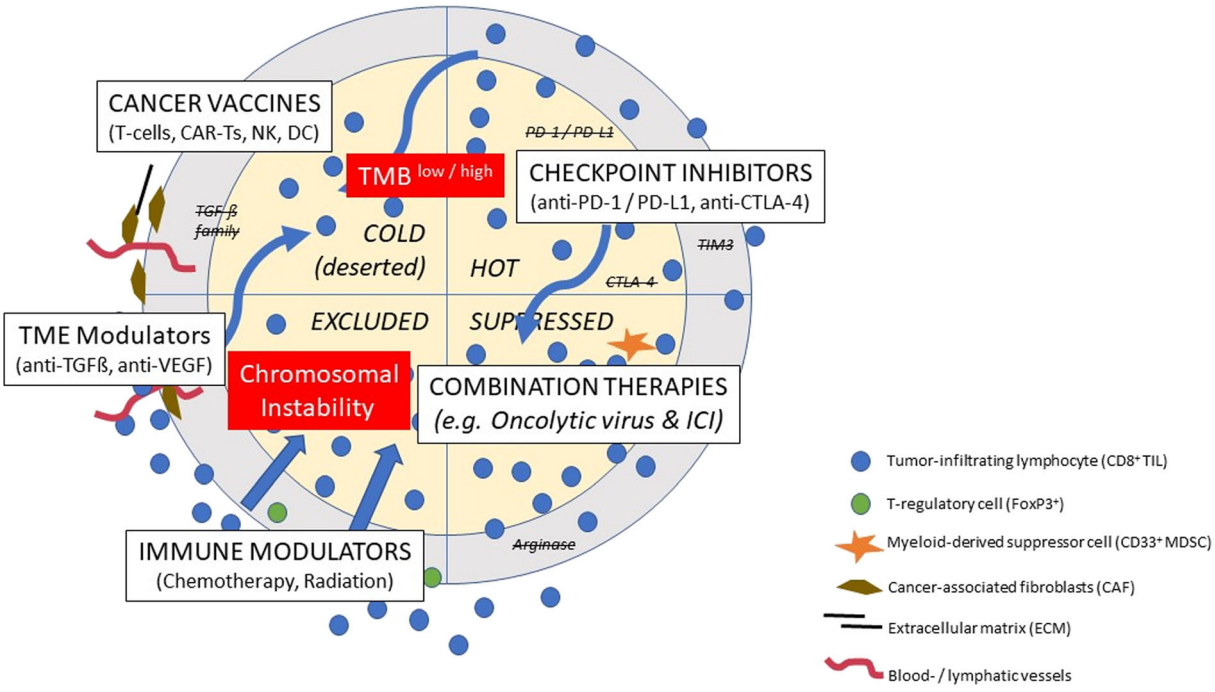

FIGURE 4 In analogy to Figures 1 and 2, we describe the possible role of advanced therapeutics especially in cold (deserted) and excluded tumors alone or in combination with other immune-modulating agents

consider the existing heterogeneity of an individual cancer. Biomarker for advanced therapies like CAR-T cells, primed DCs, NK cells, or oncolytic viruses is equally complex or still needs to be discovered and validated when the clinical applications extents to more advanced and immunologically heterogeneous tumors.

The availability of digital tools such as image analysis and machine learning allows interpreting multiplexed images with a high-dimensional complexity of information and integrating also big data from other "omics" sources to understand relevant spatial relationships even in a very heterogenetic environment and select the best and most effective treatment for an individual patient.

CONFLICTS OF INTEREST

The authors CS and BM declare no conflict of interest. M.M. is a full-time employee at Ultivue. J.T. is a full-time employee at Visiopharm. R.H. is a member of Visiopharm's scientific advisory board.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a review article with no original research data.

AUTHOR CONTRIBUTIONS

RH has written and revised the manuscript with emphasis on the tissue immune profiling and the immune microenvironment and has also provided the schematic figures for illustration. CS has written the chapter advanced therapies and reviewed the entire manuscript. MM has contributed the multiplex images and the written description and explanation. JT has written the chapter on image and data analysis. BM has contributed the part on tumor heterogeneity and has reviewed the entire manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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