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Leveraging immune resistance archetypes in solid cancer to inform next-generation anticancer therapies

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ABSTRACT

Anticancer immunotherapies, such as immune checkpoint inhibitors, bispecific antibodies, and chimeric antigen receptor T cells, have improved outcomes for patients with a variety of malignancies. However, most patients either do not initially respond or do not exhibit durable responses due to primary or adaptive/acquired immune resistance mechanisms of the tumor microenvironment. These suppressive programs are myriad, different between patients with ostensibly the same cancer type, and can harness multiple cell types to reinforce their stability. Consequently, the overall benefit of monotherapies remains limited. Cutting-edge technologies now allow for extensive tumor profiling, which can be used to define tumor cell intrinsic and extrinsic pathways of primary and/or acquired immune resistance, herein referred to as features or feature sets of immune resistance to current therapies. We propose that cancers can be characterized by immune resistance archetypes, comprised of five feature sets encompassing known immune resistance mechanisms. Archetypes of resistance may inform new therapeutic strategies that concurrently address multiple cell axes and/or suppressive mechanisms, and clinicians may consequently be able to prioritize targeted therapy combinations for individual patients to improve overall efficacy and outcomes.

INTRODUCTION

Upon antigen recognition, T cells increase expression of inhibitory receptors, constraining T cell responses. T cell receptor (TCR) and CD28 signaling during T cell activation induces expression of CTLA-4; ongoing TCR stimulation, as during chronic infection and cancer, leads to sustained expression of PD-1 and other immune-inhibitory receptors. Signaling through these receptors subsequently reduces T cell proliferation and function. Therapeutic antibodies that disrupt signaling through T cell inhibitory receptors can reinvigorate antitumor T cell responses. Known as immune checkpoint blockade (ICB), CTLA-4, PD-1, and/or PD-L1 (PD-1 ligand) blocking antibodies have significantly improved patient outcomes,[1 2] especially when given in combination.[3 4] However, additional inhibitory receptors on T cells, such as Lag-3, Tim-3, and TIGIT,[5] can limit the efficacy of approaches targeting individual receptors. Indeed, the efficacy of ICB monotherapies remains modest against most malignancies, including pancreatic ductal adenocarcinoma (PDAC), high-grade serous ovarian cancer, and glioblastoma.[6–8] Moreover, some patients who initially respond to ICB eventually develop therapeutic resistance.[9 10] Although ICB combinations may yield higher response rates, they also result in higher rates of immune-related adverse events, related to systemic immune activation that can promote self-specific responses.[11] Therefore, rational anticancer strategies are urgently needed to therapeutically target non-overlapping inhibitory pathways, overcome immune resistance mechanisms, and reduce systemic toxicities.

Broadly, to overcome poor responses to ICB and other immunotherapies, we need to: (1) correctly predict which patients will initially respond; (2) clarify the cellular and molecular mechanisms that cause primary and secondary/acquired resistance; (3) rapidly identify which mechanisms are relevant to an individual treatment-resistant tumor; and (4) expand the number of effective alternative treatments. The field could greatly benefit from a comprehensive, shared system for extensively and efficiently profiling clinical samples, producing biomarker 'signatures' that subsequently inform clinical decision-making.

Sequencing technologies have helped resolve 'immune archetypes,' the cellular immune networks that integrate signatures from multiple intratumoral cell types.

Immune archetypes have been defined by abundance and the phenotypic and functional profiles of classical immune cells, including T cells, natural killer (NK) cells, B cells, dendritic cells (DC), macrophages, and other myeloid cells.[12] Identifying 'archetypes of immune resistance' within an individual patient's tumor may rationally guide treatment selection and thereby improve that patient's clinical outcome.

Genetic engineering of tumor-specific T cells has already revolutionized cancer therapy. T cells have been engineered to express a tumor-specific chimeric antigen receptor (CAR) or high-affinity TCR, targeting acute lymphoid leukemia (ALL),[13] chronic lymphocytic leukemia[14] or acute myeloid leukemia (AML),[15] with robust numbers of highly cytolytic tumor-specific effector T cells that persist and control tumor burden in patients. However, adoptively transferred T cells remain susceptible to altered trafficking/infiltration mechanisms and the suppressive milieu of the tumor microenvironment (TME), which are particular obstacles for engineered T cell efficacy against solid tumors.

Tumor-intrinsic and tumor-extrinsic features such as oncogene expression, epigenetic dysfunction, antigen presentation, tumor cell metabolism, the host microbiome, the development of desmoplastic stroma, and the overall tumor milieu can also impact immunotherapy efficacy.[16] Here, we further explore how more comprehensive immune resistance archetypes can be leveraged to identify new immune resistance biomarkers, develop and evaluate new therapeutic strategies, and prioritize immunotherapy combinations for the clinic.

Archetypes of immune resistance

We propose that immune resistance archetypes encompass currently defined mechanisms that are active in tumors and variably use five 'feature sets' (figure 1): (1) exclusion of immune effector cells from the tumor; (2) lack of tumor antigen recognition by T cells; (3) immune cell dysfunction/death; (4) suppressive immune cells; and (5) extrinsic suppressive factors. These feature sets play a recognized role in immune evasion and escape, but the specific contribution of each feature set, and which combinations are most active, varies to generate different archetypes of immune response across different patients.

Immune effector cell exclusion

The TME is broadly described as either inflamed or non-inflamed, characterized by leukocyte infiltration, and patterns of T cell exclusion can be used to classify subcategories. Inflamed tumors exhibit measurable immune infiltration, including a high $CD8^+$ T cell:regulatory T cell (T_{reg}) ratio, with high numbers of myeloid cells, B cells, and tertiary lymphoid structures[17]; the presence of B cells and tertiary lymphoid structures are notably associated with ICB response in melanoma.[18] Inflamed tumors may also exhibit high levels of regulatory immune cells, such as T_{reg}, as a compensatory response to a robust endogenous antitumor immunity, and retain responsiveness to immunotherapy despite the presence of inhibitory cells.[19 20] This may account for the association of T_{reg} with improved prognosis in some cancer types, such as gastrointestinal cancers.[21] A complete mechanistic understanding of variable tumor inflammation is missing, but innate immune responses are revealing. For example, early activation of the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS/STING) pathway, initiated by cytoplasmic tumor-derived DNA, promotes a type I interferon (IFN) response, macrophage activation, polarization, and accumulation, and DC recruitment and activation. This cascade results in an innate immune response that facilitates antitumor T cell recruitment and function.[22]

In comparison, non-inflamed tumors lack T cell infiltration and are classified as either an immune-excluded or immune-desert phenotype. Immune-excluded tumors, including many PDAC and microsatellite stable/mismatch repair proficient colorectal cancers (MSS/p-MMR CRC), retain T cells in the surrounding TGF-β-rich stroma, containing activated fibroblast and immunosuppressive myeloid cell populations.[23] Immune-desert tumors, such as hormone receptor-positive breast cancer, prostate cancer, glioblastoma, and small cell lung cancer, are devoid of T cell infiltration, related to a lack of antigen presentation, a hostile metabolic and hypoxic environment, or tumor intrinsic WNT/β-catenin signaling.[24] For example, tumor cell-intrinsic β-catenin activation reduces DC recruitment to tumors, limiting tumor antigen uptake and cross-priming of tumor-specific $CD8^+$ T cells, producing immune 'ignorance' and low intratumoral T cell infiltration.[25] In addition, tumor cell-intrinsic *PTEN* loss has been associated with reduced neoantigen expression[26] and increased PI3K/AKT/mTORC pathway expression,[27 28] which can influence intratumoral T cell infiltration and activation. ICB remains largely ineffective in non-inflamed tumors, prompting the need for novel strategies to enhance immunogenicity and tumor immune infiltration.

Lack of tumor antigen recognition

Mounting a robust antitumor immune response requires tumor antigen expression and recognition by cognate T cells. Tumor antigens can include neoantigens, endogenous or exogenous viral antigens, cancer germline or testis antigens, and tumor-associated antigens, including overexpressed and lineage-restricted proteins. High tumor mutational burden (TMB) implies the presence many tumor neoantigens and correlates with response to ICB.[29] However, mechanisms must exist for tumor cells to limit immune recognition of tumor antigens as TMB is not a definitive biomarker for therapy response.[30]

One class of high-TMB tumors are DNA microsatellite instability-high/MMR-deficient tumors (MSI-H/d-MMR), which respond exceptionally well to ICB. d-MMR tumors are thought to have a high number of neoantigens due to inherent DNA repair defects, but evidence suggests that additional characteristics drive ICB responses. For

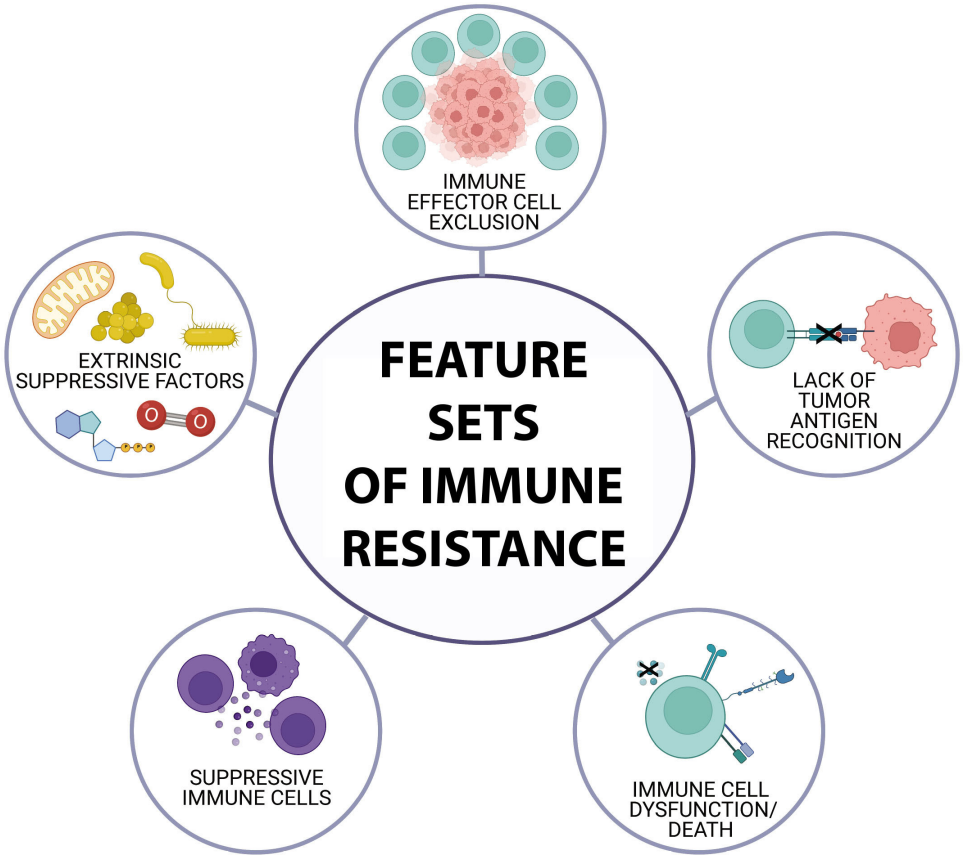

Figure 1 Feature sets of immune resistance. The currently understood immune resistance mechanisms operative in solid tumors can be broadly classified into five major feature sets: immune effector cell exclusion, lack of tumor antigen recognition, immune cell dysfunction/death, suppressive immune cells, and extrinsic suppressive factors. Each feature set encompasses tumor cell intrinsic and extrinsic pathways that have been characterized during primary and/or acquired immune resistance. For example, the immune effector cell exclusion feature set encompasses resistance mechanisms that lead to poor T cell infiltration of tumors, such as a lack of cGAS/STING activation, the presence of TGFβ-rich stroma, or tumor-intrinsic WNT/β-catenin signaling. The lack of tumor antigen recognition encompasses mechanisms that prevent tumor recognition by T cells, such as a paucity of tumor antigens, antigen loss, HLA or β2M deficiency, or changes in proteasome processing machinery within tumor cells. The immune cell dysfunction/death feature set includes mechanisms that drive T cell death, such as Fas ligand expression, or loss of cytolytic effector function, such as inhibitory receptor/ligand interactions and differentiation toward an exhausted state. The suppressive immune cells feature set includes cells that either prevent T cell activation or inhibit T cell function. The extrinsic suppressive factors feature set includes mechanisms that restrain antitumor immune responses, such as nutrient limitation, the presence of suppressive metabolites, or microbiota. As tumors often engage more than one immune resistance mechanism to escape killing by immune effector cells, we propose that cutting-edge technologies should be leveraged to identify the immune resistance features active in tumors, which altogether define an immune resistance archetype. Subsequently, novel therapeutics and/or combination approaches should aim to address immune resistance archetypes by addressing multiple mechanisms/feature sets concurrently. This figure was created with BioRender.com. β2M, beta-2-microglobulin. cGAS/STING, cyclic GMP-AMP synthase-stimulator of interferon genes.

example, DNA repair deficiency may predispose d-MMR tumors to high levels of cytoplasmic DNA, driving type-I interferon production downstream of cGAS/STING or other DNA-sensing pathways.[31] In mouse models, IFN production promotes DC maturation and increased $CD8^+$ T cell function, producing exceptional ICB responses in d-MMR tumors.[31] Immune cells must, therefore not only recognize tumor antigens but also receive the correct environmental signals to respond to ICB. TMB alone may not accurately predict immunotherapy response because it does not also consider the activation state of the immune system.

While $CD8^+$ T cells are considered the main antitumor cytotoxic effectors, $CD4^+$ T cells are increasingly recognized for their role in orchestrating antitumor immunity and ICB responses. $CD4^+$ T cells provide help signals to $CD8^+$ T cells and greatly increase the abundance and function of tumor-reactive $CD8^+$ T cells. Tumor-reactive $CD4^+$ T cells are required not only during priming to generate functional tumor-reactive $CD8^+$ T cells, but also to apparently facilitate $CD8^+$ T cell-mediated responses to ICB.[32] Moreover, a subset of tumor-specific $CD4^+$ T cells releases cytolytic granules to lyse tumor cells.[33] Thus, an

optimal antitumor immune response may require simultaneous recognition of antigens by both $CD8^+$ and $CD4^+$ T cells.

Tumor cells can evade antigenic detection through genetic or epigenetic loss of human leukocyte antigen (HLA) expression, beta-2-microglobulin (β2M) deficiency, altered proteasome processing of tumor antigens, and/or reduced tumor antigen expression. Somatic loss of HLA and/or β2M, which is required for HLA cell surface expression, occurs in 40% of non-small cell lung cancers,[34] shielding tumor cells harboring antigenic mutations from T cell-mediated killing.[35 36] HLA loss-of-heterozygosity correlates with poor ICB responses, implicating it as a mechanism of immune evasion and ICB resistance.[35] Moreover, cancer cells can evade antitumor T cell recognition by substituting components of the constitutive proteasome for the immunoproteasome, altering the repertoire of peptides presented by HLA molecules, as occurs in AML.[37] Surface antigen loss has also been described, as with CD19 and CD20 loss in B cell malignancies treated with CAR T cell therapies targeting these antigens.[38]

While HLA or β2M loss may shield tumor cells from $CD8^+$ T cell-mediated killing, this mechanism may also sensitize tumors to cytolysis by more innate-like immune cell populations. Both NK cells and gamma delta (γδ) T cells can recognize HLA class I-deficient tumor cells, and γδ T cells can participate in ICB efficacy against β2M-deficient tumors. Thus, while HLA class-I downregulation may shield tumor cells from $CD8^+$ T cell killing, it may sensitize them to other mechanisms of immune clearance.[39] Indeed, γδ T cell numbers were increased in response to ICB in β2M-deficient d-MMR colon tumors, indicating that γδ T cells contribute to antitumor responses in HLA class I-deficient cancers.[40]

Tumor heterogeneity may also limit the therapeutic benefits of high TMB. Highly heterogenic tumors show subclonal antigen expression and respond poorly to ICB compared with highly clonal tumors, with nearly universal expression of the same tumor antigens.[41] Clarifying how high tumor heterogeneity leads to poor immune responses could inform new strategies to boost the efficacy of immune-based therapies.

Strong initial recognition by $CD8^+$ T cells may also paradoxically drive tumor immune escape. Prolonged IFNγ signaling, upon tumor-specific $CD8^+$ T cell recognition of cognate antigen, can cause immune escape through IFNγ-mediated upregulation of inhibitory ligands and $CD8^+$ T cell-mediated recruitment of T_{reg}.[16 20] Immune editing, a process by which highly antigenic tumor cells are preferentially eliminated, can also select for poorly antigenic tumor cells that preferentially persist and expand.[42] In sum, productive tumor recognition is critical for effective immunotherapy responses, but both primary and adaptive resistance mechanisms may arise, necessitating novel immunotherapy approaches.

Immune cell dysfunction/death

Many tumors are infiltrated by activated, tumor antigen-specific T cells, yet virtually all will progress without treatment. TMEs induce T cell dysfunction that prevents immune-mediated tumor destruction. The best described form of T cell dysfunction is exhaustion, which is characterized by a progressive loss of T cell function that occurs when T cells are subjected to chronic antigen stimulation.[43] During T cell exhaustion, $CD8^+$ T cell functions are gradually and progressively eroded, beginning with the inability to secrete IL-2, TNFα, and IFNγ and other cytokines, and eventually impaired cytolysis, leading to antigen-specific $CD8^+$ T cells that have severely reduced functionality compared with recently activated T cells.[43] Many T cell exhaustion features were first described in chronic viral infections and have been found in tumor-infiltrating $CD8^+$ T cells. However, the functional impairments can vary; $CD8^+$ T cell expression of IFNγ and intact cytolytic ability were found in some human tumors.[44] Despite the functional heterogeneity of tumor-infiltrating $CD8^+$ T cells, it is widely accepted that activated, antigen-specific T cells that infiltrate clinically detectable human tumors are generally dysfunctional and unable to prevent tumor progression without therapeutic intervention.

Exhausted T cells display multiple inhibitory receptors that dampen T cell function, including CTLA-4, PD-1, Tim-3, Lag-3, and TIGIT.[5 43] T cell exhaustion is maintained, at least in part, through inhibitory receptor ligation, and inflamed tumors frequently express PD-L1 and other immune-inhibitory ligands that can suppress T cell responses.[45] ICB targeting CTLA-4 or PD-1/PD-L1 can lead to impressive clinical responses in tumor patients.[1 2] Combination ICB that simultaneously blocks both CTLA-4 and PD-1 can further increase response rates, indicating that inhibitory receptors use non-redundant mechanisms to inhibit T cell function.[46] Targeting additional inhibitory receptors continues to yield clinical success; for example, a phase II/III randomized controlled clinical trial demonstrated that combined PD-1 and Lag-3 inhibition improved progression-free survival for treatment-naïve patients with metastatic melanoma, compared with PD-1 blockade alone.[45] Combination ICB studies overall indicate that multiple layers of inhibitory receptor-mediated suppression can produce resistance to monotherapies, and that blocking multiple inhibitory receptors is sometimes required to overcome exhaustion and promote functional antitumor immunity.

Exhausted T cells consist of multiple subsets, including more differentiated cells with varying levels of effector function and displaying the hallmarks of terminal exhaustion, and less differentiated subpopulations that have little effector function but express the transcription factor TCF-1 and retain a stem-like capacity for proliferation and differentiation, thus providing a reservoir that maintains the exhausted antigen-specific T cell response.[47–49] $TCF\text{-}1^+$ T cells proliferate, expand, and differentiate into effector-like cells in response to PD-1/PD-L1 blockade, and are considered the major target of PD-1/PD-L1

neutralizing antibodies.[47 48] A lack of TCF-1$^+$ T cells has also been associated with resistance to ICB, which could be mediated by driving tumor-reactive T cells to terminal exhaustion.[49] However, CD8$^+$ T cells that express tissue resident memory-like features, including CD103, also predict ICB responsiveness.[50] Thus, it remains unclear whether only TCF-1$^+$ stem-like T cells respond to ICB.

Both intrinsic and extrinsic factors impact T cell differentiation states. Unique transcriptional and epigenetic signatures have been identified in stem-like and terminally exhausted CD8$^+$ T cells.[48 51] In particular, multiple groups showed that the transcription factor and epigenetic regulator Tox drives T cell exhaustion, but also sustains the survival of exhausted T cells.[52] Additionally, TME-localized interactions with different types of antigen-presenting cells appear critical for maintaining stem-like and terminally exhausted T cells. Stem-like, TCF-1$^+$ CD8$^+$ T cells are found in close proximity to DC,[53] while terminally exhausted CD8$^+$ T cells interact more frequently with tumor-associated macrophages (TAM).[54 55] While chronic antigen exposure drives T cell exhaustion, it seems that the quality of antigen exposure is also important for determining the level of exhaustion that develops. Novel immunotherapy approaches could potentially be aimed at either CD8$^+$ T cell-intrinsic or extrinsic drivers of exhaustion, but will need to balance disruption of the exhaustion program with potentially unwanted consequences, including decreased T cell survival.

A downstream consequence of chronic T cell stimulation and exhaustion is T cell apoptosis, which blunts the efficacy of both endogenous and engineered T cell immunotherapies.[43 56] Both intrinsic and extrinsic mechanisms of T cell apoptosis have been described. Fas Ligand (FasL) expression on vasculature, epithelial cells and/or stromal cells promotes T cell apoptosis by binding Fas receptor on tumor-infiltrating T cells,[57 58] and inhibition of FasL or disrupted Fas signaling in tumor-specific T cells increases the efficacy of immunotherapy in mouse models.[59–61] DNA damage has also been implicated in tumor-specific CD8$^+$ T cell apoptosis, as exhausted CD8$^+$ T cells undergoing apoptosis in the TME accumulate high levels of DNA double-strand breaks.[56] DNA damage and apoptosis can be relieved by 4-1BB co-stimulation, which results in NF-κB-mediated upregulation of DNA repair pathways and increased T cell survival,[62] providing one explanation for how 4-1BB signaling leads to increased persistence of T cells in the TME. Innovative treatment strategies are needed to overcome immunosuppressive mechanisms that reduce T cell persistence and function.

Suppressive immune cells

Myeloid cells, including mononuclear phagocyte subsets such as monocytes, myeloid-derived suppressor cells (MDSC) and TAM, mast cells, and granulocytes such as neutrophils, are key components of the suppressive TME.[63] The presence of suppressive myeloid cells is often correlated with reduced survival in most cancer types, including PDAC, breast, ovarian, and head and neck squamous cell carcinomas.[64 65] Depending on the cancer type and stage, myeloid cells have demonstrated high heterogeneity in their density, function, and transcriptional profile.[65] Some suppressive myeloid cells promote tumor progression and metastasis through TME remodeling, by releasing matrix metalloproteinases and secreting soluble factors that induce tumor cell proliferation and angiogenesis.[66] Myeloid cells may also actively promote immune suppression by producing pro-inflammatory and/or inhibitory cytokines, such as IL-10 and TGF-β.[67] Tumor-associated DC that are reprogrammed in the TME and coexpress maturation and immunoregulatory genes (mregDC) also limit antitumor responses.[68] For example, mregDC, MDSC, TAM, and tumor-associated neutrophils can directly inhibit cytotoxic T cell function by upregulating PD-L1.[63 68] Myeloid cells also suppress antitumor T cells responses by overexpressing indoleamine 2,3-dioxygenase (IDO1/2) and arginase 1 (ARG1), enzymes that drive amino acid depletion and produce metabolites that suppress T cell function (see the 'Extrinsic suppressive factors' section).[66] As myeloid cells exhibit great subset diversity both within a tumor and between tumor types,[69] a thorough and context-specific characterization of myeloid cell functional heterogeneity is critical for understanding resistance to current cancer treatments.

T_{reg} cells also infiltrate the TME and contribute directly and indirectly to immune suppression. T_{reg} cells are required for maintenance of homeostasis and prevention of autoimmune disorders. However, T_{reg} suppressive function can diminish antitumor immune responses through a variety of mechanisms, including secretion of suppressive cytokines (IL-10, IL-35, TGF-β), adenosine modulation through expression of CD39 and CD73, expression of high-affinity IL-2 receptor resulting in an 'IL-2 sink', and direct DC suppression through expression of CTLA-4 and Lag-3.[70] T_{reg} thrive in the TME where other T cells do not, due to high lactate levels and low glucose availability.[71] Many treatments have sought to target T_{reg}, but targets that are unique to intratumoral T_{reg} must be identified to avoid widespread autoimmune inflammation.

Extrinsic suppressive factors

Metabolic perturbation

Tumor cells evolve to survive in a metabolically hostile microenvironment. In contrast, immune cells are highly susceptible to changes in the surrounding milieu. Reductions in oxygen or nutrient availability, the presence of suppressive metabolites, or changes in pH can severely compromise antitumor T cell activity. Tumor hypoxia further suppresses antitumor responses by enhancing the recruitment and function of suppressive immune cells, including T_{reg}, MDSC,[72] and TAMs, increasing expression of inhibitory receptor ligands such as PD-L1 and VISTA,[73 74] and inducing suppressive cytokine production by cancer-associated fibroblasts (CAFs), including TGF-β and IL-10.[75] Moreover, accumulation of reactive oxygen species activates the adenosine-mediated

immunosuppression pathway, inhibiting effector T cell proliferation and TCR signaling.[76]

Restricted nutrient availability can directly suppress antitumor immune responses, particularly when the nutrients are essential for producing antitumor effector molecules. Indeed, increased expression of glycolysis-related genes in cancer cells correlates with impaired activity of intratumoral immune cells.[77] Glucose is essential for $CD8^+$ IFNγ production[78] and NK cell cytotoxic activity,[79] and both cell types have reduced cytolytic function in glucose-depleted settings.[80 81] Conversely, T_{reg} cells do not rely on glucose availability for survival.[71] Tumors bearing Myc mutations, or overexpressing IDO1/2 or tryptophan 2,3-dioxygenase (TDO), consume high levels of glutamine and tryptophan, respectively, impairing T cell proliferation.[78]

Cancer cells release secondary metabolites that also impede antitumor activity. Increased lactate, kynurenines, or glutamate concentrations in the TME can increase IL-17 production by $CD4^+$ cells, decrease proliferation and activation of $CD8^+$ T cells, and promote T cell dysfunction, establishing a tumor-promoting TME.[82] TME accumulation of lipids,[83] potassium (K^+),[84] and adenosine[85] can also induce $CD8^+$ T cell dysfunction.

Microbiota

Local and distant microbiota also influence immunotherapy efficacy and may contribute to immune-resistance mechanisms. Select consortia and specific bacterial families within the gut, including *Bifidobacteriaceae, Ruminococcaceae,* and *Akkermansiaceae,* have been associated with increased response to anti-PD1 treatment in melanoma patients, while *Bacteroidiaceae* is associated with poor response.[86] Dietary changes and probiotic use can also skew immunotherapy responses by impacting gut microbiota; melanoma patients consuming a high fiber diet without probiotics exhibited the greatest overall response to ICB.[87] Tumors are being shown to contain unique microbiomes, and the presence of specific bacteria has been associated with patient response.[88] Tumor resident bacteria may directly (through metabolite secretion or breakdown of certain chemotherapeutics) or indirectly (through priming or boosting a certain immune response) impact tumor progression and patient response.[89] Thus, many immune cell-extrinsic features of the TME can restrain antitumor immunity, and a clear understanding of the pathways influencing immunotherapy resistance will be critical for developing effective targeted therapeutics.

Strategies to define archetypes of immune resistance in patients with cancer receiving immunotherapy

A major gap in current research and clinical care is the ability to identify and treat the most relevant collection of these immune-resistance features in an individual tumor, here defined as an 'archetype.' Many standard genome-sequencing approaches, including tissue-based and blood-based testing, provide clinically actionable results such as microsatellite status, TMB, and specific mutations (figure 2). However, few clinical tests focus on the immune milieu of individual tumors and translate those findings to patient care.[90]

The currently available research technologies (online supplemental table 1) can serve as a foundation to address this critical need. In some cases, the technologies could be used before and during treatment to monitor the evolution of immune resistance. Other technologies may be useful for predicting which resistance mechanisms are most likely to develop. Importantly, studies correlating immune-resistance archetypes with response to currently available therapies may enable clinicians to select a combination of treatments personalized to each patient's tumor.

High-parameter technologies are needed to define complex immune-resistance archetypes. The advent of massively parallel sequencing and single-cell sequencing technologies has heralded a new era of biological discovery in cancer research and already produced clinically relevant advances, such as the identification of novel immune cell types and immunotherapy-resistance pathways.[91] The number of parameters that can be evaluated using traditional proteomic profiling assays, such as flow cytometry and immunohistochemistry (IHC), has significantly expanded with advancements in instrumentation and analyte-detection reagents, yielding important insights about immune cell phenotype, function, and location within tumors. Machine-learning algorithms and other innovative tools are also now available for microbiome sequencing, metabolomic profiling, non-invasive imaging, and modeling the complex human TME, which can be leveraged to comprehensively identify active archetypes of immune resistance in human cancers (figure 2).

Leveraging cutting-edge technologies to develop a rational immunotherapy toolkit

The logical approach to cancer immunotherapy is to select (or design) treatments that will effectively overcome the specifically active immune-resistance mechanisms in an individual patient's tumor. This is the core principle of precision medicine, but is starkly different from how care is currently delivered, in a simplified one-size-fits-all approach primarily stratified by tumor histology or anatomic site of origin. In addition to leveraging cutting-edge technologies to classify immune-resistance archetypes active in tumors, novel technologies will be needed to generate therapeutics that address the complex network of mechanisms contributing to immune resistance. Moreover, high-throughput methods for testing novel immunotherapies and optimizing current strategies are provided by new model systems that better recapitulate immune-resistance archetypes and the immunesuppressive TME features, such as ex vivo patient-derived tumor cultures or preclinical in vivo models with autologous tumor and immune cell compartments (online supplemental table 1 and figure 2). Creative and innovative technologies can simultaneously

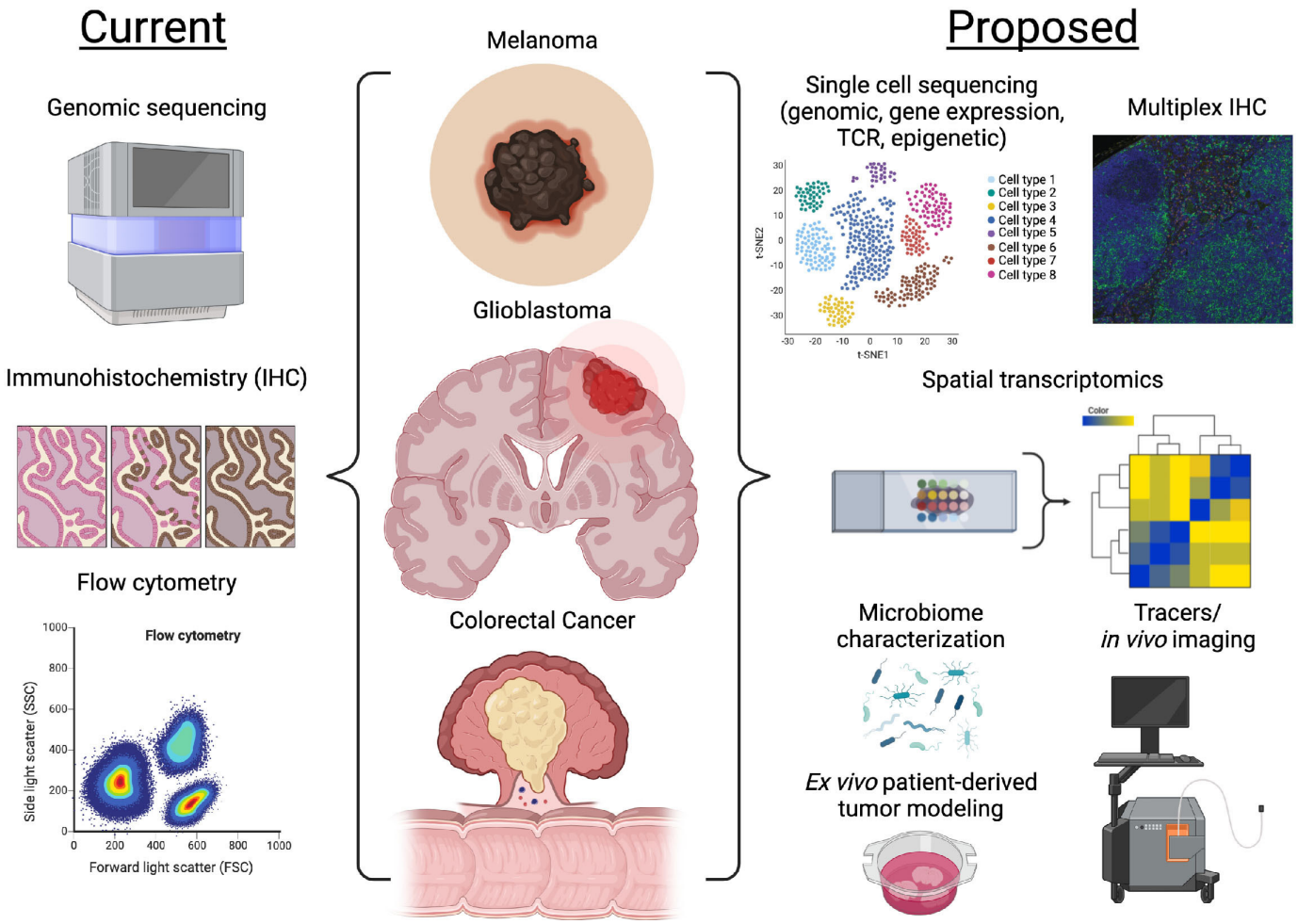

Figure 2 High-dimensional technologies for immune resistance feature set characterization. Classically, the assays used to identify mechanisms of immune response/resistance have prioritized genomic sequencing, single-color or dual-color IHC staining, and flow cytometry. Expanding this characterization to include additional mechanisms of immune resistance will require implementation of cutting-edge high-dimensional technologies, such as single cell sequencing, multiplex IHC, spatial transcriptomics, microbiome characterization, patient-derived ex vivo modeling, and in situ imaging technologies. This figure was created with BioRender.com. TCR, T cell receptor.

assess multiple resistance mechanisms, with clear therapeutic relevance. Here, we provide selected examples of immune-resistance archetypes in four solid tumors and the therapeutic opportunities for each.

Melanoma

ICB-responsive

Metastatic melanoma is the current paradigm of ICB success, with combined blockade of CTLA-4 and PD-1 reaching a 5-year survival rate of approximately 50%.[92] ICB-responsive melanomas contain tumor-specific and tumor-associated antigens. High spontaneous mutation rates produce neoantigens; melanocyte-restricted antigens, such as gp100/pmel and MART-1/melan-A, provide robust $CD8^+$ T cells targets.[30] ICB response correlates with tumor-infiltrating $CD8^+$ T cells, tumor PD-L1 expression, and an intratumoral IFNγ signature,[93] indicative of tumors in which tumor-reactive T cells have been primed, infiltrated, and differentiated into effector/exhausted T cells. T cell activation increases compensatory PD-1 receptor expression, and T cell-derived IFNγ induces PD-L1 expression on tumor cells,[20] which in turn inhibits $CD8^+$ T cell function, consistent with the Immune Cell Dysfunction/Death feature set (figure 3A). Interrupting this PD-L1/PD-1 negative feedback loop relieves T cell inhibition and may facilitate durable T cell-mediated tumor control. These inflamed melanomas exemplify the tumor-immunity cycle in ICB-responsive tumors.

Although what drives antitumor immune responses in the ICB setting have been realized, prospectively identifying responders and non-responders remains a critically unmet need. A major goal for leveraging the feature sets and technologies described in this review will be to improve our ability to accurately predict which patients will respond to current immunotherapies, and which patients should instead be directed to clinical trials focused on their tumors' specific resistance mechanisms.

ICB-resistant

Despite the success of ICB against melanoma, approximately 50% of patients will experience primary or acquired resistance to ICB.[93] Multiple forms of resistance

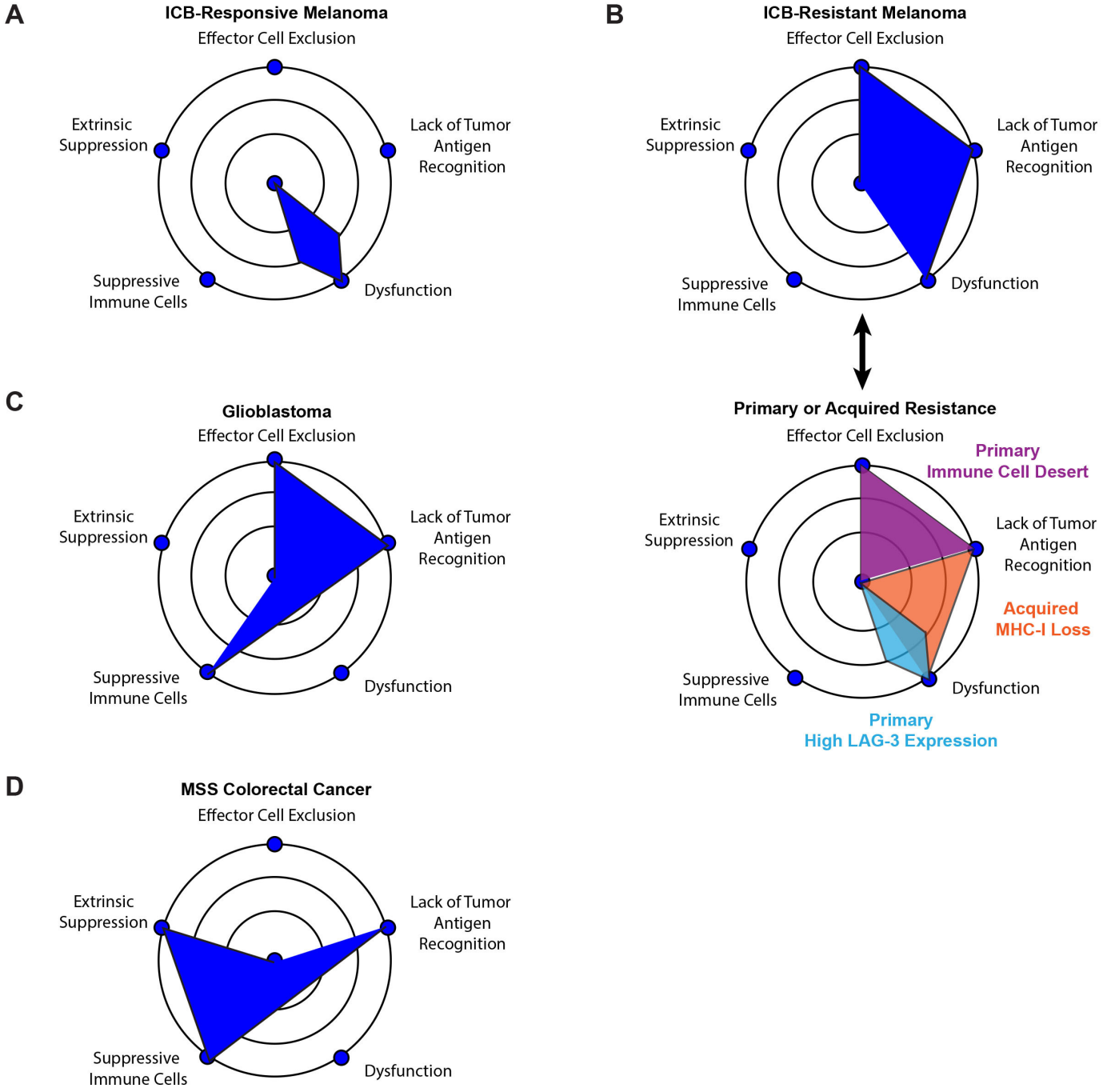

Figure 3 Immune resistance archetypes active in example tumors. (A) Immune checkpoint blockade (ICB) Responsive Melanoma represents tumors primarily engaging resistance mechanisms that drive effector cell dysfunction to evade immune-mediated killing. (B) ICB resistant melanoma represents tumors engaging immune effector cell exclusion, lack of tumor antigen recognition, and/or immune cell dysfunction immune resistance mechanisms. Because ICB-resistant melanoma may engage one or more feature sets to evade immune responses, archetypes of immune resistance in ICB-resistant melanoma include, but are not limited to, primary immune cell desert (purple), acquired MHC-I loss (orange), high Lag-3 expression (teal), a combination of any two, or of all three (dark blue). (C) Glioblastoma represents tumors engaging immune effector cell exclusion, lack of tumor antigen recognition, and suppressive immune cells immune resistance mechanisms. (D) Microsatellite stable (MSS) colorectal cancer represents tumors engaging lack of tumor antigen recognition, suppressive immune cells, and extrinsic suppressive factors as immune resistance mechanisms. MHC-I, Major Histocompatibility Class I.

exist, and ICB-resistant melanoma can often be characterized by immune exclusion, lack of tumor antigen recognition, or immune cell dysfunction/death feature sets (figure 3B).

Primary melanoma resistance to ICB is often correlated with a lack of intratumoral immune-cell infiltration, resulting in poor T cell priming and recruitment to the TME.[25] Mutational burdens in T cell rich versus T

cell poor melanomas are not significantly different,[30] suggesting that antigenicity is not the determining factor in T cell infiltration and implicating a breakdown in T cell activation or recruitment. In mouse models, DC transfer into tumors lacking T cells drove T cell recruitment and enhanced tumor control.[25] While producing therapeutic DCs remains a challenge, advances have been made,[94] raising the possibility that immune exclusion may be overcome by engineering the innate immune system to produce a productive antitumor response. The key question regarding this approach is how to best design and sequence therapies for non-inflamed melanomas. For example, does inciting inflammation via radiotherapy, tumor-targeted vaccines, or intralesional therapy produce optimal outcomes when combined with concurrent or sequential ICB? Trials testing these approaches are ongoing, primarily in the pretreated metastatic setting, rather than at the time of diagnosis when there is potentially greater chance of success.

Primary resistance may also be associated with excessive immune cell dysfunction. For example, patients who fail to respond to anti-PD-1 therapies may respond to combined blockade of LAG-3 and PD-1, demonstrating that dysfunction enforced through multiple T cell coinhibitory pathways can lead to resistance to single-agent immunotherapy.[45] The clinical success of LAG-3 neutralizing antibodies suggests that the additional development of checkpoint inhibitors beyond CTLA-4 and PD-(L)1 could further improve immunotherapy efficacy.

Another mechanism of acquired resistance involves a lack of tumor recognition by immune cells. Immune pressure exerted by ICB can select for tumor cell-intrinsic mutations in genes that mediate IFNγ signaling, especially in *Jak1/2*, leading to tumor cell IFNγ insensitivity.[95] Enforced IFNγ insensitivity downregulates HLA class-I, leaving $CD8^+$ T cells unable to detect tumor antigens and rendering tumor cells resistant to T cell-mediated killing. However, HLA class-I downregulation can sensitize tumor cells to innate-like immune cell populations, including NK cells and γδ T cells, suggesting a potential for NK cell- or γδ T cell-based therapies for overcoming acquired ICB resistance.[39 40]

Many ICB-resistant melanomas contain elements of the aforementioned feature sets. To evaluate immune exclusion, multiplex IHC or spatial transcriptomic profiling could be used to determine the number and location of immune cell subsets, likely focusing on enumerating $CD8^+$ and $CD4^+$ T cells and determining the spatial position of each relative to the tumor margin or tumor core. Consistent metrics would need to be established to similarly categorize immune-excluded versus immune-infiltrated tumors across different laboratories/institutions and to correlate these findings with objective ICB responses. Emerging radio-labeled positron emission tomography (PET) could be leveraged to non-invasively assess T cell infiltration within tumors.[96] Imaging could reveal intratumoral and peritumoral T cells across all primary and metastatic lesions at baseline, or longitudinally to evaluate T cell infiltration and immunotherapy response.

Multiplex IHC or spatial profiling could be used to interrogate a lack of immune recognition. Both imaging platforms leverage sensitive and quantitative chemical approaches that can determine surface expression of HLA molecules on tumor cells, as well as their proximity and interaction with T cell subsets. Cell-surface markers that identify tumor-reactive $CD8^+$ T cells, including CD137, CD103, and CD39, could be measured to interrogate the proportion of tumor-reactive versus 'bystander' intratumoral T cells. Large-scale genomic sequencing of single tumor cells may reveal overall TMB, oncogenic driver mutation status, and tumor mutational heterogeneity, and single cell RNA-sequencing (scRNAseq) could reveal expression levels of antigen-presentation machinery. These insights could also inform clinicians if BRAF or MEK inhibition would be appropriate second-line treatments if immunotherapy resistance occurs.

Immune cell dysfunction could be interrogated using a combination of high-dimension methodologies. RNA-seq of tumor-infiltrating T cells could reveal transcriptional states associated with function/dysfunction, which have now been widely cataloged in both preclinical and clinical studies. Multiplex IHC or spatial profiling could be utilized to simultaneously assess the presence of inhibitory receptors on T cells (such as PD-1, CTLA-4, Tim-3, and Lag-3), inhibitory ligands in the TME, and the spatial relationship between T cells, tumor cells, T_{reg}, and/or myeloid cells. Markers of functional T cells, including granzymes and cytokines, could also be measured at the RNA and/or protein level.

Glioblastoma

The brain is immunologically unique, owing to its tight barrier membranes that restrict cell movement across the brain parenchyma, and the lack of a classical lymphatic system, which limits lymphocyte trafficking. Active lymphatics are present in the brain. However, glioblastoma (glioma) brain tumors develop malformed vasculature, leading to deregulated permeability and perfusion, high interstitial fluid pressure, extensive hypoxia, and necrosis, which limits the perfusion and penetration of ICB and other immunotherapies.[97] Given the low mutation rates and low numbers of infiltrated lymphocytes, gliomas are described as 'immune deserts' and 'cold'.[90]

Most immune cells within gliomas are immunosuppressive myeloid cells (including microglia, bone marrow-derived macrophages/BMDM, and MDSCs), which often compose ~30% of the tumor mass.[98] Immunosuppressive T_{reg} are also present within gliomas, preventing the activation, expansion, and/or function of $CD4^+$ and $CD8^+$ T cells.[99]

Immune-cell infiltration has been correlated with immunotherapy responsiveness in gliomas, and technologies have been developed to identify patients with tumors containing T cells or immune-suppressive TAMs. For example, gliomas with high isocitrate dehydrogenase

(IDH) mutations have reduced $CD8^+$ and $CD4^+$ T cell infiltration and a diminished IFNγ signature,[100] whereas gliomas without IDH mutations exhibit robust T cell infiltration and PD-L1 expression.[101] RNA-seq or IHC identification of tumors with recurrent genomic mutations might enable clinicians to stratify patients according to levels of tumor-infiltrating lymphocytes (TILs) and prioritize targeted treatments, such as those that enhance TIL recruitment. While transcriptomic sequencing and imaging technologies may help identify patients with T cell-infiltrated tumors that may be more immunotherapy-responsive, advances in non-invasive analytical technologies could enable clinicians to more readily identify patients with T cell-infiltrated tumors. For example, radiolabeled anti-CD8 'minibody' is currently being evaluated in clinical trials as a PET-imaging agent to visualize TILs in patients with metastatic solid tumors.[102] In addition, the FDA-approved ultra-small superparamagnetic iron oxide nanoparticle-based (USPIO[133]) and fluorine isotope 19 (^{19}F)-based MRI contrast agents were used to quantify TAM density after radiotherapy.[103] Finally, cytometry by time of flight analysis of circulating immune cells can be used to monitor immune responses during glioma progression and treatment.[104] Together, these technologies could be used to interrogate immune responses, or lack thereof, and inform clinical decisions.

The genetic mutations in glioma are highly heterogeneous, both intertumorally and intratumorally, making it difficult to find suitable targets for tumor-specific immunotherapies. Although a series of tumor-associated antigens (eg, EGFRvIII, PDGFRA, IL13Rα2, GD2, NKG2D, HER2, CD70) are currently being tested as immunotherapy targets,[105] the loss of HLA and costimulatory molecules on antigen presenting cells (APCs) and brain parenchyma limit antigen presentation to T cells.[106] Consequently, gliomas represent tumors with lymphocyte immune exclusion (due to a tumor-specific immune desert and/or the inherently low lymphocyte levels in the brain), suppressive immune cells, and lack of tumor antigen recognition, as reflected in archetype feature sets (figure 3C). Radical strategies are urgently needed to identify therapeutics that can effectively address these resistance mechanisms. For example, patient-derived glioma organoid models (PDGOM) are a promising tool to evaluate treatment strategies targeting specific suppressive features within a tumor. When combined with spatial transcriptomics, PDGOM successfully validated that JAK/STAT inhibition prevented IL-10-mediated immune escape and rescued T cell functionality.[67] When combined with mutation analysis, $EGFRvIII^+$ PDGOM effectively predicted selective EGFRvIII CAR-T killing of $EGFRvIII^+$ tumor cells, compared with control $EGFR^+$/$EGFRvIII^-$ tumor cells.[107]

Current therapeutic approaches target the suppressive glioma TME primarily by depleting macrophage/monocyte recruitment through cytokine blockade (eg, CCL2/CCR2 and CSF1/CSF1R axis inhibition).[108 109] However, acquired resistance mechanisms have been identified,[98] and additional therapeutic approaches are greatly needed. For example, small molecule immune modulators, oncolytic viruses, or RNA-based therapeutics that activate the antitumor function of macrophages/monocytes may redirect immune-suppressive functions to immune-stimulatory pathways in the tumor milieu.[110] Other novel delivery technologies, including nanoparticle-based formulation and focused ultrasound, can improve the efficacy of therapeutic molecules by enhancing blood–brain tumor barrier penetration, increasing precise targeting, and reducing systemic toxicity. Moreover, macrophage-targeting therapeutics often work synergistically with standard-of-care treatments and/or immunotherapies. Further, CAR-engineered macrophages have been shown to phagocytose tumor cells with antigen specificity, demonstrate enhanced antigen-presentation, and increase the release of proinflammatory cytokines,[111] addressing all three relevant immune resistance feature sets.

Importantly, glioma immune-resistance feature sets (and encompassing archetypes) evolve with treatment and disease status. For example, recurrent gliomas tend to have higher BMDMs than newly diagnosed primary gliomas.[112] Treatment selection must consider that brain-resident microglia and BMDM respond differently to the same treatment. Immune exclusion may be addressed by enhancing recruitment of effector lymphocytes to the tumor through: (1) tumor vascular normalization, by targeting angiogenesis with anti-VEGF or anti-Ang-2 antibodies[113]; (2) promoting lymphangiogenesis, using VEGF-C[114]; and/or (3) enhancing T cell chemotaxis by increasing expression of T cell recruiting chemokines.[115] A lack of antigen specificity may be addressed using T cell engineering, such as with EGFR-vIII and GD2 CAR T cells.[116 117] However, recent clinical trial data indicated that overcoming the adaptive changes in the local TME and addressing antigen heterogeneity are critical to the success of CAR T cell-based strategies against glioma.[118] Dual-targeting therapeutics, such as bispecific antibodies or bispecific CAR T cells, or universal immune receptors, may address immunotherapy resistance caused by antigen-negative clonal escape and reduce the risk of relapse.[119] Together, these approaches may address the immune-resistance mechanisms driven by the heterogeneous antigen landscape and antigen downregulation in glioma.

Microsatellite stable colorectal cancer

CRC is a leading cause of cancer deaths worldwide and a prototypical ICB-resistant gastrointestinal adenocarcinoma.[120] While CRCs can be defined by many classification systems, a notable therapeutically relevant distinction is a tumor's DNA MMR status; that is, MSS/MMR proficient (MSS/p-MMR) versus microsatellite instability-high/d-MMR (MSI-H/d-MMR). MSS tumors make up the vast majority (~85%) of CRC and generally have low TMB due to intact MMR. While ICB typically has limited to no benefit against CRC, there are notable exceptions

in specific MSS cases.[120 121] Patients with MSI-H CRC have an estimated 40% response rate to anti-PD1 ICB. Further study of CRC biology is warranted.

Initial attempts to further classify CRC include the consensus molecular subtypes (CMS) taxonomy that is based on whole tumor gene expression data, and the Immunoscore method that uses IHC to evaluate the phenotype and frequency of intratumoral immune cells.[122 123] While these classifications can robustly phenotype and predict outcomes in CRC, recent advances in scRNA-seq and spatial transcriptomics technologies enable high-resolution dissection of specific immune cells and signals associated with a favorable or unfavorable TME.[124 125]

Numerous clinicopathological CRC studies confirm that MSS tumors are infiltrated by $CD8^+$ T cells, although to a lesser extent than MSI-H tumors,[126] and that the nature of T cell-[127] and myeloid-[128] infiltration predicts survival. Immune infiltration, rather than exclusion, was demonstrated in recent high-dimensional scRNA-seq studies of primary and metastatic CRC.[124 125] Together, these data suggest that immune effector cells are present but ineffective at recognizing and killing tumor cells. Therefore, immunotherapy resistance in MSS CRC can be characterized by the suppressive immune cells, extrinsic suppressive factors, lack of tumor antigen recognition, and, to a lesser extent, immune effector cell exclusion feature sets (figure 3D). It is not surprising, therefore, that ICB is generally ineffective.

Immunosuppressive drivers in MSS CRC are beginning to be revealed with novel high-resolution technologies. Myeloid populations in primary CRC and liver metastases are heterogeneous, including subsets such as *SPP1*$^+$ cells with M2-like suppressive transcriptional patterns that correlate with worse patient survival.[124] The mechanisms by which myeloid cells suppress effective anti-CRC T cell responses are not fully defined in patients, but preclinical studies implicate IL-10 and downstream STAT3 signaling,[129] and FAS/FASL-mediated T cell death.[130] TGF-β, another major suppressive cytokine driving immune dysfunction and immune exclusion, is a defining feature of an aggressive CRC molecular subtype (CMS 4),[123] produced in large part by stromal CAFs.[131] Moreover, the paucity of intratumoral DCs capable of priming antitumor T cells,[132] plus low TMB/neoantigen expression, contribute to poor tumor recognition by effector T cells.

Finally, the gut and tumor microbiome are of particular interest in CRC, and new tools enable deep profiling of the microbiome. Spatial imaging has been applied to visualizing the microbiota through fluorescent in situ hybridization (FISH) imaging.[133] More recently, 'live-FISH' has been developed, in which bacteria can be stained with bacterial probes without a fixative, so that live bacteria can be sorted based on these same fluorescent probes.[134] While FISH imaging can identify the spatial location of bacteria, it does not reliably provide quantitative data. To better determine how much of the gut or tissue microbiota is dominated by a specific bacterial family, 16S rRNA sequencing (16S)[135] can be used with downstream analyses through established pipelines, such as QIIME2.[136] Whole Genome Shotgun sequencing can provide additional species-level information, by evaluating the entire DNA genome rather than just specific regions.[137] There are currently no clinically approved microbiome-based therapies or biomarkers for CRC, but preclinical studies support potential new therapeutic targets.[138]

Taking these resistance mechanisms into account, a compelling therapeutic approach involves reprogramming suppressive myeloid cells into more effective T cell-stimulating APCs, potentially addressing multiple feature sets of immune evasion: (1) removing a major driver of suppressive immune cells, including M2-like TAMs or MDSC and their secreted cytokinome, enhancing APC-mediated stimulation of cytotoxic T cells, either directly, or indirectly through paracrine IL-12 signaling or $CD4^+$ T cell help; (2) thereby enhancing tumor antigen recognition. In addition to targeting myeloid cells directly, targeting T_{reg} may be efficacious as these cells can support an immunosuppressive TME by driving progression of monocytes to protumoral TAMs, and limit the function and migration of potentially antitumoral DC subpopulations (cDC2).[139 140] An alternative approach would be to target CAFs or the immunosuppressive TGF-β cytokine. The optimal approach will have to be tested with clinically relevant cytotoxic chemotherapies, such as FOLFOX or FOLFIRI first-line therapies against metastatic CRC. Novel immunotherapies will also require iterative testing in models of the TME and immune effector function, as immune cell dysfunction/death and other resistance feature sets may become relevant after an initial increase in T cell activation, tumor recognition, and killing.

DISCUSSION

ICB immunotherapies have revolutionized anticancer treatment, but most patients either do not respond or eventually relapse after ICB. Immune-resistance mechanisms may be present pretreatment (primary resistance) or develop (adaptive or acquired resistance),[16] and a thorough understanding of the dynamic interactions between immune cell-intrinsic and immune cell-extrinsic resistance mechanisms can provide critical insights to guide clinical care.

High-dimensional analytical technologies have already elucidated the TME of multiple cancer types. A natural extension, therefore, would be to incorporate such analyses into prospective immunotherapy clinical trials, with the goal of uncovering the cellular and molecular underpinnings of therapeutic response and resistance (figure 4). However, while there is clear biological (and likely clinical) value in incorporating high-dimensional analytic methods into clinical trials, there are numerous challenges that currently limit the feasibility of such clinical implementation on a large scale.

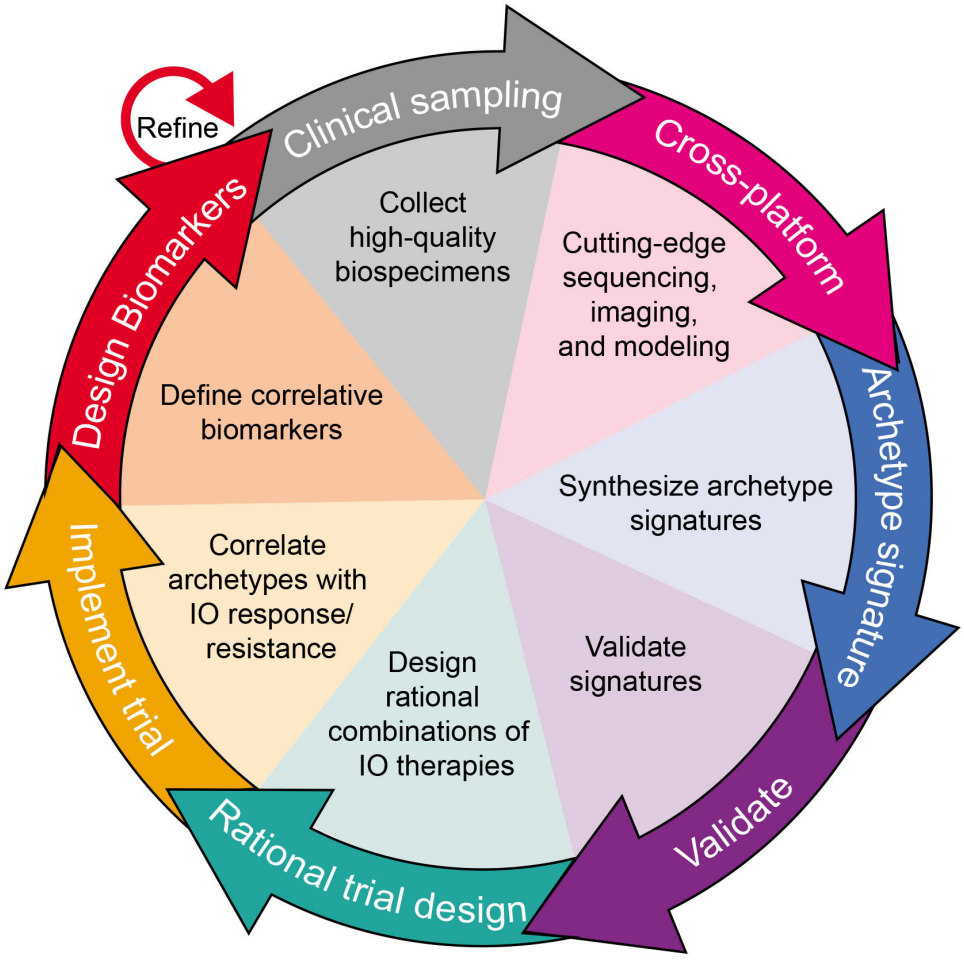

Figure 4 Implementation of immune resistance archetypes for clinical decision-making. We envision the process of implementing immune resistance archetypes for clinical decision-making as an iterative cycle starting with high-dimensional, cross-platform profiling to develop and validate of immune resistance signatures. Subsequently, rational immunotherapies that address immune resistance archetypes (rather than individual immune resistance mechanisms) would be developed and implemented for clinical evaluation. Results of these trials would be correlated with immune response or resistance and evaluated for a smaller number of informative biomarkers that could be used for clinical decision-making. This pipeline could continue to be refined as new technologies emerge, new resistance mechanisms are discovered, and novel immunotherapy technologies are developed.

While tumor material is readily accessible for many hematologic malignancies, additional research biopsies are typically needed to obtain solid tumor tissue for correlative analyses. Such research biopsies may need to be performed at multiple time points, such as prior to treatment, on treatment, and postprogression. While such biopsies could yield valuable scientific insights, research biopsy inclusion will need to consider not only the potential benefits but also the risks to the individual patient (which vary, based on disease site and patient-specific factors) and patient autonomy. Even when research biopsies are feasible, intratumoral and intertumoral heterogeneity and/or the presence of extensive fibrosis/stromal components in certain tumor types (such as PDAC) may pose additional challenges to gaining biological insights from a small sampling of a single tumor site.

Moreover, there are additional technical challenges and cost considerations related to the implementation of high-dimensional analyses in the clinical setting. For scRNA-seq, fresh tumor tissue is typically required, which must be mechanically and enzymatically dissociated into a single-cell suspension, rapidly, to avoid cell death and transcriptional changes.[141] While cryopreservation methods may reduce the upfront tissue-processing requirements and enable centralized enzymatic dissociation in a highly skilled laboratory setting, transcriptional changes can still occur with tissue processing.[142] Finally, current high-dimensional analysis methods have a substantially higher financial cost than conventional 'bulk' sequencing approaches or traditional IHC/immunofluorescence. While the cost of such technologies will likely decrease over time, methods are also being developed to 'multiplex' samples, enabling the analysis of multiple samples simultaneously for a reduced cost.

Given these challenges in tissue access, technical processing, and cost, is there a strategy for implementing these analytic methods in clinical trials? One approach is to implement detailed analyses on only a subset of patients

within a clinical trial, and then attempt to validate specific findings on available samples from the remaining trial patients.[143] For a relatively small subset of patients in a clinical trial who are amenable to the additional sample collection, clinicians and scientists could perform detailed molecular analysis using numerous high-dimensional analytic technologies, including scRNA-seq, scTCRseq, and spatial and microbiome phenotyping methods. This group of patients would essentially serve as a 'discovery' cohort for uncovering determinants of immunotherapeutic response and resistance. Specific hypotheses could then be tested in available samples from the remaining patients in a trial ('validation' cohort). Of note, it will be important to ensure that analyses performed in the discovery cohort are relevant to 'real world' patients, and specific efforts should be made to include diverse patients with respect to comorbidities, age, gender, and race/ethnicity.

While multiomic tissue analysis is already used to evaluate immunotherapy response and resistance mechanisms, data integration to identify response signatures and inform patient selection is increasingly difficult and labor intensive. Artificial intelligence (AI) has been applied to predict immunotherapy responses based on medical imaging, histological analysis, and immune signatures. More specifically, AI has successfully used radiological imaging to identify responders and non-responders[144] and to integrate imaging and genomic sequencing to predict clinical outcomes with ICB.[145] AI-based histopathology analyses have shown predictive value in determining response to immunotherapy in MMR-defective tumors.[146] Additional tools have been developed to identify TIL density, cellular integrations, and genomic and epigenetic alterations.[147] One of the major challenges to the advancement of AI in immunotherapy is the need for robust datasets to train and iterate machine learning algorithms. Prospective randomized clinical trials of an immunotherapy are often small, and larger data sets are necessarily derived from retrospective analyses. To implement AI on a larger scale, investigators may need to compare real-world evidence with clinical trial data sets to validate AI algorithms. Further, creation of a centralized data repository that includes analysis of biospecimens and outcomes for clinical trials could advance the use of AI in immunotherapy analysis. However, clear methods to protect intellectual property and patient confidentiality will be needed.

Once archetypes of immune resistance have been defined in patient samples, clinical strategies will need to effectively address multiple resistance mechanisms or feature sets, sometimes concurrently (figure 4). Currently available reagents, such as ICB, targeted cell depletion, radiotherapy, or anti-angiogenic treatments, may be combined to effectively address coexisting resistance feature sets.[148] However, combining systemic therapies that activate the immune system can lead to immune-related toxicities; and in the future, targeted engineering technologies may be able to address multiple resistance features while avoiding immune-related adverse events. For example, cell engineering technologies are being evaluated to simultaneously address lack of tumor antigen recognition and immune cell dysfunction/death. T cells engineered to express both a tumor-specific receptor (CAR or TCR) and a synthetic switch receptor (a fusion of the ectodomain of an inhibitory receptor with the signaling domain of a costimulatory molecule, to convert a suppressive signal to a proliferative/survival or activation signal) can improve therapeutic efficacy over T cells expressing the CAR or TCR alone.[60 149] Engineering NK cells to express a CAR has overcome obstacles to allogeneic and autologous T cell transplantation in the clinic,[150] and preclinical engineering approaches incorporating switch receptors or disrupting negative regulators render NK cells less susceptible to suppression in the TME.[151] Preclinical studies also suggest that T cells engineered to overcome metabolic suppression have improved persistence and antitumor efficacy,[83 152] which may be combined with CAR or TCR engineering to address lack of tumor antigen recognition and extrinsic suppressive factors. Additionally, tethering cytokines like IL-12 to infused T cells can overcome myeloid cell suppression to enhance the function of transferred T cells in the TME.[153] Engineering approaches may also offer opportunities to convert non-inflamed/cold into inflamed/hot tumors. For example, oncolytic viruses engineered to express granulocyte-macrophage colony-stimulating factor (GM-CSF) can increase DC recruitment and T cell priming.[154] In addition, CAR-macrophages can modulate the phenotype of suppressive myeloid cells, increase T cell priming and recruitment, and are resistant to suppressive cytokine signaling,[111] simultaneously addressing immune effector cell exclusion, lack of tumor antigen recognition and suppressive immune cells.

Thus, we propose approaching the hurdle of immunotherapy resistance from the perspective of immune-resistance archetypes. Cutting-edge technologies can retrospectively identify archetypes using banked samples from completed trials, which could then be correlated with clinical response or resistance outcomes, increasing our understanding of the complex interplay between different pathways (figure 4). This approach may not only inform the development of novel therapies that simultaneously address multiple resistance mechanisms, but may also guide prioritization of treatments (and treatment combinations) more effectively for patients.

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Technology	Example Methods	Advantages	Limitations	References
Genomic Sequencing	Whole exome sequencing (WES)	• Provides comprehensive coverage of coding regions and is a cost-effective alternative to whole-genome sequencing • Identifies copy number variants • Can determine mutational signature and total mutation burden • Ability to detect subclonal population with high depth of sequencing	• WES covers only a small fraction of the genome (does not detect variants in most non-coding regions) • Not validated for the detection of structural variations (SVs) • Cannot demonstrate functional relevance of abnormal findings	[1]
Genomic Sequencing	Whole genome sequencing	• Captures mutations in non-coding regions, including potentially important regulatory regions • Detects structural variants (e.g., translocations)	• Higher cost • Relatively low depth (compared to WES) limits ability to detect smaller subclonal populations • Cannot demonstrate functional relevance of abnormal findings	[2]
Transcriptomic Sequencing	Whole transcriptome sequencing	• Provides comprehensive understanding of phenotypes and identifies biomarkers across the broadest range of transcripts. • Captures known and novel gene fusions • Can infer the frequency of	• Dependent on RNA quality, which is variable to poor from FFPE rather than frozen tissue. • Expensive and high turnaround times in clinical settings. • Lack of single cell information limits the ability to define cellular composition and determine relevant pathways/gene signaling within	[3–5]

Technology	Example Methods	Advantages	Limitations	References
		immune cell types can aid in determining immune archetypes	immune cell types • Cannot demonstrate functional relevance of abnormal findings	
Single cell RNA sequencing	Whole transcriptome sequencing on a single cell basis (scRNA-seq)	• Comprehensive assessment of cellular composition and phenotypic states • Computational methods can also be applied to infer repertoire sequences from scRNA-seq not specifically enriched for V(D)J sequences • Methods for multiplexing allow for high sample throughput • Can be paired with chromatin accessibility sequencing (ATAC-seq) • Methods available for lineage tracing (ex: MAESTER)	• High cost • Technically challenging, which limits feasibility, and requires immediate dissociation of fresh tissue • Sequencing depth • Requires familiarity with bioinformatics for data analysis and large amount of data generated, leading to slow adoption for clinical use • Lack of spatial information • Cannot demonstrate functional relevance of abnormal findings	6–10
Immune repertoire sequencing	Whole transcriptome sequencing with paired sequencing of T and B cell receptors	• Can be used with single cell or bulk RNA sequencing • Paired receptor information (variable heavy and light chains or TCR α and β chains) can be obtained • Identification of antigen-specific receptors in some cases	• Antigen specificity not available for most receptor sequences • Cannot demonstrate functional relevance of abnormal findings	11–13

Technology	Example Methods	Advantages	Limitations	References
Proteomic Profiling	Flow cytometry Mass cytometry (CyTOF)	• Inexpensive • Validated laboratory and clinical tests • Quantifies expression of multiple parameters/analytes on per-cell basis	• Requires immediate dissociation of fresh tissue • Requires large quantity of tissue / cells (CyTOF) • Parameters limited by detection technology • Validated and conjugated detection antibodies needed • Cannot demonstrate functional relevance of abnormal findings unless combined with extra labor-intensive steps, e.g., intracellular cytokine staining	[14]
Proteomic Profiling	Secreted cytokine assessment (e.g., ELISA, Luminex)	• Inexpensive • Functional information • Multiplex capability (Luminex)	• Requires supernatant from fresh tissue/cell culture or immediate processing of fresh tissue • Limited to 1 parameter for traditional ELISA	
Proteomic Sequencing	Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq)	• Enables the simultaneous analysis of protein and RNA-level expression data by combining traditional scRNA-seq with staining of cells with DNA oligo-tagged antibodies	• High cost • Many techniques still limited in number of proteins analyzed • Cannot demonstrate functional relevance of abnormal findings	[15,16]
Spatial Imaging (traditional)	Immunohistochemistry/ Immunofluorescence	• Preserves spatial architecture and heterogeneity	• Limited multiplexing capabilities • Inefficient for analyzing immunotherapy response	[17]

Technology	Example Methods	Advantages	Limitations	References
		• Validated laboratory and clinical tests • Robust catalog of available antibodies • Easy and standard tissue/ cell processing • Low cost	• Cannot demonstrate functional relevance of abnormal findings	
Spatial Imaging (High dimensional)	Mass cytometry imaging (Imaging Mass Cytometry [IMC] or Multiplex Ion Beam Imaging [MIBI])	• Preserves spatial architecture and heterogeneity • >40 parameters (antibodies conjugated with stable isotopes) • High sensitivity, resolution, and throughput • Uses archival specimens	• Tissue is ablated during imagining • Difficult analysis • Cost, specialized platforms are needed • Suggests but cannot demonstrate functional relevance of abnormal findings	[18,19]
Spatial Imaging	Cyclic immunofluorescence (t-CyCIF)	• Preserves spatial architecture and heterogeneity • ~60 parameters (fluorescently labeled barcodes or secondary antibodies) • High sensitivity and resolution • Uses archival specimens	• Time, hours to 1 day per cycle per tissue section • Cost, specialized platforms are needed • Difficult analysis • Suggests but cannot demonstrate functional relevance of abnormal findings	[18]

Technology	Example Methods	Advantages	Limitations	References
Spatial Transcriptomics	Conventional methods (seqFISH, MERFISH, RNAscope, SABER)	• Serial imagining, or branched amplification • Good spatial resolution	• Lack of tools that can be easily used for multiplexing • Detects 3-4 targets • Error-prone, time consuming, laborious, and costly to scale up • Repeated processing can affect tissue integrity • Suggests but cannot demonstrate functional relevance of abnormal findings	[20]
Spatial Transcriptomics	Advanced methods (Visium, GeoMx, CosMx)	• Increased multiplexing capabilities • Can profile up to 10,000 genes • Cellular and subcellular 3D resolution • Compatible with FFPE and fresh frozen tissues	• Reduced spatial resolution and detection of low abundance targets compared to conventional methods • Optical crowding can limit the molecules that can be detected efficiently and accurately • Low mRNA detection efficiency • Cost, specialized platforms often are needed • Not high-throughput and difficult analysis • Suggests but cannot demonstrate functional relevance of abnormal findings	[20]
Proteomic and transcriptomic imaging	Multi Omic Single-scan Assay with Integrated Combinatorial Analysis (MOSAICA) and CosMx	• Visualization of 1,000 RNA and 100 proteins on one slide	• Reduced spatial resolution and detection of low abundance targets compared to conventional methods	[20]

Technology	Example Methods	Advantages	Limitations	References
	Spatial Molecular Imager	• Cellular and subcellular 3D resolution • Compatible with FFPE and fresh frozen tissues	• Optical crowding can limit the molecules that can be detected efficiently and accurately • Low mRNA detection efficiency • Cost, specialized platforms often are needed • Not high-throughput and difficult analysis • Suggests but cannot demonstrate functional relevance of abnormal findings	
Microbiome imaging	Fluorescent in situ hybridization (FISH)	• Single bacterial imaging technology that provides spatial information	• Not high throughput, tedious, must have known bacterial probes to test • Cannot demonstrate functional relevance of findings	21–24
Microbiome quantification	16S rRNA sequencing, Metagenomics	• Provides a quick look at relative abundances of microbes	• No species level specificity (16S rRNA sequencing) • difficult to analyze (Metagenomics) • Cannot demonstrate functional relevance of findings	25,26
Metabolomics	Mass Spectrometry-based methods	• Small samples • High amount of information • High sensitivity • Definition of metabolic fingerprints before and after therapy	• Data analysis requires high dimensional computational resources • Cannot demonstrate functional relevance of abnormal findings	27,28

Technology	Example Methods	Advantages	Limitations	References
Metabolomics Isotope-labeled probes and PET:	• PD1, PDL1, CTLA4, LAG3 conjugated with ^{89}Zr or ^{64}Cu • ^{18}F-FDG PET/CT • FET • ^{18}F-Gln • ^{18}F-Glu • FLT • ^{18}F-choline • ^{11}C-acetate • ^{18}F-MISO • ^{68}Ga-DOTATOC • ^{68}Ga-PSMA	• More sensitive than IHC determining patient basal expression. • Monitor response predictive changes in metabolic activities before and after ICB treatment. • Some FDA approved • Non-invasive, i.e., does not require tissue biopsy • For ^{18}F-FDG PET: widely used clinical test to assess treatment response, provides functional readout of tumor metabolism	• Their large molecular size implies a long time for biodistribution and optimal image background control. • Still in development	[29–31]
Metabolomics Energy metabolism	• Microplate analyzers • Clark-type electrode chambers	• Small samples for microplate analyzers • Simultaneous measurement of different substrates • Friendly data analysis software • Allows tissue pieces	• Cellular structure is disrupted • Specific analysis software required • Different readouts require addition of detectors sensitive to other analytes. • High quantity of sample required for Clark-type electrode chambers	[32]

Technology	Example Methods	Advantages	Limitations	References
Nanotechnology	Can be integrated with current diagnostic methods. For example, two common methods are MRI imaging and biomarker assay based on human bio-fluid samples.	• Improve the sensitivity of current diagnostic methods • Non-invasive and longitudinal assessment • Track immune resistance early on treatment • Enable real-time monitoring of adoptive cell therapy	• Often requires injection of imaging agent or substrates, therefore clinical trials are required to establish safety • Biomarkers being assessed in a single assay is limited • cGMP manufacturing and clinical trials require large investments, which can lead to high cost for the patients.	33–36
Artificial Intelligence	Integrated analysis of medical imaging, histological analysis, genomics/ epigenomics, and clinical outcomes	• Automates analysis from multiple different sources • Has been successful at predicting responders and non-responders to immunotherapy	• Field is underdeveloped • Need of robust data set to train and iterate machine learning algorithms • Prospective randomized clinical trials are often small; larger data sets derived from retrospective analyses • Patient confidentiality concerns	37
Human *ex vivo* tumor models	Organotypic slice culture	• Biologic surrogate • Tumor 3-D architecture and all stromal and immune components maintained in similar spatial and stoichiometric relationship to patient's tumor • Provides a platform in which to test functional relevance of a therapeutic target	• Slice-to-slice variation, difficult to normalize readouts unless enough biologic slice replicates • Variable yield dependent on tumor type, viability, and preoperative chemo- or radiotherapy administered to the patient • Short viability (1-2 weeks depending on tumor type and starting viability) • Cannot be propagated • Large quantities of fresh tumor are	38–40

Technology	Example Methods	Advantages	Limitations	References
			needed from surgical resection specimen rather than core needle biopsy	
Human *ex vivo* tumor models	Organoid culture (short-term)	• Heterogeneous, maintain all cellular components • Small amount of tissue is needed • Provides a platform in which to test functional relevance of a therapeutic target	• Cell-cell spatial relationships and tissue architecture are lost • Short viability (~5 days), propagation leads to reduced immune and stromal cell composition • Fresh tumor digests are preferred	[41]
Human *in vivo* tumor models	Humanized allogeneic PDX mouse models	• Developed through various sources (PBMCs, $CD34^+$ hematopoietic stem cells, surgical transplant of fetal liver and thymus fragments) • Cellular diversity • Ease of development and cost varies between models • Provides a platform in which to test functional relevance of a therapeutic target	• High cost • Time required to generate model • Cells are typically naive and lack tumor antigen specificity • HLA-mismatched • Allogeneic response may be misinterpreted as anti-tumor response • Models develop GVHD	[42,43]
Human *in vivo* tumor models	Humanized autologous PDX mouse models	• HLA-dependent, autologous response • Patient-specific response can be evaluated • Provides a platform in which	• Tissue is often limiting • TIL expansion may alter TIL maturation/exhaustion phenotype • Current models lack full immune cell reconstitution	[44–47]

Technology	Example Methods	Advantages	Limitations	References
		to test functional relevance of a therapeutic target	• Time and cost for development limits use as a clinical surrogate	

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