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Facts and hopes in using omics to advance combined immunotherapy strategies

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Abstract

The field of oncology has been transformed by immune checkpoint inhibitors (ICI) and other immune-based agents, however many patients do not receive a durable benefit. While biomarker assessments from pivotal ICI trials have uncovered certain mechanisms of resistance, results thus far have only scraped the surface. Mechanisms of resistance are as complex as the tumor microenvironment (TME) itself, and the development of effective therapeutic strategies will only be possible by building accurate models of the tumor-immune interface. With advancement of multi-omic technologies, high-resolution characterization of the TME is now possible. In addition to sequencing of bulk tumor, single-cell transcriptomic, proteomic, and epigenomic data as well as T cell receptor profiling can now be simultaneously measured and compared between responders and non-responders to ICI. Spatial sequencing and imaging platforms have further expanded the dimensionality of existing technologies. Rapid advancements in computation and data sharing strategies enables development of biologically interpretable machine learning models to integrate data from high-resolution, multi-omic platforms. These models catalyze the identification of resistance mechanisms and predictors of benefit in ICI-treated patients, providing scientific foundation for novel clinical trials. Moving forward, we propose a framework by which *in silico* screening, functional validation, and clinical trial biomarker assessment can be used for the advancement of combined immunotherapy strategies.

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Keywords

immune checkpoint inhibitors; immunotherapy; multi-omics; single-cell sequencing; spatial omics; imaging; computational biology; machine learning; artificial intelligence

Introduction: The need for advanced immunotherapy combinations

Immune checkpoint inhibitors (ICI) have revolutionized the treatment of many cancer types, though the majority of patients do not receive a durable benefit from monotherapy alone.(1,2) Since the FDA approval of CTLA4 and PD1 antibodies for the treatment of advanced melanoma, ICIs have been studied in nearly every malignancy. These trials provided an early paradigm of response criteria to explain an ever-widening gap between long-term responders and those with primary or secondary resistance.(3) The "immunogenicity" of the tumor is the backbone of this paradigm, with highly mutagenic cancers (e.g. lung and skin) initially believed to derive the most benefit.(4) However, biomarkers aimed at defining a tumor's immunogenic potential, including tumor mutational burden (TMB), PD-L1 immunohistochemistry (IHC) and tumor infiltrating lymphocyte (TIL) assays, do not always correlate strongly with ICI response.(5) Instead, markers based on static, unidimensional parameters of the tumor microenvironment (TME) only highlight the overall need for integrated, high-dimensional measures that capture the immense complexity of the tumor-immune interface. Dual CTLA4 plus PD1 blockade is used in multiple cancers, with LAG3 inhibition recently approved alongside anti-PD1 therapy in advanced melanoma.(6) However, a lack of advanced biomarkers has limited the identification of populations most likely to receive benefit, leading to high toxicity and limited efficacy of other combination strategies, such as co-stimulatory agonists and intratumoral therapies.(7–9)

Similar to ICI, multi-omics technologies have transformed the field of immuno-oncology (IO) on scientific exploration and clinical translation. Historically, high costs and resource barriers prevented these technologies from being applied broadly toward the discovery of novel IO targets.(10) Nowadays, omics production is no longer a rate-limiting step, with data analytics and computation now charged with harnessing these high-dimensional data to better characterize the TME. As an example, next-generation sequencing (NGS) of the tumor transcriptome allowed the development of inflammatory gene signatures based on interferon-gamma (IFNγ) signaling and cytolytic scores.(11) Though imperfect, these signatures provide a more comprehensive depiction of T cell activity as compared to clinically used biomarkers.(12) Given the relative paucity of ICI-treated cohorts with expression data, these inflammatory signatures also serve as more accurate, predictive surrogates for ICI response.(11,13) Most importantly, by comparing non-T cell-inflamed versus T cell-inflamed phenotypes, a model can be established by which molecular drivers of immune exclusion may be identified.(14) Apart from bulk tissue sequencing, multi-omics now encompasses nearly all aspects of cancer biology, including single-cell RNAseq/CITEseq/ATACseq, T cell/B cell receptor (TCR/BCR) sequencing, microbiome sequencing, metabolomics, spatial transcriptomics/proteomics, pathomics, and radiomics, among others.

In order to expand the efficacy of ICI and other immune-based agents, several critical hurdles need to be addressed. First, both ICI and co-stimulatory agonists require T cell-infiltration of the TME. While current histological analyses can detect rudimentary TIL levels, they do not necessarily qualify the degree of cytotoxicity or exhaustion.(5) Additionally, most analyses and associated biomarkers are focused on immune cell activity, despite strong evidence for both tumor cell-intrinsic and stromal cell-mediated immune evasion.(15,16) Characterizing the interaction and signaling mechanisms among all cells in the TME will be critical for augmenting ICI therapy. Further, the heterogeneity of each cancer type and within individual tumors themselves has been difficult to capture, yet this heterogeneity is a fundamental source of resistance via subclonal evolution.(17) Other sources of ICI resistance include intracellular proteins that can act as bypass mechanisms to extracellular checkpoint blockade; these downstream mediators are difficult to detect and target.(18) Lastly, neoantigenicity has long been heralded as a key marker of immune activity, yet the ability to detect tumor-specific antigens and translate this measurement to T cell cytotoxicity has been limited.(19)

By defining this needs assessment, the field of IO is poised to apply novel, multi-omic platforms to advance combined immunotherapy strategies. Ideally, the overall objective should be to comprehensively and accurately characterize the TME based on the interaction among immune, stromal, and tumor cells. Using these high-dimensional assessments from tumors of IO-treated patients, the interactions between non-responding versus responding cohorts can be compared to identify novel and translationally relevant resistance mechanisms. Advances in multi-omics technology and robust clinical trial tissue banking have led to an exponential growth in data acquisition, highlighting the primary need for clinically-oriented data analytics and integration. By combining these multi-omics data from IO-treated patients using computational methods and AI-driven strategies, we may be able to more accurately identify and target molecular pathways of resistance and augment existing immune-based therapies in future clinical trials (Fig. 1). Finally, an emphasis on equitable data sharing has enabled large scale AI models from published cohorts, catalyzing the discovery of novel IO combinations.(20)

Established multi-omic platforms used in translational immunotherapy

Whole genome/exome & somatic mutation profiling

On the heels of the Human Genome Project, The Cancer Genome Atlas (TCGA) was launched, laying the groundwork for publicly available, cancer-specific genomic and transcriptomic reference data.(21) With the exponential decrease in NGS costs and collaborative efforts across academic institutions and industrial partners, over 20,000 samples across 33 malignancies were sequenced. At the same time, ICIs were reaching widespread clinical use, and the first generation of high-dimensional tumor data have been mined towards ICI response and prediction strategies. For example, whole-exome sequencing (WES) from an anti-CTLA4 cohort revealed an association between TMB and long-term response.(22) As a prelude to future studies, however, this association alone was not found to be predictive of durable benefit. Instead, nonsynonymous mutations were filtered according to MHC I binding potential and categorized into mutant versus

nonmutant translated peptides. Using both hierarchical clustering and supervised learning platforms, a set of tetrapeptide sequences defined a “neoepitope signature” that successfully predicted response in a validation cohort.(22) Despite a limited number of tumors and utilization of only one omics platform, this study exemplified the strength of combining high-dimensional data with predictive modeling to more accurately connect tumor biology with treatment outcomes. A similar study of PD1 inhibition in NSCLC also demonstrated an association between TMB and therapeutic response, however neither TMB nor a smoking-specific mutational pattern were specific for a durable benefit.(23) Rather, an exome-wide differential analysis of deleterious mutations revealed an explicit pattern in genes involved in DNA repair, such as *POLD1, POLE*, and *MSH2*, the latter tied to mismatch repair deficiency (MMRd). This exome-wide analysis not only identified specific pathway aberrations tied to ICI response, but also foreshadowed a series of studies leading to the tumor-agnostic approval of pembrolizumab for dMMR and microsatellite instability-high (MSI-H) cancers.(24) Though not performed in this study, a computational model combining mutational signatures, total TMB, and histopathological variables may have offered higher predictive power in validation cohorts.

From a clinical standpoint, somatic mutational profiling is now routine practice for most solid tumors. Ten unique oncogenic drivers are now targetable in the treatment of non-small cell lung cancer, for example, and many of these targeted therapies have been approved alongside specific gene panels. In addition to MSI-H, a pan-cancer approval of pembrolizumab was granted for TMB-high (>10 mutations/megabase) solid tumors alongside a companion diagnostic based on the results of KEYNOTE-158.(25,26) While this approval provides an additional therapeutic option in patients without a reasonable alternative, additional biomarkers are still needed to help strengthen the predictive utility of TMB.

Whole transcriptome and gene expression signatures

While genomics can detect deleterious mutations associated with therapeutic response, high-resolution quantification of the transcriptome can provide a more global characterization of tumor biology, part of the overall goal in identifying tumor-intrinsic and extrinsic drivers of immune suppression and immune exclusion. A precursor to RNAseq, researchers first used microarray technologies to perform differential expression analyses between anti-PD1 resistant and wild-type murine models.(27) BMP7, a member of the TGFβ family, was found to be significantly upregulated. Additionally, BMP7 was shown to modulate IFNγ, IL2, MAPK14, and SMAD1; shRNA-mediated BMP7 knockdown restored ICI efficacy, and BMP7 transcript levels were found to negatively correlate with survival in multiple NSCLC cohorts.(27) These results helped build a model by which a biological mechanism of immune suppression was deduced, targeted inhibition was shown to reverse anti-PD1 resistance, and predictive biomarkers were validated from publicly available expression cohorts.

Using banked tumor samples from early phase trials with pembrolizumab, another study developed a gene expression signature after comparing a set of tumor- and immune-related genes in responders versus non-responders.(11) Nearly all top-ranked genes were related

to IFNγ signaling; unsupervised clustering, regression modeling, and validation among nine additional PD1-treated cohorts produced a final 18-gene T cell-inflamed signature. In comparing this signature to the clinical outcomes of a multi-tumor, anti-PD1 clinical trial (KEYNOTE-012), both response and progression-free survival were associated with higher gene expression. This study also confirmed a seemingly paradoxical but homeostatic upregulation of inhibitory molecules (IDO1, PDL1, TIGIT, LAG3) in T cell-inflamed tumors,(28) a finding with important implications for therapeutic combination strategies. Lastly, a similar but separate study took advantage of transcriptome-wide expression data from TCGA, identifying a 160-gene, pan-cancer signature that clustered tightly with established T cell effector genes.(12) This signature, denoting an IFNγ-enriched, inflamed phenotype, was used to stratify tumors into T cell-inflamed and non-T cell-inflamed subgroups.(14) Over 9000 tumors across 30 cancer types were analyzed from publicly available expression cohorts; and as opposed to other studies focused on specific immune cell processes driving T cell suppression, this analysis focused on tumor-intrinsic drivers of immune evasion and identified distinct pathways shared by non-T cell-inflamed tumors across cancer types. For example, the CTNNB1 (Wnt/b-catenin), KLF4, and MYC were found to be upregulated transcriptional programs from non-T cell-inflamed tumors across multiple cancers, with expression significantly higher in PD1-responding patients from a separate melanoma cohort.(14)

Single-cell transcriptomics, TCR sequencing, metabolomics, and the microbiome

Based on these foundational studies and with further advances in technology, a second generation of multi-omic platforms has provided additional insight into the biology of immune excluded tumors. Perhaps of greatest impact, single-cell RNA sequencing (scRNAseq) has enabled high-resolution characterization of cellular heterogeneity in the TME and provided a foundation for additional multiplexing methods.(29) That being said, scRNA sequencing depth is limited for each cell whereas bulk RNAseq can provide an entire transcriptome with fewer logistical barriers. Utilizing both bulk and single-cell technologies, researchers identified a significantly higher proportion of $TREM2^{hi}$ macrophages in non-responding versus responding melanoma cohorts treated with PD1 blockade.(30) These PD1-resistant tumors also comprised a significantly higher subset of γδ T cells, an identifiable target with therapeutic implications. Using predictive modeling and the feature genes from these differentially represented cell types, an ‘ICI outcome signature’ was developed and subsequently validated in multiple bulk expression datasets.(30) In separate studies, however, another subset of γδ T cells was shown to strongly correlate with ICI response in a cohort of dMMR colon cancer patients, and increasing evidence now points to a TCF7+CD8+ T cell population highly correlating with improved clinical outcomes to IO therapy.(31,32) Thus, even with the enhanced resolution of scRNAseq, the results from these studies continue to emphasize the immense complexity of the TME, heterogeneity among cancer types, and need for continued advancement of high-resolution platforms. One of these advancements included sequencing of the T cell receptor (TCRseq) itself as a step towards neoantigen prediction. In a proof of concept study, targeted TCRseq showed a significant correlation between TCR clonality and response, with TCR diversity more prognostic of overall survival in an anti-PD1 treated melanoma cohort.(33) With important implications for biomarker development, additional work demonstrated a greater proportion

of shared clonotypes between intratumoral and peripheral T cells among NSCLC tumors achieving major pathologic response with neoadjuvant ICI.(34) Moving forward, however, more personalized assays would be needed to identify specific sequences tied to response.

Apart from nucleic acid sequencing, an array of other multi-omic platforms have been developed to further characterize the immune microenvironment and identify targetable mediators of ICI response, including metabolomic profiling and microbiome sequencing. Numerous metabolic processes have been tied to T cell dysfunction and ICI resistance, including macrophage-specific arginine catabolism(35) and tumor-intrinsic oxidative phosphorylation.(36) With advances in liquid chromatography–mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR), however, high-dimensional metabolic profiles can be compared between ICI-resistant and -responding populations. For instance, NMR spectroscopy identified a significant enrichment of pyruvate from anti-PD1-resistant patients with NSCLC,(37) LC-MS detected significantly higher levels of kynurenine in a multi-cohort study of anti-PD1 treated patients with decreased survival,(38) and a specific lipid profile was identified in antigen-stimulated type 1 dendritic cells via untargeted mass spectrometry.(39) Lastly, metagenomic profiles of patient stool samples have revealed significant associations between microbial organisms and ICI efficacy.(40) An intratumoral analysis of melanoma samples similarly revealed distinct bacterial taxa correlating with ICI response.(41) Using a murine tumor model, fecal microbial transplant (FMT) with specific bacterial strains from responding patients was shown to reverse ICI resistance,(42) findings recently validated in early phase clinical trials.(43,44)

Novel multi-omics technology and artificial intelligence (AI)

Multiplexing technologies at the single-cell level

The next generation of omics technology will augment the dimensionality of existing platforms, add spatial resolution to expression data, integrate pharmacologic screening programs, and utilize machine learning (ML) and deep learning (DL) algorithms for optimized neoantigen discovery. With the increased accessibility of scRNAseq, multiplexing technologies now provide tiered levels of biological data using the same high-throughput platform. Examples include CITEseq (cellular indexing of transcriptomes and epitopes) and ATACseq (assay for transposase-accessible chromatin) which can tag specific proteins and epigenetic modifications, respectively, alongside single cell transcript expression. Applying this multiplex technology to models of immune resistance, researchers targeted hundreds of immune-related genes and compared high-resolution expression data using a patient-derived, tumor-TIL co-culture platform.(45) This Perturb-CITE-seq screening assay identified known drivers of immune exclusion, including defects in the IFNγ and antigen presentation pathways, but also novel immune modulators such as CD58, an adhesion protein binding macrophages and T cells, whose knockout decreased both T and NK cell effector activity.(45) In another experiment utilizing single-cell ATACseq, specific regulatory networks were identified and shown to promote the exhaustion of cytotoxic T cells from anti-PD1 treated tumors.(46) While perhaps less applicable to ICI augmentation, the results from these studies carry important implications for the design of adoptive cellular therapy in future trials.

Spatial profiling

As NGS technologies continue to evolve, it has become increasingly clear that the spatial context of these data are needed to fully characterize the tumor-immune interface.(47) For example, factors harboring the metastatic potential of different tumors have remained elusive, but advances in digital spatial profiling (DSP) may provide key insights necessary for therapeutic intervention.(48,49) Given the unique biology and high morbidity associated with brain metastases (BrM), researchers used DSP to compare spatial transcriptomics landscape between primary tumor and BrM tissue from a cohort of NSCLC patients.(50) Multiple “environments” were collected for each patient, including primary tumor, tumor-immune interface, brain metastasis, and brain tumor-immune interface, providing a transcriptome landscape across disease states. Compared to primary tumors, BrMs were enriched in cancer associated fibroblasts (CAFs) and M2-type macrophages, while the brain tumor-immune interface showed decreased lymphocyte infiltration and expression of antigen presentation genes. Additionally, transcripts from the primary tumor-immune interface were compared between fast and slow metastasis cohorts, revealing a ‘metastasis gene signature’ which included S100A11 (associated with the pro-tumor cytokine IL-8) and genes encoding adhesion molecules. By localizing transcript data within the tumor-immune interface, specific pathways can be identified and targeted to potentially decrease the risk of BrM development.(50) Further, with PD1 inhibitors used as first-line treatment for advanced NSCLC lacking driver mutations, these results carry important implications for combination strategies in high-risk patients. An emerging biomarker for ICI response, tertiary lymphoid structures have also been identified with DSP technology, with increasing evidence pointing to a critical immunomodulatory role of oligoclonal B cell populations in the peritumoral region.(51)

Multispectral immunofluorescence (mIF) and other imaging modalities also offer high-resolution spatial information,(52–54) yet establishing meaningful patterns within the exorbitant amount of three-dimensional output data has been problematic. Adapting imaging algorithms from the field of astronomy, researchers established relational databases linking single-cell mIF markers with annotated pathology slides of the tumor-host interface.(55) With this impressive breadth of histopathologic variables, predictive modeling identified biomarkers associated with anti-PD1 response from a cohort of melanoma patients. CD8+FoxP3+ T cells were strongly tied to response, despite prior studies showing suppressive activity, while $CD163^{+}PD\text{-}L1^{-}$ myeloid cells, a common phenotypic marker of M2-type macrophages, were associated with therapeutic resistance.(55) While this study accumulated an overwhelming amount of data (>40 TB), it provided a framework by which single-cell proteomic data can characterize tumor-immune interactions and identify drivers of immune exclusion—a critical step forward as computational power continues to grow.

Pharmacologic screening

Though high-dimensional proteomic data can now be used to identify drivers of ICI resistance, pharmacologic targeting remains another challenge for effective combination strategies.(56) Transmembrane proteins can be targeted with cell-surface antibodies and tyrosine kinase inhibitors, but many intracellular proteins (e.g. KRAS) have been deemed “undruggable” for decades.(57) Cbl-b, a ubiquitin-ligase and downstream mediator of CD28

and CTLA4 signaling, is an intracellular protein strongly tied to immune resistance in preclinical models.(58,59) Until recently, pharmacologic inhibition was not possible, but with the advent of DNA encoded libraries (DEL), billions of small molecules can be screened for inhibitory activity against proteins of interest.(60) Using this platform, specific allosteric inhibitors against Cbl-b have been identified for use in both monotherapy and ICI combination trials.(61) Overall, DEL screening helps translate the proteomic data from IO-treated cohorts to novel therapeutic strategies.

Neoantigen identification

While multiple attempts have been made to predict the neoantigenicity of a tumor, specific neoantigen detection has remained challenging. DNAseq and RNAseq can identify thousands of mutations and unique sequences between tumor and host tissue, yet selecting peptides that are both recognized by autologous T cells and elicit a tumor-specific cytotoxic response require additional tools.(62) To develop a predictive model, researchers screened thousands of mutant peptides from individuals with metastatic cancer and identified a subset of 185 neoantigens upon recognition by MHC class I-restricted T cells.(63) Numerous features were profiled and compared between these verified neoantigens and negative candidates, including MHC binding potential, relative expression, variant allele frequency, subcellular localization, and microbial similarity, among others. The resulting ML model was used to calculate an ‘immunogenicity score’ for candidate neoantigen sequences with the goal of more rapid therapeutic targeting.(63) The TCR repertoire can also be used to identify immunogenic antigens in the TME, providing direct access to neoepitope plus HLA data while bypassing the numerous filtering steps between WES to final peptide presentation. Further, the predictive accuracy of HLA genotyping using NGS data alone has been limited.(64) Based on this principle, researchers used a DL algorithm, denoted DeepTCR, to compare the TCR repertoire between ICI responders and non-responders.(65) Notably, antigen-specific signatures were identified in both cohorts, but non-responders were found to exhibit a high turnover of tumor-specific T cells while responders demonstrated a maintenance pool of effector and memory T cells following ICI therapy. Thus, even with a high rate of antigen-specific T cell infiltration, the functional/exhaustive state of these T cells were more predictive of ICI response as compared to the engagement of any one specific neoantigen.(65) As personalized vaccines against tumor associated antigens enter clinical trials,(66) combination strategies may be needed to prevent T cell exhaustion and maintain a neoantigen-specific effector response.

Artificial intelligence

Lastly, ML and DL algorithms can be used to reliably extract features from high-dimensional data and predict treatment response among various anti-neoplastic and immunotherapeutic agents. Starting with a syngeneic murine model, researchers developed a joint dimension reduction framework to incorporate whole transcriptome data, phenotypic variables, and ICI response outcomes across hundreds of tumors.(67) Upon training and validation, this model accurately predicted ICI response in 88% (SE = 1.1%) of treatment-naïve murine samples and also achieved meaningful predictive power with a clinical ICI dataset (AUC = 0.74).(67) Another computational pipeline, ENLIGHT, evolved from an algorithm utilizing synthetic lethality and synthetic rescue genetic interactions to predict

treatment outcomes.(68) Again utilizing transcriptomic data, the ENLIGHT model predicted response (OR > 1) in 19 of 21 cohorts treated with ICI or targeted therapies. While NGS technologies provide the historic standard of biological features, digital imaging has emerged as a more cost-efficient platform for high-throughput, high-resolution data extraction.(69) With a vast increase in feature quantity, however, DL has been utilized as a more sophisticated and efficient route for feature transformation and dimensionality reduction. This form of "self-supervision" permits a substantial number of cell-based features (with or without genetic or drug perturbations) to be used for image-based profiling in a new era of response prediction.(69) In addition to cell-based imaging, DL has been used to build CT-based imaging signatures, extracting radiomic features as an independent biomarker for IO response.(70,71) Moving forward, these tools could inform clinical trial design by eliminating patients least likely to benefit from novel IO agents, increasing the likelihood of reaching a statistical benefit in early phase IO trials.

Conclusions: Data integration and framework for developing immunotherapy combinations

While each advancement in omics technology adds another dimension in characterizing the TME, the greatest benefit towards combined immunotherapy strategies involves the integration of these platforms. A computational, multi-omics assessment of genomic mutations, RNAseq, and proteomic data in classically non-IO responsive tumors (e.g. pancreas, breast) found that lymphocyte infiltration and neoantigen predictions did not correlate with immune invasion.(72) Rather, a DNA damage response protein, ATM, was the primary predictor of immunogenic potential, providing rationale for ongoing trials with innate immune agonists in combination with ICI.(72,73) Another study of 80,000 TIL from pancreatic tumors integrated scRNAseq, scTCRseq, and *in vivo* assays to identify specific features of dysfunctional T cell phenotypes, potentially targetable features to augment adoptive TIL therapy.(74) Importantly, certain aspects of IO resistance remain difficult to measure, including T cell exhaustion and anti-inflammatory immune cell phenotypes—i.e. omic platforms are only useful if there are identifiable data points to collect. By integrating multiple, indirect features associated with various immune phenotypes, however, a more global and accurate characterization can be gathered. Even key clinical variables (e.g. sex) have become controversial predictors of IO response, requiring a more comprehensive analysis of molecular and demographic profiles using multi-omic and AI platforms, with critical implications for trial design.(75)

Moving forward, we propose a framework (Fig. 2) by which *in silico* screening of integrated, multi-omics data can nominate resistance pathways from existing IO-treated cohorts. Next, genetic knockdown assays using specialized organoids and even organ-on-a-chip models can provide functional validation.(76) Clinical trials utilizing these novel targets in combination with existing IO therapy will also collect multi-omics data in addition to safety and response assessment. Finally, in repeating this cycle, computational models can be built using prospective outcomes data and multi-omic variables to identify biomarkers of response and resistance to these novel combination therapies.

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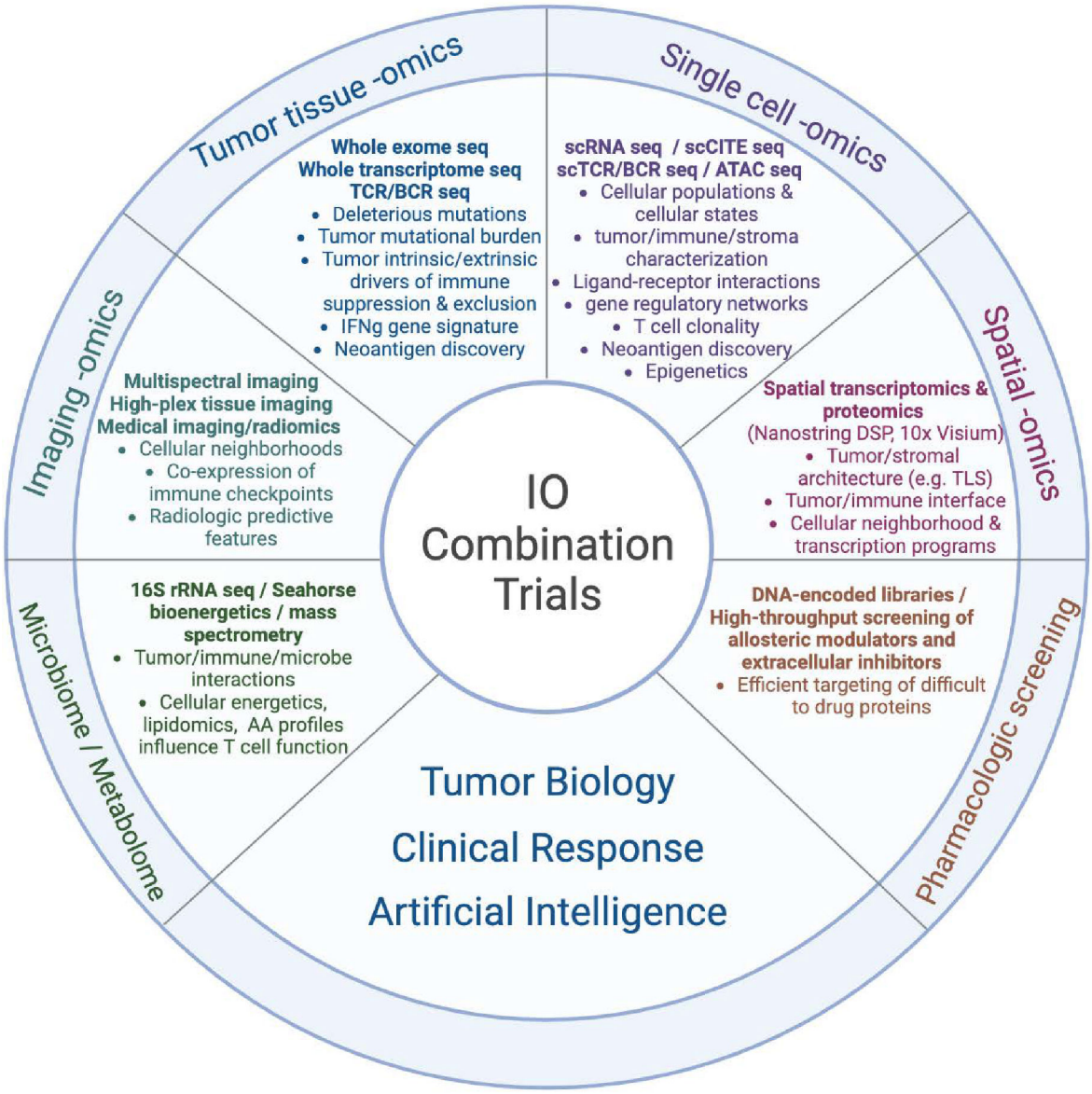

Figure 1.

An array of multi-omics technology and AI platforms can be used to characterize the tumor-immune-stromal interface, identify resistance pathways, predict clinical response, and catalyze a new era of combined immunotherapy strategies.

Tumor tissue omics: Includes high-throughput sequencing of the tumor genome, exome, and transcriptome. Specific mutations in the tumor DNA can be detected through whole-genome sequencing (WGS), WES, targeted sequencing (e.g., via companion diagnostic (CDx)), among others. RNAseq of the tumor transcriptome can detect differentially expressed genes in clinically relevant samples that may correspond to targetable pathways. The expression of IFN**γ** based gene signatures can also be used to categorize tumor samples into T cell-inflamed versus non-T cell-inflamed phenotypes and identify the upregulation of pathways specific to immune exclusion or ICI resistance. Sequencing of the T cell receptor

(TCR) or B cell receptor (BCR) may indirectly identify tumor-specific T cell or B cell clonal populations. Neoantigens can be predicted from integration of DNAseq (mutations), RNAseq (expression), and potentially mass spectrometry-based proteogenomics.

Single cell omics: High-throughput profiling of individually isolated cells provides transcript sequence and abundance from specific cell types, providing a high resolution of the tumor microenvironment. Advances in multiplexing technologies can further quantify cell-surface protein expression (CITEseq), TCR/BCR repertoire, and chromatin accessibility (ATACseq) in addition to single cell transcriptomics. By increasing the resolution of gene and protein expression to the cellular level, differentially expressed pathways can be specifically identified within the tumor, immune, and stromal cell populations to further characterize molecular mediators of immune exclusion in the TME. Further, cell surface protein expression via CITEseq can provide additional phenotypic data to determine cell types. TCR sequencing of infiltrating lymphocytes can identify neoantigen-specific TCRs and allow engineering of neoantigen-specific TCRs *in vitro*.

Imaging omics: An array of technologies including multiplex immunofluorescence, codetection by indexing (CODEX), iterative indirect immunofluorescence imaging (4i), and imaging mass cytometry (among others) now permit dozens of unique protein markers to be digitally measured from fixed tumor tissue. On the macroscopic level, thousands of features from radiologic body imaging can be computationally extracted to build machine learning models in prediction of clinical outcome.

Spatial omics: As another platform to characterize tumor-immune-stromal interactions, both Nanostring digital spatial profiling (DSP) GeoMx and 10X Visium can be used to map gene expression values to fresh frozen or formalin-fixed paraffin embedded (FFPE) tissue, identifying pathways of immune exclusion at a geographical level along with cellular neighborhoods that may be associated with clinical outcomes. Moreover, single-cell resolution has been achieved by Nanostring CosMx single-molecule imaging (SMI), 10x Xenium, and Vizgen MERSCOPE (Multiplexed Error-Robust Fluorescence in situ Hybridization, MERFISH). These technologies profile several hundred to a few thousand targeted genes within individual cells in a spatial context. This advancement allows for a deeper understanding of cell-to-cell communication and interactions between ligands and receptors in cellular neighborhoods of TME.

Metabolomics and the Microbiome: In addition to gene and protein expression, a number of metabolic platforms have been developed to measure parameters of cellular energetics, including oxygen consumption, extracellular acidification, and lipid and amino acid profiling. Comparing the microbiota of ICI responders versus non-responders using 16s rRNA sequencing, metagenomics, and metatranscriptomics has also identified unique taxa correlated with clinical outcomes.

Pharmacologic screening: While multi-omic platforms can be used to identify novel pathways of IO resistance, advances in pharmacologic screening are required to target these pathways in clinical trials. DNA encoded libraries allow high-throughput screening of millions of compounds to efficiently identify candidate agents to target historically “undruggable” proteins.

Artificial intelligence: Machine learning (ML) and deep learning (DL) have been pivotal in driving our understanding and therapeutic approaches to diseases at a personalized level, including but not limited to, multimodal data integration across radiology scans,

pathology images, electronic health records (EHR), and multi-omics; predictive modeling on patient stratification/selection, adverse event monitoring, and immunotherapy treatment response; new immunotherapeutic agent discovery or drug repurposing from in vitro screens and/or cell imaging; and foundation models for medical imaging segmentation, single-cell phenotypic annotation, and drug discovery from chemical language representations. As technology and data evolve, it's anticipated that AI will play an even more significant role in propelling these fields forward.

IO, immuno-oncology; sc, single-cell; seq, sequencing; CITE, cellular indexing of transcriptomes and epitopes; TCR, T cell receptor; BCR, B cell receptor; ATAC, assay for transpose-accessible chromatin; DSP, digital spatial profiler; TLS, tertiary lymphoid structure; IMC, imaging mass cytometry; CODEX, co-detection by indexing; AA, amino acid

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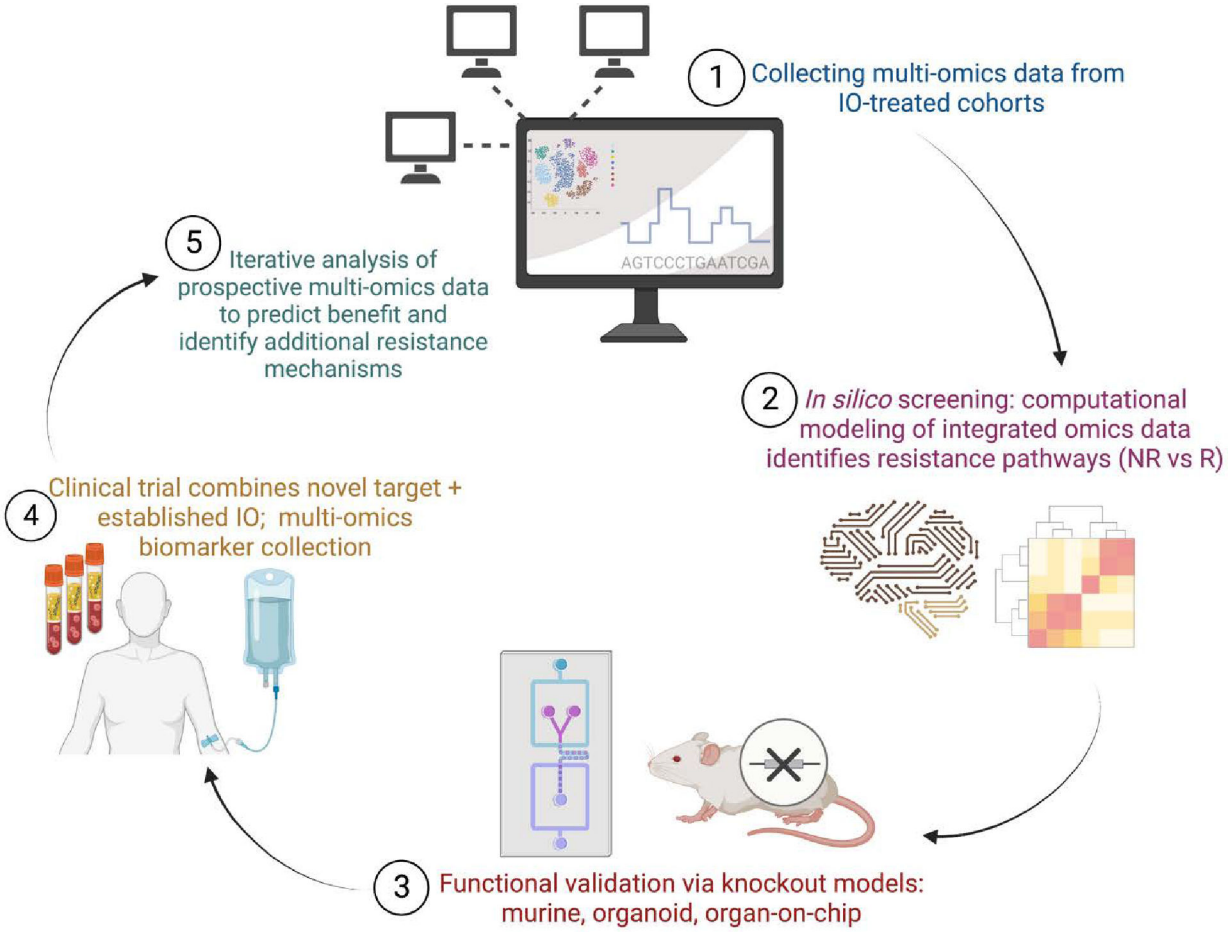

Figure 2.

Framework for utilizing multi-omics data to advance immunotherapy combination strategies. An iterative cycle of *in silico* screening, functional validation, and clinical trial biomarker collection can be used to identify novel resistance pathways, develop predictive models of benefit, and highlight additional resistance mechanisms.

In step 1, multi-omics and/or image data is collected from clinically annotated or biologically distinct patient cohorts, using bulk RNAseq, single-cell RNAseq, multispectral imaging, spatial profiling, among others. These data are computationally integrated and compared between IO-non-responders and IO-responders along with non-T cell-inflamed versus T cell-inflamed tumors samples. This *in silico* screening identifies candidate genes and molecular pathways that may be associated with IO-resistance and/or immune exclusion for therapeutic target nomination (step 2). In step 3, functional assays are used to experimentally validate the immune-exclusive role of these candidate genes/pathways and assess novel inhibitors and other agents. Based on these preclinical data, early phase clinical trials combine novel pathway agents with established IO therapies; clinical outcome along with translational biomarker data is collected (step 4). In returning to the start of the cycle (step 5), multi-omics biomarker data from these novel clinical trials are used to identify predictors of response as well as additional genes/pathways tied to therapy resistance.

IO, immuno-oncology; NR, non-responder; R, responder

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