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TCR sequencing in cancer immunology and immunotherapy: what, when, where, why, and how

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ABSTRACT

T-cell receptors (TCRs) are generated through somatic recombination of variable (V), diversity (D), and joining (J) gene segments, resulting in an extraordinarily diverse receptor repertoire that is essential for immune surveillance and host defense. TCR sequencing (TCR-seq) has emerged as a powerful tool for comprehensive characterization of the adaptive immune repertoire, offering deep insights into T-cell diversity, antigen specificity, and clonal dynamics.

TCR-seq enables the tracking of T-cell clones across both temporal and spatial dimensions. From a longitudinal perspective, it allows for the monitoring of clonal dynamics before and after therapeutic interventions or over the course of disease progression. Temporal shifts in clonal composition can reveal the persistence, contraction, or expansion of specific T-cell populations, thereby providing valuable information on the durability of immune responses and the efficacy of treatments. From a spatial standpoint, TCR-seq facilitates comparative analyses of repertoires across distinct anatomical compartments, including tumors, blood, and lymph nodes. Such analyses yield insights into tissue-specific immune responses, T-cell trafficking, and infiltration patterns. Moreover, the ability to track antigen-specific T-cell clones enables the visualization and quantification of tumor-specific immune responses. Advances in spatial TCR-seq now integrate spatial context with clonal identity and repertoire diversity, further illuminating complex immune architecture within tissue microenvironments. Nonetheless, despite the development of various approaches for antigen specificity prediction, further advances are needed to improve their accuracy and generalizability.

A wide range of TCR-seq platforms are currently available, including DNA-based and RNA-based protocols, short-read and long-read sequencing technologies, and bulk and single-cell approaches. Each method presents unique advantages in terms of resolution, throughput, cost, and biological relevance. For instance, DNA-based TCR-seq is well suited for longitudinal tracking of clonal populations, whereas RNA-based approaches are advantageous for detecting actively transcribed, antigen-responsive clones. Short-read sequencing offers high-throughput capabilities, while long-read and paired-chain sequencing provide comprehensive structural and functional information on TCRs. Additionally, computational methods, including machine learning algorithms and motif-based clustering, are increasingly employed to infer antigen specificity directly from TCR-seq data.

In this review, we examine the current landscape of TCR-seq through the lenses of what, when, where, why, and how, highlighting recent technological developments and emerging applications that are shaping the field of immune repertoire analysis.

INTRODUCTION

The what: defining the T-cell receptor and its generation

The T-cell receptor (TCR) is an antigen-specific receptor expressed on the plasma membrane of T cells. It is a transmembrane protein complex composed of two chains: either an α and β chain or a γ and δ chain.[1 2] αβ-T cells, including $CD4^+$ and $CD8^+$ T cells, express αβTCR. When these TCRs bind to antigens presented on major histocompatibility complex (MHC) molecules of antigen-presenting cells, they trigger an immune response.[3 4] αβ-T cells play central roles in adaptive immunity by recognizing antigens via TCRs.[5] In contrast, γδ-T cells express γδTCRs and recognize non-peptide antigens in an MHC-independent manner.[6] Thus, γδ-T cells are key components of innate immunity and contribute critically to the early response against invading pathogens.[7] Each T cell typically expresses a unique TCR that determines its antigen specificity. TCRs consist of variable and constant regions. This variable region contains three complementarity-determining regions (CDR1, CDR2, and CDR3) that form the antigen-binding site. The germline-encoded CDR1 and CDR2 loops contact the conserved helical residues of the MHC, whereas the highly variable, somatically recombined CDR3 loops primarily interact with the peptide (figure 1a).[8]

The variable region is generated during T-cell development in the thymus through genomic rearrangement of multiple gene

segments: variable (V), diversity (D; β chain only), joining (J), and constant (C) segments.[3 9 10] This V(D)J recombination process creates extensive TCR diversity. Notably, the diversity of the CDR3 region largely depends on this recombination process. Additionally, nucleotide insertions and deletions at junctions during recombination further increase this diversity.[11] Although CDR2β can play an important role in cross-reactivity and recognition patterns for specific epitopes,[12] the most variable region, the CDR3, located within the antigen-binding domain, largely determines the TCR's antigen specificity.[13] Therefore, TCR sequencing (TCR-seq) primarily assesses the diversity of T-cell clones by analyzing the CDR3 sequence.[14] During T cell development, β chain recombination is initiated first, and a successfully rearranged β chain subsequently triggers α chain recombination. This sequential order ensures the proper assembly and expression of the TCR. Following successful β chain rearrangement, recombination at the second β allele is inhibited, a mechanism referred to as allelic exclusion, thereby allowing each T cell to predominantly express a single β chain. In contrast, α chain allelic exclusion is incomplete, and a subset of T cells may express two α chains.[15]

Although V(D)J recombination can theoretically generate up to 10^{15} to 10^{18} possible TCRs, only 10^{7} to 10^{8} unique clonotypes are actually observed in humans.[16] This discrepancy reflects multiple biological constraints, including thymic negative selection, recombination efficiency, the finite number of T cells in the body, and clonal expansion biases in the periphery. An important compensatory mechanism is TCR cross-reactivity, in which a single TCR can recognize multiple distinct peptides, thereby enabling the limited TCR repertoire to cover an enormous diversity of antigens.[16–18]

The why: impact of evaluating TCR on immunology questions

Since the TCR determines the capacity of T cells to recognize pathogens and cancer antigens, analyzing the complete TCR repertoire is critical for understanding the immune system in contexts such as cancer immunotherapy, vaccine response assessment, autoimmune diseases, and infectious diseases.[3 19 20] In particular, in the field of cancer immunology, TCR-seq enables a wide range of analyses, including the assessment of TCR diversity and clonality, longitudinal and spatial tracking of the specific T-cell clones, evaluation of immune responses during immunotherapy such as T-cell clonal expansion and repertoire skewing, and inference of antigen specificity (figure 1b). In summary, leveraging the unique identifiers of each T cell, the TCR, enables a high-resolution dissection of the immune system's "fingerprint."

There are two types of TCR-seq: bulk TCR-seq and single-cell TCR-seq.[3] Bulk TCR-seq is cost-effective and can sequence millions of cells, but except for certain statistical inference approaches, it is generally unable to determine which TCR α and β chains are paired. Consequently, accurate tracking of antigen-specific clonotypes is challenging, and since some TCR clusters are TCR α-dominant while others are β-dominant, bulk sequencing relying solely on TCR β chains may fail to capture certain T cells.[21] Single-cell TCR-seq, while more expensive and limited to a few thousand cells, can identify paired TCR chains.[22] The advent of single-cell technologies has brought two major advantages to TCR analysis. First, the integration of RNA sequencing (RNA-seq) with TCR-seq has enabled the association of TCR gene pairs with specific cell types and functional states[23 24] and allows the identification of low-frequency clones and rare subsets that would likely be masked in bulk analyses. Second, evaluating TCR clonality and diversity at the single-cell level facilitates molecular tracking of T-cell trajectories, clonal dynamics, and phenotypic changes during immunotherapy.[25 26] While bulk TCR-seq can quantify clonal expansion by measuring changes in clone frequencies, single-cell TCR-seq enables the simultaneous assessment of clonal expansion and the phenotypic states of the expanded clones, providing deeper insights into their biological relevance. Moreover, this approach allows for the monitoring of neoantigen-specific T cells across different time points and tissues before and after treatment with immune checkpoint inhibitors.[27 28] In recent years, throughput-intensive rapid TCR library sequencing (TIRTL-seq) has been introduced as an approach that addresses the limitations of both bulk and single-cell TCR-seq, enabling paired TCR α and β chain sequencing without single-cell isolation and facilitating quantitative analyses of antigen-specific TCR clones in large-scale samples.[29] Thus, TCR-seq has become a powerful tool for comprehensively understanding immune system dynamics and aiding disease diagnosis, treatment, and prognosis prediction.[26 30 31] Looking ahead, advancements in TCR-seq analytical techniques will improve the precision of personalized immunotherapy and disease monitoring, significantly impacting future medical care. This review will present the latest findings and data analysis methods related to the potential of TCR-seq, focusing mainly on tumor immunity.

The how: TCR-seq profiling methodologies, DNA versus RNA, short versus long reads, and αβ+Vβ versus Vβ-alone

Several experimental and computational approaches are available for analysis of TCR-seq; however, each is optimized for specific research and clinical applications.[22] These methods can be categorized based on the template used as source material for sequencing (DNA vs RNA), read length (short vs long read), and chain resolution (paired αβ sequencing vs Vβ-only profiling or, more rarely, Vα sequencing).[32]

DNA-based TCR-seq amplifies recombined TCR genes directly from genomic DNA (gDNA), providing a stable representation of all T-cell clonotypes, including non-expressed or recently inactivated TCRs. The quantification of total clonotype abundance is independent of transcriptional activity (PMC10163020). While DNA provides stable copy numbers per cell, it includes non-rearranged V and J segments that reduce amplification

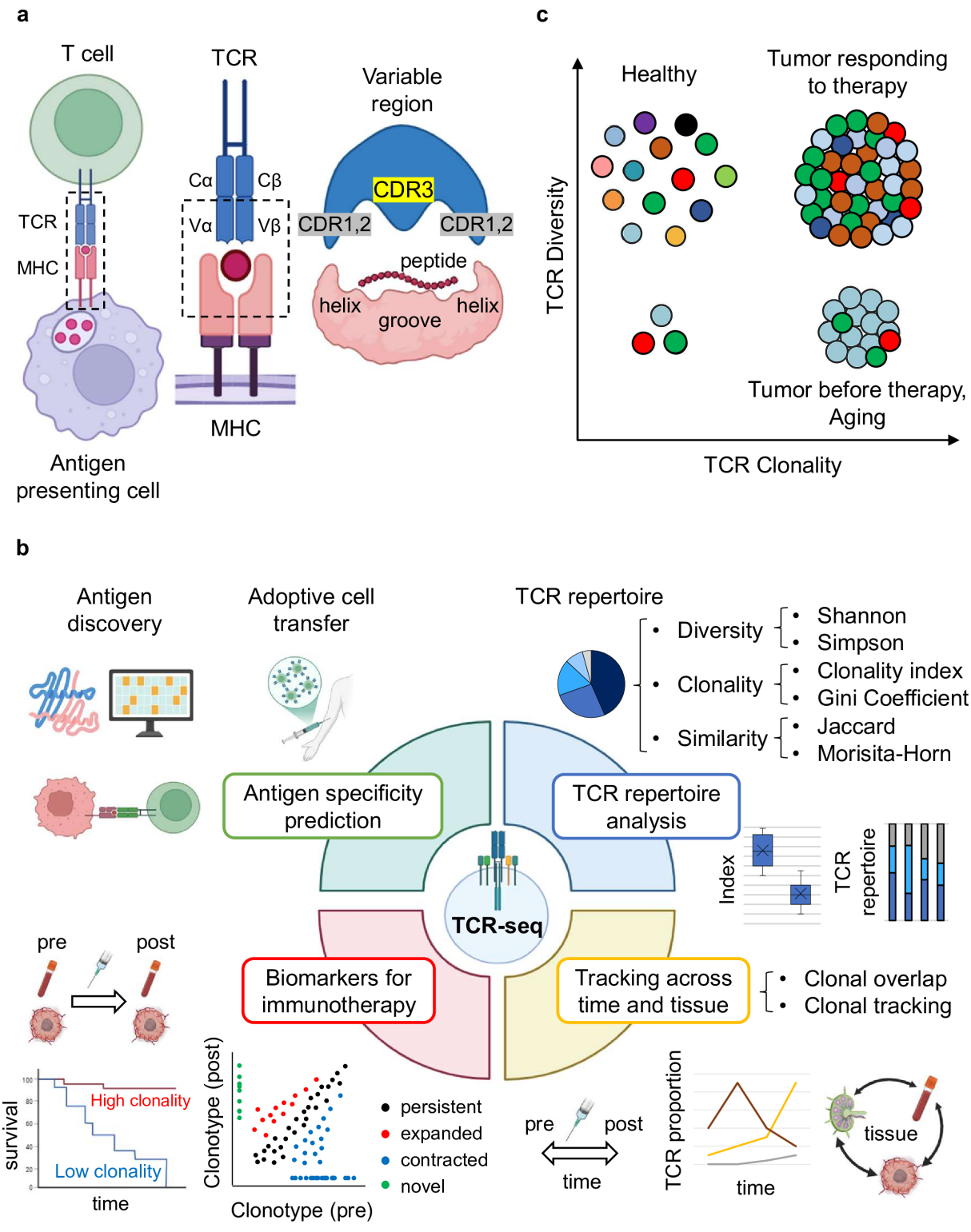

Figure 1 Overview of T cell receptor (TCR) structure and the potential of TCR sequencing (TCR-seq) for repertoire analysis. (a) Schematic representation of the TCR bound to a peptide–MHC complex. The variable region of TCR contains three complementarity-determining regions (CDR1, CDR2, and CDR3) that form the antigen-binding site. The germline-encoded CDR1 and CDR2 loops of both TCR α and β chains mainly contact the α-helices of the MHC molecule, while the highly variable CDR3 loops predominantly interact with the central region of the presented peptide, conferring antigen specificity. (b) Overview of the potential of TCR-seq and TCR repertoire metrics. TCR-seq primarily enables TCR repertoire analysis, TCR tracking, prediction of immunotherapy response, and antigen specificity prediction. Each section presents an overview diagram and commonly used plots for that specific aspect. (c) The concept of individual TCR clones unique to each T cell is illustrated using TCR clonality on the X-axis and TCR diversity on the Y-axis. Specific characteristic states associated with each region are also indicated. Dots of different colors represent distinct TCRs. MHC, major histocompatibility complex.

efficiency. RNA-based approaches, in contrast, reflect the expressed TCRs and allow the quantification of clonal frequencies and abundances.[33] Thus, DNA-based TCR-seq is commonly used in immune repertoire tracking, minimal residual disease detection, and TCR evolution studies. A significant limitation is that DNA-based TCR-seq cannot distinguish functional (expressed) vs non-functional TCRs.[33] On the contrary, RNA-based TCR-seq uses full-length TCR transcripts from messenger RNA, capturing only actively expressed or transcribed TCRs. RNA is also less stable, which limits its use from frozen or fixed samples. This approach provides better sensitivity for detecting rare or expanded clones compared with DNA-based approaches. It can be integrated into single-cell immune profiling, tumor-infiltrating lymphocytes, and vaccine response assessments. The largest drawback is that this method is prone to bias due to variable TCR gene expression levels across different T-cell subsets.[33]

Short-read TCR-seq uses Illumina sequencing (~150–300 bp reads) to cover V(D)J regions, often combined with UMI barcoding for quantitative clonotype analysis and longitudinal tracking when possible. This is the most widely used approach because it is high-throughput and cost-effective, besides its compatibility with bulk and paired single-cell TCR-seq. A potential limitation for this approach is the moderately intensive computational assembly of V(D)J sequences, which can also lead to errors in hypervariable regions when not carefully performed.[34 35] Also, another complicating variable consists of amplification biases due to primer efficacy. A technology still in development is long-read TCR-seq (PacBio, Oxford Nanopore), which captures full-length TCR-seq (>600 bp) without fragmentation, preserving complete V(D)J junction information. In theory, this is a more accurate approach in resolving hypermutated or complex TCR rearrangements and can be used for detailed clonotype analysis, repertoire reconstruction, and identifying novel TCR variants. However, currently, this technology is limited to lower throughput and higher error rates compared with short-read approaches.[36]

Paired αβ TCR-seq is the preferred approach to simultaneously sequence TCRα and TCRβ chains, preserving the natural pairing between the two chains. This is essential for TCR functional analysis since correct αβ pairing determines antigen specificity.[37] However, amplification of only the TCRβ chain, which has greater diversity compared with TCRα and contributes most to antigen recognition, is a simpler, cheaper, and widely used alternative to paired αβ with the limitation of reduced antigen specificity.[38] The choice of TCR-seq method depends on the specific hypothesis. DNA-based methods provide a stable record of T-cell clonality, while RNA-based methods target active TCRs. Long-read sequencing enables full-length TCR reconstruction, while short-read sequencing enables high-throughput repertoire profiling. Finally, paired αβ TCR-seq provides functional pairing information, while Vβ-only sequencing offers a cost-effective solution for diversity studies. A more complete reconstruction of CDR3 regions and their associated genes offers the advantage of going beyond mere diversity metrics, providing insights into antigen specificity by identifying similarities in complementary regions at the protein level, even when nucleotide sequences differ. Machine learning (ML)-based repertoire reconstruction and long-read single-cell TCR-seq will continue to improve precision and depth of TCR profiling for immunotherapy, vaccine development, and immune monitoring.

The what: TCR repertoire metrics (evaluation of diversity, clonality, and similarity)

The major metrics used to evaluate TCR repertoire are diversity, clonality, and similarity (figure 1b upper right).[30 39] TCR diversity and clonality are influenced by multiple factors such as genetics, development, immune responses, disease states, treatments, and aging. First, diversity measures the breadth of TCR variation, reflecting how many different antigens the immune system can recognize. High diversity indicates a robust ability to respond to a wide range of pathogens and tumor antigens.[40 41] More specifically, diversity comprises two components: *richness*, which is the total number of unique clones, and *evenness*, which represents how evenly distributed the clone frequencies are. Clonality, by contrast, quantifies the dominance of specific TCR clones, reflecting the proliferation and expansion of particular T-cell clones during an immune response. High clonality is often associated with a focused and strong immune reaction to a specific antigen, including antitumor activity.[41–43] Importantly, diversity and clonality are not simply opposite ends of a spectrum but rather complementary metrics, where a balance between them is crucial. Generally, high clonality with low diversity typically reflects the selective expansion of specific T-cell clones, as seen in cancer, infectious diseases, or aging.[44] Conversely, low clonality with high diversity corresponds to a healthy immune system capable of mounting broad responses to diverse antigens.[45] When immunotherapy is effective in cancer patients, specific T-cell clones proliferate selectively while overall TCR diversity is maintained, resulting in high clonality and high diversity being achieved simultaneously in some cases[46 47] (figure 1c). Finally, TCR similarity (overlap) measures the degree of resemblance between TCR repertoires, often used to assess similarities within the same population before and after treatment or between different tissues.[48 49]

Diversity metrics

Rényi entropy, which encompasses both the Shannon and Simpson indices, is commonly used to evaluate TCR diversity in cancer immunology. The measure of diversity provided by Rényi entropy depends on the variable α[50], which adjusts the emphasis placed on clones of different abundances. As α increases, the diversity measure increasingly reflects the influence of highly abundant clones.[51] When α approaches 1, Rényi entropy converges to the Shannon index, and when α equals 2, it corresponds to

the Simpson index.[26 52] The Shannon index evaluates the uniformity of clone distribution and is typically used to assess overall clonal diversity. In contrast, the Simpson index focuses on the dominance of specific clones and is useful for examining clonal expansion. Additionally, when rare clones are of particular interest, the Chao index can be employed to estimate the number of undetected rare clones.[26]

Clonality metrics

Clonality is commonly evaluated using metrics such as the Clonality index and Gini coefficient.[26] The Clonality index is defined as 1 minus Pielou's evenness index, which is a normalized form of Shannon index (H).[53] Specifically, the Clonality index reflects the dominance of particular T-cell clones, while Pielou's evenness index measures the uniformity of the TCR clone distribution. A low Pielou's evenness index indicates clone skewing due to the biased expansion. The Gini coefficient, on the other hand, quantifies the inequality or imbalance in clone sizes. The Gini coefficient is commonly used as an indicator of inequality in the distribution of income or wealth. Specifically, countries with greater social disparities tend to exhibit higher Gini coefficients, whereas those with lower inequality display lower Gini coefficient values.[54] Thus, the Clonality index quantifies the overall degree of clonality, Pielou's evenness index assesses how evenly clones are distributed, and the Gini coefficient captures the extent to which certain clones dominate the repertoire.[55]

Similarity (overlap) metrics

The Jaccard index, which evaluates the proportion of shared clones between two repertoires, and the Morisita-Horn index, which measures the degree of overlap considering clone frequency distributions, are commonly used to assess TCR repertoire similarity between pairs of populations in immunology.[26 30 50 56 57] The Jaccard index focuses on whether clones are shared but does not account for clone frequency, making it sensitive to rare clones. In contrast, the Morisita-Horn index incorporates clone frequency distribution, thus giving more weight to high-frequency clones.

Various metrics exist for analyzing T-cell repertoires, and their optimal use depends on factors such as sample type and size, requiring careful validation. Given that various TCR repertoire metrics and evaluation methods capture different facets of the repertoire, employing multiple complementary metrics rather than relying on a single measure is advisable to mitigate potential oversights and biases in TCR repertoire assessment. Accurate evaluation of TCR repertoire metrics requires careful consideration of several factors.[18] Cross-reactivity and convergent recombination mean that the number of observed clonotypes does not directly reflect the number of functional antigen specificities. Sampling limitations, such as restricted sample volume, the practical upper limit on the number of cells, and sample loss during experimental procedures, can lead to underrepresentation of low-frequency clones, and statistical inference methods have intrinsic constraints. Thus, adjusting sequencing depth across samples and combining empirical data with refined models of clone size distributions are essential for a more precise understanding of repertoire diversity and immune coverage.

The when and the where: role of TCR-seq as a tracking tool for T cells, across time or across tissues

The TCR repertoire can serve as lineage tags to track T cells (figure 1b lower right).[58] As T cells dynamically proliferate, differentiate, and migrate across different time points and tissues, TCR-seq provides a valuable tool to capture these immune responses by distinguishing individual T-cell clones based on their unique TCR-seq.[16 25] Common tracking approaches include TCR clonal overlap analysis and TCR clonal tracking. Clonal overlap analysis, using metrics introduced earlier, evaluates the similarity of TCR repertoires between different time points and tissues. This overlap can also serve as a biomarker for the therapeutic efficacy of immunotherapy, as discussed in the next chapter. Clonal tracking, on the other hand, visualizes how specific clones expand or contract over time by following identical CDR3 sequences in longitudinal data or by depicting trends in clone frequencies.[31 59] Additionally, specialized TCR tracking methods combine spatial transcriptomics and imaging techniques to map the precise location of TCR clones within tissues.[60 61] Such spatial analyses of T-cell clones offer promising new dimensions for immunological research.

Tracking tool for T cells across time

TCR-seq is a useful tool for longitudinally tracking dynamic changes in T cells. Overall clonal trends can be assessed by quantifying the similarity of TCR repertoires within the same samples across different time points.[48 55 62–65] Conversely, the trajectory of individual clones can be monitored through TCR tracking, which follows specific CDR3 sequences and their clone frequencies over time.[31 47] More recently, simultaneous single-cell analysis of TCR-seq and gene expression has enabled detailed characterization of functional changes in subsets such as tumor antigen-specific, effector, and memory T cells.[28 66]

Tracking tool for T cells across tissues

TCR-seq enables comparison of TCR repertoires across different tissues, allowing us to determine the tissue origins of T cells and identify which tissues are actively responding to treatment.[23 42] Specifically, analyzing TCR overlap between tissues can indirectly reveal T-cell migration patterns,[55 67–71] while examining the enrichment of skewed clones can highlight tissue-specific clonal expansions.[72] Additionally, antigen-specific T-cell localization can be inferred by matching TCR-seq with public databases.[73] Beyond comparisons across tissues and time points, integration with single-cell RNA-seq facilitates

detailed characterization of T-cell phenotypes and transcriptional profiles.[27 59 74] More recently, combining TCR-seq with spatial transcriptomics has enabled simultaneous analysis of T-cell spatial distribution and gene expression.[61] For example, T-cell clones identified as hyperexpanded by TCR-seq have been visualized within tissue using a combined multiplex immunofluorescence–RNA in situ hybridization approach.[75] Furthermore, the STARTRAC method (single T-cell analysis by RNA-seq and TCR tracking) provides a powerful approach to analyze T-cell clonal expansion, migration, differentiation, and diversity, offering deep insights into the temporal and spatial dynamics of immune responses.[58 76] In summary, by comparing TCR repertoires across time and tissues through TCR-seq, we can now determine which T cells respond, when and where they act, and to what extent, thereby mapping the temporal and spatial behavior of T cells.

The why: role as a biomarker and monitoring for immunotherapy

ICI therapy has revolutionized oncology and is now an established therapeutic option for cancers.[77] However, its efficacy varies significantly among individuals, underscoring the need to identify patient subgroups most likely to benefit from ICI treatment. With advances in TCR repertoire analysis, numerous studies have reported associations between TCR repertoire features and treatment outcomes. In this context, we have summarized previous findings on the relationship between the TCR repertoire metrics and ICI efficacy or survival, categorizing each study by the type of TCR repertoire metrics used, the timing of assessment (pre-treatment or post-treatment), and the tissue source (tumor or blood) (tables 1 and 2, and figure 1b, lower left).

Diversity and response to immunotherapy

Patients exhibiting high TCR diversity in blood before ICI therapy have been suggested to demonstrate improved therapeutic responses, likely due to the increased probability of possessing T-cell clones capable of recognizing tumor-associated antigens within a broader TCR repertoire. Indeed, several studies have reported that higher peripheral TCR diversity at baseline is associated with improved ICI efficacy and prognosis.[62 78–82] Moreover, analyses of tumor and blood samples before and after ICI treatment have shown that responders exhibit a significant increase in TCR richness and a decrease in evenness.[43 46 83–86] Conversely, other studies have suggested that a reduction in TCR diversity, or a limited increase in diversity, is associated with favorable treatment outcomes.[69 87 88] Additionally, there are reports indicating no clear association between TCR diversity and treatment efficacy, either at baseline or after therapy.[47 63 89–92] Reports on changes in TCR diversity before and after treatment remain limited. Furthermore, many of these studies have methodological limitations, such as ambiguous definitions of TCR metrics, reliance on non-significant trends, extremely small sample sizes, or inclusion of cohorts with prior anti-CTLA-4 therapy. Therefore, these findings should be interpreted with caution.

Clonality and response to immunotherapy

The association between baseline TCR clonality and treatment response appears to differ depending on the tissue. In tumors, higher baseline TCR clonality has been associated with improved treatment outcomes,[39 56 64 65 93–95] whereas in blood, lower baseline clonality has been linked to better responses.[90 96 97] However, several studies have reported no significant association between baseline TCR clonality and treatment efficacy.[48 56 57 64 84 96 97] These contrasting findings may reflect dynamic T-cell trafficking between tissues before and after therapy. Importantly, many studies have emphasized changes in TCR clonality post-treatment, often observing increased clonality in both blood and tumor tissues among responders to ICIs.[42 48 62 65 93 94 97 98]

In summary, the majority of studies have reported that high baseline TCR diversity and low clonality in blood are associated with favorable treatment outcomes and prognosis, whereas in tumors, high baseline TCR clonality is more frequently linked to better clinical responses and prognosis. Subsequently, numerous studies have demonstrated that, following immunotherapy, selective expansion of specific T-cell clones occurs in responders, leading to increased TCR clonality in both blood and tumors. In contrast, changes in TCR diversity after treatment require the most careful interpretation. Although TCR diversity is generally inversely correlated with clonality, when focusing on changes before and after treatment, it is important to consider not only the expansion of pre-existing T-cell clones but also the influx of newly recruited clones. Thus, diversity and clonality should be regarded as complementary parameters. Indeed, immunotherapies may promote the expansion of specific T-cell clones while preserving broad antigen recognition, potentially leading to concurrent increases in both metrics (figure 1c). Furthermore, dynamic patterns have been observed in mice, where TCR diversity transiently decreases following anti-CTLA-4 antibody treatment, followed by a subsequent increase.[99] Accordingly, the interpretation of TCR repertoire dynamics is highly influenced by factors such as the timing of therapy and sample collection, methodology used to quantify repertoire metrics, treatment regimen, age of patients, and the limited sample sizes. As a result, a definitive consensus has yet to be established. Therefore, it is essential to clearly define the indices used for each metric and to evaluate TCR repertoires using multiple complementary parameters.

Temporal changes in clonotypes (expanded, contracted, and novel clones)

By profiling expanded, contracted, persistent, and novel T-cell clones before and after treatment, the dynamic changes in the T-cell repertoire can be characterized (figure 1b, lower left). A key area of ongoing debate is

Table 1 Pretreatment TCR repertoire metrics in relation to treatment efficacy and prognosis

cancer type	num	ICI type	SMP	pre/post	metrics	results	depth	ref
melanoma	n=12	CTLA4	blood	pre	diversity	responder: high richness, high evenness	N/A	80
melanoma	n=38	PD-1	blood	pre	diversity	responder: low diversity	N/A	78
melanoma	n=42	CTLA4	blood	pre	diversity	responder: high diversity	N/A	78
melanoma	n=17	CTLA4	blood	pre	diversity	responder: high restricted TCR Vβ-gene (CD4)	N/A	79
NSCLC*	n=40	PD-1 or PDL1	blood	pre	diversity	responder: high diversity (PD-1^{+}CD8)	N/A	62
NSCLC	n=93	PD-1/CTLA4	blood	pre	diversity	responder: high richness	normalization	81
Solid tumor	n=14	PD-1 or PDL1	blood	pre	diversity	responder: high diversity	Frequency-based correction	82
GI* cancer	n=125	PD-1	blood	pre	diversity	no difference	N/A	63
MDS*	n=37	CTLA4 and/or PD-1	blood	pre	diversity	no difference in richness	N/A	90
melanoma	n=34	PD-1	blood	pre	diversity	no difference in richness	standardization	43
NSCLC	n=27	PD-1 (neoadjuvant)	blood	pre	diversity	no difference in evenness and diversity	normalization	89
NSCLC	n=19	PD-1 (neoadjuvant)	tumor	pre	diversity	responder: low evenness, no difference in diversity	normalization	89
UC*	n=29	PDL1	blood	pre	clonality	responder: low clonality	normalization	96
MDS	n=37	CTLA4 and/or PD-1	blood	pre	clonality	responder: low clonality	N/A	90
PDAC*	n=25	CTLA4	blood	pre	clonality	responder: low clonality	standardization	97
PDAC	n=32	PD-1	blood	pre	clonality	no difference	standardization	97
melanoma	n=33	CTLA4	blood	pre	clonality	no difference	N/A	56
various*	n=22	CTLA4	blood	pre	clonality	no difference	standardization	57
NSCLC	n=71	PDL1	blood	pre	clonality	no difference	N/A	84
RCC*	n=15	PD-1	blood	pre	clonality	no difference	normalization	64
melanoma	n=23	PD-1	tumor	pre	clonality	responder: high clonality	N/A	39
melanoma	n=18	PD-1 (after CTLA4)	tumor	pre	clonality	responder: high clonality	N/A	93
melanoma	n=30	PD-1/CTLA4	tumor	pre	clonality	responder: high clonality	normalization	94
melanoma	n=18	CTLA4	tumor	pre	clonality	responder: high clonality	N/A	56
RCC	n=14	PD-1	tumor	pre	clonality	responder: high clonality	normalization	64
melanoma	n=25	PD-1	tumor	pre	clonality	responder: high clonality	N/A	95
SGC*	n=18	PD-1 or PD-1/CTLA4	tumor	pre	clonality	responder: high clonality	N/A	65
melanoma	n=19	PD-1 or PD-1/CTLA4	tumor	pre	clonality	no difference	N/A	48
UC	n=24	PDL1	tumor	pre	clonality	no difference	normalization	96
NSCLC	n=14	PD-1 (neoadjuvant)	blood	pre	similarity (tissue)	responder: high overlap between blood and tumor	normalization	42

Continued

Table 1 Continued

cancer type	num	ICI type	SMP	pre/post	metrics	results	depth	ref
GI cancer	n=8	PD-1	blood	pre	similarity (tissue)	responder: high overlap between blood and tumor	down-sampling	68

depth: control for variable sequencing depth (If the adjustment for sequencing depth was not explicitly indicated, it was noted as 'N/A'.)
various: prostate+melanoma+adenocarcinoma.
GI cancer, gastrointestinal cancer; ICI, immune checkpoint inhibitor; MDS, myelodysplastic syndromes; N/A, not available; NSCLC, non-small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma; RCC, renal cell carcinoma; SGC, salivary gland cancer; SMP, type of sample; TCR, T-cell receptor; UC, urothelial carcinoma;

whether the expansion of pre-existing clones or the induction of novel clones plays a more critical role in the therapeutic efficacy of ICIs. Several studies suggest that the expansion of pre-existing T-cell clones is more important, as many of the clones proliferating post-treatment were already present at baseline.[39 48 62–65 69 91 97 100]

Conversely, other reports highlight the importance of newly induced T-cell clones, which emerge only after treatment, in mediating effective responses.[55 90 101 102] These seemingly contradictory findings suggest that ICIs may exert their effects through dual mechanisms: sustaining and amplifying pre-existing T-cell clones, as well as recruiting and expanding novel clones.[23 25 64 103]

Similarity (overlap) and response to immunotherapy

TCR similarity (overlap) refers to the extent of shared TCR clonotypes across different time points before and after treatment or different tissues such as tumor, blood, and lymph nodes. High TCR similarity before and after ICI treatment suggests minimal change in clonal composition, whereas low overlap indicates significant reshaping of the TCR repertoire. As such, TCR similarity is largely influenced by the dynamics of clonal expansion or contraction. When pre-existing T-cell clones expand, the overlap increases due to their persistent presence before and after treatment. Conversely, when novel clones are induced and expand following therapy, or when pre-existing clones contract, TCR overlap tends to decrease.

Regarding the association between TCR overlap and treatment response, several studies have reported that the expansion of pre-existing clones is critical for therapeutic efficacy, with increased pretreatment and post-treatment TCR similarity observed in responders.[48 64–66] In contrast, other reports highlight the importance of newly induced clones, in which case lower TCR overlap is associated with better outcomes.[55 68 90 101 102 104 105] Taken together, these conflicting findings, likely influenced by limited sample sizes and cancer type heterogeneity, suggest that the qualitative nature of clonal changes may be more informative than overlap metrics alone.

Analyzing shared TCR clones across tissues can also provide insight into the origin of novel clones observed after treatment. For instance, higher baseline TCR overlap between tumor and blood has been associated with improved therapeutic responses,[42 68] possibly reflecting a pre-existing antitumor immune response and active T-cell trafficking. Additionally, post-treatment responders often exhibit increased sharing of TCR clones between tumor and blood, which may serve as a biomarker of clinical benefit.[42 49 98 106] Within the framework of the cancer-immunity cycle, lymph node-derived T cells may infiltrate tumors following ICI therapy,[70 107] contributing to increased inter-organ TCR overlap, an indicator of systemic anti-tumor immunity.[28 68] Finally, the TCR repertoire has also been implicated in immune-related adverse events associated with ICIs.[47 79 108–112]

Table 2 Post-treatment or dynamic changes in TCR repertoire metrics in relation to treatment efficacy and prognosis

cancer type	num	ICI type	SMP	pre/post	metrics	results	depth	ref
melanoma PC	n=16 n=25	CTLA4	blood	pre–post change	diversity	responder: low decreased clones (maintain)	normalization	46
NSCLC	n=71	PDL1	blood	pre–post change	diversity	responder: high increased diversity	N/A	84
HL	n=9	PD-1	blood	pre–post change	diversity	responder: high increased diversity	N/A	85
RCC	n=25	PD-1	blood	pre–post change	diversity	responder: high decreased diversity	N/A	69
NSCLC	n=20	PD-1	blood	pre–post change	diversity	responder: high decreased diversity	N/A	87
melanoma	n=21	CTLA4	blood	pre–post change	diversity	no difference	N/A	47
NSCLC	n=7	PDL1	blood	pre–post change	diversity	no difference	N/A	92
melanoma	n=69	PD-1 or PD-1/CTLA4	blood	post	diversity	no difference	statistical adjustment	91
NSCLC	n=32	PD-1 (neoadjuvant)	blood	post	diversity	no difference in evenness and diversity	normalization	89
melanoma	n=16	PD-1 (after CTLA4)	tumor	pre–post change	diversity	responder: high increased richness	standardization	43
melanoma	n=17	PD-1	tumor	pre–post change	diversity	responder: high decreased evenness	standardization	43
MCC	n=18	PD-1 or PDL1	tumor	post	diversity	responder: high richness (trend)	N/A	83
glioblastoma	n=24	PD-1	tumor	post	diversity	responder: high diversity	N/A	86
NSCLC	n=38	anti-PD-1 (neoadjuvant)	tumor	post	diversity	no difference in evenness and diversity	normalization	89
melanoma	n=10	PD-1	tumor	pre–post change	diversity	responder: high decreased diversity (trend)	N/A	88
NSCLC	n=19	PD-1 or PDL1	blood	pre–post change	clonality	responder: high increased $PD\text{-}1^{+}CD8^{+}$ T cell clonality	N/A	62
RCC	n=25	PD-1	blood	pre–post change	clonality	responder: high increased clonality	N/A	69
PDAC	n=32	PD-1	blood	post	clonality	responder: high clonality	standardization	97
PDAC	n=25	CTLA4	blood	post	clonality	no difference	standardization	97
RCC	n=12	PD-1	blood	post	clonality	no difference	normalization	64
melanoma	n=22	PD-1/CTLA4	tumor	pre–post change	clonality	responder: high increased clonality	normalization	94
melanoma	n=9	PD-1 (after CTLA4)	tumor	post	clonality	responder: high clonality	N/A	93
melanoma	n=19	PD-1 or PD-1/CTLA4 (neoadjuvant)	tumor	post	clonality	responder: high clonality	N/A	48
NSCLC	n=11	PD-1 (neoadjuvant)	tumor	post (at surgery)	clonality	responder: high clonality	N/A	98
NSCLC	n=14	PD-1 (neoadjuvant)	tumor	post	clonality	responder: high clonality	normalization	42
SGC	n=18	PD-1 or PD-1/CTLA4	tumor	post	clonality	responder: high clonality	N/A	65
RCC	n=15	PD-1	tumor	post	clonality	no difference	normalization	64
UC	n=22	PDL1	blood	pre–post change	expanded	responder: high expanded clones from tumor	normalization	96
NSCLC	n=20	CTLA4	blood	pre–post change	expanded contracted	responder: high expanded and contracted clones	normalization	100

Continued

Table 2 Continued

cancer type	num	ICI type	SMP	pre/post	metrics	results	depth	ref
PDAC	n=13	CTLA4	blood	pre–post change	expanded	responder: high expanded clones	standardization	97
melanoma	n=15	CTLA4	blood	pre–post change	expanded	responder: high expanded clones from tumor	N/A	56
NSCLC	n=12	PD-1 (neoadjuvant)	blood	pre–post change	expanded contracted	responder: high expanded clones from tumor	normalization	42
melanoma	n=49	PD-1 or PD-1/ CTLA4	blood	pre–post change	expanded contracted	responder: high expanded clones	statistical adjustment	91
RCC	n=25	PD-1	blood	pre–post change	expanded	responder: high expanded clones	N/A	69
melanoma	n=18	PD-1/CTLA4	blood	pre–post change	expanded	responder: high novel T cell clones	N/A	101
MDS	n=37	CTLA4 or PD-1	blood	pre–post change	expanded contracted	responder: high novel T cell clones	N/A	90
NSCLC	n=60	PD-1 or PDL1	blood	pre–post change	expanded contracted	responder: high novel clones expansion	N/A	102
melanoma	n=11	PD-1	tumor	pre–post change	expanded contracted	responder: high expanded clones	N/A	39
melanoma	n=24	CTLA4	blood	pre–post change	similarity (time)	no difference	N/A	56
RCC	n=12	PD-1	blood	pre–post change	similarity (time)	no difference	normalization	64
GI cancer	n=47	PD-1	blood	pre–post change	similarity (time)	responder: high overlap pre and post treatment	N/A	63
NSCLC	n=19	PD-1 or PDL1	blood	pre–post change	similarity (time)	responder: high overlap pre and post treatment	N/A	62
melanoma	n=7	PD-1 or PD-1/ CTLA4	blood	pre–post change	similarity (time, tissue)	responder: reduction of TCR clonal relatedness from tumor-derived PBMC	N/A	55
GI cancer	n=8	PD-1	blood	pre–post change	similarity (tissue)	responder: high increased overlap between blood and tumor	down–sampling	68
RCC	n=15	PD-1	blood	post	similarity (tissue)	responder: high number of shared clones with tumor	N/A	69
melanoma	n=9	PD-1 (neoadjuvant)	tumor	pre–post change	similarity (time)	responder: high overlap pre and post treatment	N/A	48
RCC	n=12	PD-1	tumor	pre–post change	similarity (time)	responder: high similarity pre and post treatment	normalization	64
SGC	n=18	PD-1 or PD-1/ CTLA4	tumor	pre–post change	similarity (time)	responder: high overlap pre and post treatment	N/A	65

GI, gastrointestinal; HL, Hodgkin lymphoma; ICI, immune checkpoint inhibitor; MCC, merkel cell carcinoma; MDS, myelodysplastic syndromes; N/A, not available; NSCLC, non-small cell lung cancer; PC, prostate cancer; PDAC, pancreatic ductal adenocarcinoma; RCC, renal cell carcinoma; SGC, salivary gland cancer; SMP, type of sample; TCR, T-cell receptor; UC, urothelial carcinoma.

The why: role in defining the specificity of T cells, either for adoptive transfer or for antigen discovery

Adoptive cell transfer

Adoptive cell transplantation (ACT) is a highly personalized cancer therapy in which T cells and other immune cells are collected from a patient, enhanced and expanded ex vivo, and then reinfused to boost their ability to target pathogens and tumor cells (figure 1b, upper left). ACT encompasses several approaches, including Tumor-Infiltrating Lymphocyte therapy and TCR-engineered T-cell therapy.[113] TCR-seq is central to the implementation of ACT, playing key roles in treatment design, evaluation, tracking the T-cell repertoire, and prediction of therapeutic response. Initially, TCR-seq enables the identification of tumor antigen-specific T-cell clones required for ACT. Additionally, TCR-seq characterizes T-cell diversity and clonality, facilitating the selection of optimal T-cell populations for transfer.[114 115] TCR-seq can also serve as clonal barcodes, enabling the tracking of the distribution and functional fate of transferred T cells following ACT.[116 117] Finally, TCR repertoire profiling has been employed to predict the therapeutic efficacy of ACT-based strategies.[118 119] More recently, a clinical study demonstrated the feasibility of generating and

administering T cells engineered to express neoantigen-specific TCRs, derived from a patient's own TCR-seq data.[120] Notably, the median time from neoTCR identification to T-cell infusion was only 102 days, highlighting a promising approach to overcoming one of ACT's major limitations, namely the lengthy time required for treatment initiation.

Antigen discovery

T-cell antigen discovery refers to the identification of antigens or peptide–MHC (pMHC) complexes recognized by T cells. This can be achieved through various strategies, including antigen-directed approaches, TCR-directed approaches, and computational methods.[121] Among antigen-directed methods, one of the most representative is the identification of antigen-specific TCRs by exposing T cells to a panel of pMHC complexes and performing TCR-seq on pMHC-positive cells.[122] Recently, innovative techniques have emerged, such as tetramer-associated TCR-seq (TetTCR-seq), which enables high-throughput linkage of antigen specificity with TCR-seq at the single-cell level,[123] and microfluidic antigen–TCR engagement sequencing, which facilitates the isolation and TCR-seq of neoantigen-specific T cells.[124] In TCR-directed approaches, antigen screening platforms such as baculoviral and yeast display libraries, as well as T-Scan technology, use extensive peptide libraries to assess reactivity with T cells, enabling identification of antigen-specific TCRs through subsequent TCR-seq analysis.[125 126] Computational (in silico) approaches have also gained prominence. The first study to combine tetramer-based sorting with single-cell TCR-seq for antigen prediction was reported in 2017.[9] Since then, various ML and deep learning-based methods have been developed to predict antigen–TCR interactions.[5 119–121] However, the existing datasets cover only a small subset of the immense diversity of potential TCR–antigen combinations (online supplemental table 1). Future work should aim to expand TCR–antigen databases, particularly for rare HLA alleles and non-viral epitopes, to enhance predictive power and coverage.[127] In summary, TCR-seq is instrumental in identifying the antigenic peptides recognized by T cells, including prediction of neoantigens based on TCR associations.[128] Such antigen identification can inform ACT and vaccine development, enable personalized T-cell therapies based on each patient's antigen repertoire, and facilitate immune monitoring using TCR and antigen data. Thus, by capturing the TCR-seq of antigen-reactive T cells, TCR-seq serves as a critical tool in the field of antigen discovery. Finally, it is worth noting that tumor-specific TCRs have high commercial value due to their therapeutic potential, and many high-affinity or clinically promising TCRs are retained within proprietary corporate databases, making them inaccessible to the academic community.

The next: novel computational approaches that try to predict what TCR recognizes

While recent sequence-based models are increasingly informative, predicting αβ TCR antigenic specificity from sequence alone remains an open problem, and reported performance often depends on epitope/HLA familiarity and dataset composition. Understanding TCR antigen specificity, defined as the precise recognition of peptide–MHC complexes, is pivotal to uncover the functional roles of T cells in immune responses, particularly within the tumor microenvironment. Traditional TCR-seq analysis, while robust in capturing clonotype diversity, does not inherently provide information about antigen specificity. Recently, significant advancements in computational biology have paved the way for predictive approaches to decipher what TCRs recognize directly from their sequences. A recent perspective underscores these constraints, noting that current datasets sample only a tiny fraction of possible TCR–peptide–MHC combinations and that performance commonly drops on unseen epitopes/HLAs, motivating standardized benchmarks, higher-quality negatives, and paired αβ inputs.[127]

Database-driven methods constitute one classical approach, where the specificity of newly sequenced TCR clonotypes is inferred by matching to experimentally validated TCR-antigen pairs stored in curated repositories such as VDJdb[129 130] and McPAS-TCR (online supplemental table 1).[131] In recent years, beyond these reports, a variety of public databases have been developed to predict TCR–antigen combinations.[132–137] These databases compile extensive experimentally determined TCR–peptide pairs, facilitating rapid identification of known pathogen-specific or tumor antigen-specific clonotypes. However, the predictive power of this approach is inherently limited by the breadth and quality of the reference data.[138] Clonotypes recognizing uncommon or novel epitopes may remain unidentified, underscoring the need for complementary prediction strategies. Addressing this limitation, ML methods have emerged, leveraging large-scale TCR-seq datasets to computationally predict antigen specificity. ML-driven approaches such as GLIPH/GLIPH2 (Grouping of Lymphocyte Interactions by Paratope Hotspots) cluster TCR-seq based on conserved motifs in their CDR3, thereby identifying clonotypes that likely share antigen specificity, even without prior experimental annotation.[139 140] Similarly, advanced deep learning models such as DeepTCR[141] and ERGO[142 143] have integrated convolutional neural networks and sequence-based modeling to predict antigen specificity directly from the primary TCR-seq. These algorithms can identify subtle sequence features associated with antigen recognition, substantially expanding the potential for novel antigen discovery and characterization of rare clonotypes.[144] The predictive accuracy of these

computational methods varies significantly depending on the sequencing technology and library preparation method used. Bulk TCR-seq approaches using short-read sequencing (eg, Illumina platforms) commonly sequence only a single chain, typically the β chain due to its greater combinatorial diversity, but lack paired-chain information, thus restricting antigen specificity predictions.[145 146] Conversely, single-cell TCR-seq technology provides paired-chain sequencing of α and β TCR chains from individual cells, significantly improving prediction accuracy and enabling precise antigen specificity identification.[3 147] Consistent with broader evaluations, high area under the curves (AUCs) tend to cluster around well-represented viral epitopes in common class I HLA backgrounds, whereas unseen-epitope generalization is modest and variable across datasets and negative-sampling schemes. This pattern highlights the importance of independent, harmonized benchmarks and paired αβ modeling to curb overfitting and improve out-of-distribution reliability. In addition, explicit reporting of true-negative construction and cross-cohort testing would strengthen claims of generalizability.[127]

Long-read sequencing technologies, such as PacBio and Oxford Nanopore, offer distinct advantages by capturing full-length TCR transcripts without fragmentation, preserving complete V(D)J junctions and α–β pairing information within individual T cells. These features facilitate accurate clonotype resolution, robust reconstruction of complex TCR-seq, and enhanced antigen specificity prediction, particularly beneficial for characterizing rare or novel antigen-specific T cells.[148 149] However, long-read sequencing currently faces limitations, including higher error rates and lower throughput compared with short-read methods, requiring specialized bioinformatics approaches and careful error correction to achieve accurate results. Moreover, the choice of library preparation further influences prediction capabilities. Multiplex PCR-based library preparations, despite their high throughput and cost-efficiency, introduce biases toward specific TCR V/J segments, potentially overlooking rare antigen-specific clonotypes.[150] In contrast, 5' RACE-based methods, due to minimal primer biases, are particularly suited to uncover novel clonotypes and improve subsequent computational prediction.[33] Therefore, careful selection of sequencing methodologies aligned with computational prediction approaches is essential to optimizing antigen specificity analyses.

Structure-aware and hybrid approaches

A complementary direction incorporates explicit structure of the TCR–peptide–MHC ternary complex to better ground sequence-level correlations in biophysics. Early pipelines using predicted complexes (eg, AlphaFold-Multimer-style modeling) suggest structure-guided re-ranking of candidate TCRs to a given pMHC may add value in seen-epitope settings, though broad benchmarks remain limited. The recent AlphaFold 3 framework extends diffusion-based prediction to multimolecular complexes, theoretically enabling large-scale TCR–pMHC modeling; however, its utility for prospective ligand discovery will hinge on task-specific validation across diverse HLAs and antigens. In practice, hybrid workflows that (1) prefilter peptides with peptide–HLA presentation predictors (eg, NetMHCpan), (2) apply sequence-based TCR models, and (3) optionally score/dock the top candidates structurally are emerging as pragmatic routes for ligand nomination in constrained HLA/proteome settings, although performance on unseen-epitope/novel-HLA still requires rigorous blinded testing.[151 152]

Recent advances in computational biology and artificial intelligence have markedly expanded our capacity to model and predict TCR–antigen interactions, offering unprecedented opportunities for hypothesis generation, candidate screening, personalized immunotherapy strategies, vaccine development, and disease monitoring.[30 95 153] Nevertheless, their predictive scope remains narrow outside well-represented epitopes and HLA contexts, and most current models still rely heavily on data availability and curated training sets. Sustained progress will likely depend on integrating paired αβ TCR information, structure-aware features, and rigorously curated negative datasets, alongside independent, standardized benchmarking frameworks to ensure generalizability across unseen epitopes and donors. As emphasized by Hudson *et al*,[127] such combined efforts, linking high-quality data, transparent evaluation, and structural interpretability, are essential before computational predictions of TCR specificity can be considered ready for translational or clinical application.

CONCLUSIONS

We have discussed the critical role of TCR-seq in cancer immunotherapy across various domains, including the fundamental understanding of TCR biology, methodologies, its application as a tracking tool and biomarker in immunotherapy, and emerging computational approaches (figure 2). In summary, the TCR is central to antigen recognition, and its high specificity allows for precise tracking of T-cell clones reactive to given antigens. This capability is especially valuable for monitoring immune dynamics before and after ICI therapy, as well as for assessing the spatial distribution of T cells across different tissues. Moreover, analyses of diverse TCR repertoire metrics hold promise as biomarkers for predicting treatment efficacy and patient prognosis. TCR information also contributes to clinical applications such as adoptive T-cell transfer and novel antigen discovery. Notably, recent advances in single-cell analysis, integration with spatial transcriptomics, and the use of artificial intelligence and ML to predict antigen specificity

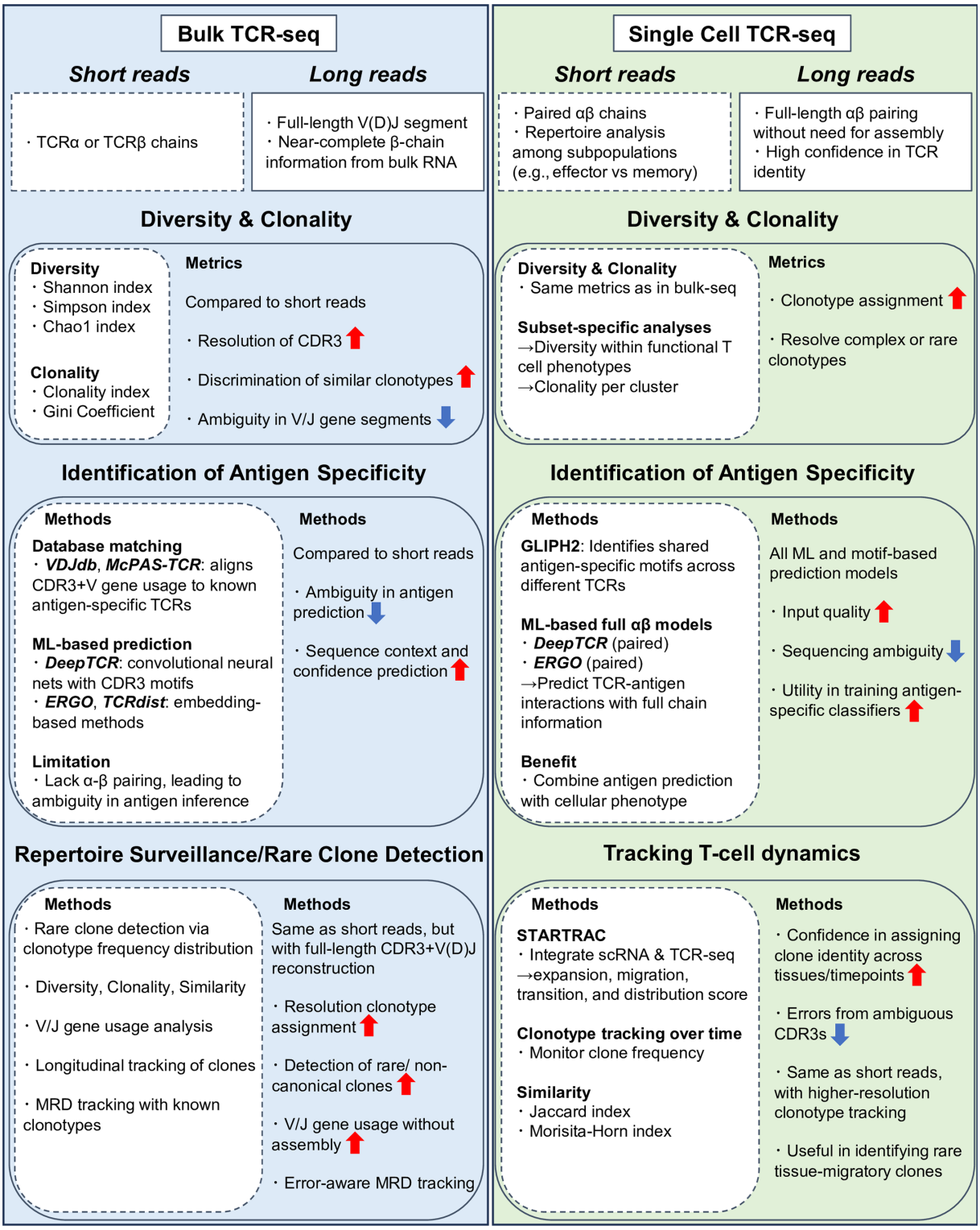

Figure 2 Summary chart of short and long reads in bulk TCR-seq and single-cell TCR-seq. Dendrogram of biological questions we want to answer and the computational methods that address them. These concepts are comprehensively organized by dividing them into bulk TCR-seq and single-cell TCR-seq, and further subdividing into short-read and long-read approaches. MRD, minimal residual disease; TCR, T cell receptor.

have greatly enhanced the depth and utility of information obtainable from TCR-seq. Nonetheless, significant challenges remain, including technical biases, interpretation of large-scale data, and high costs. As immunotherapy indications expand rapidly across various cancer types, a solid understanding of TCR-seq is increasingly essential for elucidating immunotherapy mechanisms, forecasting therapeutic

responses, and enabling personalized treatment strategies.

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