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Immune dysregulation in immunodeficiency disorders: The role of T-cell receptor sequencing

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ABSTRACT

Immune dysregulation is a prominent feature of primary immunodeficiency disorders, which commonly manifested as autoimmunity, cytopenias and inflammatory bowel disease. In partial T-cell immunodeficiency disorders, it has been proposed that the imbalance between effector and regulatory T-cells drives the breakdown of peripheral tolerance. While there is no robust test for immune dysregulation, the T-cell receptor repertoire is used as a surrogate marker, and has been shown to be perturbed in a number of immunodeficiency disorders featuring immune dysregulation including Omenn's Syndrome, Wiskott-Aldrich Syndrome, and common variable immunodeficiency. This review discusses how recent advances in TCR next-generation sequencing and bioinformatics have led to the in-depth characterization of CDR3 sequences and an exponential growth in examinable parameters. Specifically, we highlight the use of junctional diversity as a means to differentiate intrinsic T-cell defects from secondary causes of repertoire perturbation in primary immunodeficiency disorders. However, key questions, such as the identity of antigenic targets for large, expanded T-cell clonotypes, remain unanswered despite the fact that such clones are likely to play a pathogenic role in driving immune dysregulation and autoimmunity. Finally, we discuss a number of emerging technologies such as in silico reconstruction, high-throughput pairwise αβ sequencing and single-cell RNAseq that offer the potential to define the antigenic epitope and function of a given T-cell, thereby enhancing our understanding in this field.

Contents

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1. Introduction

Antigen receptor (VDJ) rearrangement is a defining feature of our adaptive immune system, unique to B-lymphocytes and T-lymphocytes [1]. During the development of B-lymphocytes and T-lymphocytes, each cell must produce a functional antigen receptor through a process of somatic DNA recombination, in which germline gene segments – falling into V, D or J families – are recombined together; if successful, this rearrangement can produce an antigen receptor, then used to receive crucial survival signals from epithelial cells lining the bone marrow or thymic epithelium [2]. This VDJ rearrangement process first recruits a protein complex composed of recombinase activating genes 1 and 2 (RAG1/2), which mediates the recombination [3], before the DNA lesions are repaired by components of the non-homologous end joining (NHEJ) repair pathway [4]. The combinatorial diversity created by recombination selecting from large pools of V-, D- and J-gene segments is further augmented by junctional diversity (non-templated addition and deletion of nucleotides at the recombining edges), as well as pairing of immunoglobulin heavy (IgH) and light (IgL) chains, TCR α and β chains, or TCR γ and δ chains. The section of the variable antigen receptor encoded by the recombined junction is termed the complementarity determining region 3 (CDR3), and forms the primary contacts with the antigen, and is thus typically the site of interest for most studies.

The diversity in naïve B-cell receptor (BCR) and T-cell receptor (TCR) repertoires generated by these mechanisms is enormous – as has been demonstrated in both animal and human studies [5,6] – which allows our adaptive immune system to recognize a vast number of potential epitopes [1]. It is estimated that the human VDJ recombination machinery could produce as many as $10 \times {}^{14-20}$ different TCRs [1,5,7]. Of note, the diversity of the human B-cell repertoire also benefits from somatic hypermutation following antigen recognition [1,5,7]. While some CDR3 sequences are more likely to be synthesized due to convergent recombination or biases in the recombination machinery [8,9], the theoretical permutations of CDR3s for both B-cells and T-cells mean that the majority of naïve cells are unlikely to share identical CDR3 sequences, making it a suitable unique molecular signature for each lymphocyte. This approach has proven highly valuable in demonstrating clonality in patients with suspected leukemia and lymphoma since proliferating cells will retain their original CDR3 sequences [10,11].

Successful VDJ rearrangement is central to a functioning adaptive immune system. The consequence of its failure is clearly seen in immunodeficiencies such as $T^{-}B^{-}NK^{+}$ Severe Combined Immunodeficiency (SCID), in which components of the VDJ recombination machinery are defective or absent, and patient prognosis is poor without bone marrow transplantation [12]. Monogenetic mutations of a number of key enzymes and molecules have now been linked to $T^{-}B^{-}NK^{+}$ SCID [13]. However, abnormalities in antigen receptor diversity, particular in the T-cell receptor repertoire, have also been appreciated in a number of other immunodeficiency syndromes such as Omenn's Syndrome, Wiskott-Aldrich Syndrome (WAS), and immune dysregulation, polyendocrinopathy, enteropathy and X-linked Syndrome (IPEX) [14–16]. In these immunodeficiencies, which have been linked to both single gene defects and polygenic inheritance patterns, there are often features of autoimmunity, which is thought to be driven by T cell dysfunction [17]. There are a number of hypotheses for the generation of autoimmunity, which include that patients with hypomorphic mutations have autoreactive TCRs and BCRs due to impaired selection and low frequency [17]. Additionally, although there are increasing efforts to target T-cell pathology and autoreactivity in patients with autoimmunity in primary immunodeficiencies, there is still much that is unknown about the pathogenesis and progression of these processes [18]. Historically, assessing the TCR repertoire was much more challenging than assessing the immunoglobulin repertoire. While the immunoglobulin repertoire could be examined with relative ease by serum immunofixation, the study of the TCR repertoire demands more sophisticated molecular approaches. This article will focus on discussing the recent advances in TCR sequencing and how they have furthered our understanding of immunodeficiency disorders, and will outline some of the challenges ahead. In particular, we will discuss how emerging new technologies are bringing us one step closer to overcoming the historical barriers of antigen specificity and functional correlation, allowing us to better understand immune dysregulation in immunodeficiency disorders.

2. Methods for assessing TCR repertoire diversity

The stochastic nature of VDJ rearrangement means CDR3 sequences of various lengths will inevitably occur in an immune repertoire containing millions of T-cells. This property has been exploited to define the 'shape' of our TCR repertoire, as the length distribution tends to demonstrate a Gaussian distribution of CDR3 lengths in healthy individuals.

Unlike immunoglobulins, TCRs do not exist as soluble proteins and the characterization of TCRs is best achieved by molecular approaches. DNA or mRNA may be extracted from either blood or tissue samples for analysis, which can then undergo a variety of tests to infer clonality. One popular technique involves amplifying across the CDR3 and visualizing the length distribution ("spectratyping"), while alternative strategies exist to infer diversity from other molecular properties, such as the electrophoretic shift in rapidly denatured/cooled PCR amplicon pools as a function of heteroduplex formation [19]. CDR3 sequences can be amplified using panels of sense and anti-sense primers targeting different V- and J-gene segments, which can be pooled together into multiplex PCR reactions. If using RNA (cDNA)-based approaches, the constant region (onto which VDJ recombinations are spliced) may be targeted for the reverse primer, instead of multiple J regions [19].

These techniques have found their role in routine clinical practice in the evaluation of hematological malignancy by demonstrating clonality. Standardized international consensus for clinical testing is now available, and results may be classified as clonal, pseudoclonal (oligoclonal) or polyclonal according to the BIOMED2 consortium agreement [10]. While these approaches have proven to be very useful in evaluating clonality, results are still largely qualitative, and comparison between samples is incredibly difficult even with the use of a complex scoring system that defines the heights of clonal peaks in relation to the remaining repertoire [20]. At the same time, PCR bias may over- or under-represent clonotypes of interest due to differences in primer annealing and amplification properties.

Alternatively, flow cytometric analysis using a panel of Vβ-specific antibodies can be used to assess TCR repertoire diversity [21,22]. While this technique has the advantage of assessing the actual protein expression on the cell surface, it is limited by revealing only the frequency of V-family usage, making this technique relatively insensitive. Additionally, it does not cover the entire range of available TCR Vβ families, and even fewer Vα genes.

The major breakthrough came when high throughput sequencing (HTS) protocols were modified to perform massive parallel sequencing of thousands to millions of CDR3 amplicons from a single sample simultaneously [23–25]. By profiling the immune repertoire down to the actual nucleotide sequence, an unprecedented level of information could be generated at the

highest possible resolution. Large, expanded clonotypes could be accurately defined and cross-referenced between samples. This technique also led to our better understanding of the nature of the TCR repertoire of healthy individuals, and in particular the biology of public clonotypes and convergent recombination, where certain CDR3 sequences may be frequently shared between HLA unmatched individuals due to intrinsic bias in the way we synthesize our TCRs [8,26–28].

With such large data sets, HTS-TCR data require sophisticated bioinformatics support. Not only could T-cell clonotypes then be more accurately defined, but the exact V-, D- and J-gene segment usage and junction diversity of each clonotype is revealed [29]. Repertoire diversity is no longer a subjective assessment but can be accurately calculated, commonly using entropy or diversity indexes, though many other methods also exist [30–32]. The numerical definition of repertoire diversity hence allows a statistical comparison to be made between groups in biological studies. As with all deep-sequencing, HTS-TCR may still be subject to PCR bias and errors [33]. By creating a synthetic TCR-γ repertoire, Carlson et al. showed that PCR bias may be corrected computationally [34]. Similarly, bias may be reduced by an additional step of nested-PCR [35], and both error and bias may be reduced through the addition of unique molecular indexes or barcodes [36,37]. In the past few years, there has been a tremendous growth in the available TCR bioinformatics software with increasingly more complex mathematical models applied, and the full potential of HTS-TCR technology is still to come [38–43].

3. Primary immunodeficiency disorders associated with abnormal TCR repertoires

Profiling immune repertoires offers another dimension of insight when studying lymphocytes, and has been explored in a number of immunodeficiency disorders both in research and clinical settings (Table 1). Unsurprisingly, the majority of the efforts focused on T-cell-related diseases including Omenn's Syndrome, MHC class II deficiency, Lck deficiency, WAS, IPEX and common variable immunodeficiency (CVID) [14–16,30,44,45]. Using RAG deficiency, WAS, CVID and Xrcc4-like factor (XLF) deficiency as examples of various degrees of T-cell failure, we will discuss how the TCR repertoire may be affected.

3.1. Omenn's syndrome

Omenn's Syndrome is a severe combined immunodeficiency syndrome that shares a similar molecular basis with $T^{-}B^{-}NK^{+}$ SCID where mutations in genes involved in the recombination machinery (RAG1, RAG2) or NHEJ DNA repair pathway (e.g. Artemis) can severely disrupt VDJ rearrangement. Mutations at different sites in the gene can result in variable residual enzyme activities, producing a wide spectrum of disease. RAG1 of Omenn's patients exhibits intermediate enzyme activity when compared to wild-type [14,46]. This residual enzyme activity is likely to allow a low level of VDJ rearrangement to occur, giving rise to circulating T- and B-lymphocytes in Omenn's patients. However, circulating T-cells are abnormal, and spectratyping, heteroduplex analysis and Vβ flow cytometry have all shown an oligoclonal repertoire in these patients, with preferential V-gene usage driven by expanded clonotypes [47–49]. Although the exact mechanism for reduced repertoire diversity is not known, mutation in murine RAG1 (R972Q) demonstrated selective deficiency at certain coding flanks near the recombination signal sequences (RSSs) which led to failure of hairpin formation during VD or DJ joining; a process that could significantly limit the total permutations of successful VDJ rearrangement [50]. However, one case report has suggested that circulating dominant clonotypes may be of maternal origin, contributing to skewing in repertoire diversity [49]. The advances in HTS technology now allow us to examine VDJ rearrangement in greater detail. In addition to reduction in repertoire diversity, Omenn's Syndrome patients have now been shown to have a shorter CDR3 length and reduced junctional diversity when compared with other T-cell immunodeficiencies such as atypical DiGeorge and ZAP70 deficiency, suggesting a primary defect within the recombination process in this disease [14]. TCR sequencing can even be used in Omenn's Syndrome patients and more severe SCID patients with extremely low to undetectable peripheral levels of rearranged TCRs, by generating induced pluripotent stem cells (iPSCs) and differentiating them into immature T-cells, allowing inspection of a pre-selection repertoire and demonstration of a strongly biased TCR generation process [46]. Overall, there is compelling evidence to support the notion that oligoclonality in Omenn's Syndrome represents an intrinsic failure of T-cell development.

3.2. Wiskott- Aldrich syndrome

In addition to an intrinsic defect within VDJ rearrangement, many partial T-cell immunodeficiencies may also have a restricted TCR repertoire [51]. In these disorders, the abnormal TCR repertoire is most likely to be driven by altered T-cell signaling which in turn promotes the development of immune dysregulation. Of all partial T-cell immunodeficiencies, the TCR repertoire has been most

Table 1
Primary immunodeficiency disorders associated with T-cell receptor repertoire defects. Immune dysregulation, polyendocrinopathy, enteropathy and X-linked Syndrome (IPEX). Major histocompatibility complex (MHC). Common Variable Immunodeficiency (CVID). X-linked agammaglobulinaemia (XLA). High throughput sequencing (HTS).

Primary Immunodeficiency Disorder	Severity of T-cell immunodeficiency	TCR repertoire diversity	Preferential V-usage	Junctional diversity defect	Methods of investigation	References
Omenn's syndrome	Severe	Severely reduced	Yes	Reduced nucleotide editing	Spectratyping, heteroduplex analysis, GeneScan, flow cytometry and HTS of β-chain	[14,46–50]
Lck deficiency	Severe	Reduced	Yes	Unknown	RT quantitative PCR of α, β, δ & γ-chains	[45]
Wiskott-Aldrich Syndrome	Intermediate	Mild to moderately reduced	Yes	Reduced nucleotide editing	Spectratyping, GeneScan and HTS of β-chain	[16,52–56]
Cerrnunnos XLF deficiency	Intermediate	Reduced only in TCRδ reperotire	None	Normal	HTS of β and δ-chain	[62]
IPEX	Intermediate	Reduced- severity unknown	None	Unknown	Flow cytometry	[15]
MHC II deficiency	Intermediate	Reduced	None	Unknown	Flow cytometry, GeneScan (TCRγ)	[44]
CVID	Mild	Normal to moderately reduced	Possible	Possible	Spectratyping, flow cytometry and HTS of β-chain (whole blood)	[30,32,59,60]
XLA	Sub-clinical	Normal	None	Reduced nucleotide editing	Spectratyping, HTS of β-chain (whole blood)	[61]

intensely studied in WAS. WAS is characterized by impaired cytoskeleton remodeling and impaired antigen receptor signaling, leading to a range of immune dysfunctions including decreased repertoire diversity [16,52–54]. While the exact causes for these phenotypes are not fully understood, one hypothesis proposed by Wada et al. suggests that a reduction in TCR repertoire diversity may be age-dependent and driven by factors extrinsic to T-cell development such as infections and autoimmunity [53]. The greatest diversity loss was later noted in the CD8 memory and TEMRA compartments by HTS studies [54]. CD4 T-cells also demonstrated a lower degree of abnormality. However, later reports did not agree on the extrinsic influence of TCR repertoire and showed that Vβ restriction can occur at an early age [16,54]. Again using TCR sequencing O'Connell et al. identified a number of patients with strong preferential usage of the TRBV6-5 gene segment among their hyperexpanded clonotypes, which is particularly noteworthy as this V-gene has also been reported to be over-represented in the repertoires of HIV patients [16,36]. Also, immune repertoire diversity was corrected in a patient following gene therapy suggesting that the WAS-protein may have an actual role in shaping TCR repertoire diversity [55]. However, progressive lymphopenia may contribute to additional loss in repertoire diversity in WAS patients, promoting immune dysregulation over time via the imbalance of effector and regulatory T-cells as Liston et al. proposed [51,56,57]. Overall, both intrinsic and acquired factors are likely to have a role in shaping the TCR repertoire in WAS patients. Interestingly, no correlation between disease severity and the degree of oligoclonality has so far been suggested.

3.3. Common variable immunodeficiency

Although CVID has historically been considered an antibody deficiency syndrome, there is growing evidence of global disruption of the adaptive immune response, in both the B and T cell compartments, and many consider CVID to be within the spectrum of T-cell immunodeficiency [58]. Although heterogeneity has hampered the understanding of its etiology, group analyses of CVID patients have consistently demonstrated subtle defects in T-cell functions [58]. Restricted CD8 repertoire diversity, largely as a result of $CD8^{+}CD28^{-}$ T-cell expansion, was first demonstrated through spectratyping. The abnormalities were also observed to be stable over time, hence reactive changes were improbable [59]. In addition to the expansion of antigen experienced CD8 T-cells, other studies showed that the reduction of naïve CD4 T-cells also contributes to the alteration in TCR repertoire [30,60]. Preferential V-gene usage was reported in two studies, but it was intriguing that agreement of specific V-families was not seen between the two studies [32,60]. Unlike WAS, two HTS-TCR studies provided conflicting evidence regarding V-gene usage and germline junctional diversity, which may in part be explained by disease heterogeneity between study participants. Both studies were also limited by only analyzing whole blood [30,32]: future studies analyzing T-cell subsets in specific subgroups of patients may help better address the current uncertainties. While comparing repertoire diversity between studies can be precarious, the defect in general is more subtle in CVID than in the other T-cell immunodeficiencies.

3.4. Other immunodeficiencies

TCR sequencing has also been applied beyond conventional T-cell immunodeficiencies, with broadly comparable results. Surprisingly, an investigation into X-linked agammaglobulinemia (XLA), also revealed decreased junctional diversity in patients relative to controls [61]. This could be attributed to a lack of non-templated nucleotide insertions and deletions leaving a more germline-like repertoire and a concomitant increase in inter-individual sharing of TCR sequences. Interestingly, this defect in diversification did not appear to be due to a lack of B-cell driven T-cell selection, as the non-productive repertoire is also perturbed, suggesting intrinsic defects in the VDJ recombination process. Immunodeficiency caused by defects in the NHEJ protein XLF (also known as Cernunnos) was found to correlate with a reduction in the number of non-templated additions and diversity in the TCRδ but not the TCRβ repertoires, which may in part explain the milder symptoms observed in these patients [62]. Another study investigated the TCRα repertoire of both XLF-deficient patients and model mice, demonstrating that while XLF is dispensable for VDJ recombination, doing so in its absence is associated with a DNA-damage related decrease in cell viability [63]. Murine experiments suggest that the TCRα locus is able to undergo multiple successive rounds of V-J recombination in its attempts to generate a product receptor, using progressively more distal gene segments [64]. The decreased viability upon recombination in XLF-deficient patients is therefore less likely to produce rearrangements using these α genes, some of which are used in phenotypically distinct T-cell subsets, such as mucosal associated invariant T (MAIT)-cells and invariant natural killer T (iNKT)-cells, providing a novel model for depletion of such subsets in immunodeficiencies [63].

Using the above examples, we have highlighted a number of mechanisms, such as intrinsic VDJ rearrangement failure, abnormal TCR signaling, hyperexpanded CD8 T-cells and progressive lymphopenia, which are encountered in primary immunodeficiencies that could lead to a reduction in TCR repertoire diversity. As TCR repertoire diversity ultimately must correspond to the breadth of antigenic challenges an immune system can respond to, this is clearly an important parameter for all such studies to measure. We evaluated these mechanisms with a focus on dissecting whether an intrinsic or extrinsic cause was involved. To a certain degree, the severity of T-cell deficiency dictates the TCR repertoire, with the most severe cases demonstrating a global impairment in repertoire diversity, while less severe diseases are more likely to have defects that are confined to the memory CD8 T-cell compartment. Some promise has already been shown in thymic transplantation and gene therapy, and it will be interesting to see whether the monitoring of the TCR repertoire would be useful in the clinical arena [65,66]. As shown in Omenn's Syndrome, WAS and CVID, the analytical power of HTS-TCR not only allows us to better calculate clonality using more sophisticated indexes to reduce subjectivity, but the examination of junctional diversity of primary cells as well as iPSC-derived thymocytes promises to differentiate intrinsic T-cell defects from secondary changes.

However, it is important to point out that findings from bioinformatics analyses have not been vigorously tested and the biological significance remains to be seen. Also, there are not yet sufficient age-matched data to define the normal range of repertoire diversity by HTS-TCR and it is clear that different T-cell subsets behave differently even in health donors [54]. Clinically, we would like to stress that the TCR repertoire forms an information rich biomarker, which should be part of an overarching assessment in order to capture the full complexity of an individual's immune parameters. For example, while a large CD8 clonotype may be perfectly consistent with antibody deficiency as discussed above, every effort should be made to exclude a lymphoproliferative disorder, such as large granular leukemia (LGL) given the significant overlap between the two disorders [67].

4. Can we determine T-cell antigen specificity?

Perhaps one of the key questions in immunodeficiency TCR research is whether expanded clonotypes are causally connected to

the development of autoimmunity or immune dysregulation. Antigen recognition is the fundamental function of a TCR. mRNA (cDNA) sequencing is debatably more representative of the actual protein surface expression than gDNA sequencing, which will contain a greater proportion of nonproductive sequences (which are produced during failed attempts to recombine each receptor chain). While we are now better at defining clonality and repertoire diversity with HTS-TCR, profiling of the TCR-β chains offers very little information regarding the antigen specificity of clonotypes of interest. Unlike B-cells, T-cells recognize antigens presented on MHC molecules, adding an additional layer of complexity to the equation [68]. To the best of our knowledge, the antigenic targets of large clonotypes in immunodeficiency disorders have not yet been successfully identified. Defining the antigen specificity of a T-cell clone still requires laborious screening using vast peptide-MHC libraries under difficult culture conditions, especially for class II peptides [69]. Meanwhile, knowledge of the TCR-α chain, TCR-β chain, HLA-types and the T-cell subset (CD4 or CD8) is believed to be required in order to narrow down the putative peptide targets. However, new insights have shown that repertoire formation, for example CD8 Vβ usage following EBV and CMV infections, may be predominantly guided by peptides as opposed to the rest of the MHC complex [27,69–71]. Therefore the identification of shared clonotypes between patients with similar clinical presentations may actually be of some value even when the other information is not known.

The systemic circulation acts as a major highway for lymphocytes to circulate in the body, homing to their designated location [72]. For example, in Sézary Syndrome, matching malignant T-cell clonotypes are often detectable in both peripheral blood and skin biopsy samples, underlining the common origin of the entire population [73,74]. Furthermore, TCR tissue-blood pairing may be observed in inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease [75,76]. A related observation has also been made in solid tumors where anti-tumor T-cells are found in greater frequency at the site of the immune response than in peripheral blood [77], presumably reflecting increased local retention and division of cells. Interestingly, a tissue-based T-cell clonotype was found even when only a small number of HLA-unmatched patients with Crohn's were examined, supporting the notion that the signal is enhanced at the site of inflammation compared to blood [76]. Should this be true, immune dysregulation research in immunodeficiency should refocus on tissue-based TCR sequencing with the aim of identifying a list of T-cell clonotypes responsible for tissue damage. The presence of autoantibodies often precedes the development of autoimmune diseases and the same has been hypothesized for TCRs [78]. Therefore, in the future, clonotypic analysis in the blood may forewarn of the development of autoimmunity - a highly valuable tool for those who are antibody deficient.

Test vaccination challenge is a common strategy during the diagnostic work up of antibody deficiency. Using both polysaccharide and protein conjugated antigens, the health of the humoral immune system is determined by the host's ability to synthesize antibodies against these antigens [79]. A number of studies have now demonstrated the possibility of tracking herpesvirus specific T-cell responses following vaccination or primary infection [80,81]. Similar approaches have also been used in peptide-based cancer immunotherapy to demonstrate long lasting T-cell memory in melanoma and Merkel cell carcinoma [82,83]. However, the use of TCR profiling to test in vivo T-cell antigen responses will remain largely conceptual until more is known about T-cell clonotypic response to vaccination in an immunocompromised host and the immune-dominant epitopes of common vaccines [84].

Overall, the identification of full T-cell epitopes for immune dysregulation or autoimmunity in immunodeficiency still seems far away. Despite that, important steps have taken place to bring us

Table 2
Emerging technologies for obtaining paired α and β-chain TCR sequences.

	Description	Advantages	Estimated throughput	References
Mini-pool sequencing and αβ matching through probabilistic analysis	Cells from a given sample are separated into mini-pools (~1% each) for DNA extraction. The α and β repertoires of each pool are individually sequenced. αβ matching of T-cell clones is then calculated through probabilistic analysis.	Paring of α and β-chains using existing platforms	<500,000 reads	[85,86]
Emulsion droplet RT-PCR with barcode specific microbeads of the α and β- chains	Using a microfluidic pump, single cells are captured within emulsion oil droplets together with microbeads containing unique barcoded primer of the TCRα and TCRβ chains. Following RT-PCR, which occurs within the emulsion droplets, all droplets may be sequenced together. The origins of each α and β sequence are traced back according to their unique barcodes.	High accuracy and throughput	1×10^6 reads	[88]
Emulsion droplet RT-PCR with overlap extension PCR of the α and β- chains	Instead of using microbeads, overlapping reverse and forward primers of the α and β-chains can be designed to capture both sequences in a single amplicon.	High accuracy and throughput	1×10^6 reads	[89,90]
Single cell RNA-seq	Data are retrieved via bioinformatics analysis	Large amount of concurrent data, no additional experiment required and suitable for retrospective analysis,	Tens to hundreds	[91,92]
Single cell RNA-seq with functional gene enrichment	Specific sets of primers may be designed to capture and enrich transcripts of interest by RT-PCR prior to conventional RNA-seq protocols	Matching TCRαβ sequences to function/phenotype of T-cells	Tens to hundreds	[95]

closer to this goal, with TCR sequencing of multiple tissue sites presenting exciting opportunities.

5. Future directions and emerging technologies

Regardless of the power of bioinformatics, current HTS-TCR approaches are still restricted to single-chain analyses and the pairing of TCR α and β chains cannot be readily inferred. Determining the full TCRαβ clonotype traditionally requires T-cell cloning followed by the identification of the α and β chain sequences independently via Sanger sequencing, a laborious method with a high failure rate. However, exciting new technologies are now being developed to offer hope in high-throughput αβ paired sequencing (Table 2). For example, by dividing a T-cell sample into multiple wells prior to separate bulk sequencing, the α and β chains can be inferred computationally with high accuracy through probabilistic analysis of which chains occur together in multiple pools [85,86]. Another technique takes advantage of the advances in microfluidics and nanotechnology, where single cells can be captured in emulsion droplets for RT-PCR. Specifically, the RT-PCR can be tailored to target the CDR3 sequences of both α and β chains simultaneously within the droplets [87–90]. To tag the α and β chain amplicons from a single cell, Hanson et al. demonstrated that microbeads with a unique DNA barcode linked to TCR primers may also be used [88]. Alternatively, Turchaninova et al. and McDaniel et al. were able to fuse the α and β chain amplicons into a single amplicon within the droplet by overlap extension PCR [89,90]. The throughput of emulsion pairwise TCR sequencing was estimated to be as high as 1.2×10^6 per analysis [90].

With the increasing use of single-cell RNAseq, it is also possible to retrieve TCR sequences from the transcriptome without performing additional experiments [91,92]. While some techniques described above purposely amplify TCR transcripts or incubated T-cells overnight with IL-2 to boost the TCR mRNA level, some reports show that murine TCR genes may be naturally highly expressed in T-cells [92]; various laboratories have now demonstrated that one can assemble and analyze the CDR3 regions of both α and β chains from unadulterated RNAseq data of single cells. However, single-cell RNAseq is limited by its throughput as most commercially available microfluidic devices typically can only process tens to hundreds of cells at a time [93]; those technologies that are capable of higher cellular throughput – such as 'Drop-seq' being unsuitable for TCR analysis, as the reads are biased towards the mRNA 3′ and would not cover the CDR3 [94]. Despite this limitation, single-cell RNAseq does offer a significant advantage over other techniques, which is the ability to link T-cell function to its receptor [91]. By enriching the TCRαβ transcripts and 34 other functional genes such as cytokines and transcription factors via an additional RT-PCR step, Han et al. showed that tumor-infiltrating T-cells from a colorectal cancer patient were mostly IL-17A, IFNγ, IL-2, TNFα, RUNX1 and RORγT expressing. Furthermore, T-cells that carry the same TCR may be of opposing phenotypes such as $FOXP3^+$ regulatory T-cells (Tregs) [95]. With increasing recognition of Tregs located in inflamed tissue in autoimmune diseases such as rheumatoid arthritis [96], it will be interesting to test whether they share homologous TCR with those responsible for tissue damage.

The ability to accurately characterize the paired TCRαβ clonotype brings us another step closer to identifying the antigenic target of a T-cell, leaving the MHC component as the final piece of the puzzle. But how much value would it add? As discussed above, there is now a shifting paradigm to suggest a high degree of plasticity in T-cell antigen recognition, in which a T-cell may recognize homologous peptides presented on different MHC molecules. By testing T-cells against yeast and baculovirus display pMHC libraries, Birnbaum et al. showed that it is possible to predict cross-reactive targets of a given TCRαβ clonotype [69]. It remains to be seen whether continuing development in this field could help identify the antigenic targets of a T-cell without, or with only limited, knowledge of the HLA types of an individual. Research in this area is also likely to further our understanding regarding the link between autoimmunity and molecular mimicry in immunodeficiency and other immune-related disorders.

6. Conclusion

Over the past decade, significant technological advances have been made in the field of TCR sequencing. With the help of sophisticated bioinformatics, the TCR repertoire can now be accurately described and increasing parameters and information are being revealed in a single experiment. The ability to examine junctional diversity has allowed us to differentiate intrinsic causes of repertoire perturbation, such as VDJ rearrangement and T-cell signaling defects, from secondary causes. There is a wide spectrum of TCR repertoire perturbation in immunodeficiency disorders, mirroring the severity of T-cell deficiency. However, immune dysregulation remains a relatively unexplored area, with antigenic targets of large T-cell clonotypes still being unknown. Emerging technologies bring great promise and the answer is becoming ever more tangible: the challenge perhaps moves from not being able to access a suitable technology, to selecting the right one. Future work should also focus on defining the normal repertoire diversity in age-matched populations, as well as for other parameters, and examining the TCR repertoire in other immunodeficiency disorders to further our understanding of the biology of these rare diseases.

Author contributions

GKW, JMH, SB & MC wrote the paper.

Conflict of interest

G.K.W. is an employee of UCB Celltech.

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