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T cell receptor therapeutics: immunologic targeting of the intracellular cancer proteome

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Abstract

The T cell receptor (TCR) complex is a naturally occurring antigen sensor that detects, amplifies, and coordinates cellular immune responses to epitopes derived from membrane-associated and intracellular proteins. Thus, TCRs enable the targeting of proteins selectively expressed by cancer cells, including neoantigens, cancer-germline antigens, and viral oncoproteins. As such, they have become an emerging class of oncology therapeutics. Herein, we review the current cancer treatment landscape using TCRs and TCR-like molecules. This includes adoptive cell transfer of T cells expressing endogenous or engineered TCRs, TCR bi-specific engagers, and antibodies

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CAK wrote an initial draft of the manuscript. All authors edited and revised the final manuscript.

Competing interests:

C.A.K. and S.S.C. are inventors on patents related to TCR discovery and public neoantigen-specific TCRs and are recipients of licensing revenue shared according to MSKCC institutional policies. C.A.K. has consulted for or is on the scientific advisory boards for Achilles Therapeutics, Affini-T Therapeutics, Aleta BioTherapeutics, Bellicum Pharmaceuticals, Bristol Myers Squibb, Catamaran Bio, Cell Design Labs, Decheng Capital, G1 Therapeutics, Klus Pharma, Obsidian Therapeutics, PACT Pharma, Roche/Genentech and T-knife. C.A.K. is a scientific co-founder and equity holder in Affini-T Therapeutics. B.M.B. is an inventor on patents related to TCR engineering and neoantigen discovery, has consulted for Eureka Therapeutics and EnaraBio, and is on the scientific advisory board of T-cure Bioscience. S.A.Q is an inventor in patents related to the use of T cell therapies targeting tumour clonal neoantigens in cancer. S.A.Q. is also a founder, CSO, and equity holder of Achilles Therapeutics. A.R. reports personal fees from Amgen, Chugai, Genentech, Merck, Novartis, Roche, Sanofi, Vedanta, 4C Biomed, Appia, Apricity, Arcus, Highlight, Compugen, ImaginAb, Kalthera/ImmPACT Bio, MapKure, Merus, Rgenix, Lutris, Nextech, PACT Pharma, Synthekine, Tango, Advaxis, CytomX, Five Prime, RAPT, Isoplexis, and Kite/Gilead and has received grants from Agilent and Bristol Myers Squibb outside the submitted work.

specific for human leukocyte antigen (HLA)-bound peptides (TCR mimics). We discuss the unique complexities associated with TCR clinical development such as HLA restriction, TCR retrieval, potency assessment, and the potential for cross-reactivity. In addition, we highlight emerging clinical data that establishes the antitumour potential of TCR-based therapies, including tumour infiltrating lymphocytes, for the treatment of diverse human malignancies. Finally, we explore the future of TCR therapeutics, including emerging genome editing methods to safely enhance potency and strategies to streamline patient identification.

Introduction.

The T cell receptor (TCR) is a lineage-defining, membrane-anchored, clonotypic immune receptor that plays a central role in the ligand-dependent activation of T lymphocytes[1,2]. Nearly all FDA approved cancer immunotherapies promote the activation and expansion of T cells expressing TCRs that confer recognition to tumour antigens[3–5]. Unlike antibodies, which physiologically bind solely to cell surface and soluble epitopes, TCRs can respond to antigens derived from the entirety of the cancer cell proteome[6]. This includes the ~88% of proteins that reside exclusively within intracellular compartments, such as the cytoplasm, nucleus, and mitochondria[7]. The unique capacity of TCRs to engage intracellular antigens, a property that dramatically expands the landscape of actionable immunologic targets, stems from the fact that they do not bind to intact proteins. Rather, they recognize proteolytically degraded polypeptides bound to **human leukocyte antigen (HLA) [G]** molecules that have been trafficked to the surface of human cells for extracellular presentation[8].

TCRs display high sensitivity for ligands. The number of specific peptide/HLA (p/HLA) complexes on the surface of tumour cells is typically within the range of 1–100 molecules[9,10]. This value is orders of magnitude smaller than the number of molecules required by therapeutic antibodies[11,12]. Nevertheless, only one to three p/HLA complexes are sufficient to trigger T cell effector functions[13,14]. Simultaneously, TCRs are capable of remarkable specificity. They can discriminate between peptides that differ by a single amino acid, such as those resulting from a somatic point mutation[15] or germline polymorphism[16], as well as different stereoisomers of the same amino acid[17].

Despite these virtues, the first TCR therapeutic (tebentafusp) only entered the standard of oncologic care in 2021[18]. By contrast, antibodies and their derivatives, including antibody-drug conjugates, bispecific proteins, and chimeric antigen receptor (CAR)-modified T cells, have been a mainstay of cancer treatments since the mid-1990s[19]. In this Review, we provide a comprehensive overview of the opportunities and challenges associated with developing TCR-based therapies for the treatment of human cancers. We describe the architecture of naturally occurring TCRs and discuss strategies to rationally modify different structural domains of the TCR to create receptors with distinct biochemical, functional, and pharmacologic properties. Next, we summarize how TCRs can be retrieved and compared with respect to potency and off-target toxicity risk. Finally, we conclude by assessing recent clinical developments using the TCR as a drug and provide a vision for how TCRs will be further integrated into clinical care. On the subjects of **HLA-restricted tumour antigens**

[G] and the molecular mechanisms of resistance to T cell-based therapies, we refer the reader to several recent reviews[6,20,21].

Architecture of TCR-based therapeutics

Endogenous TCR.

The TCR is not a single molecule; rather, it is a complex of proteins in which the functions of antigen recognition and signal transduction are divided among distinct subunits. The functional unit of a TCR is an octameric complex composed of six proteins: the clonotypic TCRα/TCRβ hemichains and the invariant CD3γ, δ, ε, and ζ chains (Fig. 1a)[22]. These proteins assemble with a 1:1:1:1 stoichiometry comprised of the dimeric subunits TCRαβ:CD3δε:CD3γε:CD3ζζ. In lieu of TCRαβ, a minority of circulating T cells express **TCRγδ [G]** [23]. Neither TCR hemichain possess a signal transduction domain. Rather, the receptor depends on non-covalent interactions with the CD3 molecules to initiate intracellular signaling, T cell activation, and cell fate decisions. The CD3γ, CD3δ, and CD3ε subunits are genetically related and contain a single **immune receptor tyrosine-based activation motif** (ITAM) **[G]**, whereas the CD3ζ subunit is genetically unrelated and contains three ITAMs.

Structurally, each TCR hemichain is composed of a variable (V) domain, a constant (C) domain, a transmembrane domain, and a short cytoplasmic tail[24]. TCR diversity, and in turn specificity, is generated through combinatorial and junctional diversity involving the Vα/Vβ domains. Like an immunoglobulin's variable heavy chain (V_H), combinatorial diversity of the TCR Vβ domain results from the **somatic recombination [G]** of germline encoded variable (*V*), diversity (*D*), and junctional (*J*) gene segments[1,2]. The Vα domain, like an immunoglobulin's variable light chain (V_L), forms through the recombination of chain-specific *V* and *J* gene sequences. The recombined Vα and Vβ sequences are in turn linked to a Cα domain (encoded by the *TRAC* locus) and one of two Cβ domains (encoded by *TRBC1* and *TRBC2*). Additional combinatorial diversity results from pairing of recombined TCRα and TCRβ hemichains. The two TCR hemichains become covalently linked through a single disulfide bond formed by conserved cysteine residues located in the Cα and Cβ domains[24].

Each TCR heterodimer contains six regions of sequence hypervariability, termed complementarity-determining regions (CDRs). The CDRs are looped structures that project from a TCR's variable domains to form the principal sites of contact with a p/HLA complex[22]. Between the CDRs, each variable chain possesses three framework (FR) regions that facilitate interchain packing of the Vα/Vβ domains and intrachain interfaces of the Vα/Cα and Vβ/Cβ domains[25]. The CDR1 and CDR2 loops are peripherally located in the solvent-exposed terminus of a TCR and are germline encoded. By contrast, the CDR3 loops are centrally located and generated through combinatorial and junctional diversification to create the most polymorphic sequences of each TCR. Beyond the juxtaposition of different V(D)J gene segments, additional diversity in the CDR3 loops is created through the deletion and addition of non-template encoded nucleotides[26].

Most TCRs dock diagonally over the p/HLA complex, placing the Vα and Vβ domains over the N- and C-terminus of an HLA-bound peptide, respectively[8]. This configuration establishes a broad interface between the TCR and p/HLA complex. Further, it positions the regions of greatest TCR sequence diversity, the somatically rearranged CDR3 loops, over a peptide's central core where they contribute to the receptor's fine specificity. The germline-encoded CDR1 and CDR2 loops, by contrast, primarily (although not exclusively) contact the two α-helices that define the binding groove of an HLA molecule. These interactions ensure that a TCR recognizes the p/HLA complex in a peptide-dependent manner. Most naturally occurring TCRs possess comparatively weak binding affinities with a typical disassociation constant (K_d) measured in a micromolar range[27–29]. By comparison, the affinities for mature antibodies are generally in a nanomolar to picomolar range[30].

Exogenous TCRs and genome editing.

The specificity of primary human T cells can be genetically redirected to recognize p/HLA complexes displayed by tumour cells through expression of an exogenous TCRαβ gene sequence (Fig. 1b)[31,32]. To date, integrating retroviral[33–41] and lentiviral[42–45] vectors have been the most common means of introducing exogenous TCRs for clinical applications. However, non-viral genome integration technologies, including the ***Sleeping Beauty* transposon/transposase system [G]** and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9[46,47], can also be used. Despite an extensive safety track record[48], integrating viral vectors possess certain limitations. Viruses integrate semi-randomly with variable copy numbers resulting in heterogenous transgene expression[49,50]. Additionally, viruses are expensive to manufacture[51], can cause insertional mutagenesis[52,53], and possess a relatively limited cargo capacity. Finally, integrating viral vectors leave the endogenous TCR hemichains intact. This can result in mispaired (α'/β, α/β') heterodimers in addition to the properly paired endogenous (α/β) and exogenous (α'/β') TCRs. Expression of endogenous and mispaired TCRs may compromise therapeutic potency by competing for a limited pool of signaling molecules[54–56]. Moreover, because mispaired TCRs have not undergone thymic selection, they can possess novel reactivities, including to self-antigens[57]. T cells expressing mispaired TCRs trigger lethal graft versus host disease (GVHD) in syngeneic mice[58]. Fortunately, GVHD resulting from TCR mispairing has not been observed in human clinical trials[59].

To reduce TCR mispairing, nuclease-based genome editing techniques, including zinc finger nucleases[60], transcription activator-like effector endonucleases[61], and CRISPR/Cas9[49,62,63] can be used to disrupt the endogenous *TRAC* and/or *TRBC1/2* loci. However, these strategies induce double-strand DNA breaks that risk off-target genomic effects, including insertions/deletions and chromosomal translocations[64]. Inhibitory RNAs can silence expression of the native TCR without inducing double-strand DNA breaks, but this approach cannot completely mitigate the risk of mispairing as knock-down is often incomplete[65]. DNA base editors have recently been developed that disrupt protein expression without causing double-strand DNA breaks through splice site inactivation or introduction of a premature stop codon[66]. Manipulation of the *TRAC*[67] and *TRBC1/2*[68,69] loci using DNA base editors might provide a safer alternative for genetic removal of the endogenous TCR.

Beyond disrupting the endogenous TCR, CRISPR/Cas9 may also facilitate the targeted genomic integration of exogenous TCRαβ genes at the *TRAC* locus using template-guided homology directed repair (HDR)[47,62,63]. Targeted integration simultaneously provides for enhanced functionality and a more predictable safety profile compared with TCRs inserted semi-randomly into the genome[70]. Targeted integration enables physiologic antigen receptor regulation using the gene's endogenous promoter, a feature that may prevent immunologic exhaustion[49,70]. Importantly, targeted knock-in using CRISPR/Cas9 can be performed in a non-viral manner through co-electroporation of Cas9 ribonucleoprotein (RNP) and a DNA HDR template[47,62]. This innovation dramatically reduces the time and cost required to generate TCR constructs for clinical use. Non-viral TCR integration has historically been less efficient than viral approaches. Process improvements, including single-stranded DNA donor templates[62,71], nanoplasmids[72], intranuclear template shuttling[73], Cas9 RNP stabilization[73], and small-molecule cocktails[71] may increase editing rates.

Domain engineering.—TCR domain engineering is an alternative means of enhancing exogenous TCR potency and safety. Alterations to the constant and transmembrane domains are especially versatile because they are applicable across therapeutic candidates. For example, placement of additional cysteine residues along the Cα/Cβ interface facilitates the creation of a second interchain disulfide bond, enhancing proper hemichain pairing[74,75]. Murinization of all[54] or selected[76,77] amino acid residues in place of the human Cα/Cβ sequences can also minimize mispairing. Although this approach introduces potentially immunogenic foreign sequences, analysis of patients who received TCRs with murine constant regions has failed to find a correlation between immunogenicity, cellular persistence, and clinical outcomes[41,78]. If no xenogeneic sequences are desired in a cell product, inversion of the human Cα/Cβ domains (domain swapping) can be used as an alternative method to minimize mispairing[79]. The constant chains contain residues that undergo post-translational glycosylation[80]. Removal of glycosylation sites through site-directed mutagenesis enhances the functional avidity of exogenous TCRs, possibly by improving TCR clustering within the immunologic synapse. Finally, strategic replacement of non-ionizable amino acids with aliphatic residues in TCRα's transmembrane domain can increase TCR surface expression[81].

Modifications to the TCR variable domains, including the framework and CDR regions, can also improve TCR function. Unlike constant and transmembrane domain alterations, these modifications require empiric testing and optimization. Exogenously expressed TCRs with identical constant regions are not equally expressed at the cell surface[25,55], a finding that suggests the variable domains contribute to TCR assembly and stability. Comparison of recurrent amino acid sequences in the framework regions of highly versus weakly expressed TCRs revealed three optimal residues at the interface of the variable and constant domains[25]. For TCRs containing suboptimal residues, substitution with optimal amino acids enhances surface expression and *in vivo* antitumour efficacy. Importantly, because framework regions do not participate in antigen binding, changes to these sites will not alter a TCR's cross-reactivity profile.

Mutagenesis of selected CDR residues may enhance a TCR's binding affinity. This can be accomplished through empiric testing[82] or directed evolution techniques[83–85]. Single or

combinatorial substitutions to the CDR1, CDR2, or CDR3 loops can result in as much as a million-fold increase in affinity compared with the native sequence[83]. Beyond modulating binding affinity, alterations to the CDR sequences may also alter TCR specificity. Two clinical trials using affinity-enhanced receptors have resulted in lethal off-tumoru/off-target toxicities attributable to changes in the TCR's cross-reactivity profile[36,86]. Thus, a detailed assessment of the cross-reactivity profile for an affinity-enhanced TCR is requisite before clinical development. Notwithstanding early setbacks, many TCR therapeutics currently in advanced clinical development have undergone affinity enhancement[9,82,87]. A recent strategy that draws from fields in which specificity concerns are paramount (such as DNA binding and enzyme catalysis) is to engineer TCRs with improved specificity[88]. Rather than altering affinity, this structure-guided approach seeks to redistribute attractive interactions across the TCR:p/HLA interface to reduce off-target peptide recognition[89].

Finally, proof-of-concept studies have illustrated how **catch bond [G]** engineering can increase TCR potency without altering binding affinity[90]. Catch bonds are created by the transient formation of hydrogen bonds and salt bridges under shear force conditions immediately prior to receptor disengagement[91]. This results in extended bond lifetimes leading to augmented TCR signaling and T cell activation. Catch bond engineering can be achieved through introduction of polar and charged amino acids into CDR loops located in proximity to but not directly in contact with the p/HLA surface. The goal of this approach is to facilitate the creation of favourable interactions during TCR disengagement from the p/HLA complex akin to a fishhook engaging its prey. Because the affinity of catch bond engineered TCRs generally remains within a physiologic range, the cross-reactivity profile of these receptors should be unaltered as residues that directly contact the ground state p/HLA complex remain fixed.

Soluble bispecific TCRs.

TCRαβ heterodimers can be expressed as a soluble, single-chain, recombinant protein, enabling the development of off-the-shelf reagents that do not require genetic engineering. Soluble TCRs can be fused to a single-chain variable fragment (scFv) derived from an agonistic anti-CD3ε antibody to create bispecific proteins termed 'immune-mobilizing monoclonal TCR against cancer' (ImmTAC) and 'T cell engaging receptor' (TCER) (Fig. 1c)[9,85,92]. ImmTACs and TCERs create a synthetic immunologic synapse between polyclonal T cells and target cells that express a specific p/HLA complex. However, to bind a p/HLA complex in a stable manner as a monomer requires CDR mutagenesis to enhance the TCR's binding affinity into a picomolar range, a value ~10^6 times higher than naturally occurring TCRs. Altering a TCR's binding affinity to this degree may lead to a loss in antigen specificity[93–95]. In the extreme, this can result in a highly promiscuous receptor that binds to a particular HLA molecule independent of the sequence of the bound peptide. Careful assessment of the specificity of affinity-enhanced TCRs, including the use of large panels of antigen and HLA mismatched target cells, is therefore obligatory. T cells redirected using a bispecific TCR exhibit antigen-specific cytolysis of target cells expressing as few as 10 p/HLA molecules[9]. ImmTACs have a smaller molecular weight compared with monoclonal antibodies (75 kDa versus 150 kDa); therefore, they require re-iterative

infusions to maintain potency. Serum half-life extension, such as fusion to a modified IgG Fc molecule, might overcome this limitation[96].

TCR-mimics.

An alternative class of bispecific T cell engaging proteins, termed TCR-mimics, use two antibody-derived scFvs covalently linked through a peptide linker[10,97]. One scFv binds an epitope presented as a p/HLA molecule on cancer cells while the other binds a member of the CD3 complex (Fig. 1d) on T cells. An advantage to using an scFv as the antigen binding domain is that they can be generated *de novo* using high-throughput techniques such as phage[98] and yeast display libraries[98,99], a feature that presently cannot be done for TCRs. This attribute streamlines the time required to develop candidate molecules. Like soluble TCRs, TCR-mimics are small (~55k kDA), enabling close interactions between target cells and T cells. Functional, structural, and molecular dynamic comparisons of TCRs and TCR-mimics has revealed that the two antigen receptors engage their targets in distinct ways[100,101]. These differences are attributable in part to the different orientation of the variable domains of antibodies versus TCRs[102]. TCRs typically bind a broad region of the p/HLA complex, centered along the peptide's core, using energetically balanced interactions involving peptide side chains and invariant regions of the HLA molecule[8]. By contrast, many TCR-mimics dock with a bias towards the extreme terminus of the HLA-bound peptide or to one of the HLA helices using a limited number of highly focused 'hot spot' interactions[100]. A similar binding mode has been observed in conventional TCRs associated with a high degree of cross-reactivity[103,104]. Therefore, TCR-mimics seem to dock with p/HLA in manner that is permissive of a greater degree of peptide sequence variability compared with most TCRs, a property that can result in increased cross-reactivity. This limitation might be overcome by screening for ultra-rare scFvs which bind in a peptide-centric manner[101,105] or using existing TCR-mimic antibodies that dock with a TCR-like topology as a template for mutagenesis[99]. In addition to bispecific engaging proteins, TCR-mimics can be incorporated into various CAR designs[99,101,105].

Designs based on TCR signal transduction

Beyond strategies that use the TCR as a tumour antigen-binding domain, several novel therapies repurpose the physiologic signal transduction machinery of the TCR complex. These alternative TCR-based approaches can enhance receptor sensitivity while potentially reducing cytokine-related toxicities and T cell exhaustion associated with conventional CAR designs.

T cell antigen coupler (TAC).—TAC is a modular, bispecific transmembrane protein expressed as a transgene within T cells that incorporates two binding domains[106]. One binding domain is used for tumour antigen recognition and the second is used for recruitment of signaling components from the endogenous TCR complex (Fig. 1e). Unlike conventional CARs that integrate domains for T cell activation, co-stimulation, and antigen binding into a single molecule, a TAC lacks the intrinsic capacity to signal. Functionally, TAC-modified T cells exhibit antigen-specific cytokine production and cytolytic activity against target cells in the absence of tonic-signaling, a feature of some CAR designs that results in accelerated T cell exhaustion[107]. *In vivo*, TAC-modified T cells demonstrate

comparable antitumour efficacy to conventional CAR-modified T cells but without cytokine-related toxicities.

TCR fusion construct (TRuC).—TRuC is an alternative antigen receptor design that, like a TAC, also engages the endogenous TCR signaling complex[108,109]. However, unlike a TAC, the TRuC covalently links an antigen-binding scFv to the extracellular region of the CD3ε molecule using a non-immunogenic $(Gly_4Ser)_3$ linker (Fig. 1f). Biochemical studies demonstrate that up to two TRuC molecules are incorporated into the TCR complex, a finding that is consistent with the natural stoichiometry of CD3ε within the TCR complex[22]. Like TAC-modified T cells, TRuC-modified T cells do not exhibit tonic-signaling and release significantly lower levels of cytokines compared with CARs while retaining antigen-specific cytolytic potency.

Synthetic TCR antigen receptor (STAR)/HLA-independent TCR (HIT).—STAR/HIT is a non-HLA restricted receptor that replaces the variable domains of the TCR with the V_H/V_L domains of an antibody (Fig. 1g)[12,110]. Because the TCR variable regions have a similar size and tertiary fold as V_H/V_L, these domains can be replaced interchangeably. By retaining the TCR constant domains, the STAR/HIT receptor recruits the full complex of CD3 signaling molecules. Like exogenous TCRs, the STAR/HIT receptor is a heterodimer capable of mispairing with endogenous TCR hemichains. Mispairing can be minimized using the same strategies as exogenous TCRs. Following antigen-stimulation, the STAR/HIT receptor induces TCR-like signaling, enabling enhanced responsiveness to target cells with lower antigen site densities.

Discovery of therapeutic TCR candidates.

From tumour infiltrating lymphocytes (TILs).

Cancers are frequently infiltrated by T cells, a subset of which are reactive against various classes of tumour antigens. These include tissue differentiation antigens[111], cancer germline antigens[112,113], antigens associated with transforming oncoviruses[112], and mutation-derived neoantigens[111,112,114–116]. Consequently, TILs can serve as a source for TCR gene sequences that confer antitumour immunity (Fig. 2a). Despite being enriched in tumour-reactive T cells, TCR frequency alone is generally insufficient to predict tumour-reactivity[117,118]. This is because most TILs are passive bystanders with specificity for viruses or other pathogens[111,116,119]. Strategies to identify and isolate tumour-specific TIL have therefore been developed which fall into three categories: phenotypic, functional, and transcriptomic.

Phenotypic markers are constitutively expressed membrane-associated proteins, making them straightforward to measure and convenient to use in large-scale isolations. Detection of phenotypic markers is accomplished with fluorescently-labeled monoclonal antibodies using FACS analysis or molecularly barcoded antibodies followed by **cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) [G]**[120,121]. Expression of the ATPase CD39[119,120,122–124], the tissue-resident marker CD103[120,122], or the immune checkpoint receptors PD-1[118,125–127], lymphocyte activation gene 3 protein (LAG-3)[125], and T-cell immunoglobulin mucin receptor 3 (TIM-3, also known as Hepatitis A virus cellular

receptor 2 (HAVCR2)) [125] identify tumour-reactive TIL populations. In the case of $CD4^+$ TIL, cells with a regulatory T (Treg) cell phenotype may serve as an additional source of TCR sequences that confer reactivity to HLA class II-restricted tumour antigens[121,128].

Functional markers either directly assess the antigen-specificity of TIL or measure TCR ligation-induced changes in surface marker expression. Although technically more challenging to assess than phenotypic markers, functional markers enable enrichment of tumour-reactive TIL with greater specificity. TCRs specific for cancer antigens can be identified through binding to synthetic HLA multimers loaded with tumour-associated peptides. Fluorochrome-conjugated peptide (p)/HLA multimers facilitate the sorting of antigen-specific TIL using FACS or magnetic bead isolation[129]. However, this approach requires a large starting population and has a limited capacity to simultaneously screen multiple antigen specificities. CITE-seq using DNA-barcoded p/HLA multimers combined with single-cell sequencing enables high-throughput screening and retrieval of antigen-linked TCR sequences using smaller sample sizes[130]. Alternatively, the specificity of TCR clonotypes from TILs of unknown specificity can be de-orphaned using yeast display or antigen-presenting cells (APCs) displaying highly diverse p/HLA libraries[131–135]. Despite the utility of each of these approaches, practical limitations restrict the total number of HLA alleles, epitopes, and peptide lengths that can be screened at a time. Two members of the tumour necrosis factor receptor superfamily, 4–1BB (also known as TNFRSF9 and CD137) and OX40 (also known as TNFRSF4 and CD134), are not expressed on resting T cells but are upregulated after antigen stimulation. Unlike cytokine expression, expression of 4–1BB and OX40 is dependent on TCR-ligation and occurs independently of T cell differentiation. TIL selection based on expression of these two markers can therefore serve as a functional strategy to retrieve tumour-reactive TCRs without *a priori* knowledge of the epitopes they recognize[136,137].

Finally, single-cell transcriptomic signatures can identify tumour-reactive TCR clonotypes directly *ex vivo* without the need for expansion or functional testing. Expression of the chemokine *CXCL13*[120,127,138,139] and multiple exhaustion-related genes, including *ENTPD1* (encoding CD39), *PDCD1* (encoding PD-1), *HAVCR2*, and *TIGIT* are higher in cancer-specific T cells compared with bystander T cells[111,116,120,121,127,139]. Bystander T cells, by contrast, often express high levels of memory-associated genes such as *IL7R* and *TCF7*[111,116].

From circulating T cells in cancer patients.

TCR clonotypes expressed by TILs can be found among circulating T cells, albeit at significantly lower frequencies[111,116,140]. Thus, peripheral blood can serve as a minimally invasive source of tumour-reactive T cells (Fig. 2a). In select cases, T cells that are specific for cancer antigens and have undergone clonal expansion are detectable directly *ex vivo* using p/HLA multimers[129]. More commonly, strategies that enrich for rare TCR clonotypes are required. The co-inhibitory molecule PD1[112,140] and T-cell memory marker CD45RO[141] identify circulating populations containing antitumour T cells. However, because tumour-reactive T cells comprise a minor fraction of these pools, *ex vivo* T cell expansion is required for reactivity screening and TCR sequencing. This can be accomplished by *in vitro*

sensitization (IVS) using monocyte-derived dendritic cells pulsed with tumour-associated peptides or electroporated with RNA encoding tumour antigens.

If the peptide sequence and restricting HLA allele for a tumour antigen are known, antigen-specific T cells can be isolated directly using synthetic p/HLA complexes. Conjugation of the p/HLA complex to a fluorescent dye[129], ***Strep*-tagII sequence [G]**[142], or **paramagnetic artificial APC [G]**[143] permits enrichment and expansion of rare T cell populations. Alternatively, tumour-reactive T cells can be captured directly *ex vivo* and their corresponding TCR gene sequences retrieved in a single step using DNA-barcoded magnetic nanoparticles displaying p/HLA multimers[47].

From healthy donors.

A significant proportion of HLA-presented peptides fail to elicit T cell responses in patients with cancer[144,145]. This may occur because of ineffective priming or because prior lines of cancer treatment negatively influence tumour-reactive T cell fitness[146]. To overcome these limitations, TCR discovery can be 'outsourced' using the naïve T (T_N) cell repertoire of healthy donors (Fig. 2a). In this approach, T_N cells are isolated to maximize TCR repertoire diversity prior to IVS using APCs. APCs can be pulsed with peptide[63,147] or transfected with mRNA encoding a tumour antigen to enforce more physiologic antigen presentation requiring proteolytic degradation and endogenous HLA loading[144,148,149]. Alternatively, antigen-specific T_N cells may be isolated directly without prior IVS using p/HLA multimer enrichment followed by single-cell sorting[150]. Screening multiple healthy donors increases the likelihood of detecting a T cell response and the diversity of T cell clonotypes generated[63,144,148]. In the case of tumour-associated antigens, in which expression is shared by cancer cells and normal tissues, HLA mismatched donors may be used as a strategy to retrieve high-affinity TCR sequences[32,149,151]. This is because the T cell repertoire reactive against allogeneic HLA molecules is not subject to negative thymic selection. However, because TCRs retrieved from mismatched donors may exhibit promiscuity for generic allogeneic HLA molecules[152], systematic screening for potential off-target reactivities is required prior to clinical testing[153].

From HLA transgenic mice.

Transgenic (Tg) mice that express HLA molecules and have been immunized with human tumour antigens can be used as a source of TCR candidates (Fig. 2b). There are several advantages to this approach. First, it exploits differences in the protein sequences of mice and humans as a strategy to overcome the negative influence of thymic selection on the pool of TCRs targeting self-antigens[154]. Second, because rodents are relatively easy to immunize, HLA Tg mice are a time and cost-efficient way of generating diverse TCRs. Variable sequences of T cell clones retrieved from immunized HLA Tg mice have been used as the source of TCRs for several clinical trials[34–36]. Evidence of on-target antitumour immunity in patients who received TCRs obtained from HLA Tg mice validates the therapeutic potential of this approach. However, a major limitation of TCRs sourced in this manner is that the receptor's murine variable sequences can contain immunogenic epitopes[78].

From humanized mice.

To overcome the limitations of using TCRs with murine variable sequences, Tg mouse strains that express the entire human TCRαβ genomic loci but are deficient in murine TCR sequences have been generated[155,156]. Conceptually, this approach builds on prior work demonstrating that Tg mice engineered with the human immunoglobulin V_H/V_L gene loci can serve as a source of fully human antibody sequences[157]. Crossing TCR humanized mice with HLA Tg mice enables the sampling of a diverse human TCR repertoire that is HLA-restricted. Immunization of TCR humanized mice with cancer antigens generates TCR candidates with fully human variable sequences (Fig. 2b). In some cases, TCRs cloned from humanized mice demonstrate higher functional avidity compared with human-derived TCRs[158,159].

Vetting TCRs for safety and potency.

Assessing on-target/off-tumour toxicity risk.

On-target/off-tumour toxicities are expected when tumour cells and normal tissues share expression of the same p/HLA complexes[160]. Consequently, toxicity risk mitigation for therapeutic TCR candidates begins with rigorously assessing antigen expression by healthy cells (Fig. 3a). In the case of epitopes resulting from somatic mutations and transforming oncoviruses, off-tumor toxicity risk is minimized because normal tissues do not express these proteins. For other antigen classes, an initial assessment of expression can be made using publicly available transcriptomic and proteomic databases. RNA-sequencing (seq) has high sensitivity, high specificity, and a large dynamic range[161]. RNA-seq databases, such as the Genotype-Tissue Expression (GTEx) project[162], provide high-quality sequencing results from multiple normal tissues. Human single-cell RNA expression atlases complement bulk RNA-seq data by facilitating the identification of rare cell populations[163]. Because the correlation between gene and protein expression is imperfect[164], detectable RNA transcripts within critical tissues is alone insufficient to conclude a candidate antigen represents an unsafe target. Analysis of protein level expression is therefore advisable as a confirmatory step. Finally, it is important to consider that select tissues, such as the testes, do not express HLA molecules and therefore are impervious to direct T cell recognition[165].

Assessing cross-reactivity potential.

TCRs can also mediate off-target toxicities resulting from the degeneracy potential of any individual TCR (Box 1). The safety profile of a TCR is not driven by the absolute number of peptide sequences it can potentially bind; rather, it is determined by the capacity of a TCR to engage peptides resulting from the endogenous processing and presentation of off-target human proteins in the context of an HLA molecule. Unacceptable toxicities will result only in cases in which an off-target protein is expressed by healthy tissues that perform critical functions. Differences in protein sequences between laboratory animals and humans combined with the absence of HLA expression limit the utility of *in vivo* toxicology studies for assessing on- and off-target TCR toxicity risks. *In vitro* strategies are therefore generally required to quantify the degeneracy potential and safety profile of TCR candidates. It remains an outstanding question in the field which assays should be performed and under what circumstances to assess for cross reactivity. Below, we present an overview of several

techniques which have been used to support **Investigational New Drug applications [G]** for TCRs as well as evolving technologies which may be useful for de-risking candidates.

Alloreactivity occurs when a TCR displays cross-reactivity to HLA molecules that were not encountered during thymic development[166]. Using a panel of HLA mismatched cell lines that express prevalent HLA alleles but lack expression of the target antigen can establish whether a TCR possesses alloreactivity (Fig. 3b)[87]. If a TCR does not display alloreactivity, additional methods should be used to assess the specificity of a TCR for targets that express the restricting HLA allele but are bound by alternative self-peptides. This can be accomplished by screening candidate TCRs against a panel of normal cells obtained from HLA-matched healthy donors[87,92,153]. However, even large panels may fail to include highly specialized or rare cell types. One strategy to enhance representation of cellular subsets from vital organs, such as the heart or kidney, is the use of induced pluripotent stem cells or normal tissue organoids[86,167].

A complementary technique to define a TCR's cross-reactivity potential, termed amino acid positional scanning, begins by establishing which peptide residues form critical contacts with the receptor (Fig. 3c)[86,168]. In this approach, each amino acid in the cognate peptide is sequentially replaced with alanine, the smallest chiral amino acid. An alternative compact amino acid, such as glycine, can be used in cases in which the native residue is alanine. The recognition motif of a TCR is defined by peptide positions in which an amino acid substitution results in significant loss of function (typically >50%) compared with the native amino acid. The clinical utility of amino acid scanning was demonstrated when it identified an off-target peptide derived from titin, a cardiac structural protein, as the cause of lethal cardiac toxicities resulting from an affinity-enhanced TCR targeting an epitope in the melanoma-associated antigen (MAGE)-A3 protein[86]. Positional scanning can be extended to include all 20 standard amino acids to measure a TCR's permissiveness for peptides containing chemically similar residues[94]. Once a TCR's recognition motif has been determined, these data are used to perform *in silico* searches to establish whether homologous motifs exist elsewhere in the human proteome.

Although positional scanning provides useful information on which peptide positions contribute to TCR recognition, the technique can miss important cross-reactivity. For example, some peptide sequences function as potent agonists yet display minimal homology with the native peptide recognized by a TCR[169]. This can occur as a result of intrapeptide coupling whereby modification to residues outside a peptide's central core facilitates the generation of new TCR contacts[103,170]. Combinatorial peptide libraries (CPLs) are highly diverse (often 10^8 to $>10^{11}$ variants) and contain peptides in which one amino acid position is sequentially fixed while the remaining positions are substituted with all 20 amino acids. CPLs have shown promise in identifying peptide targets[153,171], especially when combined with bioinformatic database screening[172]. Alternatively, yeast display[170], phage display[92], and molecularly-barcoded artificial APCs[132–135] can enhance the speed and sensitivity of cross reactivity screening. In all instances where potential cross-reactive peptides are identified, additional studies are required to establish physiologic significance.

Measuring potency.

A minimal measure of potency for TCRs being considered for clinical development is the capacity to recognize polypeptides resulting from endogenous processing and presentation of a protein (Fig. 3d–i). For HLA-I-restricted TCRs, this should ideally be complemented with assays that establish whether physiologic expression levels of the full-length protein and restriction element are sufficient for recognition. In addition to cytokine secretion, it is important to also compare the capacity of different TCR candidates to cause tumour cell lysis[90]. Public databases can identify commercially available tumour cell lines that co-express a target antigen and the restricting HLA allele[173]. However, it is not always possible to identify established lines that express a p/HLA pair of interest. One solution to overcome this limitation is the creation of an ‘avatar’ that represents a tumour’s HLA-restricted antigenic landscape. This can be accomplished using tandem minigenes (TMGs) or synthetic long peptides containing somatic mutations, gene fusions, insertions/deletions, integrating viruses, or cancer-germline antigens[114,174]. Transfection of TMGs or pulsing of synthetic long peptides onto autologous APCs enforces antigen processing and presentation by a patient’s complement of HLA molecules. More recently, patient-derived **tumour organoids [G]** have been used to sample the repertoire of naturally processed peptides displayed on a cancer cell’s surface[175,176]. Relative to established cancer cell lines, cancer organoids better approximate the genomic heterogeneity, phenotype, and three-dimensional characteristics of human tumours *in vivo*[177]. Further, unlike patient-derived xenografts, normal tissue organoids may be established in parallel, providing a complementary approach for assessing off-target effects. Notwithstanding these virtues, organoids are time consuming and expensive to generate. Characterization of TCRs retrieved from $CD4^+$ T cells poses additional challenges because many solid cancers lack steady-state expression of the genes required for HLA class II presentation[178]. This barrier can be overcome by exogenous expression of class II major histocompatibility complex transactivator (CIITA)[179], a master transcriptional regulator for HLA II expression.

Functional avidity, the capacity of a T cell to respond to progressively lower concentrations of cognate peptide, is a commonly used and technically straightforward method to compare different TCRs. In general, functional avidity correlates with the magnitude of tumour cell recognition[180–182]. However, functional avidity measurements are influenced by multiple factors independent of the intrinsic properties of a TCR, including T cell differentiation[183] and activation history[184]. Moreover, distinct T cell functions (such as cytokine production and cellular proliferation) are trigged by different antigen thresholds[185]. Alternative assays that assess TCR potency which are quantitative, reproducible, and independent of T cell state are therefore highly desirable.

Binding affinity is the strength with which a single TCR molecule interacts with a single p/HLA complex and is typically represented by the disassociation equilibrium constant (K_d). The binding affinity of naturally occurring TCRs has correlated with antitumour efficacy in preclinical models[180,186] and human clinical trials[33,34,40,41]. Under equilibrium conditions, K_d is defined by the ratio of the rates of dissociation and association (k_{off}/k_{on}). Most thymically-selected TCRs have K_d values in a 1–200 μM range[27–29]; however, directed evolution techniques can generate TCRs with picomolar binding affinities[83,84]. TCRs that

bind self-antigens often possess K_d values at a higher (weaker) end of the physiologic range, likely because of negative thymic selection. By contrast, TCRs that target mutation-derived neoantigens tend to have lower (stronger) K_d values in a range that overlaps with pathogen-associated receptors[148,187–189]. K_d values are commonly measured using surface plasmon resonance (SPR), a three-dimensional technique in which a recombinant single-chain soluble TCR is flowed over a sensor chip containing an immobilized p/HLA complex (or vice versa)[190]. Binding affinity correlates to varying degrees with TCR functional responses[90,191]. However, this correlation is not universally true. In the extreme, two TCRs can bind the same p/HLA complex with near equivalent K_d values yet display disparate signaling responses (for example, one receptor may trigger T cell activation while the other does not)[91]. The uncoupling of a TCR's three dimensional binding affinity from its signaling capacity has in some cases been attributed to the inability of SPR to account for force-dependent interactions, such as catch bond formation[90,91]. Two dimensional methods directly measure TCR affinity and/or binding kinetics on living T cells, bypassing the need for recombinant TCR expression and purification[192]. Although two dimensional affinities correlate more closely with biological outcomes[192,193], this technique remains relatively low throughput and requires specialized instrumentation.

To enable more rapid throughput, flow cytometry-based approaches have been developed that either infer TCR affinity or directly measure the dissociation kinetics of monomeric TCR-p/HLA complexes on living T cells[194–196]. The degree to which a T cell depends on the CD8 co-receptor for activation is inversely correlated to a TCR's affinity and its disassociation half-life from a p/HLA molecule[197]. Thus, a TCR that binds p/HLA multimers[182,197] or triggers antigen-specific effector functions in a co-receptor independent manner[148,182] qualitatively implies the receptor is relatively high-affinity. A quantitative flow cytometry-based measurement is structural avidity, the capacity of an individual membrane-associated TCR to bind monomeric p/HLA molecules. Unlike functional avidity, structural avidity measurements are agnostic of T cell differentiation state and therefore demonstrate a high degree of concordance between T cell clones and TCR transduced T cells[198]. Methodologically, structural avidity measures the TCR-p/HLA dissociation rate (k_{off}) using p/HLA multimers that dissociate into monomers following addition of an inert chemical compound. Adoptive cell transfer (ACT) of T cells expressing TCRs with slower k_{off}-rates provide superior antitumour efficacy compared with TCRs with faster k_{off}-rates[198–200].

Clinical efficacy of TCR-based cancer immunotherapies.

TIL.

Because TILs are frequently enriched in tumour-reactive TCR clonotypes, they can be used as a source of therapeutic T cells for non-receptor engineered ACT. TIL therapies are generated through the surgical resection of a metastatic tumour followed by *ex vivo* T cell expansion to achieve treatment numbers (up to 10^{11} cells). T cells can either be expanded in bulk or following selection based on evidence of tumour reactivity. TILs are typically administered following chemotherapy pre-conditioning to deplete bystander lymphocytes that function as 'sinks' for homeostatic cytokines, such as IL-15, and remodel

the immunosuppressive tumor microenvironment[201–203]. TIL infusion is often followed by a multi-day course of the **common gamma chain cytokine [G]** IL-2 to promote T cell engraftment[204]. Melanoma[111,121,205,206] and cancers of the bladder[207], breast[208], cervix[112,209], gastrointestinal (GI) tract[115], head and neck[209], kidney[210], lung[113,116,211], and ovary[136,206] contain TILs with HLA-dependent reactivity to cancer antigens. For many of these cancers, TIL ACT caused objective tumour regression in early phase clinical trials[113,174,205,208,209,212], a registration enabling single-arm phase II trial[213], and a randomized phase III trial[214]. These findings establish proof of principle that TCR-based therapies can mediate cancer regression in a broad range of human malignancies.

The largest clinical experience with TIL comes from patients with metastatic cutaneous melanoma[215]. In patients who have not received prior treatment with anti-PD-1/PD-L1 immune checkpoint inhibitors (ICIs), both the overall and complete response rates (ORR and CR, respectively) are relatively high (ORR: 40–62%; CR: 7–24%)[212,214,216]. Importantly, ~96% of patients with a CR to TIL therapy do not have disease recurrence[217]. Response to TILs in melanoma can occur following progression on other therapies, including ICIs [213,214,217]. However, whereas prior anti-CTLA-4 does not appear to impact TIL efficacy[217,218], response rates often are lower following progression on antibodies targeting the PD1/PD-L1 axis[213,217,219]. This suggests that TIL and anti-PD1/PD-L1 therapies have partially overlapping mechanisms of response and resistance.

Consistent with this hypothesis, meta-analyses of >1000 patients treated with ICIs established a significant association between tumour mutational burden (TMB) and the likelihood of ICI response[138,220]. In parallel, TIL studies have similarly discovered correlations between response rate and TMB[219,221] or response and the frequency of neoantigen-reactive T cells in the infusion product[222]. These data suggest that the success of TIL and ICI therapies is likely dependent on the abundance of neoantigen-reactive T cells. Consequently, next-generation TIL approaches in which neoantigen-reactive T cells are selectively expanded and/or enriched might allow for more consistent tumour control.

Beyond cutaneous melanoma, TIL therapies have clinical activity in **uveal melanoma [G]** [205] and several common epithelial malignancies[174,208,209]. Unlike cutaneous melanoma, uveal melanomas are modestly mutated and largely refractory to ICIs[223]. In a phase II clinical trial, ACT of uveal melanoma TILs caused cancer regression in seven out of 20 patients, including one CR (ORR: 35%; CR: 5%)[205]. Among responding patients, three had previously progressed on ICIs. In a *post hoc* analysis, responding patients received significantly higher numbers of tumour-reactive T cells compared with non-responders. In a second study involving 16 patients with non-small cell lung cancer who received TIL after tumour progression on an anti-PD1 antibody, three had evidence of cancer regression including two durable CRs (ORR: 19%; CR: 13%) ongoing 1.5 years later[113]. Responders in this study were significantly more likely to have received TIL containing neoantigen and cancer germline-reactive T cells compared with non-responders. Similar results were observed in a third trial that tested TIL therapy in human papillomavirus (HPV)-associated cancers. Among 18 patients with HPV^+ cervical cancer who received TILs, five had objective responses including two durable CRs (ORR: 28%; CR: 11%) ongoing 4 years later[209]. Immune-monitoring studies revealed that the infusion products

of the two patients with CRs contained T cells specific for neoantigens, cancer germline antigens, and peptides derived from the HPV E6/E7 oncoproteins[112]. In a cohort of patients with HPV^+ oropharyngeal, anal, and vaginal cancers, two out of 11 subjects had a partial response (ORR: 18%; CR: 0%). Across both cohorts, the frequency of HPV E6/E7-reactive T cells in the infusion product and the persistence of tumour-reactive T cells in the peripheral blood correlated with the likelihood of response.

Finally, case reports have documented responses to TIL ACT in modestly mutated epithelial malignancies. For example, ACT of neoantigen-selected TILs resulted in tumour regression in two patients with gastrointestinal malignancies associated with **DNA mismatch repair proficiency [G]**[174,224]. One patient with cholangiocarcinoma, an aggressive bile duct cancer, had a prolonged PR following infusion of a near clonal $CD4^+$ TIL population that recognized a patient-specific private neoantigen[174]. Of note, this patient failed to respond to an unselected TIL population before receiving >10-fold higher dose of neoantigen-selected T cells. A second patient with metastatic colorectal cancer also had a partial response following infusion of neoantigen-selected $CD8^+$ T cells[224]. In this case, ~75% of transferred T cells recognized a shared, or public[225], neopeptide derived from a recurrent $KRAS^{G12D}$ hotspot mutation. All metastases regressed in this patient except for a single lung lesion. Analysis of this escape lesion revealed loss of heterozygosity (LOH) for the HLA allele that presents the $KRAS^{G12D}$-derived peptide, establishing a mechanism of targeted immune escape. Another patient in this study received TILs with reactivity to the same $KRAS^{G12D}$ public neoantigen with no response. In this case, only 0.002% of the infusion product was neopeptide-specific. Finally, three patients with metastatic breast cancer experienced objective tumour regression following neoantigen-selected TIL therapy[208]. In one patient who achieved a durable CR, ~23% of T cells in the infusion product targeted neoepitopes resulting from four somatically mutated genes that were restricted by HLA-I and HLA-II alleles. The other two patients had PRs lasting 6 and 10 months, respectively. Together, these examples provide further evidence that the infusion of neoantigen-reactive T cells can trigger tumour regression in humans.

T cell clones.

Although TILs are relatively enriched in tumour-reactive T cells, they typically contain large numbers of bystander T cells that do not contribute to antitumour immunity[111,116,117,119]. One strategy to enrich for a homogenous cell product is ACT of expanded T cell clones of a single, well-defined specificity. T cell clones have been generated from TILs and the circulating repertoire of cancer patients using IVS alone[226], IVS followed by p/HLA sorting[227], or IVS followed by limited dilution cloning[228–232]. Initial clinical experience of ACT with T cell clones focused on the targeting of non-mutated tissue differentiation antigens. Most early T cell clone clinical trials targeted epitopes derived from the shared melanocyte/melanoma differentiation antigens **MART-1 [G]**, gp100, and tyrosinase[228,232,233]. Overall, patients experienced limited treatment-related toxicities in these studies, with most side effects attributable to expected on-target/off-tumour destruction of normal melanocytes. However, clinical activity has generally been modest (ORR <10%).

Another important antigen class are the cancer-germline antigens (CGAs), a family of >100 immunogenic intracellular proteins whose normal tissue expression is typically, although not universally, restricted to germ cells and fetal tissues[234]. Because germ cells lack HLA expression[165], they are immune privileged and impervious to T cell-mediated attack. Consequently, targeting CGAs can afford a wider therapeutic index compared with tissue differentiation antigens. Many CGAs are epigenetically silenced in somatic tissues through promoter methylation[235]. Epigenetic dysregulation during tumourigenesis can lead to de-repression of CGA genetic loci, resulting in expression of a cancer-selective target. In a case study, a patient with refractory metastatic melanoma who received an HLA-II restricted $CD4^+$ T cell clone targeting the CGA New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1) experienced long-term and complete tumour regression[230]. Similar to findings using TILs, a correlation exists between the *in vivo* persistence of transferred T cell clones and the likelihood of clinical response[236]. The limited clinical efficacy of T cell clones observed across most trials is likely attributable to the extended clone manufacturing process resulting in enhanced cellular differentiation and poor *in vivo* persistence[237]. Strategies that enhance cellular fitness by altering cytokines during T cell priming[227], selection of clones with memory-like attributes[238,239], and optimize co-stimulation during expansion[226] may improve outcomes.

TCR-engineered T cells.

TCR gene transfer streamlines many practical challenges associated with TIL and T cell clone therapies. These benefits include: 1) a minimally invasive procedure to procure autologous T cells (such as **leukapheresis [G]**); 2) a high probability of developing potent cell products in a relatively short time; 3) the opportunity to pre-select TCRs with optimal potency and off-target profiles; 4) the capacity to introduce TCRs into minimally-differentiated T cell populations with superior engraftment and proliferative potential[240–242]; and 5) the opportunity to concurrently introduce genetic manipulations that enhance T cell function through augmented T cell survival[243–245], resistance to inhibitory ligands[45,245,246], or enhanced antigen-driven signaling through the TCR complex[247–250]. As summarized below, TCR clinical trials have targeted diverse classes of antigens and, in many cases, distinct epitopes derived from the same antigen (Table 1; Supplementary Table 1).

Tissue differentiation antigens.—As in studies using T cell clones, initial TCR gene therapy studies targeted non-mutated tissue differentiation antigens. The first published human TCR clinical trial tested an HLA-A*02:01-restricted receptor specific for a MART-1 peptide that was cloned from melanoma TILs and transduced into an allogeneic T cell line[251]. The first two TCR clinical trials using autologous T cells sequentially tested a pair of receptors (DMF4 and DMF5) specific for the same MART-1 peptide but cloned from the TILs of a separate patient. In the first autologous trial, out of 31 patients with melanoma who received T cells retrovirally transduced with the CD8 co-receptor dependent DMF4 TCR, four achieved a PR (ORR: 13%)[33,34]. No patients developed toxicities related to TCR-modified cells. In a second trial, patients received T cells modified with the DMF5 receptor, a CD8 co-receptor independent TCR with an affinity ~5-fold higher than DMF4[252]. Among 20 patients who received the DMF5 TCR, six had an objective tumour response (ORR: 30%)[34]. However, a significant proportion developed on-target toxicities

related to destruction of MART-1 expressing melanocytes present in the skin, eye, and inner ear. Antitumour activity and on-target toxicities were observed in a third trial testing a CD8 co-receptor independent TCR targeting an HLA-A*02:01-restricted epitope derived from the melanocyte-associated protein gp100[34]. Unlike DMF4 and DMF5, this receptor was generated in an HLA-A*02:01 Tg mouse. Among 16 treated patients, three had responses (ORR: 19%; CR: 6%). Significant on-target toxicities related to melanocyte targeting was also observed in this study. The liability of targeting tissue differentiation antigens was established outside the context of melanoma in a fourth trial targeting a peptide derived from the gastrointestinal lineage marker carcinoembryonic antigen (CEA)[35]. Three patients with colorectal cancer received T cells engineered with an affinity-enhanced TCR generated in an HLA Tg mouse. Following T cell infusion, all patients had significant reductions in serum CEA, indicating on-target engagement, and one had a PR (ORR: 33%). However, all patients developed severe inflammatory colitis resulting from T cell mediated destruction of CEA^+ colonic epithelial cells. Collectively, these early clinical trials established that TCR gene therapy can trigger cancer regression but simultaneously highlighted the critical importance of target selection and receptor affinity.

Overexpressed, non-mutated antigens.—Non-mutated epitopes derived from transcriptional regulators, including Wilms' tumour 1 (WT1) and P53, are frequently overexpressed by haematologic and solid malignancies[253–255]. Although not cancer-specific[167], expression of these proteins can differ by as much as a 1000-fold between normal and transformed tissues, providing a potential therapeutic window. Several HLA-I restricted WT1 epitopes have been targeted using TCRs cloned from healthy donors with no evidence of normal tissue toxicity[39,44]. In one study which tested a CD8 co-receptor dependent TCR in patients with leukaemia[39], no objective antitumour responses were observed. Two CD8 co-receptor independent WT1 TCRs[44,63] have entered therapeutic trials for liquid and solid cancers. In one study, the receptor is integrated into the *TRAC* locus followed by disruption of the *TRBC*1/2 loci using CRISPR editing (NCT05066165)[63]. Efficacy results for these studies are forthcoming. A CD8 co-receptor independent TCR targeting a wild-type P53 epitope generated in an HLA Tg mouse[256] was tested in an early phase clinical trial. Although allogeneic T cells transduced with this TCR demonstrated reactivity to a diverse range of cancers *in vitro*, autologous T cells expressing this receptor could not be efficiently expanded[257]. This finding correlates with increased wild-type P53 expression by activated T cells and TCR-dependent fratricide. These data indicate that differences in wild type P53 expression by normal cells compared with cancer cells is likely insufficient to permit safe immunologic targeting.

Cancer germline antigens.—The first CGA TCR trials targeted an HLA-A*02:01-restricted epitope derived from NY-ESO-1 using an affinity-enhanced, CD8 co-receptor independent receptor cloned from a patient with melanoma[82]. An initial trial in patients with melanoma reported a >50% overall response rate (ORR: 55%; CR: 20%) without off-tumour toxicities[258]. This response rate is comparable to that observed in a contemporaneous group of patients with melanoma treated with TILs[212], suggesting that single epitope targeting may be comparable to approaches targeting multiple antigens. Antitumour efficacy using this TCR has also been observed in synovial cell sarcoma and myxoid/round cell liposarcoma

(ORR: 40–61%; CR: 0–8%)[43,258,259], two mesenchymal cancers that respond poorly to other immunotherapies.

The MAGE-A family of CGAs is comprised of 12 genes[260], several of which have been targeted in TCR trials. The first two MAGE TCR trials targeted the A3 isoform using HLA-I restricted receptors. One trial used an affinity-enhanced, CD8 co-receptor independent TCR generated in an HLA-A*02:01 Tg mouse[261]. Objective responses occurred in 5 out of 9 patients (ORR: 56%; CR: 11%) treated as part of a dose-escalation study[36]. However, 3 out of 5 patients who received the highest T cell dose developed severe neurologic toxicities resulting in two treatment-related deaths. These toxicities resulted from the off-tumour destruction of a previously unappreciated neuronal population expressing MAGE-A12, a protein with >95% homology to MAGE-A3. At the same time, a second trial targeting MAGE-A3 reported lethal toxicities using an affinity-enhanced TCR restricted by HLA-A*01:01[42,86]. Two patients in this study developed cardiogenic shock following T cell infusion resulting from the off-tumour/off-target recognition of an epitope derived from titin. In this case, affinity-enhancement altered the native receptor's specificity in a manner that was initially not detected in preclinical studies using a diverse panel of normal cells that express the restricting HLA allele. A third trial also targeted a MAGE-A3 epitope[38]. Unlike the other studies, the patient-derived TCR used in this trial was restricted by HLA-II and did not undergo affinity-enhancement. As part of the manufacturing process, $CD4^+$ T cells were isolated to test the safety and antitumour efficacy of an HLA-II-restricted TCR in a physiologic context. Among 17 treated patients, none developed off-tumour toxicities and four had objective tumour responses (ORR: 24%; CR: 6%). Interestingly, many patients experienced prolonged fevers following treatment, a finding that may be attributable to enhanced cytokine secretion by $CD4^+$ T cells.

Both MAGE-A4 and MAGE-A10 have also been targeted in TCR gene therapy trials. An initial trial targeting MAGE-A4 using an HLA-A*24:02-restricted, CD8 co-receptor dependent, non-affinity enhanced receptor failed to demonstrate antitumour activity[37]. By contrast, an affinity-enhanced TCR targeting this CGA showed efficacy in soft tissue sarcomas[262] but limited activity in other solid cancers[263]. A multi-cistronic vector that co-expresses CD8α and the TCR seems to improve responses in common epithelial malignancies (ORR: 36%; CR: 5%), including ovarian, head and neck, and gastroesophageal cancers[264]. Several trials have targeted MAGE-A10 with an affinity-enhanced HLA-A*02:01-restricted TCR[94]. To date, there has been no evidence of off-tumour toxicities with this receptor; however, there has been minimal evidence of clinical activity[265,266].

Despite its name, preferentially expressed antigen in melanoma (PRAME) is a CGA that is frequently expressed by both melanoma and non-melanoma cancers[267]. A dose-escalation trial using a naturally occurring TCR targeting an HLA-A*02:01-restricted PRAME epitope recently reported responses in multiple solid cancers (ORR: 50%; CR: 0%) without evidence of off-target toxicities[268]. A TCR targeting an alternative HLA-A*02:01-restricted PRAME epitope has entered clinical studies in patients with haematologic cancers (NCT03503968).

Viral oncoproteins.—Viral proteins are immunologically foreign, not expressed by normal tissues, and in certain cases directly contribute to malignant transformation[269].

Therefore, they represent an attractive source of shared cancer-specific epitopes. Several clinical trials have tested the safety and antitumour activity of TCRs targeting virally encoded oncoproteins.

An association between TCR avidity and clinical activity was highlighted in two clinical trials targeting HLA-A*02:01 restricted epitopes derived from the human papillomavirus (HPV)-16 E6 and E7 oncoproteins. In one phase I/II study, patients with HPV-16-associated cancers received T cells transduced with a TCR that binds an epitope derived from the E6 oncoprotein[40]. The patient-derived TCR used in this study functions in a CD8 co-receptor independent manner and contains no modifications to its variable domains. Among the twelve patients treated, none had evidence of off-target toxicities and two had cancer shrinkage (ORR: 17%). Analysis of post-treatment tumour samples from non-responding patients identified one with *HLA-A*02:01* LOH and a second with a frameshift mutation in *IFGNR1*, a critical gene involved in antigen presentation. By contrast, no mutations in antigen processing and presentation genes were observed in a responding patient.

In a second study, patients received T cells transduced with a patient-derived TCR targeting an epitope derived from the HPV-16 E7 oncoprotein[41]. Like the E6 TCR, the E7 TCR functions in a CD8 co-receptor independent manner and contains unaltered variable domains. However, the E7 TCR has a significantly slower K_{off} rate compared with the E6 TCR[199], indicating higher structural avidity. Among 12 patients who received E7 TCR-modified T cells, six had PRs (ORR: 50%), including four who had progressed on prior anti-PD1 therapies. No patients experienced off-target toxicities. Four patients had tumours with loss of function mutations in interferon-signaling, HLA-I presentation, or HLA-A*02:01 expression. When considered together with results from the E6 TCR trial, these data indicate that immune-editing occurs frequently in HPV-associated cancers.

Merkel cell carcinoma is a rare and aggressive form of skin cancer that in ~80% of cases is caused by the transforming Merkel cell polyomavirus (MCPyV)[270]. Multiple epitopes derived from MCPyV-encoded oncoproteins drive T cell responses in patients[271], including an HLA-A*02:01 restricted epitope[272]. A patient-derived, CD8 co-receptor independent TCR targeting the HLA-A*02:01 epitope is now being tested in a phase I/II clinical trial[273].

Private neoantigens.—A recent clinical trial tested a highly personalized cell therapy approach that introduced private neoantigen-reactive TCRαβ sequences into polyclonal T cells using non-viral CRISPR/Cas9 genome editing[47]. In this study, tumours from each patient underwent whole exome sequencing and RNA-seq to define its unique expressed mutational landscape. Then, a curated list of *in silico* predicted HLA-I neoantigens was selected for incorporation into a custom p/HLA capture library. Gene sequences for TCRs that bound to these p/HLA complexes were retrieved from circulating T cells and the functionality of these TCR was tested for the capacity to trigger antigen-specific cytokine release. Patients received autologous T cells modified with up to three unique TCRs. The exogenous TCRs were inserted into the *TRAC* locus by HDR following CRISPR editing of *TRAC*/*TRBC* to remove the endogenous TCR. Immune monitoring revealed that TCR edited cells engrafted in the peripheral blood and trafficked to metastatic tumour sites. Among 16 treated patients, five demonstrated stable disease and 11 had progressive disease.

Although no objective responses were observed, this study established the feasibility and safety of targeting multiple neoantigens simultaneously using a fully non-viral TCR genome editing approach.

Public neoantigens.—Two proof of principle clinical trials have recently established the therapeutic potential of targeting public neoantigens resulting from recurrently mutated driver genes using TCR gene therapy. In one trial, patients were co-infused with T cells individually transduced with two HLA-C*08:02-restricted TCRs specific for a 9mer or 10mer peptide resulting from the *KRAS*^{G12D} hotspot mutation[274]. Both TCRs were cloned from a patient with *KRAS*^{G12D} colorectal cancer who had experienced an objective response following neoantigen-selected TIL therapy[224]. One patient with pancreatic cancer achieved a durable PR lasting >6 months following infusion of TCR engineered T cells in the absence of toxicities attributable to the cell product. A second patient with pancreatic cancer who also received TCR engineered T cells developed cytokine release syndrome (CRS) and did not have tumour regression. In a second trial, a patient with treatment-refractory breast cancer received T cells transduced with an HLA-A*02:01-restricted TCR specific for an epitope resulting from a hotspot mutation (R175H) in the tumour suppressor gene *TP53*[275]. The TCR, which functions in a CD8 co-receptor independent manner, was cloned from an HLA-A*02:01^{+} patient with metastatic colorectal cancer who received neoantigen-selected TIL[276]. Immediately following T cell infusion, the patient developed CRS; however, these symptoms promptly resolved with administration of intravenous steroids. The patient then achieved an objective PR that lasted six months. Genomic sequencing of a new metastatic tumour site revealed LOH for *HLA-A*02:01* as a likely resistance mechanism.

Soluble bispecific TCRs and TCR mimics.—Multiple ImmTACs and TCR-mimics have entered human clinical trials (Table 2). Tebentafusp, an ImmTAC that targets an HLA-A*02:01-restricted gp100 epitope, recently received FDA approval for patients with unresectable or metastatic uveal melanoma[18]. ImmTACs targeting additional HLA-A*02:01-restricted epitopes resulting from NY-ESO-1, MAGE-A4, MAGE-A8, and PRAME are now in early clinical development. In phase I-III trials, the overall response rate following tebentafusp administration has been modest (4.7–9.1%)[18,277,278]. Nevertheless, in a randomized phase III trial, patients who received tebentafusp had a significant overall survival benefit compared with patients in the control arm[18]. The fact that conventional response criteria did not strongly correlate with overall survival is reminiscent of other immunotherapies[279,280] and suggests that tebentafusp might alter tumour growth kinetics. If the uncoupling of radiographic responses from survival benefit is a class effect, the clinical development of soluble bispecific TCRs may be relatively prolonged and expensive. Unlike T cell-based therapies that have received FDA approval in the relapsed/refractory setting on the basis of high overall response rates in single-arm phase II trials[281–287], a soluble bispecific TCR registration trial would require a randomized design. Overall, the toxicity profile of ImmTACs seems similar to other bispecific T cell engaging proteins. This includes a reversible and generally mild CRS that abates after the first few doses[18,278]. Expected on-target/off-tumour cutaneous toxicities, such as rash and vitiligo, have been observed with tebentafusp. Importantly, no significant neurotoxicity has been observed in patients receiving ImmTACs thus far.

Conclusions and future perspective.

TCR-based therapies represent a new class of precision oncology treatments. Through a unique mechanism of action, TCRs enable the intracellular proteome to become a source of actionable cancer-selective and cancer-specific immunologic targets. Simultaneously, TCRs pose new challenges compared with conventional molecularly targeted therapies because they require co-expression of two biomarkers: a target protein and a specific HLA molecule. Because the *HLA* locus is the most polymorphic region of the human genome[288], identifying patients who express both biomarkers can be inefficient when performed in an unguided fashion. Prospective clinical next-generation sequencing (NGS) has revolutionized the ability to match approved and investigational treatments to specific molecular abnormalities identified in a patient's tumour[289]. Many widely used clinical NGS platforms capture sequencing reads that permit inference of a patient's HLA haplotype[290,291]. However, these data are currently not systematically reported in a patient's medical record, partly because of a perception that they do not represent actionable information. The FDA's recent approval of tebentafusp, which requires confirmation of *HLA-A*02:01*[18], challenges this notion and provides a rationale to begin routinely ascertaining every cancer patient's HLA haplotype.

Several drugs targeting specific genomic alterations have recently gained approval regardless of the site of disease origin. This new paradigm contrasts with the historical norm of developing cancer therapies in a tissue-specific but genome agnostic manner. Examples of tissue-agnostic therapies include the *NTRK* gene fusion inhibitors[292,293] and ICIs for malignancies associated with a high TMB[294,295]. Single-arm studies that lack a conventional control arm have recently been used in support of tissue-agnostic small molecule and antibody drug approvals. This study design is defensible in light of high response rates and acceptable toxicities and has dramatically accelerated the timelines for developing new treatments. A similar approach might be applied to TCR-based therapies, particularly those that target public neoantigens or viral oncoproteins, in which target antigen expression can be determined through NGS. With one notable exception[47], nearly all genetically engineered TCR, bispecific TCR, and TCR-mimic clinical trials have targeted only a single p/HLA complex. Concurrent targeting of multiple epitopes restricted by distinct HLA alleles might be a future strategy to minimize therapeutic resistance resulting from antigen heterogeneity or HLA LOH.

Thus far, most TCR-engineered cell therapies have used autologous αβ T cells. Although autologous T cells have a track record of antitumour efficacy and avoid the risk of GVHD, their use poses challenges to scalability and cost efficiency[296]. Third party sources of T cells, including allogeneic viral-specific T cells[44], allogeneic *TRAC*-edited naturally occurring or induced pluripotent T cells[297], γδ T cells[23], and CD3 complex engineered-NK cells[298] can each generate 'off-the-shelf' products that support TCR signaling. Whether allogeneic TCR-modified lymphocytes will match the potency of autologous T cells, particularly in patients with solid malignancies, remains unknown and will be an important area of ongoing clinical research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary:

Human leukocyte antigen (HLA)
A family of highly polymorphic, germline encoded transmembrane proteins that bind proteolytically degraded polypeptides. In vertebrate species, related proteins are referred to as the major histocompatibility complex (MHC)

HLA-restricted tumour antigens
Cancer-specific and cancer-associated polypeptides resulting from proteasomal, endosomal, or lysosomal protein degradation. These polypeptides are bound non-covalently within the binding groove of an HLA class I or class II molecule and facilitate the activation of antigen-specific T cells.

γδ T cells
~1–5% of circulating T cells express a somatically recombined γδ TCR that associates with the CD3 subunits and mediates antigen-specific cellular immunity. γδ T cells recognize a limited number of ligands presented in the context of non-polymorphic antigen presentation molecules.

Immune receptor tyrosine-based activation motif (ITAM)
A conserved four amino acid sequence (YxxL/I) contained in the cytoplasmic tails of non-catalytic tyrosine-phosphorylated receptors found in immune cells.

Somatic recombination
The genes for the *V*, *D*, *J*, and *C* segments of the TCRα and TCRβ hemichains do not encode functional proteins in their germline configuration. Rather, each segment undergoes site-specific recombination with the aid of recombination activation genes to assemble a single functional frame.

***Sleeping Beauty* transposon/transposase system**
A gene therapy method that uses co-transfer of two DNA plasmids to achieve stable transgene genomic integration and expression. One plasmid transiently expresses a transposase enzyme that digests the second plasmid, the *Sleeping Beauty* transposon, at inverted/direct repeats and ligates the transposon cassette containing a gene of interest into TA dinucleotide repeats within the genome.

Catch bond
A property of many low-affinity cell surface adhesion systems, including selectins, integrins, adhesins, and TCRs in which a bond's likelihood of separating is reduced as tensile force is applied. This property contrasts with the more common slip bond in which a bond disassociates rapidly following application of sheer force.

Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)
A sequencing-based method that enables the simultaneous detection and quantification of cell surface proteins, immune receptor binding specificity, and transcriptomic data at single-cell resolution.

DNA-barcoded p/HLA multimers
A synthetic p/HLA molecule conjugated to a unique oligonucleotide sequence that is detected and quantified using next-generation sequencing methods. This reagent enables the parallel detection of >1,000 T-cell specificities in a single sample.

***Strep*-tagII sequence**
A short polypeptide that binds with intermediate affinity to the biotin binding site of a mutated form of streptavidin. In the presence of excess d-biotin, *Strep*-tagII multimers dissociate into monomers. When p/HLA molecules are conjugated to *Strep*-tagII, this reagent facilitates the capture, enrichment, and release of antigen-specific T cells.

Paramagnetic artificial APC
An iron-dextran nanoparticle conjugated to a synthetic p/HLA complex and an anti-CD28 co-stimulatory antibody. In the presence of a magnetic column, this reagent simultaneously enriches for rare antigen-specific T cells and induces T-cell proliferation.

Investigational New Drug application (IND)
A request from a clinical study sponsor to obtain authorization from the FDA to administer an investigational drug or biological product to humans. An IND application is comprised of pre-clinical data establishing whether the product is reasonably safe and can be manufactured consistently alongside a protocol for the study's conduct that ensures patients are not exposed to unnecessary risks.

Tumour organoids
Multicellular *in vitro* structures that preserve the genetic diversity, phenotype, and structural features of a tumor *in vivo*. *In vitro* responses of tumour organoids to different treatments, including immunotherapies, often correlates with patient responses.

Common gamma chain cytokines
a family of six cytokines that share the common gamma chain (γ_c, CD132) as part of a receptor complex. Members of this cytokine family include IL-2, IL-7, IL-9, IL-15, and IL-21.

Uveal melanoma

a rare malignancy that arises from melanocytes within the uveal tract of the eye, which includes the iris, ciliary body, and choroid. Unlike cutaneous melanoma, uveal melanomas are modestly mutated and generally respond poorly to immune checkpoint blockade.

DNA mismatch repair proficiency (pMMR)

The vast majority (~95%) of colorectal cancers and other GI malignancies are proficient in DNA mismatch repair enzymatic function. These cancers are associated with a modest tumour mutational burden and are largely unresponsive to immune checkpoint inhibitors.

Leukapheresis

An outpatient procedure to obtain large numbers of circulating T cells, B cells, and monocytes for downstream clinical applications, including genetic engineering and *in vitro* stimulation. In this procedure, mononuclear cells are separated from red blood cells, platelets, and plasma through differential centrifugation.

Melanoma antigen recognized by T cells 1 (MART-1; Melan-A)

A transmembrane protein associated with normal melanocytes and the majority of melanomas.

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Box #1:

TCR diversity, degeneracy, and cross-reactivity.

The processes of combinatorial and junctional diversity can theoretically create as many as 1×10^{15}-10^{20} unique TCR sequences[24]. However, the human TCR repertoire is far more constrained and is typically measured within the range of 2×10^{7}-1×10^{8} unique sequences[301,302]. In part, the discordance between the theoretical and measured size of the TCR repertoire reflects the influence of negative and positive thymic selection[303]. To enable a limited T cell repertoire to respond to the largest possible universe of potential epitopes, many TCRs are capable of cross-reacting to multiple unrelated peptides, a parameter termed TCR degeneracy. The biologic requirement for TCR cross-reactivity has been established using both a theoretical framework[304,305] and experimental data[306]. The degeneracy potential of any individual TCR is highly variable. Some TCRs bind only to a limited number of structurally related peptides whereas others are capable of binding to more than 1×10^{6} unique peptide sequences[306]. Adding additional complexity, the peptides recognized by a TCR can share little to no sequence or structural homology[131,169]. Thus, TCR cross-reactivity is an expected rule rather than an exception.

Three distinct mechanisms contribute to TCR degeneracy. First, some TCRs and p/HLA complexes display flexibility in how they engage one another. Flexibility can occur as a result of plasticity in the conformations of CDR loops, enabling a single receptor to accommodate different p/HLA landscapes[307]. Alternatively, flexibility can occur through the orientation of a TCR's variable domains over different p/HLA complexes[252,308] or rearrangements in a peptide and presenting HLA protein to accommodate a TCR[169,309]. Second, some TCRs display highly focused interactions involving a minimal 'hot spot' motif displayed by an HLA-bound peptide[103]. Such TCRs retain specificity for structurally and chemically similar amino acid residues contained within this motif but otherwise tolerate multiple substitutions outside this region (a process termed molecular mimicry). Finally, because most HLA alleles can accommodate related amino acids at primary anchor positions[310], multiple peptide sequences can function as agonists for the same TCR.

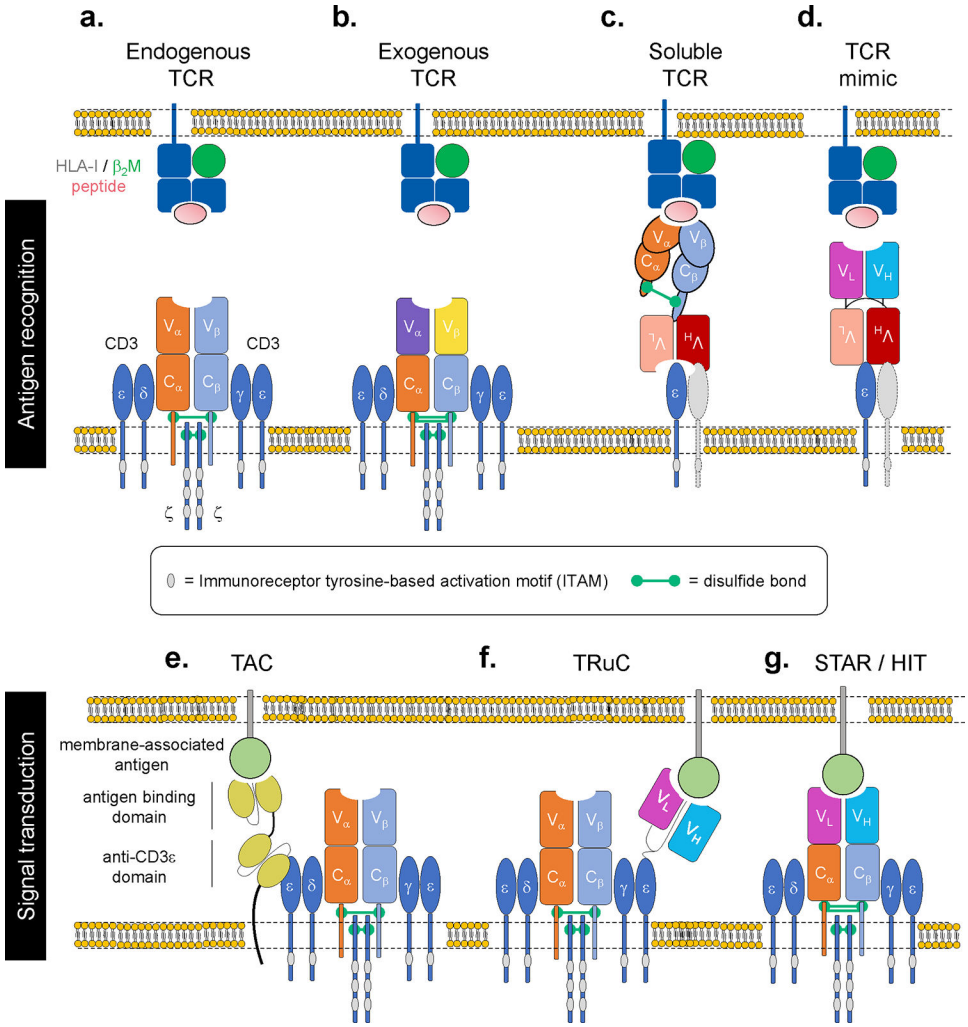

Fig. 1: The molecular architecture of TCR-based therapeutics.

(**a-d**) Comparison of the structural features of TCR and TCR-like molecules that bind specific peptide/human leukocyte antigen (p/HLA) complexes. (**a**) The endogenous TCR is comprised of an octameric complex composed of six proteins: the clonotypic TCRα (red) / TCRβ (blue) membrane-anchored heterodimer and the invariant CD3γ, δ, ε, and ζ chains. These proteins assemble with a 1:1:1:1 stoichiometry comprised of the dimeric subunits TCRαβ:CD3δε:CD3γε:CD3ζζ. Each TCR hemichain is composed of an antigen-binding variable (V) domain, a constant (C) domain, a transmembrane domain, and a short non-signaling cytoplasmic tail. The endogenous TCRα/TCRβ hemichains are covalently linked though a single interchain disulfide bond (grey spheres). Non-covalent interactions with the CD3 molecules facilitates intracellular signaling. HLA class I-restricted TCRs bind to a pHLA complex comprised of three alpha subunits (blue), beta-2-microglobulin (light grey) and a short polypeptide sequence typically 8–10 amino acids in length (red). (**b**) T-cell specificity can be genetically redirected to recognize p/HLA complexes displayed by tumour cells through expression of an exogenous TCRα (light grey) and TCRβ (dark grey) hemichain. Mispairing with the endogenous TCR hemichains can be minimized by introduction of a second interchain disulfide bond. (**c**) Soluble TCRs are recombinant

bispecific proteins that contain a TCR's α/β variable domains linked in a single-chain format on one end and an antibody-derived antigen binding variable heavy (V_H, rouge) and variable light (V_L, pink) chains specific for CD3ε on the other. (**d**) TCR-mimics are an alternative class of recombinant bispecific proteins that use an antibody's V_H/V_L domains (yellow and purple) in place of a TCRα/TCRβ to engage a specific p/HLA complex. Shown is a diabody format TCR-mimic. (**e-g**) Comparison of the structural features of next-generation antigen receptors that repurpose one or several components of the TCR's CD3 signaling complex. (**e**) The T cell antigen coupler (TAC) is a bispecific transmembrane protein expressed as a transgene in polyclonal T cells. One domain of a TAC uses an antibody-derived variable sequence to engage a membrane-associated tumor antigen (dark grey) while the other binds CD3ε. (**f**) The T cell receptor fusion construct (TRuC) is a transgene expressed in polyclonal T cells that covalently links an antibody variable sequence with specificity for a tumour antigen to an exogenous CD3ε molecule. (**g**) The synthetic TCR antigen receptor (STAR)/HLA-independent TCR (HIT) is a non-HLA restricted receptor that replaces the TCR variable domains with the tumor antigen-binding variable domains of an antibody. By retaining the TCR constant domains, the STAR/HIT receptor can recruit the full complex of CD3 signaling molecules upon ligand binding. V = TCR variable domain, C = TCR constant domain, V_H = immunoglobulin variable heavy chain, V_L = immunoglobulin variable light chain.

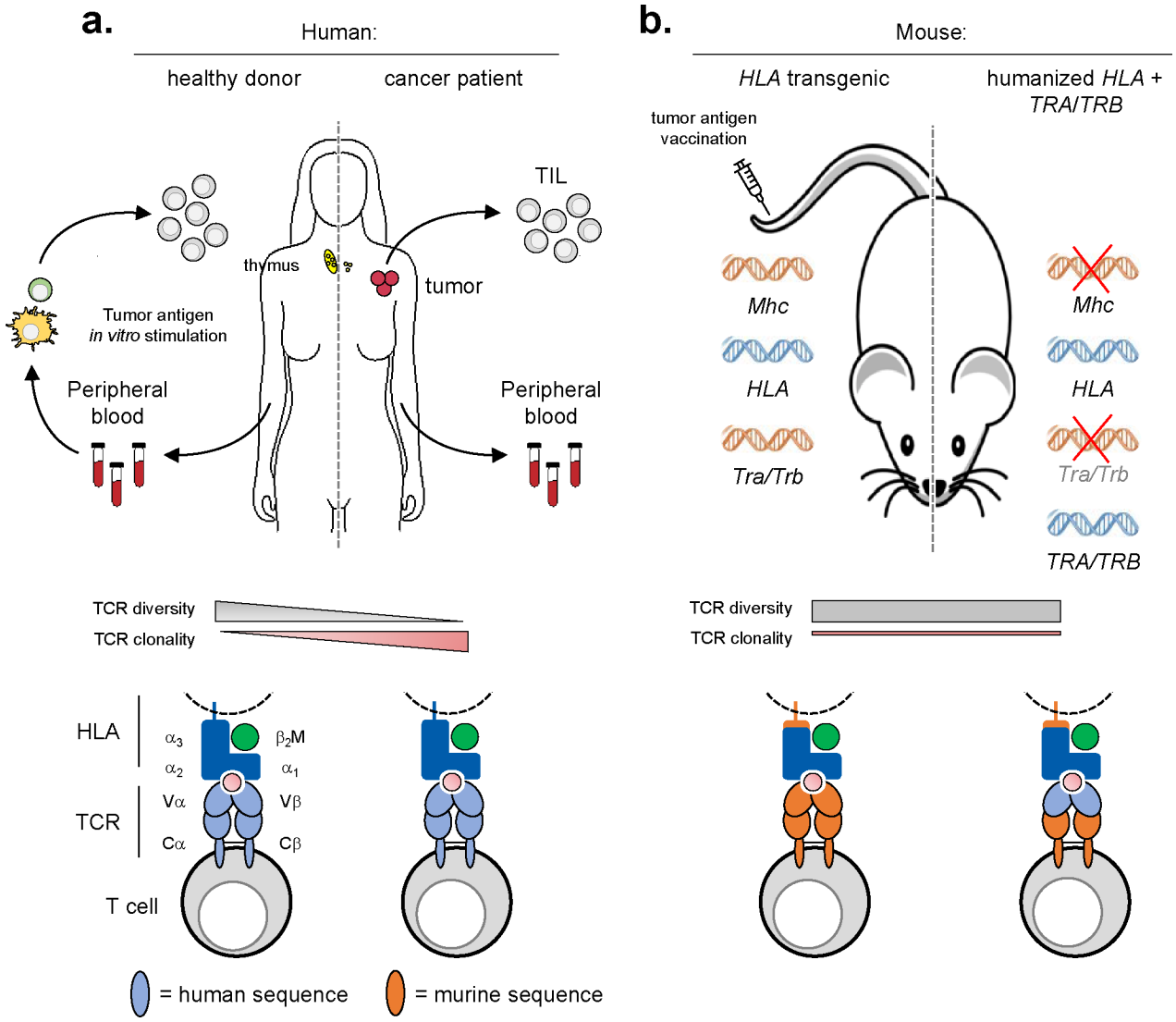

Fig. 2: Discovery of TCR therapeutic candidates.

(**a**) Fully human TCR gene sequences that confer recognition to tumour-derived peptide/HLA (p/HLA) complexes can be retrieved from healthy donors and cancer patients. Healthy donors have a broad circulating TCR repertoire that has not been subjected to the negative influence of immune-depleting cancer treatments, thymic involution, and peripheral tolerance (left). However, because the frequency of tumour-reactive TCR clonotypes is exceedingly rare within the naïve repertoire, healthy donor T cells must undergo *in vitro* stimulation (IVS) to enable detection. Tumour antigen-reactive T cells can be detected within the peripheral blood and tumour infiltrating lymphocytes (TILs) of patients with cancer (right). Although TCR diversity (represented as a grey bar) is typically lower in patients compared with healthy donors, the TCR repertoire often is enriched in tumour-reactive T cells that have undergone *in vivo* clonal expansion (represented as a purple bar). This feature may enable the retrieval of a larger number of tumour-reactive TCR clonotypes than is possible using a comparable sample volume obtained from healthy donors. (**b**) Tumour-reactive T cells can be generated through antigen-specific vaccination of HLA

transgenic mice. HLA transgenic mice possess a diverse TCR repertoire that has not been subjected to central thymic tolerance against human proteins that differ in sequence from their murine counterparts. However, TCRs retrieved from HLA transgenic mice possess immunogenic murine variable sequences capable of triggering host-versus-graft rejection when infused into humans. To overcome this limitation, “humanized” mice have been generated in which the genetic sequences encoding the human TCR variable chains have been knocked into the genetic loci encoding the murine TCR chains (light blue, right). TCRs generated in these mice therefore possess fully human TCR variable sequences. α_1/α_2 = highly polymorphic domains of HLA class I; α_3 = HLA class I constant domain; β_2M = beta-2 microglobulin (HLA light chain); V = TCR variable domain; C = TCR constant domain; *Mhc* = genetic locus encoding the major histocompatibility complex proteins (the murine ortholog of HLA).

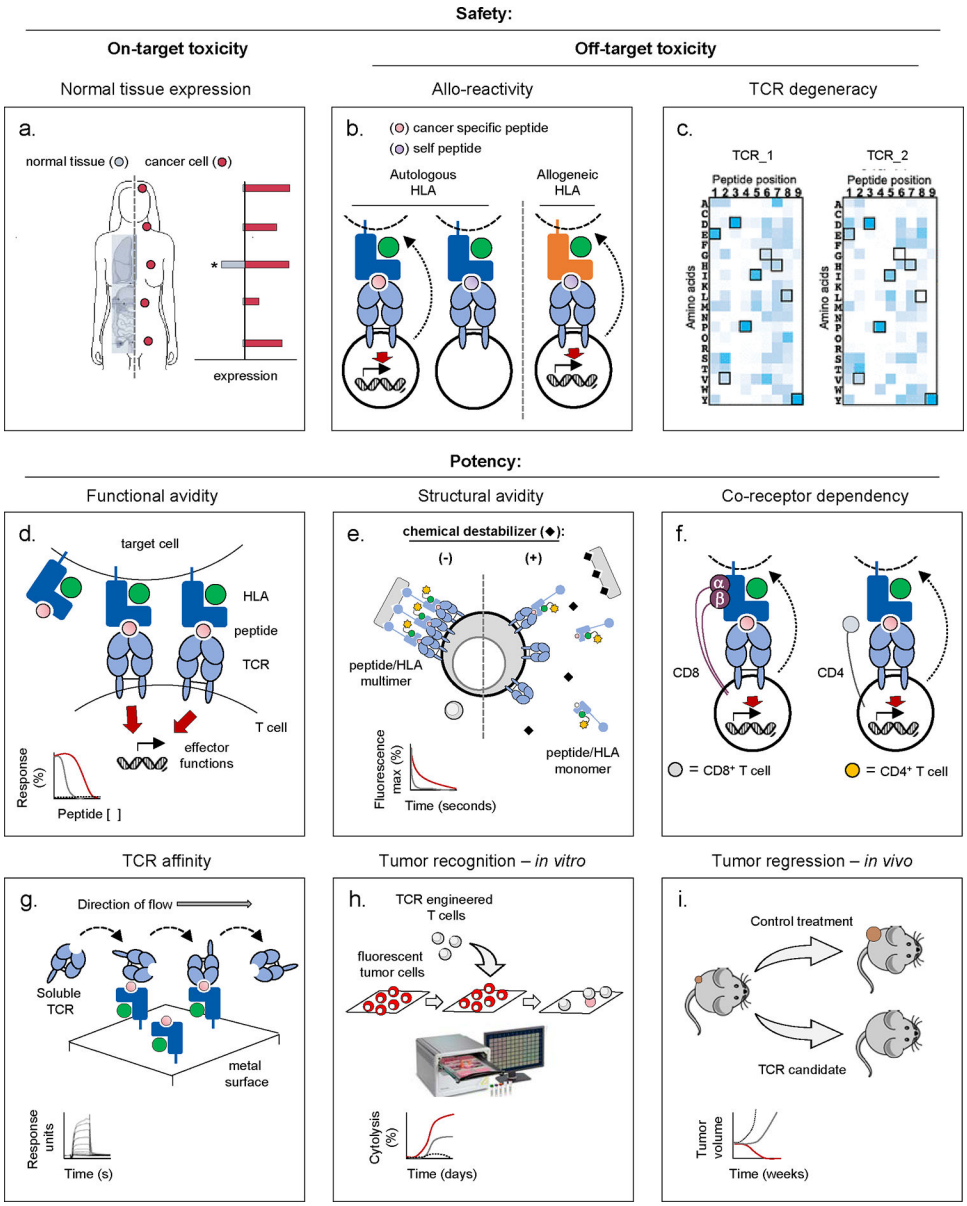

Fig. 3: Strategies to resolve the safety profile and potency and of TCR therapeutic candidates.

(**a-c**) Methods to quantify the safety profile of a TCR candidate. (**a**) Normal tissue expression assesses the presence of a target antigen in healthy tissues to assess the risk for on-target/off-tumour toxicities from a TCR candidate. Ideally, the risk profile for a novel antigen will be determined using multiple assays, including bulk tissue RNA sequencing, single-cell RNA sequencing, and measurement of protein level expression. (**b**) Allogeneic (allo) reactivity measures the capacity of a TCR to respond to a mismatched HLA molecule irrespective of the bound peptide sequence. Allo-reactivity is assessed by co-culture of T cells expressing a candidate TCR with a panel of target cells, such as EBV-transformed B-lymphoblastic cell lines (B-LCLs), that express diverse HLA alleles. (**c**) TCR degeneracy is the capacity of a single TCR to respond to unrelated peptide sequences but restricted by the same HLA molecule. The degeneracy potential of a TCR can be measured using

sequential amino acid scanning mutagenesis (eg. X-Scan) or large combinatorial libraries. In the example shown, the TCR is capable of recognizing both the cognate peptide sequence (highlighted boxes) and unrelated peptide sequences with similar or even enhanced potency. In the heatmap, dark blue represents an amino acid that results in enhanced TCR-mediated cytokine release or binding relative to other amino acids. In all instances where potential cross-reactive peptide sequences are identified, confirmatory studies are required to establish physiologic significance. (**d-i**) Methods to quantify TCR potency. (**d**) Functional avidity measures the capacity of T cells expressing multiple copies of a membrane-associated TCR to functionally respond to progressively lower concentrations of a specific p/HLA complex. Functional avidity results from the summation of all binding interactions between a T cell and target cell, including contributions from the TCR, the CD8 or CD4 co-receptors, and intercellular adhesion molecules. (**e**) Structural avidity measures the TCR-p/HLA dissociation rate (k_{off}) using fluorophore-conjugated (star) p/HLA multimers that dissociate into monomers following addition of an inert chemical (grey diamond). Unlike functional avidity, measurement of structural avidity is not affected by the differentiation state of T cells. (**f**) Co-receptor dependency measures the capacity of T cells expressing a TCR candidate to respond to target cells in the absence of the avidity and signaling contributions facilitated by the CD8α/β or CD4 co-receptors. The ability of a TCR to function in a coreceptor-independent manner suggests the TCR has a relatively high binding affinity. (**g**) TCR affinity is the strength of interaction between a single TCR molecule and a single p/HLA complex. Most commonly, affinity is measured using surface plasmon resonance by flowing a recombinant, soluble, single-chain TCR over a metal surface containing immobilized p/HLA complexes. Under equilibrium conditions, a TCR's binding affinity is inversely proportional to its dissociation constant (K_d) which in turn is defined by the ratio of the rates of dissociation and association (k_{off}/k_{on}). Plot illustrates time-dependent changes in binding (measured as relative response units) of a single-chain TCR flowed over a sensor containing immobilized p/HLA complexes at different concentrations. (**h**) *In vitro* tumour recognition measures the capacity of a TCR to trigger T cell responses to physiologic levels of an endogenously processed peptide displayed in the context of a specific HLA allele by tumour cells. Tumour recognition can be quantified by measuring T cell-mediated cytolysis or cytokine production. Solid red and grey lines represent the time-dependent cytolytic activity of two therapeutic TCR candidates while the grey dashed lines represents the cytolytic activity of a control TCR. (**i**) *In vivo* tumour regression assesses the ability of a TCR candidate (solid red line) to penetrate an established tumour mass and cause a sustained antitumour response over time. Grey solid and dashed lines represent a control TCR and no treatment control, respectively.

Table 1:

Summary of selected TCR gene therapy clinical trials.

Antigen class	HLA	Co-receptor independent	CDR modification	Tumour	ORR*	Reference
Tissue differentiation						
MART-1	A*02:01	No	No	MEL	13%	33
MART-1	A*02:01	Yes	No	MEL	30%	34
gp100	A*02:01	Yes	No	MEL	19%	34
Tyrosinase	A*02:01	Yes	No	MEL	33%	299
CEA	A*02:01	Yes	Yes	CRC	33%	35
Cancer germline						
NY-ESO-1	A*02:01	Yes	Yes	MEL	55%	258
NY-ESO-1	A*02:01	Yes	Yes	SS	61%	258
NY-ESO-1	A*02:01	Yes	Yes	SS	50%	43
NY-ESO-1	A*02:01	Yes	Yes	MRCLS	40%	259
MAGE-A3/9/12	A*02:01	Yes	Yes	various	56%	36
MAGE-A3/6	DPB1*04:01	Yes	No	various	24%	38
MAGE-A4	A*24:01	No	No	ESCA	0%	37
MAGE-A4	A*02:01	Yes	Yes	SS and MRCLS	36%	262
MAGE-A4	A*02:01	Yes	Yes	various	24%	263
MAGE-A4 + CD8α	A*02:01	Yes	Yes	various	36%	264
MAGE-A10	A*02:01	ND	Yes	NSCLC	11%	266
PRAME	A*02:01	No	No	various	50%	268
Overexpressed						
WT1	A*24:02	No	No	MDS/AML	0%	39
WT1	A*02:01	Yes	No	AML	NED	44
Viral						
HPV16 E6	A*02:01	Yes	No	$HPV16^+$	17%	40
HPV16 E7	A*02:01	Yes	No	$HPV16^+$	50%	41
Neoantigen						
Private	various	various	No	various	0%	47
TP53 (R175H)	A*02:01	Yes	No	BRCA	1/1	275
KRAS (G12D)	C*08:02	Yes	No	PDAC	1/2	274
KRAS (G12D)	A*11:01	Yes	No	NSCLC	1/1	300

Abbreviations: AML, acute myeloid leukemia; BRCA, breast cancer; CRC, colorectal cancer; ESCA, oesophageal carcinoma; HPV, human papilloma virus; MDS, myelodysplastic syndrome; Mel, melanoma; MRCLS, myxoid/round cell liposarcoma; ND, not defined; NED, no evaluable disease; NSCLC, non-small cell lung cancer; ORR, overall response rate; PDAC, pancreatic ductal adenocarcinoma; SS, synovial cell sarcoma.

*ORR reported if ≥3 patients treated.

Table 2:

Summary of soluble bispecific TCR and TCR-mimic clinical trials.

Antigen	HLA	TCR Vs. TCR-mimic	Disease	ORR	Reference or Trial identifier
Tissue differentiation					
gp100	A*02:01	TCR	MEL	8.7%	[277]
gp100	A*02:01	TCR	Uveal MEL	4.7%	[278]
gp100	A*02:01	TCR	Uveal MEL	9.1%	[18]
Cancer germline					
NY-ESO-1	A*02:01	TCR	Solid cancers	n.d.	NCT03515551
PRAME	A*02:01	TCR	Solid cancers	n.d.	NCT04262466
MAGE-A4	A*02:01	TCR	Solid cancers	n.d.	NCT03973333
MAGEA4/8	A*02:01	TCR	Solid cancers	n.d.	NCT05359445
MAGE-A4	A*02:01	TCR-mimic	Solid cancers	n.d.	NCT05129280
Overexpressed					
WT-1	A*02:01	TCR-mimic	AML	n.d.	NCT04580121

Abbreviations: AML = acute myeloid leukemia; Mel, melanoma; n.d., no data.