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FOCUSSED RESEARCH REVIEW

Understanding TCR affinity, antigen specificity, and cross-reactivity to improve TCR gene-modified T cells for cancer immunotherapy

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Abstract

Adoptive cell transfer (ACT) using T cell receptor (TCR) gene-modified T cells is an exciting and rapidly evolving field. Numerous preclinical and clinical studies have demonstrated various levels of feasibility, safety, and efficacy using TCR-engineered T cells to treat cancer and viral infections. Although evidence suggests their use can be effective, to what extent and how to improve these therapeutics are still matters of investigation. As TCR affinity has been generally accepted as the central role in defining T cell specificity and sensitivity, selection for and generation of high affinity TCRs has remained a fundamental approach to design more potent T cells. However, traditional methods for affinity-enhancement by random mutagenesis can induce undesirable cross-reactivity causing on- and off-target adverse events, generate exhausted effectors by overstimulation, and ignore other kinetic and cellular parameters that have been shown to impact antigen specificity. In this Focussed Research Review, we comment on the preclinical and clinical potential of TCR gene-modified T cells, summarize our contributions challenging the role TCR affinity plays in antigen recognition, and explore how structure-guided design can be used to manipulate antigen specificity and TCR cross-reactivity to improve the safety and efficacy of TCR gene-modified T cells used in ACT.

Keywords T cell · T cell receptor (TCR) · TCR affinity · TCR cross-reactivity · Adoptive cell transfer · PIVAC 18

Abbreviations

ACT	Adoptive cell transfer
APL	Altered peptide ligand
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
PBL	Peripheral blood lymphocyte
pMHC	Peptide-major histocompatibility complex
TCR	T cell receptor

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On- and off-target cross-reactivity of TCR gene-modified T cells

Adoptive cell transfer (ACT) using T cell receptor (TCR) gene-modified T cells is an exciting and rapidly evolving field. Its use fosters an innovative and promising approach to treat cancer, viral infections, and other immune-modulated diseases [2]. Numerous preclinical and clinical studies have demonstrated various levels of feasibility, safety, and efficacy [3]. Although evidence suggests TCR gene-modified T cells can be safe and effective, ways to maximize their therapeutic potential and minimize adverse events are still matters of investigation.

Namely, T cell recognition of targeted antigen on normal tissue (on-target, off-tumor) or off-target recognition of related or unrelated antigen caused by TCR cross-reactivity are significant barriers to optimal efficacy and safety [4]

(Fig. 1). Such adverse events have been documented since the earliest clinical trials, demonstrating how the selection of both target antigen and TCR are critical considerations in therapy design. For example, early clinical trials targeting HLA-A2$^+$/MART-1$^+$ melanoma demonstrated that TCR gene-modified T cells were generally safe and well tolerated, with some clinical benefits [5]; however, later studies using a higher avidity MART-1-specific TCR and a high affinity, murine TCR targeting melanoma antigen gp100 induced uveitis and hearing loss, caused by on-target, off-tumor destruction of normal melanocytes in the eye and inner ear [6]. A more recent clinical trial targeting melanoma using a tyrosinase-specific TCR showed no serious adverse events, but vitiligo was observed in a patient who achieved complete remission [7]. Additionally, a clinical trial using high affinity CEA-reactive TCR-engineered T cells targeting CEA$^+$ colon cancer provided objective clinical responses, but also induced inflammatory colitis by recognition of normal CEA$^+$ colonic tissue [8].

Arguably more concerning is unpredictable off-target TCR cross-reactivity against related or unrelated antigens, which has been documented in multiple clinical trials using TCR gene-modified T cells. For example, two separate studies targeting cancer-testis antigen family member MAGE-A3 demonstrated considerable off-target toxicities [9–11]. One trial using a murine, high-avidity TCR further modified by CDR3 region site directed mutagenesis induced clinical regression in 5/9 patients treated, but caused neurological toxicity including two deaths from cross-reactivity against related antigen MAGE-A12, previously unknown to be expressed in brain tissue [11]. Similarly, the use of T cells engineered with an affinity-enhanced MAGE-A3-specific TCR caused cardiogenic shock and death in the first two patients treated in a separate trial [9, 10]. It was later identified that the affinity-enhancing mutations designed in the CDR2 regions generated cross-reactivity against titin, a sarcomeric protein expressed in cardiomyocytes, causing off-target cardiac tissue destruction in these patients.

While naturally high-affinity or affinity-enhanced TCRs induced some of these adverse cross-reactivities, there remains potential benefit of cross-reactive TCRs to combat the inherent genomic instability of cancers and viral diseases targeted where antigen mutation is a driver for immune escape [12]. As such, the above clinical examples highlight the importance of understanding the potential for on- and off-target recognition and call for a more cautious approach when designing TCRs for engineering T cells for ACT.

Enhancing TCR affinity as a tool to augment T cell function

The affinity between the TCR and peptide-major histocompatibility complex (pMHC) is generally thought to play the most central role in antigen recognition [13]. As such, it has long been be predicted that T cells engineered with high affinity TCRs are better effectors than T cells engineered with lower affinity TCRs [14–16]. High-affinity TCRs do not require the presence of the CD8 co-receptor to facilitate antigen recognition [17], can detect low levels of antigen [18], and can engineer MHC-I-restricted CD4$^+$ T cells to

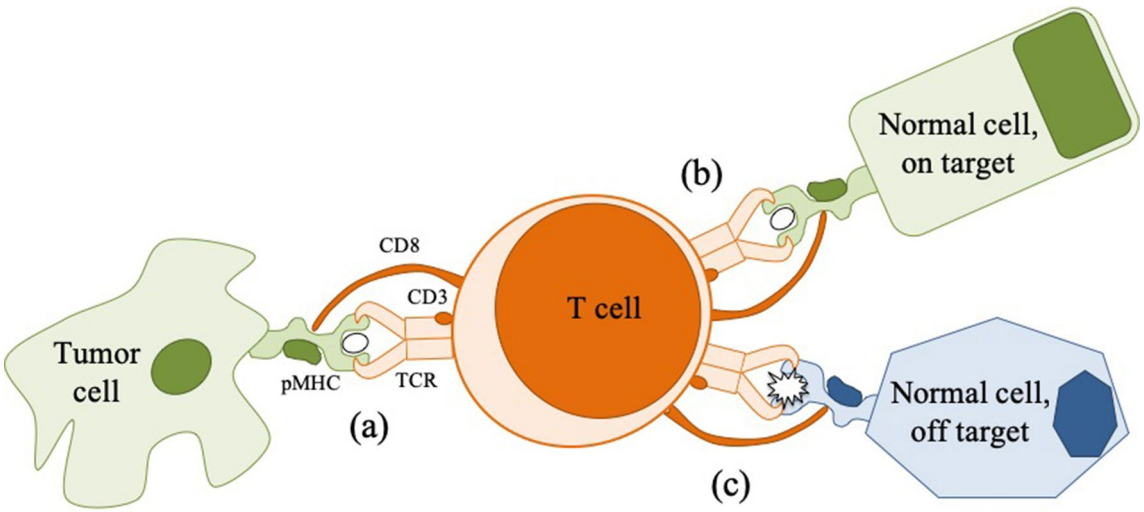

Fig. 1 On- and off-target recognition by TCR gene-modified T cells. Although TCR gene-modified T cells are designed to redirect antigen reactivity and maintain specificity, preclinical and clinical data have shown the potential for TCR-engineered T cells to exhibit (**a**) appropriate on-target, on-tumor antigen recognition (tumor eradication); (**b**) on-target, off-tumor recognition (i.e. low-level antigen on normal tissue); or (**c**) off-target cross-reactivity (i.e. related or unrelated antigen on target or non-target tissue). A more comprehensive understanding how TCR affinity and other physical and biologic parameters modulate antigen recognition will better equip the field to rationally design TCRs to maximize both safety and efficacy

provide helper cytokine support, facilitate cross-priming, and enhance tumor regression [16, 19–22]. Yet, naturally occurring high-affinity CD8-independent TCRs are relatively rare due to thymic selection [23], with natural affinities generally occurring between only 1–100 μM [24].

Thus, many in the field have resorted to screening for high-affinity TCRs or enhancing the affinity of previously characterized TCRs to improve the efficacy of TCR gene-modified T cells. Such strategies include vaccination of HLA-transgenic mice to generate high affinity, murine TCRs raised against human pMHC [25–27] and using high-throughput techniques including yeast and phage display to genetically alter TCR biophysical properties, enhancing TCR affinities to the nM or pM ranges [28–30]. However, there is concern that murine-raised TCRs can be immunogenic inducing clearance of transferred TCR-engineered T cells, as seen using murine-derived chimeric antigen receptor T cells [4, 31]. Additionally, T cells engineered with high-affinity TCRs have greater potential to undergo activation-induced cell death upon antigen encounter [32, 33], and have been shown to induce on- and off-target recognition discussed earlier, both counterproductive to their therapeutic intention. Moreover, yeast and phage display strategies rely on random mutagenesis, without the ability to rationally modulate specificity or cross-reactivity, giving blinded emphasis to a single parameter and potentially exacerbating off-target toxicity by strengthening interactions with the MHC protein at the expense of the peptide.

Given these concerns related to high-affinity TCRs and the strategies used to create them, many have remained apprehensive. We and others have focused on understanding *how* TCR affinity influences antigen recognition and TCR cross-reactivity, distinguishing TCR biochemical recognition and T cell functional recognition to more rationally design TCR-based immunotherapeutics [34]. As examples, we have utilized multiple well-defined TCR models to demonstrate the cross-reactive potential of TCRs, understand how affinity and other kinetic and cellular parameters impact TCR cross-reactivity, and propose structure-guided design may provide a means to rationally and carefully optimize TCR-engineered T cells.

Clarifying the role TCR affinity plays in antigen recognition

As mentioned earlier, TCR-pMHC affinity is generally thought to be the most critical factor dictating antigen recognition [13]. And while supporting evidence is valid, it is important to further clarify the role of TCR affinity to decide the most appropriate manipulation of kinetic or cellular parameter(s) when designing TCR gene-modified T cells. If the field can better understand and define the role TCR affinity plays in antigen recognition and TCR cross-reactivity, we may be better able to design and implement safer and more effective TCR-based immunotherapeutics. Our defined TCR models have allowed us to assess important factors dictating antigen recognition of gene-modified T cells and provide a framework for evaluating TCR cross-reactivity and manipulating specificity.

The first system in which we have published extensively is HCV1406 TCR, an HLA-A2-restricted hepatitis C virus (HCV)-reactive TCR cloned from an $A2^-$ HCV-infected individual that received an $A2^+$ donor liver allograft [35]. We validated the use of HCV1406 TCR gene-modified T cells as a potential immunotherapeutic for HCV-associated hepatocellular carcinoma (HCC) showing adoptive transfer of TCR-engineered peripheral blood lymphocyte (PBL)-derived human T cells can eradiate HCV^+ HCC xenografts in mice [36]. Interestingly, HCV1406 TCR was found among other HCV-reactive TCRs to be broadly cross-reactive, recognizing a variety of naturally occurring and epidemiologically prevalent mutant escape variant HCV peptides [12]. We showed that these and other TCRs from T cell clones in patients who spontaneously resolved HCV infection have broader cross-reactivity than those who developed chronic infection, attributing immunologic fitness to T cells with cross-reactive TCRs and proposing the use of cross-reactive TCRs may be beneficial in disease with genomic instability, including cancer and viral infections [12].

Given we could generate TCR gene-modified PBL-derived T cells with a defined antigen specificity while exhibiting cross-reactive behavior against a set of related immune-escape antigens, we measured the affinity of HCV1406 TCR with each mutant immune escape variant peptide, known as an altered peptide ligand (APL). We showed that functional recognition, determined by cytokine release and degranulation, is not solely defined by TCR-pMHC affinity. Notable examples include: of two APLs with nearly identical TCR-pMHC affinities, only one was recognized; an APL with non-detectable TCR-pMHC binding was still functionally recognized; and the magnitude of cytokine release or percent degranulation was not clearly correlated with relative changes in TCR-pMHC affinity [37]. This was later confirmed with polyfunctional assays, demonstrating that APL interactions modulated the number or diversity of functional phenotypes, but again not directly related to TCR-pMHC affinity [38].

We assessed other parameters thought to influence antigen recognition and cross-reactivity, showing that antigen density and varying levels of TCR transgene expression had profound effects on cross-reactivity, which was not always reconciled by differences in TCR-pMHC affinity [38]. We also showed that the CD8 co-receptor's recruitment of lck and signaling potentiation was also critical for low-affinity interactions, but its role in stabilizing TCR-pMHC binding

was not [38]. This observation confirmed earlier studies in other TCR systems which attempted to define the role CD8 plays in antigen recognition [39, 40]. We also showed that modifications to the TCR that enhanced chain pairing, limited mispairing with endogenous TCR chains, or reduced competition for CD3 alleviated CD8-dependence, enhanced cross-reactivity, and increased T cell polyfunctionality [41, 42]. These small modifications also facilitated antigen recognition at low or even undetectable levels of transgene, evaluated by tetramer or TCR vβ antibody binding, demonstrating a profound impact on biological function.

We coupled these functional and kinetic studies with structural approaches, solving the crystal structure of the wildtype TCR-pMHC interaction and using computational modeling to predict what TCR, peptide, or MHC motifs or amino acid residues may facilitate cross-reactivity. Unremarkably, modeling based off the crystal structure predicted that HCV1406 TCR achieves small conformational shifts to accommodate certain peptide alterations. But, such small conformational changes at the TCR-pMHC interface have been previously suggested to substantially alter TCR-initiated signaling events and downstream propagation, which may rationalize substantially different functional outcomes, despite similar binding affinities [43–45]. More strikingly, through alanine scanning, we found that a single amino acid residue of the HCV cognate peptide NS3:1406-1415 was such a critical binding "hot spot" for HCV1406 TCR engagement, that its substitution into a previously unrecognized HLA-A2-restricted self-antigen tyrosinase-induced functional recognition by T cells [46].

Together, these data suggest that TCR affinity does not play the sole defining role in antigen recognition and that affinity coupled with other kinetic, cellular, and structural parameters may cooperatively influence antigen specificity and TCR cross-reactivity. These data also caution against the use of random mutagenesis to generate high-affinity TCRs as means to create more potent effectors and suggest more rational structure-guided approaches manipulating TCR-pMHC interface may provide a better tool for engineering specificity. Moreover, screens for reactivity must be broad and not limited to target antigens or closely related homologs. While these observations are an important first step in re-evaluating the role of TCR-pMHC affinity on antigen recognition, we acknowledge they describe the behavior of a single TCR; however, ongoing studies in our group evaluating other well-characterized TCRs, including the HCV1073 [12, 47], TIL 1383I [16, 39], and DMF5 TCRs [48, 49] suggest that these observations are not unique to HCV1406 TCR.

In fact, other groups using additional TCRs have also supported our observations. For instance, one study showed five TCRs with similar binding affinities to the same pMHC exhibited different spectra of cross-reactivity to other antigens, which was rationalized by varying structural interfaces and CDR3 loop dynamics of the TCRs [50]. They showed that despite similar affinities, the structural mechanisms of binding and degree of hydrogen bond formation governed the relative level rigidity or flexibility influencing antigen specificity and cross-reactivity, respectively. Others propose that inconsistencies between TCR affinity and antigen sensitivity is because traditional methods used to measure affinity using soluble TCRs and pMHCs in three-dimensions (3D) do not accurately reflect physiologic regulations of TCR-pMHC interactions are anchored on two-dimensional (2D) membranes of opposing cells [51]. Reduced spatial degrees of freedom of molecular motion and the presence and contribution of co-receptors are also not taken into account using traditional 3D methods [52]. In fact, comparing 2D and 3D techniques revealed dramatically different kinetic parameters, with 2D measurements displaying a better correlation with T cell functional responses in vitro [53, 54]. Interestingly, a clinical trial using autologous melanoma-reactive TCR-transduced T cells found that patients who showed clinical or biological responses with reduced tumor burden or development of vitiligo (on-target, off-tumor) received engineered T cells displaying higher 2D affinity compared to the non-responder, despite identical 3D affinity measurements [7]. Others suggest alternative binding parameters including forces exhibited during TCR-pMHC engagement [55] and the amount and rate of serial triggering [56, 57] may also provide parameters to better assess antigen sensitivity or predict biological function. Further evaluation and modulation of these parameters may be of benefit when designing and assessing TCRs used in ACT.

Making a case for structure-guided TCR design

It is clear that various physical and cellular parameters can significantly modulate antigen specificity and TCR cross-reactivity. In response, we and others have proposed a more rational, structure-guided design of TCRs using fine manipulation of the structural topography of TCR-pMHC interactions rather than large affinity increments, which may offer a more predictable and safer approach to TCR design [49, 58–61]. As noted above, enhancements in TCR affinity by random mutations may indiscriminately do so by improving interactions between TCR and MHC protein at the expense of the peptide, inducing off-target cross-reactivity. Strategies that are more educated, understanding *how* the TCR binds its antigen rather than merely binding a peptide/MHC complex more tightly may better predict and avoid on- and off-target effects.

Building on our observations with HCV1406 TCR, we examined the clinically relevant DMF5 TCR, highlighting

unexpected binding mechanisms for TCR cross-reactivity and employing novel structure-guided methods to manipulate antigen specificity. DMF5 TCR was initially characterized as specific to the MART-1 melanoma antigen restricted by HLA-A2 and used in clinical trials [6, 62], but was later unexpectedly found, through yeast display of peptide-MHC libraries, to bind to two distinct classes of peptides with opposing central cores (one hydrophobic, one dominated by charged amino acids) [63]. We found rather than small TCR conformational adaptability (akin to HCV1406 TCR), cross-reactivity of DMF5 TCR is accommodated by dramatic shifts in peptide, HLA-A2, and TCR side-chain rearrangements [48]. Rather than molecular mimicry of the MART-1/HLA-A2 complex, the DMF5 TCR unexpectedly accommodates new pMHC surfaces, demonstrating new principles of TCR cross-reactivity and emphasizing the challenges associated with predicting TCR-engineered T cell reactivities.

We exploited the cross-reactivity of DMF5 TCR to evaluate a novel structure-guided approach to modulate TCR specificity [49, 60]. Structure-guided computational design included both "positive design" to enhance peptide-centric binding, and "negative design", which weakened interactions with the MHC. While positive design mutations induced stepwise improvements in binding affinity, they also introduced new cross-reactivities and weakened T cell potency [60]. However, DMF5 variants incorporating both positive and negative design maintained potent MART-1 recognition, eliminated cross-reactivity against a more divergent class of epitopes, and reduced cross-reactivity towards many other MART-1 homologues.

While this system tests a limited number of experimental and computational structure-guided screens and does not evaluate all known physiological targets for DMF5 TCR, these observations provide a proof of principle for the use of structure-guided design to improve antigen specificity, while minimizing TCR cross-reactivity. Further efforts are ongoing to build on these foundational observations to refine computational design, evaluate additional peptide targets, and assess the impact on T cell polyfunctionality.

Future perspectives

The therapeutic promise of high-affinity TCR gene-modified T cells has been dampened by on- and off-target adverse events documented in multiple clinical trials. A better understanding of the fundamentals governing antigen specificity and TCR cross-reactivity is necessary to optimize these immunotherapeutics. We and others have recently identified a number of biological and physical parameters, summarized in Table 1, that modulate antigen specificity and TCR cross-reactivity. Our observations, discussed in this Focussed Research Review, establish a new working model highlighting a previously unappreciated and complex relationship between kinetic, cellular, and structural parameters governing antigen recognition and T cell function, which should be considered when designing TCRs for ACT (Fig. 2).

With these observations in mind, approaches using a more educated design of TCRs and comprehensive predictive analysis of in vivo cross-reactivity should be implemented to generate more efficient and safer effectors. We propose that efforts solely designed to enhance TCR affinity in a random or blinded way, such as yeast and phage display, should be used with caution. Instead, rational structure-guided modulation of the TCR-pMHC interface may better productively and predictably modulate antigen specificity and TCR cross-reactivity [49, 58–61, 64]. Some strategies rely on selecting targeted mutations using computational design with high-fidelity predictive modeling to predict conformational change and subsequently characterize binding parameters and structure to reinform and improve algorithmic performance [60]. Others use known TCR-pMHC structures to rationally design sequence substitutions in contact areas between TCR and pMHC to improve surface shape, energetic complementarity, and T cell potency [59]. More recently, deep mutational scanning of TCR-pMHC interactions samples the effects of every amino acid alteration at multiple positions [64]. Coupled with in silico binding analyses, this provides a more informed high-throughput screening of engineered TCRs than traditional yeast and phage display technologies [65].

Table 1 Factors influencing productive TCR-pMHC engagement and T cell polyfunctional responses

Kinetic parameters	Signaling propagation	TCR and antigen density	Biophysical adaptations
Two- vs three-dimensional binding affinity	TCR-pMHC serial triggering	TCR transgene expression	Structural topography of peptide and MHC
On- and off-rates	TCR/CD3 complex signaling	Endogenous/introduced TCR mispairing	Peptide- vs MHC-centric binding
TCR-pMHC binding force magnitude and duration	Lck-recruitment by CD8	TCR competition for CD3	Conformational adaptability of TCR
TCR-pMHC stabilization by CD8	Additional co-receptor expression and signaling	Antigen expression, processing, and presentation	Antigen mutation/molecular mimicry

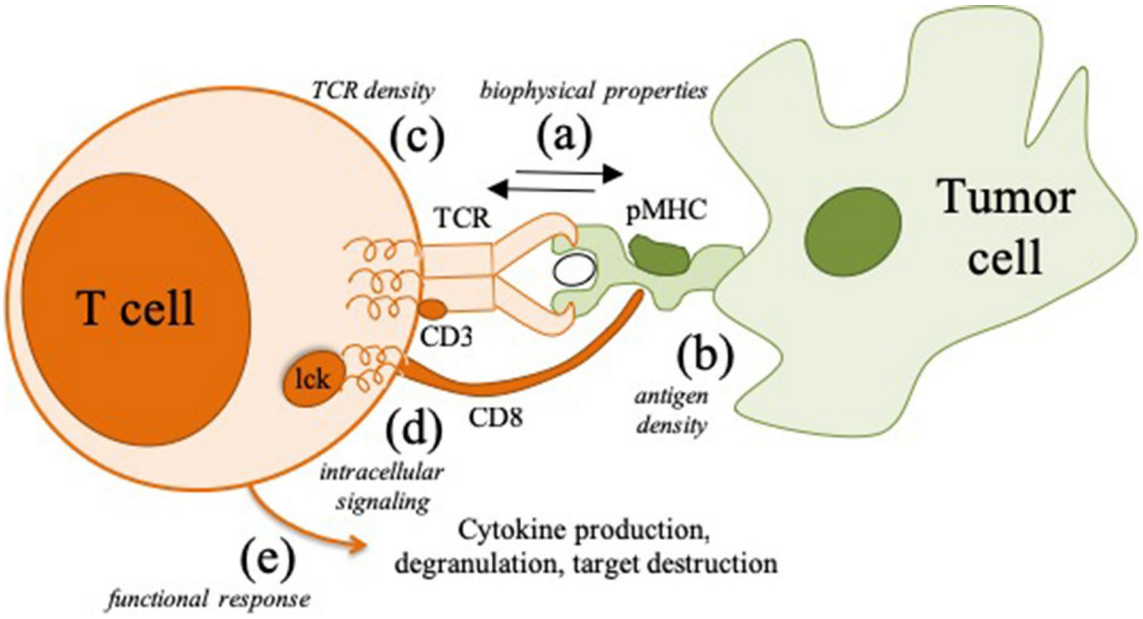

Fig. 2 Model of physical and biologic parameters governing TCR specificity and cross-reactivity leading to a functional T cell responses. Rather than TCR affinity dictating antigen recognition and defining a candidate TCR for genetic engineering, when designing a TCR-based immunotherapeutic the field should consider the interaction among (**a**) biophysical parameters: 2D and 3D binding affinity, on-/off-rates, magnitude and duration of forces exerted on the TCR-pMHC complex; (**b**) ligand densities; (**c**) TCR densities; and (**d**) serial triggering or signal propagation by TCR, CD3, and co-receptors. Together, these factors play a cooperative role in facilitating (**a**–**d**) a biochemical TCR recognition into (**e**) a functional T cell response

Aside from more informed TCR engineering, the use of cross-reactivity surveying may better screen for or anticipate on- or off-target antigen recognition by high-affinity TCRs before their in vivo use. Some have implemented novel in vitro specificity screens, or "X-scans", to survey TCR cross-reactivity against epitopes where each residue of the target peptide is mutated to all possible amino acids. This allows for more sensitive and specific predictions of tolerated residues compared to alanine scanning, and better assesses cross-reactive differences among TCRs with similar affinities to cognate antigen [66]. However, this strategy is limited to single residue substitutions and does not take into account broader shared motif similarities that may still be recognized. Others have instead generated low-affinity de novo peptides known as "velcros" as a tool to probe broadly for specificities of engineered TCRs and anticipate promiscuous peptide recognition [67]. Additionally, many have shifted focus to peptide target selection as a means of modulating or predicting TCR specificity. In particular, one study demonstrated that the binding affinity of peptide to MHC played a critical role in dictating the overall potency and specificity of a T cell, even when comparing TCRs with similar binding parameters to either pMHC complex [68]. Identifying or manipulating certain peptide-MHC binding parameters may provide an additional tool to better inform target selection and predict unanticipated TCR-pMHC interactions.

Overall, the future of these novel strategies has significant implications for predicting and customizing antigen recognition in a given TCR system. As we learn more about parameters influencing antigen recognition, unlock the structural and biophysical interfaces of TCR-pMHC, and refine technology to predict and modulate antigen specificity, we may be able to greatly advance the safety and efficacy profiles of TCR gene-modified T cells. This may additionally enhance the way in which we study and manipulate other immune cell and receptor types, approach epitope discovery, and evaluate vaccine design.

Author contributions TT Spear, BD Evavold, BMB, and MIN designed the research, performed experiments, and analyzed the data summarized in the Focussed Research Review as well as wrote and critically revised the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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