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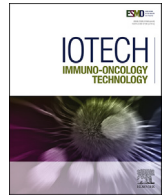
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Review

T-cell-receptor cross-recognition and strategies to select safe T-cell receptors for clinical translation



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ABSTRACT

Adoptive transfer of T-cell-receptor (TCR)-transduced T cells has shown promising results for cancer treatment, but has also produced severe immunotoxicities caused by on-target as well as off-target TCR recognition. Off-target toxicities are related to the ability of a single T cell to cross-recognize and respond to several different peptide–major histocompatibility complex (pMHC) antigens; a property that is essential for providing broad antigenic coverage despite a confined number of unique TCRs in the human body. However, this degeneracy makes it incredibly difficult to account for the range of targets that any TCR might recognize, which represents a major challenge for the clinical development of therapeutic TCRs. The prospect of using affinity-optimized TCRs has been impeded due to observations that affinity enhancement might alter the specificity of a TCR, thereby increasing the risk that it will cross-recognize endogenous tissue. Strategies for selecting safe TCRs for the clinic have included functional assessment after individual incubations with tissue-derived primary cells or with peptides substituted with single amino acids. However, these strategies have not been able to predict cross-recognition sufficiently, leading to fatal cross-reactivity in clinical trials. Novel technologies have emerged that enable extensive characterization of the exact interaction points of a TCR with pMHC, which provides a foundation from which to make predictions of the cross-recognition potential of individual TCRs. This review describes current advances in strategies for dissecting the molecular interaction points of TCRs, focusing on their potential as tools for predicting cross-recognition of TCRs in clinical development.

Adoptive T-cell transfer

The field of adoptive T-cell transfer was established with the therapeutic application of *ex vivo* expanded tumor infiltrating lymphocytes that effectively mediate tumor regression when re-infused into patients with cancer [1]. Despite the success of these treatments, which has been widely demonstrated with impressive response rates of 20–50% [2–4], their broad application has been impeded due to extensive production time and limited commercial potential [5]. Importantly, the specificities of such T-cell products are unknown, which makes it difficult to predict the outcome of treatment in individual patients. This has driven interest in the development of T-cell therapies that are amenable for a broader group of patients, such as the transfer of T cells transduced with a chimeric antigen receptor [6,7] or a TCR that recognizes known cancer-associated targets [8,9]. The proposal that TCR-based gene

therapy of cancer would be feasible came from studies showing the effective redirection of T-cell specificity through transduction with $\alpha\beta$ TCR genes that could provide antiviral and antitumor immunity [8–15]. Early trials that used the DMF4 TCR [16] or the DMF5 TCR [17], which both target a human leukocyte antigen (HLA)-A0201 MART-1 melanoma peptide, revealed that a TCR with greater functional avidity and higher affinity towards its target seemed to have greater promise for cancer treatment but also caused autotoxicities [17–19]. These studies imply that successful TCR-based gene therapy is facilitated by transfer of T cells expressing high-affinity TCRs, which is also corroborated by early studies correlating TCR binding affinity with functional responses [20–26]. This, in turn, has encouraged strategies for the design of high-affinity TCRs intended for adoptive cell transfer schemes [24, 27–29], but may simultaneously increase the risk for TCR cross-recognition of endogenous tissue [30,31].

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The affinity optimization strategy has primarily been applied to TCRs selected for their ability to recognize shared cancer antigens; however, for TCRs targeting foreign antigens, such as viral or neoantigens, which are expected to be of higher affinity, the need for such engineering may be obviated.

Cross-reactivity

Several trials have been initiated to evaluate the safety and efficacy of adoptive transfer of TCR-transduced T cells [16,17,32–37]. Although the strategy seems extremely attractive for treating various cancers effectively with an ‘off-the-shelf’ T-cell product to be used across patients with the same HLA haplotype, the occurrences of immunotoxicity have challenged the development of such treatment strategies. In particular, two clinical trials highlight the need to approach the use of TCRs with extreme care. The first trial applied a high-avidity TCR generated from vaccinated HLA-A0201 transgenic mice that targeted a melanoma antigen-encoding-A3 (MAGE-A3)-derived peptide. Of nine enrolled patients, five had objective clinical responses; however, three experienced neurological toxicity and two eventually died [38]. The toxicities were subsequently explained by the ability of the TCR to cross-recognize a related peptide, derived from MAGE-A12, which is expressed in the human brain. This peptide only varies at the position 2 anchor residue, and might therefore indicate that although natural TCRs should not target healthy tissue, the strategies applied in adoptive cell therapy might override peripheral tolerance mechanisms [39]. The second trial applied a TCR targeting a MAGE-A3-derived peptide that binds HLA-A0101 [30]. The TCR had been obtained from a vaccinated patient [40,41] and engineered to obtain higher affinity [24]. Two patients were treated in this trial, and both developed cardiac arrest and died shortly after T-cell infusion. Extensive investigations revealed that despite a restricted sequence similarity with the MAGE-A3 peptide (55% sequence overlap), the TCR had cross-reacted with the protein, titin, which is expressed in beating cardiomyocytes (Figure 1A) [30].

TCR binding degeneracy

Extrapolations from experimental data estimate that a single TCR can recognize at least 10^6 different MHC-bound peptides [42], which is crucial for immunological protection of virtually all encountered pathogens and cellular malignancies [43–45]. Such TCR degeneracy is necessary because if each TCR could recognize only one target, the number of required single naïve T cells carrying a different TCR would far exceed the number of cells of the human body [44,46] (Figure 1B). Reflecting on the peptide targets of CD8 T cells (a sequence of eight to 14 amino acids [47,48]), the total number of different peptides that could theoretically be generated from the 20 naturally occurring amino acids would exceed 10^{18} [43]. Moreover, this does not account for post-translational modifications, which are believed to alter peptides sufficiently to generate an even greater number of T-cell targets [49–57]. Although this will be reduced to only a few percent (1–3%) when accounting for peptide processing and HLA presentation [44,58,59], it still amounts to an astounding number of potential peptides that our TCR repertoire are able to recognize to protect against pathogens of any type. The advantage of having a confined repertoire of highly cross-reactive TCRs is that various clonal T cells can respond to a given pathogenic pMHC [43]. This reduces the risk that pathogens will produce escape variants, as a single mutation in a peptide sequence is unlikely to bypass recognition by several different TCRs that could recognize the original pMHC [60,61].

Individual TCRs are generated through somatic recombination of V(D) and J gene segments in developing T cells, and are clonally selected before they develop into mature T cells. This produces a repertoire of TCRs that can interact specifically with targets that are formed as randomly as the range of pathogenic peptides, while not responding inappropriately to self. However, the dual nature of the TCR recognition motif, comprised of both peptide and MHC, represents an inherent challenge for specific recognition as only a small area of the peptide is exposed for direct contact with a TCR. Thus, much of the T-cell specificity is conferred by the more invariant interaction with MHC, which explains

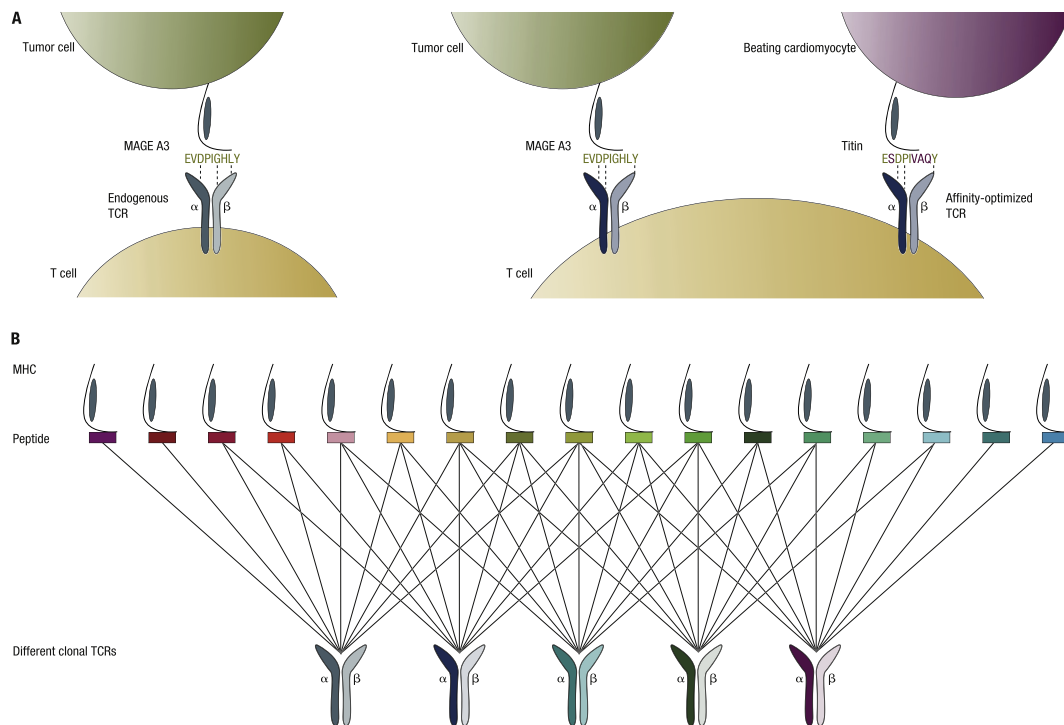


Figure 1. T-cell receptor (TCR) degeneracy. (A) Engineering of a TCR targeting a MAGE-A3-derived peptide (EVDPIGHLY) that binds HLA-A0101 to obtain higher affinity resulted in cross-reactivity of a peptide (ESDPIVAQY) derived from the protein, titin, expressed in beating cardiomyocytes. (B) TCR degeneracy provides broader antigenic coverage than if each TCR only recognized one epitope. Moreover, each peptide-major histocompatibility complex (pMHC) is recognized by several TCRs, which reduces the risk that a virus or malignancy will produce escape variants.

how TCRs can be highly promiscuous to different targets presented by the same MHC haplotype [62,63], while rarely being cross-reactive between MHC haplotypes [64,65]. It has been reported that TCRs that require a lower number of direct peptide interactions will have a greater binding degeneracy in terms of the number of different pMHCs that they may recognize [63,66,67]. Such promiscuity enables T cells to respond to a large variety of target peptides, and allows recognition of peptides with minimal sequence overlap [68,69].

Implications for immune therapy

The extent of TCR binding degeneracy is corroborated through studies providing evidence of structural flexibility of TCR–pMHC interactions [61,70–79], and the continuous emergence of new examples of clonal T cells recognizing peptides of varying sequence homology [30, 42,63,67,80–85]. These studies emphasize that although TCR

cross-reactivity is essential for providing effective immunological surveillance [44], it is also implicated in the development of autoimmune diseases [66,86–89], and represents a major challenge for the development of new TCR-based therapeutics. Currently, much concern is associated with the potential cross-reactivity of affinity-optimized TCRs as these TCRs have not been through clonal selection. However, due to the extensive binding degeneracy inherent in all TCRs, both endogenous and modified TCRs may lead to cross-reactivity of healthy tissue when applied in adoptive cell therapy, where peripheral tolerance mechanisms are revoked. Consequently, critical adverse events may arise from the use of natural TCRs [39]. Technologies are emerging which enable extensive characterization of the patterns decisive for TCR engagement [67, 90–96], along with the prospects of describing and understanding some of the elementary relationships between antigen specificity and the cross-recognition potential of a TCR [97].

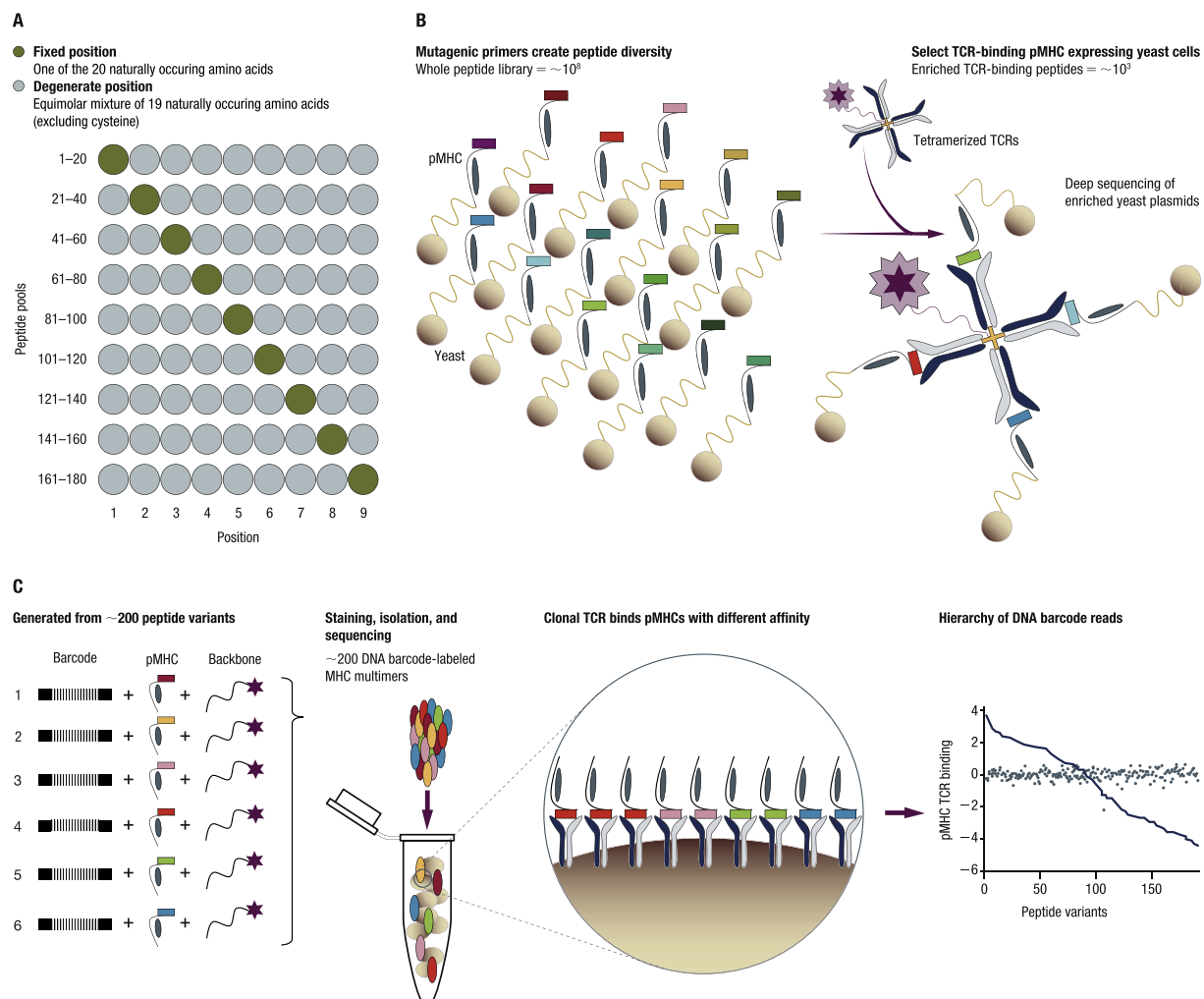


Figure 2. Overview of the main experimental strategies to describe the cross-recognition potential of T-cell receptors (TCRs). (A) The 9mer combinatorial peptide strategy (CPL). The 5×10^{11} peptides of the CPL are distributed in 180 different pools. Each pool contains one specific amino acid at a fixed position (blue circles), while the remaining eight positions comprise a random equimolar composition of all other naturally occurring amino acids except cysteine (grey circles). The CPL comprises a new peptide pool for each of the 20 naturally occurring amino acids at each position. T cells are incubated with each peptide pool, and a subsequent functional interrogation reveals the binding preferences of the given TCR. (B) The yeast display strategy. Gene constructs encoding peptide–major histocompatibility complexes (pMHCs) are diversified through the use of mutagenic primers. This produces a library of $\sim 10^8$ different peptides presented in the context of a given human leukocyte antigen. A tetramerized TCR of interest is incubated with the pooled library of pMHC-displaying yeast cells, and the interacting cells are isolated. The peptide sequences expressed by the yeast-displaying cells are recovered through deep sequencing of the isolated yeast cells. (C) DNA barcode-labelled MHC multimers. MHC multimers are produced from peptide variants of a known target. The variants comprise the peptides generated from sequentially substituting each amino acid with the remaining 19 naturally occurring amino acids, which produce ~ 200 different peptides from an original 9–11mer peptide. Each MHC multimer is individually labelled with a unique DNA barcode, and the total pool of multimers can be incubated with a T-cell clone or TCR-transduced T cells. The clonal TCR will bind the multimers in a hierarchy governed by the TCR–pMHC affinity, which is reflected in the hierarchy of DNA barcode reads after sequencing.

Dissecting the requirements for TCR recognition

The initial safety evaluation of the affinity-optimized MAGE-A3/titin cross-reactive TCR (a3a), which proved incapable of identifying potential toxicities despite thorough ‘specificity testing’ against primary cells from a broad range of normal tissue, illustrates the need for technologies that can effectively assess the cross-recognition potential of TCRs. Ideally, their approach of assessing T-cell reactivity towards numerous cells and tissue types would be the optimal way of determining biologically relevant cross-reactivity, as this also accounts for antigen presentation and post-translational modifications. However, this would require testing of all tissue types within all stages of development, which is not feasible at present. In the case of the a3a TCR, it was only after the observed fatalities that actively beating cardiomyocyte cell cultures were investigated more extensively, and reactivity was discovered towards this tissue. After careful examination of the TCR specificity, the authors identified the cross-recognized titin-derived peptide–HLA-A0101-target [30]. Several such approaches have since been developed, summarized in Figure 2 and Table 1. Collectively, these aim to determine the exact amino acids at given positions of the peptide sequence that are required for TCR recognition, which, when correlated with knowledge of the entire human proteome and potential HLA presentation, provides a valuable strategy for predicting cross-recognition and hence potential cross-reactivity.

Combinatorial peptide libraries

One such approach to identify the amino acid requirements for TCR recognition, which have been adapted to several laboratories, utilizes large combinatorial peptide libraries (CPLs) [42,92,98,99]. The peptides are synthesized such that one position of a 9mer peptide is fixed, while the rest is composed of stochastic equimolar distribution of all naturally occurring amino acids, except cysteine. The fixed positions are exchanged sequentially so that the complete pool of peptides contains all 20 naturally occurring amino acids at all fixed positions, and T-cell recognition is assessed based on functional interrogation of T cells expressing a clonal TCR after exposure to individual peptide pools. The absence of T-cell reactivity will thus reflect the requirement for a different amino acid at the fixed position, and collectively the data can inform on those amino acids and positions that are essential for TCR interaction. The assay also enables interrogation of how changing variables, such as presence or absence of the CD8 co-receptor, will affect

functionality [92]. The CPL strategy requires large amounts of TCR-expressing cells to be included in each individual pool, which is feasible when investigating TCR-transduced T cells. However, assays that require fewer cells would be valuable for early assessment of potential cross-recognition, providing an initial selection criterion to ensure that those TCRs with the greatest risk of cross-recognizing endogenously derived peptides are excluded at an early stage. Moreover, because the interrogation is based on a functional readout derived from recognition of individual peptide pools, it does not provide a relative hierarchy of the interactions, but rather a binary (yes or no) answer to the importance of the different amino acid positions.

Peptide–MHC display and target cell selection

Another approach that provides a more accurate description of the TCR binding degeneracy, because it relates to direct pMHC–TCR interactions, applies a yeast display system where the pMHCs are encoded in gene constructs with a linker between the peptide and the MHC molecule [90]. This enables the generation of a random peptide sequence through the use of mutagenic primers that produce degenerate codons, allowing all 20 naturally occurring amino acids. pMHCs displaying yeast cells are probed with tetramerized TCRs of interest, and interacting cells are isolated through rounds of magnetic bead enrichment and fluorescence-activated cell sorting. Subsequent sequencing informs about the peptide sequence expressed by the enriched yeast-displaying cells, and thus reveals the TCRs’ amino acid requirements for interaction with pMHCs. The great advantage of this strategy, enabled by the random formation of an incredible number of peptides, is associated with the ability to perform largely unbiased screens, and is illustrated by the identification of pMHC targets of TCRs with otherwise unknown specificities [100]. Moreover, a recent study has interrogated the a3a TCR using the methodology on an HLA-0101 yeast display library. From the TCR recognition pattern, it is evident that the amino acids at positions 3, 4 and 9 comprise the main interaction points with the receptor. This can help explain the observed cross-recognition between the MAGE-A3- and titin-derived peptides, as these positions are all shared between the peptides [101]. Disadvantages of the technique are associated with the inability to equally display all positions of the peptide sequence, and cover all peptide variants. Furthermore, to date, the system has only been developed for a few MHC molecules and is restricted to specialized laboratories. A related strategy, based on a mammalian display system, utilizes a minigene-based approach to encode peptide ligands of MHC-I

Table 1
Strategies for resolving the recognition motif of T-cell receptors (TCRs)

Method	Readout	Require a pre-established TCR epitope	HLA availability	Reflects natural peptide–HLA binding	Disadvantages	Advantages	First reported
Combinatorial peptide library	Functional readout that reflects the amino acid requirement one position at a time	No	Independent	Yes	No direct measure of the relative importance of each peptide position	Can include variables and synthetic molecules, e.g. peptide mimetics	[92]
Yeast display ^a	Quantitative readout based on mutual epitope competition. All positions analysed in one reaction	No	New yeast display library required for every new HLA. Currently only developed for a few HLAs	Moderately	Peptide positions may not be equally well represented. Method restricted to a few specialized laboratories	Unbiased screening based on direct pMHC recognition	[90]
DNA barcode-labeled MHC multimers	Quantitative readout based on mutual epitope competition. All positions analysed in one reaction	Yes	Possible for all foldable HLAs. Currently ~35	Marginally	New peptide library required for each analysed pMHC specificity	Easy to implement in laboratories working with MHC multimers	[67]

The methods listed have been applied to understand the molecular interaction points of TCRs, which can be used to estimate their cross-recognition potential. HLA, human leukocyte antigen; pMHC, peptide–major histocompatibility complex.

^a The strategies reported in References 93–96, which have been applied to discover the antigen specificities of TCRs, also have the potential to resolve their recognition motif. Such implementation will share many characteristics with the yeast display method.

that are not subjected to peptide processing. Such a system enables pooled screenings of TCR interactions with different pMHCs, and hence, determination of potential cross-recognition [96].

A number of recently reported strategies for detecting T-cell antigens [93–95] conceptually resemble the yeast display approach, but provide an advantage because TCR interactions are determined from a cellular system (either T-cell clones or TCR-transduced T cells), whereas the yeast display strategy requires production of soluble TCRs for tetramerization. Since these strategies enable the investigation of a large number of different peptides, they have great potential for describing TCR binding degeneracy, but have only been reported for detection of T-cell antigens to date. One approach utilizes signaling and antigen-presenting bifunctional receptors (SABRs), where a signaling domain has been introduced to the MHC class I molecule, leading to green fluorescent protein (GFP) expression following pMHC–TCR engagement. Hence, interacting target cells expressing a cognate antigen can be isolated based on GFP expression. Antigens applied in this system include large libraries of defined peptide sequences transduced into the NFAT-GFP-Jurkat cells together with SABRs [94]. A similar strategy has been developed for MHC II antigen presentation [95]. The flexibility of these approaches allows for customizations required for antigen discovery for public or private TCRs, and can also be used to evaluate the breadth of recognition for a given TCR. An alternative to the SABR approach, published by the same group, takes advantage of a phenomenon known as ‘trogocytosis’, a process whereby lymphocytes and target cells exchange surface molecules upon TCR–pMHC engagement. When pMHC is expressed at supraphysiological levels, this process occurs bi-directionally, hence transferring surface molecules from T cells to the cells presenting a cognate epitope. This marks the target cells, thereby enabling their isolation and, ultimately, the discovery of the cognate TCR epitope. The process has been used for discovery of the antigen specificity of orphan TCRs transduced into Jurkat cells [93]. Like the SABR technique, this approach enables the identification of antigens expressed from large predefined peptide libraries transduced into the target cells, and might become valuable for assessing potential cross-recognition of TCRs.

TCR fingerprinting using DNA barcode-labeled MHC multimers

We have recently developed a ‘one-pot’ tool for determining the amino acid requirements at specific peptide positions for TCR recognition of pMHCs [67]. This method leverages DNA barcode-labeled MHC multimers [102,103], which allows determination of the relative affinity of a clonal TCR to libraries of MHCs that carry peptides with substantial sequence overlap. Peptide libraries are constructed from the originally identified targets, and include all variants generated from sequentially substituting each position with the 20 naturally occurring amino acids. This generates libraries of approximately 200 peptide variants from an original 9–11mer peptide. Individual DNA barcode-labeled MHC multimers are generated from each peptide, and the multimers are pooled and incubated with T cells expressing a clonal TCR. Sorting of all MHC multimer-binding T cells and subsequent sequencing reveals a distribution of DNA barcode reads that reflects the pMHC–TCR binding hierarchy. The experimental design, which includes all amino acids at all peptide positions, enables the translation of the binding hierarchy into a positional scoring matrix for each TCR (Figure 3). This, in turn, is used to determine the relative amino acid preferences of the TCR at individual positions of the peptide sequence.

A disadvantage of the TCR fingerprinting strategy relates to the number of peptides included in a parallel screen. The strategy of substituting a single amino acid position at a time underscores the possibility that several simultaneous amino acid substitutions might promote TCR interaction, or that a TCR might even be able to recognize a largely different peptide [104,105]. Thus, an optimal assay would include many more peptide variants to reflect the great variety of pMHC targets that a TCR may potentially meet *in vivo*. However, this is not feasible because peptide synthesis represents a considerable financial

cost of the total MHC multimer analysis, and the strategy requires a new peptide library for every new TCR that is interrogated. To investigate the extent of this drawback, we interrogated one TCR using a larger peptide library (~800 peptides) that included peptides with two simultaneous amino acid substitutions, and obtained results that were comparable with those obtained from substituting one position at a time. Having only investigated the effect of multiple substitutions on one TCR, we cannot conclude that simultaneous substitutions will never generate new possibilities for a TCR to interact, but the result implies that, for peptides with some sequence overlap, single substitutions can potentially describe most of the amino acids required for TCR interaction. Another drawback relates to the need to have a pre-established peptide target of the interrogated TCR from which to construct the substitution library. Thus, in the current embodiment of the strategy, it would not be possible to identify new pMHC targets of TCRs with unknown specificities. Advantages include flexibility (in terms of the possibility of interrogating TCR recognition restricted to all foldable MHC molecules) and a relatively straightforward methodology, which should make it possible to implement the technology in most laboratories familiar with molecular biology and MHC multimers. Moreover, the pooled format of the screening provides a sensitive readout for specific TCR interactions that describe the relative importance of the individual amino acids at each position in the peptide.

An alternative use of DNA barcode-labeled MHC multimers, which also addresses the issue of TCR cross-recognition, has enabled the straightforward identification of those TCRs responsive to a mutation-derived neoepitope with the least risk of also cross-recognizing the wild-type counterpart [106]. In this approach, one fluorochrome is used to label all DNA-encoded MHC multimers holding a neoepitope, and another fluorochrome is used for all the corresponding wild-type peptides. T cells that cross-recognize both types of peptide will thus stain in two colors. By single-cell sorting and capturing the TCR transcripts associated with the single-color neoepitope-responsive T cells, the TCRs with a lower risk of cross-recognizing wild-type peptides can be isolated, while the simultaneously captured DNA barcode will inform on the exact antigen specificities. The TCRs identified with such a methodology will still require comprehensive analysis of the complete cross-recognition potential before seeing therapeutic uses, but the strategy provides an intelligent first-selection criterion of neoepitope-responsive TCRs intended for the clinic.

Although all these strategies [combinatorial peptide libraries, pMHC display strategies and DNA barcode-labeled MHC multimers (Figure 2)] are advantageous compared with previous strategies that applied single-position alanine or glycine substitutions [30], they are still experimentally limited compared with the number of possible ligands that might be encountered. Therefore, interpretations of the results should reflect that it is merely a window of estimated cross-reactivity. Structural information and *in silico* modeling [107,108] may, in turn, enhance the utility of the experimental approaches for assessing TCR binding degeneracy.

Translating molecular interaction points of TCRs into cross-recognition potential

Knowing the molecular interaction points of a TCR might assist the identification of cross-recognized peptides derived from endogenous proteins, and hence provide a tool to assess the cross-recognition potential of a given TCR before taking it to the clinic (Figure 3), ultimately avoiding severe side-effects caused by TCR therapy. To assess this risk, the knowledge acquired from dissecting the TCR interactions with pMHC can be further analysed *in silico*, where a number of tools, including find individual motif occurrences [109] or ScanProsite [110], can be utilized to identify peptide sequences in the human proteome that match the molecular interaction points of a TCR, and hence are at risk of being cross-recognized (Figure 3).

However, from the point of understanding TCR recognition and predicting the potentially cross-recognized peptides using any of the

methods described in this review, further assessments are required to determine whether such cross-recognition will pose an actual clinical risk, as multiple mechanisms are in play controlling the ability of our immune system's T cells to initiate cellular cytotoxicity. Important factors to take into consideration include: (i) peptide processing and presentation; (ii) pMHC affinity; (iii) protein expression level; and (iv) peripheral tolerance mechanisms maintained by regulatory T cells, and surface expressed and secreted molecules [111–115]. Peptide processing and presentation in the context of patients' HLA molecules will substantially

limit the number of potential peptides presented [58,59]. However, prediction of antigen presentation is not trivial due to the many variables involved in the processing machinery, and is, therefore, currently not capable of sufficiently identifying the peptides that are truly presented. The methods described in this review reflect differently on natural presentation of peptides; while the ability of peptide binding to MHC is an integrated part of the CPL strategy, this method and the DNA barcode-based methods do not reflect intracellular processing of longer peptides. The methods utilizing antigen display to a larger degree reflect

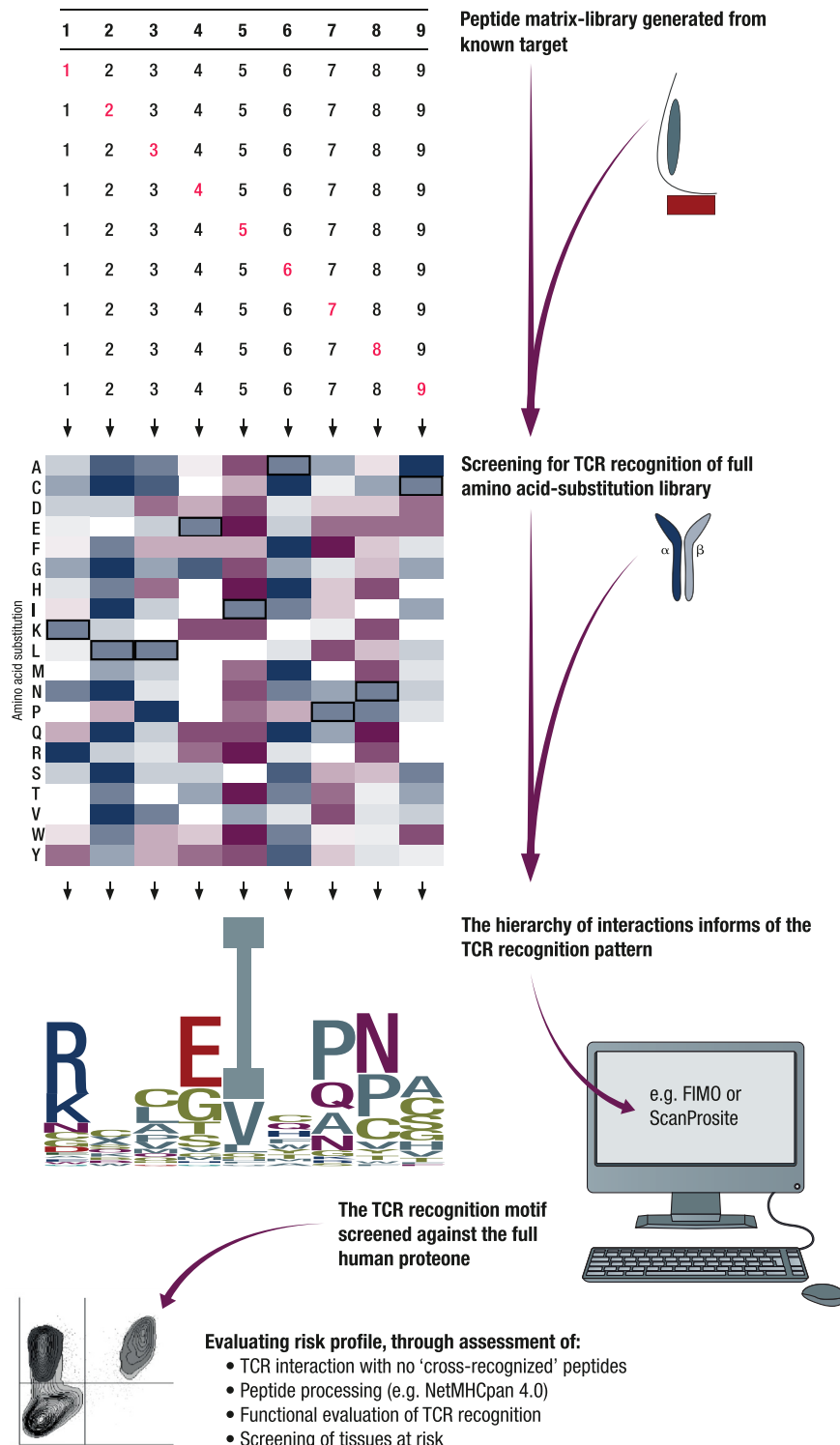


Figure 3. Schematic overview of the proposed strategy for evaluation of T-cell receptor cross-recognition potential.

the processing of peptides, as the presented pMHCs are derived from cellular systems.

A requirement for reaching a certain threshold of pMHC–TCR interactions to activate a T cell means that T-cell recognition is only translated into a functional response if the peptide is derived from a protein expressed above a certain level [26], and is moreover influenced by affinity between the peptide and MHC [116]. Thus, TCR interaction with a given pMHC does not necessarily correspond to T-cell reactivity, which makes it important to distinguish between biochemical and immunological recognition when considering potential toxicity of a TCR. Consequently, the profiling of the TCR recognition motif to the human genome should be used to guide researchers to identify tissues at risk, which can be subjected to in-depth interrogation of functional T-cell recognition to reveal whether T-cell recognition of an endogenously derived peptide will lead to cellular destruction *in vivo*.

As several strategies of immune therapy of cancer, including adoptive cell transfer strategies, work to deliberately break some level of tolerance [117,118], it is not safe to assume that natural (non-modified) TCRs, which have passed through clonal selection, will never target endogenous tissue. Based on the 12 MCC clones investigated in the fingerprinting strategy [67], which target a Merkel cell polyomavirus-derived peptide, these TCRs are non-modified and target an antigen that is foreign to the immune system, but the data imply that most TCRs cross-recognize peptides derived from healthy tissue. Currently, knowledge of the extent and consequences of TCR cross-recognition are insufficient to predict when such cross-recognition will have an effect at a functional level, and the potential of both natural and modified TCRs for recognizing endogenously derived peptides should be examined carefully prior to clinical translation.

Improving TCR design and optimization

Knowing the landscape of the cross-recognized pMHCs of individual receptors has proven particularly important for the *in vitro* affinity-optimized TCRs developed for clinical applications. Due to the severe adverse effects observed with such genetically modified TCRs, and because of the evidence that T-cell function is not enhanced above a certain TCR–pMHC affinity threshold [119–121], novel structure-guided approaches are concerned with the effect of modifying TCRs in a way that finds an optimal (rather than maximum) TCR affinity range [20,26,122, 123], and propose to incorporate changes that simultaneously enhance and decrease the affinity of the pMHC–TCR interaction [62]. It is suggested that a TCR with optimal on-target (only) affinity may be generated by modifying the TCR in a way that promotes stronger binding to the peptide part of the TCR recognition motif while decreasing the overall binding to MHC [124]. Therefore, while all modifications of natural TCRs may pose a risk, increased motivation for describing the molecular recognition patterns of a TCR to assist the process of ‘rational design’ of genetically modified TCRs intended for the clinic is emerging. Moreover, knowledge of the molecular interaction points of TCRs can feed into developing *in silico* platforms, increasing our overall capacity to understand TCR interactions and predict T-cell cross-recognition. Taken together, such measures can be applied to facilitate the generation of efficient and safe TCR gene therapy strategies for future treatment of advanced cancer.

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