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REVIEW

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Advances in identification and selection of personalized neoantigen/T-cell pairs for autologous adoptive T cell therapies

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ABSTRACT

Based on the success of tumor-infiltrating lymphocytes (TIL)-based therapies, personalized adoptive cell therapies (ACT) targeting neoantigens have the potential to become a disruptive technology and lead to highly effective treatments for cancer patients for whom no other options exist. ACT of TIL, peripheral blood or gene-engineered peripheral blood lymphocytes (PBLs) targeting neoantigens is a highly personalized intervention that requires three discrete steps: i) Identification of suitable personal targets (neoantigens), ii) selection of T cells or their T cell receptors (TCRs) that are specific for the identified neoantigens and iii) expansion of the selected T cell population or generation of sufficient number of TCR modified T cells. In this review, we provide an introduction into challenges and approaches to identify neoantigens and to select the Adoptive Cell Therapy, ACT, Neoantigen, T cell, Cancer respective neoantigen-reactive T cells for use in ACT.

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Introduction

The human immune system has developed diverse strategies to fend off diseases. Some rely on the adaptive immune system, which is antigen specific and forms immunological memory in contrast to defense mechanisms that depend on innate immunity. T cells are part of the cellular adaptive immune response and cytotoxic T cells excel at eliminating cells infected with viruses or bacteria.[1] In recent years, their ability to protect against cancer was firmly established by the success of T cell checkpoint inhibitors and T cell therapies.[2]

Usually, T cells recognize their targets via their T-cell receptor (TCR) which binds a small peptide in the context of the major histocompatibility complex (MHC), also referred to as human leukocyte antigen (HLA). We refer to this type of natural T cell recognition of their target as endogenous T-cell recognition. T cells can also be leveraged for cancer treatments by redirecting them with bispecific antibodies[3] or by genetic manipulation to express a chimeric antigen receptor (CAR), as reviewed elsewhere.[4] In such cases, the bispecific antibodies or CARs establish a synthetic recognition between a cancer cell surface target and a shared component of the TCR, independent of the endogenous specificity of the T cells. As a result, bispecific antibodies and CAR-T therapies are constrained by their target space to the cell surfaceome. Thus, relying on endogenous T cell recognition in the context of personalized T cell therapy provides major advantages and is the scope of this review.

The human HLA class I and II molecules share the ability to present antigens to T cells. However, the origin and processing of the peptides differ between the two HLA classes.[5] In brief, HLA-I presents intracellular peptides, while exogenous material is the source for peptides presented on HLA-II. Although this functional separation is generally true, exceptions to this rule occur. Antigen cross-presentation by specialized antigen presenting cells (APCs) allows the presentation of exogenous peptides in HLA-I and autophagy-driven degradation can lead to presentation of intracellular peptides in HLA-II.[6] All nucleated cells express HLA-I molecules, while HLA-II is mostly restricted to professional APCs, such as dendritic cells (DCs), macrophages, and B cells.

Each person typically expresses up to six classical HLA-I and eight HLA-II allele variants with tissue and cell type-specific expression levels.[7] The multiple HLA alleles (HLA-A, HLA-B, HLA-C, HLA-DP, HLA-DQ, and HLA-DR) encoded in the human genome are among the most polymorphic genes in the human genome. Each of these HLA alleles will restrict the presentation of peptides with specific anchor residues. TCRs only bind to their antigens in the context of a specific HLA allele. Consequently, HLA restriction confines the use of identified TCRs to the specific alleles expressed in a given patient.

In this review, we focus on T cells expressing the αβ TCR. The role of γδ T cells in cancer immunotherapy was recently reviewed elsewhere.[8] To ensure that T cells do not recognize self-proteins, self-reactive T cells undergo apoptosis in the thymus. The majority of mature T cells are specific for non-self peptides.[9] The selection of non-self-specific T cells consists of a positive HLA-specific selection, giving rise to a CD4 or CD8 positive phenotype based on HLA-I or HLA-II recognition, and elimination of self-antigen specific T cells during negative selection.[10] CD4 positive T cells, also named T helper cells, recognize HLA-II bound peptides and control the activity of the immune system through cell-cell contacts and the release of cytokines. CD8 positive cytotoxic T cells can

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target HLA class I bound peptides and directly kill malignant cells through the release of cytolytic granules. Despite many self-reactive T cells being eliminated, the presence of T cells targeting self-peptides is commonly observed and these can contribute to some autoimmune diseases.

Neoantigen-reactive T cells recognize non-self tumor-specific peptides presented on HLA

After leaving the thymus, T cells are tolerized to most of the body's own peptide HLA complexes (pHLA). Yet, to function in the context of cancer, a TCR must recognize an antigen arisen from the body's own cells. Many of the first identified tumor antigens recognized by T cells were shared antigens expressed in melanoma cells and at a lower level in healthy melanocytes.[11] As a result of the divergence of tumor cells from normal cells, tumors can process and present non-mutated and mutated peptides which can elicit T cell responses. These can be categorized into i) tumor-associated antigens with low tumor specificity. This category includes antigens that are over-expressed by tumor cells but are also present at lower levels in normal cells or specific tissues; ii) tumor-specific antigens including cancer testis antigens, which are derived from reactivated embryonic or developmental genes, or neoantigens.[12,13]

As defined by the etymology of the word, neoantigens are "new" or non-self-protein products that are capable of inducing an immune response. These can originate from mutated gene products, expression of viral oncoproteins and unconventional antigens such as aberrant splicing and translation.[14] Such antigens escape central tolerance and are exquisitely tumor-specific. These features render them exceptionally attractive to treat cancer and offer a target space that cannot easily be accessed by bispecific antibodies or CAR-T-based therapies. Recent studies suggest that T cell responses in patients with melanoma are dominated by neoantigens arising from tumor-specific somatic mutations.[15]

While there is an emerging interest in neoantigen identification either for predicting response to immunotherapy or to develop personalized clinical interventions targeting them, there has been an increasingly relaxed and inaccurate usage of this term in the last years. *Bona fide* neoantigens are naturally processed and presented on HLA and capable of eliciting a T cell response and thus require immunogenicity testing.[16] This should be distinguished from non-self peptides predicted to bind to HLA or eluted from cell surface HLA lacking a confirmed T cell response, which should be referred to as predicted, putative, or candidate neoantigens.

For T cells the presence of their cognate peptide on the cell surface may suffice to induce an immune response against the tumor cells. However, not all mutations are equally well suited for cell therapy. An important aspect is their clonal expression within the tumor.[17] Mutations can be separated into two categories: i) truncal mutations expressed in all tumor cells often including driver mutations; ii) branch mutations expressed in a subset of tumor cells frequently consisting of passenger mutations. The T cell repertoire in lung cancer was shown to consist of T cell clones specific for truncal and branch mutations.[18] Targeting truncal mutations offers the opportunity to level a response against the majority of the malignant cells. However, truncal neoantigens are rare and may have escaped immune recognition due to early immune selection. As mentioned before, truncal neoantigens are not necessarily driver genes. Nevertheless, Rosenberg and his colleagues have identified immunogenic mutations in RAS, TP53, and other drivers of tumorigenesis.[14]

Non-synonymous amino acid substitutions including deletions, gene fusions, chromosomal translocations, and alternative splicing events may give rise to truly novel peptides. Recent technological advances in sequencing allow to identify these abnormalities from tumor biopsy material (Figure 1a) and enable the identification of candidate neoantigens in a personalized fashion. In the future, circulating tumor cells or circulating tumor DNA (ctDNA), may also be a valuable source for the detection of putative antigens. Currently, analysis of personal ctDNA is limited to monitoring of limited gene subsets.[19] The demand for DNA quantity and the cost of deep sequencing presently prevents routine implementation of this technology for identification of candidate neoantigens.

In silico prediction of potentially immunogenic peptides is commonly used to identify tumor-specific candidate neoantigens from non-synonymous mutations (Figure 1b). The advances in predicting T cell epitopes were comprehensively summarized recently.[20] The main challenges identified by the authors include benchmarking prediction algorithms in large data sets and across multiple alleles, the limitations of whole exome sequencing (WES) compared to whole genome sequencing (WGS), and the default addition of RNA sequencing (RNAseq) to workflows to exclude candidate variants that are not expressed. The authors proposed as final goal to reverse the approach by predicting the TCR-specific epitope from a TCR sequence of unknown specificity.

Novel methodologies like deep learning and artificial intelligence are used to further increase the accuracy of predictions.[21] A delicate balancing between sensitivity and specificity is required in the pipeline for the prediction of candidates as it was substantiated by the comparative study of the TESLA consortium.[22] While filtering out 98% of non-immunogenic peptides with more than 70% precision is a leap forward, indications with low mutational burden which were not studied might require an even higher precision.

An additional methodology to identify candidate neoantigens is based on immunopeptidomics.[23] In this approach, pHLAs are immunoprecipitated, the peptides eluted and analyzed by a tandem MS/MS workflow and matched to a personalized database including all known coding proteins and the non-synonymous amino acid sequences to identify the candidate mutated minimal epitope. In contrast to the previously described technologies, immunopeptidomics also allows the discovery of post-translationally modified peptides such as phosphopeptides.[24] While such methods are not dependent on predictions, they lack sensitivity and are therefore biased to underestimate the true neoantigen burden. They also suffer from relatively high tissue demand required for the analysis. Thus, mutated peptides are only rarely detected from patient materials using immunopeptidomic-based approaches.[25]

Both in silico prediction and immunopeptidomics are capable of identifying putative neoantigens, which are more likely

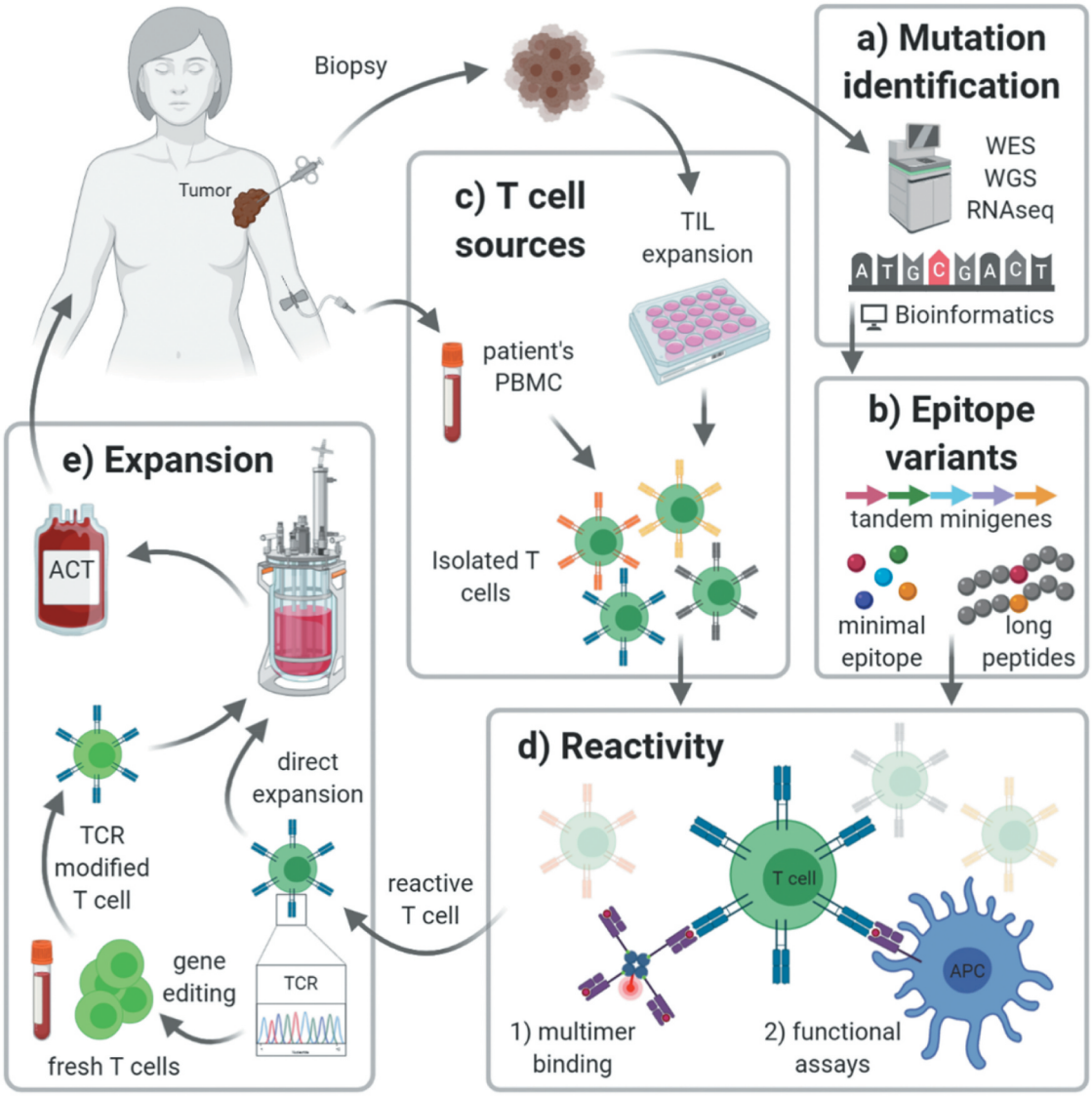

Figure 1. Overview of the ACT process. a) Mutations in the tumor are identified by sequencing of the biopsy material (WES, WGS and/or RNAseq). Mutations are analyzed and possible epitopes are discovered. b) Epitopes may be encoded on a DNA/RNA level, for example as tandem minigenes, or peptide level as minimal and long variants depending on the requirements of the selection technology used c) Two major sources are currently used for T cells: TILs expanded from tumor biopsies or lymphocytes isolated from blood. d) Reactivity of T cells to epitopes can by analyzed by binding of pHLA multimers (1) or functional assays (2). e) Eventually, T cells are expanded for infusion. Either reactive cells are directly grown to huge numbers with the risk of exhaustion and terminal differentiation or the TCR sequence is extracted and freshly sourced cells are genetically modified to express the desired TCR.

to be presented on HLA of tumor cells. However, for both approaches functional T cell assays are required to evaluate the immunogenicity of the identified peptides and confirm them to be true neoantigens. In the next sections, we will summarize the most recent advances in the development of such T cell-based high-throughput screening platforms. These platforms not only enable the identification of immunogenic peptides but can also be used to enrich for neoantigen-specific T cells or to isolate neoantigen-specific TCRs for therapeutic use in ACT.

T-cell epitope identification using high-throughput T cell antigen discovery platforms

We start with highlighting recent technologies, which are particularly suited for identifying the exact immunogenic epitope targeted by a specific T cell or the TCR in a cellular context. In one such approach, libraries of antigen-presenting bifunctional receptors (SABRs) are introduced into target cells and used to screen Jurkat cells modified to express a TCR of interest[26] (Figure 2a). SABRs are composed of a pHLA complex and an additional intracellular signaling domain which upon SABR engagement with a TCR induces a TCR-like signaling in the target cells. The reporter gene GFP under control of NFAT is consequently expressed and GFP positive cells are sequenced to determine the cognate peptide. A similar approach is followed by pMHC–TCR (MCR) hybrid molecules[27] (Figure 2b). The hybrids comprise the extracellular part of MHC with directly tethered peptides and fused to the transmembrane and intracellular domain of the TCR chain. In TCR-deficient T cell hybridomas, the MCRs are associated with the native CD3 complex and used to induce an NFAT reporter system. With this setup, the Kopf lab was able to identify CD4 T cell epitopes from libraries covering whole tumor peptidomes.[27] However, the aforementioned technologies require the

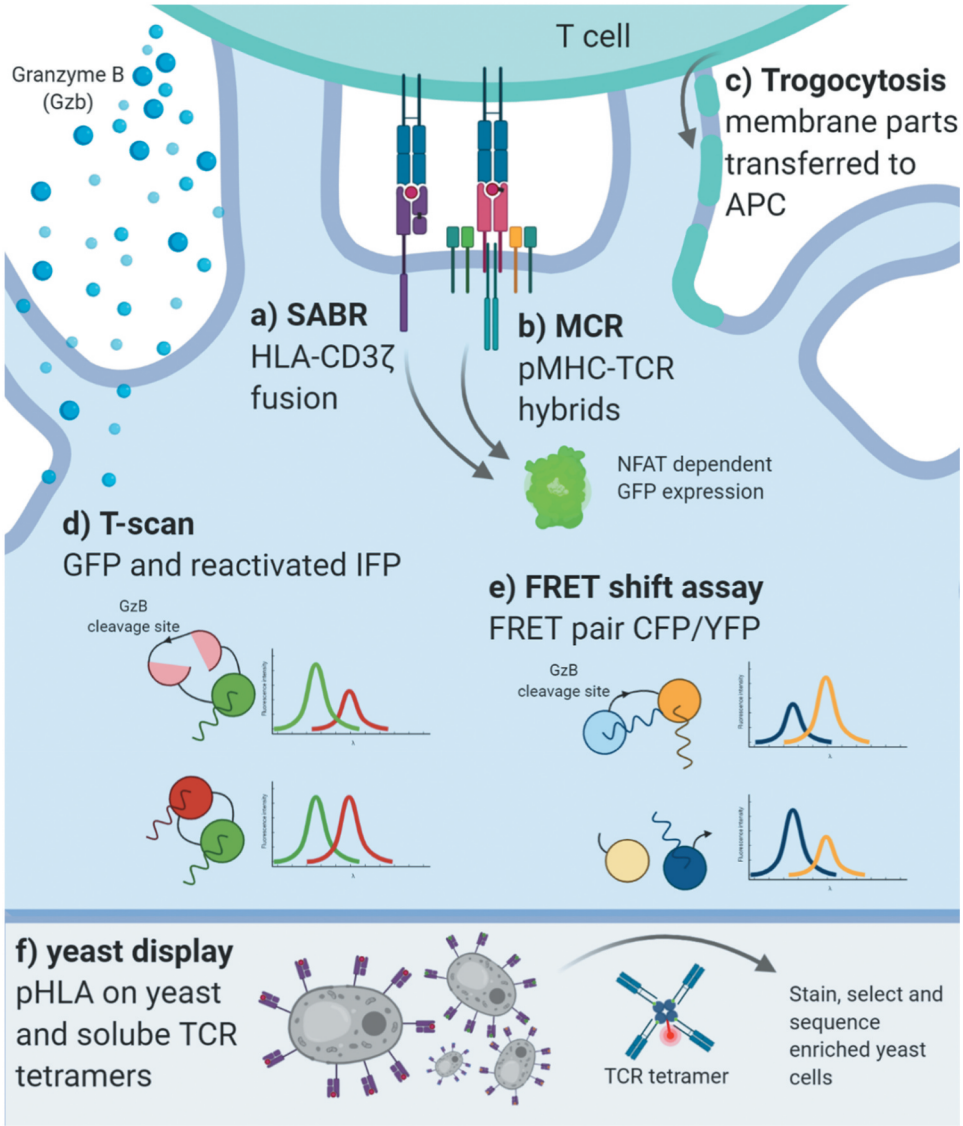

Figure 2. Several T cell epitope discovery technologies based on encoding antigens in a cellular library were developed. a) Antigen-presenting bifunctional receptors (SABRs) extend the function of HLA to also elicit an intracellular signal upon engagement with a TCR from a T cell. Consequently, NFAT dependent GFP expression is induced in cells of the library coding for T cell epitopes. b) pMHC–TCR (MCR) hybrid molecules have the target peptide directly tethered to MHC and the transmembrane and intracellular domain have been replaced by the TCR chain. The hybrids therefore engage in the CD3 complex and binding of a TCR to the MCR hybrid results in NFAT dependent GFP expression. c) Trogocytosis is the transfer of membrane fragments including their protein content during cellular contacts in the immune synapse. APCs are selected based on the presence of transferred T cell parts for the analysis of their presented antigens and thus allowing to discover the T cell epitope. d) Reactive T cells deliver serine proteases such as granzyme B (GzB) to induce apoptosis in their target. The T-scan technology utilizes the enzymatic activity of GzB to distinguish cells presenting T cell epitopes from those that don't. IFP is engineered with a linker splitting the protein and as a result keeping it inactive. However, enzymatic cleavage of the linker triggers the fluorescence of IFP in productively recognized cells. e) The enzymatic activity of GzB can also be used in a FRET shift assay using CFP/YFP. The functional activity of GzB here spatially separates the FRET pair und thus members of the library presenting a T cell epitope lose their FRET activity. f) The yeast display system can be modified to present pHLA on the surface. TCRs of interest are tetramerized and labeled with a fluorochrome. The yeast cells displaying the TCR epitope can so be stained and selected. After multiple rounds the enriched yeast populations is sequenced for the displayed antigens.

generation of T cell clones or isolation of TCRs with confirmed tumor recognition. Whether they can be used to perform high-throughput detection of several T cell antigens starting with a mixed population of cells with variable frequency of tumor reactivity remains to be seen.

Another approach to identify epitopes without cell engineering is based on trogocytosis (Figure 2c). During trogocytosis, small membrane parts including their protein content are transferred from T cells to target cells during the duration of the immune synapse.[28] Li and colleagues used the phenomenon to sort out target cells from a library of pHLA variants that were positive for T cell membrane proteins after co-incubation.[29] By sequencing those positive cells, they were able to identify the specific epitopes targeted by the T cells.

T cells induce apoptosis in their target cells by activation of the caspase cascade via delivered serine proteases such as granzyme B (GzB). In a recently described approach, Kula et al. harnessed the cytotoxic potential of T cells to discover immunogenic antigens.[30] T-scan (Figure 2d), a co-culture-based assay capable of flagging target cells productively recognized by T cells is based on GzB activation of a fluorescent marker in said target cells. The HLA-deficient target cells were transduced with a lentiviral library expressing a large number of antigens and the individual HLA allele of interest prior to

the co-culture. In addition, the endogenous CAD nuclease was replaced with its inactive form to prevent genomic fragmentation and target cell apoptosis. At the end of the co-incubation period fluorescent cells, presumably exposed to GzB secreted by T cells following antigen-specific stimulation, were sorted and sequenced to reveal the epitope of the T cell. This method was shown to enable genome-wide antigen discovery. A similar approach was developed by Sharma et al. using a Förster resonance energy transfer (FRET)-shift reporter based on GzB cleavage[31] (Figure 2e). Initially, the FRET pair is held in proximity by a linker allowing energy transfer between the two, which can be detected by flow cytometry. When the linker is cleaved by GzB, the two moieties are released and they leave the immediacy required for FRET. Thus, GzB cleavage ablates the FRET signal in cells targeted by T cells. Minigenes encoding the epitope and the FRET pair were virally introduced into target cells and co-cultured with T cells. Target cells with low FRET were isolated before GZB-induced apoptosis by FACS. Their encoded minigene can be sequenced to determine the exact epitope recognized by the T cells.

A yeast display-based approach was conceived by Chris Garcia and his coworkers (Figure 2f). By using recombinant expression of pHLA in yeast, they were able to construct a yeast display antigen library. The library was used to interrogate the specificity of orphan T-cell receptors isolated from tumor-infiltrating lymphocytes in a colorectal adenocarcinoma setting.[32] Serial enrichment of specific pHLA contained in the pHLA yeast display library was performed through consecutive flow of the library through a column containing beads coated with TCR multimers. This method underscores the promiscuity of TCRs, as this approach often led to the identification of a number of peptides recognized by a single TCR. While this can hinder the identification of the actual peptide recognized by T cells, this method can be leveraged to analyze peptide mimicry and anticipate potential toxicities.

Identification and enrichment of neoantigen-specific T cells

tologous tumor-specific T cells are commonly sourced from tumors where the neoantigens are expressed. As early as the 1980s, the field shifted from treating patients with lymphokine-activated killer (LAK) cells[33] to harnessing tumor-infiltrating lymphocytes (TIL) expanded from biopsies.[34] Pioneering immunotherapy studies were conducted at the Surgery Branch of the U.S. National Cancer Institute by Steven A. Rosenberg and colleagues.[35] In 2002, they showed complete remission in metastatic melanoma patients using a combination of lymphodepleting chemotherapy followed by TIL infusion and high dose IL-2 treatment.[36] While such an approach holds great promise, the numbers of TILs are limited in some tumors. Furthermore, homing of T cells to tumors is not solely an antigen-dependent process,[37] thus T cells with other specificities were shown to reside in tumors.[38–40] Neoantigen-reactive T cells are frequently detected in tumors, including melanoma,[41–43] gastrointestinal,[44,45] breast,[46,47] lung,[48] and head and neck cancers.[49] Their frequency is highly variable between cancer indications, within tumor indications and even within an individual tumor. Dominant neoantigen-specific clones can be detected in some tumors, but they often represent a relatively rare subpopulation of the tumor-resident repertoire.[40,44,50] A second emerging source for neoantigen-specific T cells is the blood of patients (Figure 1c). The sampling is less invasive in contrast to tumor biopsies and is thus readily available for most patients. Although less frequently pursued, tumor-specific T cells can also be derived from other body fluids such as urine,[51] ascites, and pleural effusions.[52]

Several methods and markers have been suggested for the enrichment of tumor-reactive T cell clones. PD-1 is an inhibitory receptor expressed on acutely or chronically stimulated T cells. PD-1 positive TILs grown from melanoma samples showed higher tumor reactivity after in-vitro expansion and tumor-specific IFNγ production when compared to PD-1 negative TILs.[53] PD-1 positive TILs and PD-1 positive blood-derived T cells have also been shown to be enriched in neoantigen-reactive clones, compared to the PD-1 negative counterparts.[54,55] Eventually, PD-1 negative T cells were shown to overgrow the more relevant PD-1 positive population, potentially limiting the efficacy of the therapy and highlighting the importance of selecting T cell subsets enriched for tumor recognition.[56] TIM-3 is a co-inhibitory receptor restricted to IFNγ-producing T cells. TIM-3 and PD-1 were shown to be co-expressed on tumor-resident cytotoxic lymphocytes capable of recognizing autologous tumor,[55] but not on the circulating population from which the neoantigen-reactive T cells were identified.[54] Selection based on activation-induced expression of 4–1BB after overnight rest in cytokines proved a viable strategy to select for tumor-reactive T cells.[57] 4–1BB positive TILs were also shown to contain neoantigen-reactive TCRs.[58] In another study, T cells recognizing mutated KRASG12D and KRASG12V variants were isolated from the memory pool (CD62L and CD45RO) in combination with vitro sensitization (IVS; see below) and 4–1BB as a marker to select for neoantigen-specific lymphocytes.[59] The activation marker LAG-3 was also shown to be co-expressed on PD-1 positive tumor-resident T cells.[54,55] CD39, another T cell activation marker, discriminates infiltrating lymphocytes into bystander and tumor reactive cells.[38] CD39 positive preselection was further combined with CD103, a tissue residence marker, as an additional metric.[60] An alternative approach to enrich reactive T cells is IVS. This method typically involves serial stimulation of PBMCs in presence of antigen-loaded DCs[45] or more recently using patient-derived organoids.[46]

In summary, the application of such enrichment approaches for the identification of neoantigen-specific T cells is not without apprehension. None of the methods or markers are currently able to specifically identify neoantigen-specific T cells[61] and may therefore miss tumor-specific T cells. Nevertheless such approaches may be useful to enrich neoantigen-reactive T cell prior to further downstream processing[39] in particular when aiming to identify neoantigen-specific lymphocytes in peripheral blood. Ultimately, the specificity and functionality of neoantigen-reactive T cells need to be verified by their ability to recognize and kill their target cells (Figure 1d). In the following section, we present separate technologies to verify the specificity and functionality of neoantigen-specific T cell. In reality, however, the different approaches were used as complementary

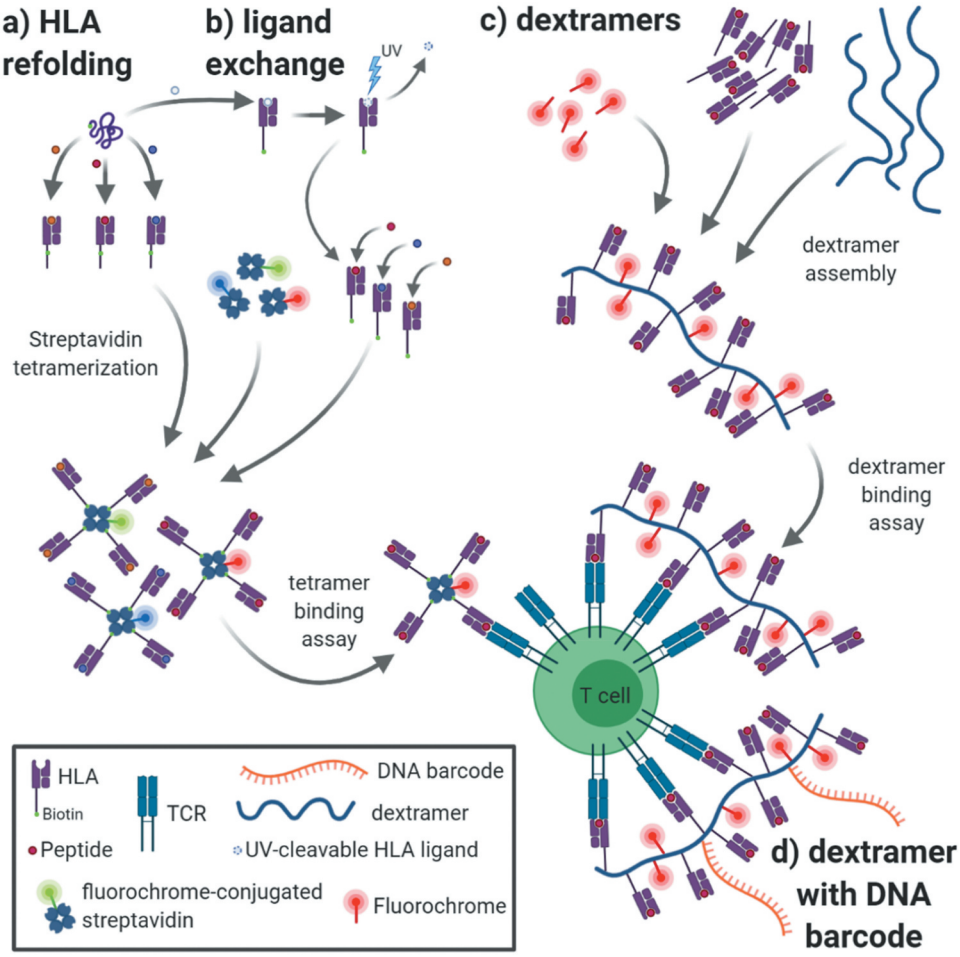

Figure 3. Reading out binding between pHLA and T cells requires multimerization of the pHLA reagent. Biotinylated HLA molecules are refolded with a) peptide or b) UV-breakable ligand present. Ligand exchange allows to replace the UV-breakable ligand with a peptide in the folded HLA. Peptide loaded HLA molecules are tetramerized via the streptavidin-biotin interaction. Fluorochromes attached to streptavidin allow to quantify binding of the tetramers to T cells in for example flow cytometry. c) Tetramers were succeeded by dextramer-based constructs to overcome low-affinity binding by higher avidities. d) Encoding the antigen information on DNA barcodes instead of fluorochromes increased the number of different epitopes analyzable in parallel.

supportive readouts in many studies. The development of these technologies often happened in parallel, but for the purpose of this review, we will present them separately.

Detection of neoantigen-specific T cells using recombinant peptide HLA multimers

The binding strength between a single TCR and its target pHLA is mostly in the range of 10 to 200 µM and thus comparably weak compared to antibodies and their ligands. Only after multimerization of soluble peptide HLAs, the Davis lab managed to study the binding of pHLA tetramers to T cells and correlate the binding capability with T cell cytotoxicity.[62] The commonly used tetrameric structure is composed of four biotinylated HLA molecules, refolded in the presence of the target peptide, and multimerized using fluorochrome-conjugated streptavidin (Figure 3a). Binding of these molecules to T cells is typically detected using a flow cytometer. The history of the pHLA multimer development was comprehensively recapitulated in a recent review.[63]

Three major advances have contributed to make pHLA multimers a useful tool for the identification of neoantigen-specific T cells. Adding the peptides for each pHLA during refolding is cumbersome when studying a large library of mutated peptides. To circumvent this problem, peptides of choice can be loaded via ligand exchange.[64,65] This technology allows to produce and refold one recombinant HLA with a UV sensitive ligand that can be cleaved and exchanged with the desired peptides as needed (Figure 3b). Empty but peptide-receptive HLA molecules can also be generated by stabilizing mutations in the HLA α-chain, as recently shown for HLA-A*02:01 by the Hadrup lab.[66]

Secondly, pHLA tetramers may underrepresent the reactive T cell population due to the high affinity required to stain positive.[67] The relatively low avidity of pHLA tetramers is especially problematic when trying to identify TCRs with low affinity. Of note, the actual binding valency may not be identical to the multimerization factor. A tetramer does thus not necessarily bind with all four possible sites simultaneously. The use of dextran as a scaffold has allowed the generation of higher-order multimer structures and valencies[68] (Figure 3c). Dextramers also enabled to place additional fluorochromes to brighten the staining.

Despite these improvements in increasing the avidity of pHLA multimers, the parallel screening of libraries of pHLAs was limited by the availability of channels in flow cytometry. To overcome this limitation, studies shifted to encoding

strategies that allowed detection of 25 to 50 different variants in parallel in a single sample using FACS[69] or CyTOF[70] and potentially unlimited variants using DNA-barcoded multimers more recently[62] (Figure 3d). These innovations have enabled the screening of large numbers of potential neoantigens. The TetTCRseq technology further advanced the concept by linking the TCR sequence and its antigens at the single cell level.[71] Binding of multiple but different pHLA variants to a single T cell can be quantitatively read out in a high-throughput manner to capture also peptide mimicry. However, current protocols require the lysis of the cells to read-out the DNA barcode rendering subsequent T cell expansion impossible.

Recent smaller improvements in the pHLA multimer technology including the use of brighter fluorochromes, the addition of protein kinase inhibitors, and anti-co-receptor antibodies have further boosted the usefulness of pHLA multimer for the identification of tumor-specific T cells.[72] The ease of use and the ability to run high-throughput assays have all contributed to the wide-spread use of pHLA multimer technology in the field. Recent publications have shown that improved protocols indeed detect more relevant T cell populations,[73] and that selection of neoantigen-reactive T cells is possible by pHLA multimer staining.[74,75] However, there are also important method-intrinsic limitations of the pHLA multimer technology, which we will discuss below.

To construct pHLA multimers for neoantigen discovery, the exact peptide sequence of the putative neoantigen is required. Frequently, candidate minimal epitopes are deduced from in silico predictions (see above). Thus, rare HLA variants pose an additional challenge since the current algorithm parameters have not been extensively optimized for many of these variants. Predictions of HLA class II peptides and the technology to generate HLA class II multimers are not as far advanced as for class I, but substantial progress has been made in the last years.[76] Notably, affordable synthesis of peptides in sufficient quality for large-scale screening of peptide libraries still remains a bottleneck today. An additional limitation arrives from the low frequency of tumor-reactive T cells and their challenging isolation. Flow cytometry-based capture was shown to only retrieve about half of the expected cells in a spike-in experiment.[77] The authors of this study hence conceived a magnetic nanoparticle-based pHLA avidity reagent to improve the recovery to 94% for T cells with low prevalence (0.1% to around 1%). Eventually, a further limitation

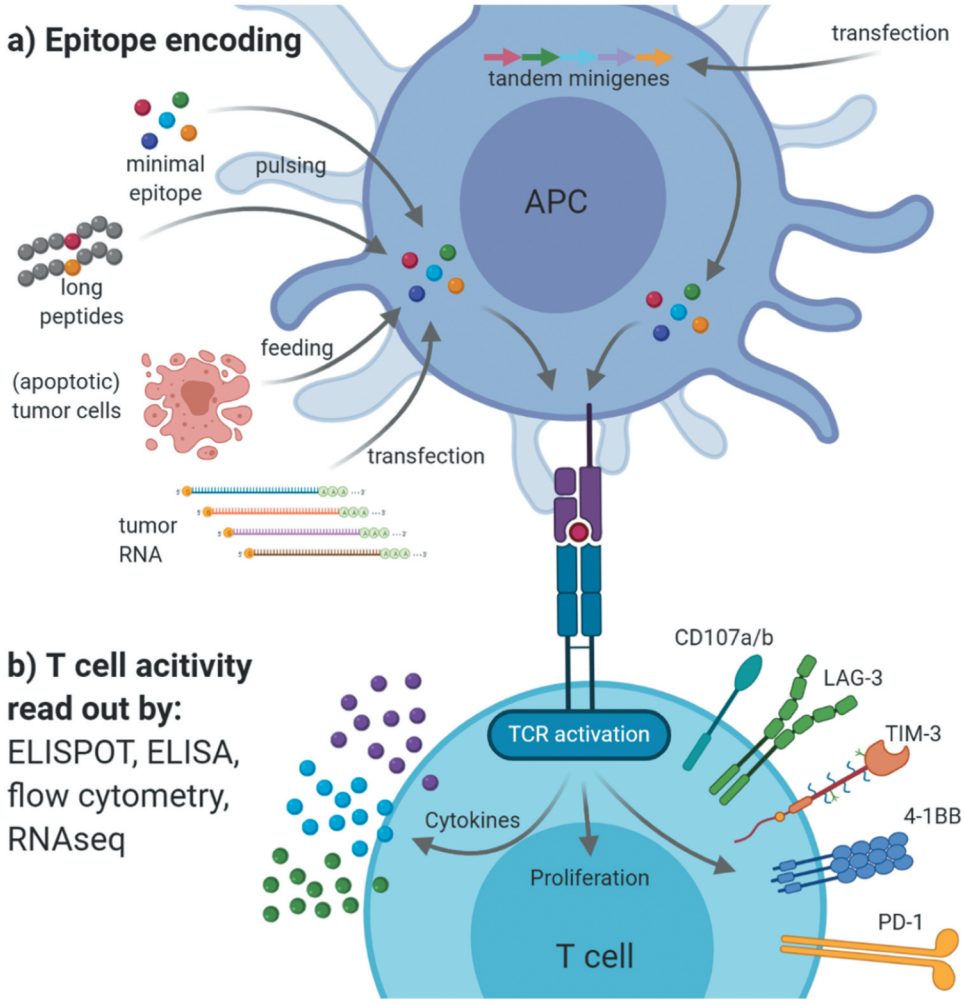

Figure 4. T cell activation upon engagement with antigen presenting cells. a) Synthetic peptides in long and short form as well as tumor material such as cells or amplified RNA can be used as sources for the epitope. Tandem minigenes can also be transfected in APCs. b) A reactive T cell can be identified by secretion of cytokines, proliferation of the T cell or upregulation of markers by various methods such as for example ELISPOT, ELISA, flow cytometry and RNAseq.

originates from biology. Binding of T cells to pHLA multimers does not automatically equal reactivity.

Functional assays to identify neoantigen-specific T cells

Identification of ex vivo acutely stimulated T cells is currently addressed with several technologies. All these assays rely on the availability of cellular targets for T cell activation (Figure 4a). In principle, three types of target cells are available: professional antigen-presenting cells (APCs), tumor cells, and artificial APCs. APCs can be pulsed with synthetic peptides, representing all mutated peptides identified by tumor WES and predicted to bind to the patient-specific HLA molecules as well as peptides eluted from cell surface HLA of tumor cells.[78] Alternatively, APCs can also be pulsed using tumor-specific material, such as apoptotic tumor cells at the cost of not knowing which antigens are recognized by T cells.[70] In multiple myeloma, it was shown that autologous APCs fed with tumor cells were able to generate a pool of T cells that recognized and killed autologous tumor cells.[79] Furthermore, transfection of renal carcinoma derived mRNA into DCs and amplified tumor DNA from prostate cancer were used to create polyclonal cancer-specific cells.[80,81] An important limitation of this approach is the amount of tumor derived material required to run such screens. With the advances of WES/WGS (see above), additional synthetic alternatives were proposed to overcome these limitations.

The development of tandem minigenes (TMG) has circumvented the need to predict or identify the exact minimal epitope presented by HLA.[41,43] In this approach, each mutated minigene encodes for the identified mutation flanked by at least twelve neighboring natural amino acids on each side. Moreover, minigenes can be assembled into a tandem minigene by concatenating them into a single ORF. Recent experimental evidence suggests that the order is of relevance in certain cases.[82] Most commonly RNA encoding for the TMG identified by WES are transfected into autologous APCs, which will express, process, and present peptides on cell surface HLA-I and, potentially, HLA-II. As an alternative, long peptides can also be synthesized and pulsed into APCs.[83]

T cells activated by above-mentioned approaches can be identified using several different assays. Isolation of doublets of T cells and APC through fluorescence-activated cell sorting (FACS) was shown to catch cells in the moment of a long-lasting immune synapse, enabling to identify the exact TCR capable of recognizing a unique antigen.[84] However, rare neoantigen-reactive T cells may be hard to capture routinely using such a method.

Both secreted cytokines and surface protein expression can be exploited to identify reactive T cells (Figure 4b). IFNγ is secreted by activated T cells and is known to promote tumor rejection. IFNγ production can be analyzed using different assays including ELISA, ELISPOT,[85] intracellular cytokine staining followed by FACS analysis.[77] The latter two assays allow measurements of IFNγ levels at the single-cell level. In one study, B cells were pulsed with 31-residue mutated peptides, identified from tumor whole-exome-sequencing and RNA-sequencing, and secretion of IFNγ by CD4 positive T cells was detected by ELISA.[83] CD107a/b has proven a valuable marker to detect degranulation, another T cell effector function acquired following antigen stimulation.[86] 4–1BB is expressed acutely after antigen encounter and was shown to be an effective way to identify T cells specific for Wilms tumor antigen[57] and mutated tumor-associated antigens.[58]

In an alternative approach, tumor-reactive TCRs were identified from activated T cells by single-cell RNA sequencing based on an IL-2 and IFNγ high signature.[87] This study also highlighted the importance of the time point to measure the different activation markers. 4–1BB and PD-1 were both shown to be poor indicators of early stimulation, while IFNγ and to a limited degree IL-2 discriminated reactive from non-reactive lymphocytes more efficiently.

Reactive T cells can also be identified by combining immune assays with large-scale sequencing. In recent developments, scientists from Adaptive Biotechnologies successfully showed that the combination of immune receptor sequencing plus immune assays is able to identify tumor-associated antigen-specific TCRs.[88,89] The applicability of this technology for identifying low abundance neoantigen-specific T cells remains to be seen.

The read-out of many of the functional markers used to identify activated T cells is very time sensitive. Their successful use requires precise understanding of the underlying signaling biology to set the ideal time window for detection of T cell activation.[90] Short-lived and non-accumulating signals further complicate this issue.

A further limitation is the availability of autologous APCs and tumor cells. Synthetic systems with engineered cells may partly overcome this issue. However, they suffer from the need to match all or many of the patient's HLA allelic variants.

Lastly, even some TCRs recognizing peptides in the context of cells (APCs) did not show reactivity to tumor cells presenting the same peptide[91] highlighting the need to ultimately use tumor cells to validate T cell reactivity. However, their availability remains a major limitation in many clinical settings. Further development of patient-derived tumor organoids may help to overcome this limitation.[92,93]

Conclusion and future perspective

ACT targeting neoantigens has come a long way, but there are still many hurdles ahead before its wide-spread use in the clinic. The technological advances of the last two decades have made ACT a priority for both academic and industry researchers. There are now over 100 clinical trials based on ACT in different cancer indications according to "clinicaltrials.gov". Currently, only a few of those trials are targeting neoantigens and most focus on melanoma.

In this review, we omitted describing the potential influence of the selection of the tumor type and biomarkers on the success of ACT. Promising results were initially observed in melanoma patients. However, melanoma is a relatively immunogenic cancer with brisk T cell infiltrate due to its high neoantigen load.[94] Immunogenic antigens are a function of the mutation frequency of the cancer and hence were postulated to serve as a biomarker.[95] Consistent with this, metastatic melanoma patients with a higher tumor mutational burden were more likely to respond to (non-selected) TIL therapy.

However, the influence of the tumor mutational burden on the success of ACT targeting specific neoantigens is unclear.

The number of different TCRs and/or target specificities in the transferred product may also play a role in the success of ACT. Unselected TIL products are regularly a mixed population and early on the limitations of single specificities were recognized.[94] However, the effect on the efficacy has to date not been systematically explored. It would be desirable to combine multiple specificities across all HLA types present on tumor cells to hinder resistance development (see below). Yet, the abundance of ideal neoantigens may be too low in certain tumor types.

We summarized the challenges to derive an autologous adoptive T cell therapy targeting personalized neoantigens. An ideal neoantigen has the following characteristics. 1) Sufficiently different from its natural counterpart to prevent reactivity against healthy tissue and overcome T cell tolerance mechanisms, 2) High expression levels, 3) Adequately processed and presented on HLA to be visible for the adoptively transferred T cells, and 4) Truncal to the tumor to allow targeting of all tumor cells. With more TCRs reactive to neoantigens arising from driver mutations being discovered, their contribution to clinical efficacy will be elucidated in the future. A recent article further suggested that the choice of the specific target antigen, particularly regarding its function and regulation, also influences the clinical outcome and resistance following ACT.[96] Currently, no single technology is capable to assay all these characteristics. This list represents a way to prioritize neoantigen candidates identified by WES/WGS using the full spectrum of available methods. The latest developments for epitope identification in target cells provide methods to screen a huge potential antigenic space, but more work is needed thereafter to identify the reactive T cells or their TCR, particularly when their frequency is relatively low.

Despite all the progress, identifying and isolating neoantigen-reactive T cells remains challenging today. The long experience with multimer staining and the advances in this technology over the last two decades, support their use for ACT. However, the prerequisite to deduce the minimal epitope and the lack of functional readouts makes them insufficient on their own. Functional assays able to detect activated T cells based on cellular readouts have the potential to overcome many of these shortcomings, but at the cost of an increased technical complexity. Furthermore, such assays have their own limitations including availability of autologous cells or the necessity to match HLA of engineered cells to the HLA of patients.

T cell expansion for infusion remains technically challenging and time-consuming (Figure 1e). It adds significant delays before an ACT product is ready to be used and time is critical for many cancer patients. In addition, rapid expansion protocols used to achieve the numbers required for transfer may drive T cell toward functional exhaustion. Of note, T cell exhaustion is a broad term with blurry definition borders.[97] Although this is a sufficiently complex topic on its own, there is a trend toward the development of TCR modified T cells supported by the advances in cell single TCR sequencing[98] and the progress of non-viral gene editing of T cells.[99] Especially, T cells with orthotropic T-cell receptor replacement, which preserve near-physiological function, seem like a promising way forward.[100] Worth mentioning, a recent study revealed a dynamic dysfunctional immune macroenvironment, the immune system beyond the tumor microenvironment, due to the cancer burden.[101] Hence, it is possible that even freshly sourced T cells from the blood of some cancer patients may be dysfunctional. So the immune system in cancer patients is affected in such a way that even freshly sourced T cells from the blood may be unsuitable for ACT. Allogenic adoptive T cell therapy and healthy donor-derived T cells for neoantigens may provide an alternative therapeutic approach for such patients.[102]

Another important point to consider in the production of T cells is the metabolic state of the transferred product. Metabolic fitness and T cell function are closely related and ways to target the T cell metabolism were recently reviewed.[103] For example, transient glucose restriction[104] and using stem-like CD8 cells[105] were shown to improve the efficacy of T cells in ACT approaches.

The immune response to neoantigens can also be mounted by vaccination, preferentially in combination with checkpoint inhibitors and promising early stage data have been reported recently.[106] Tumor vaccines may consist of cells, peptides, or mRNA, and their role in immunotherapy was recently reviewed.[107] Notably, the technologies applied for such neoantigen vaccines[108] have also enabled the development of the first approved SARS-CoV-2 vaccine.[109] For vaccines, neoantigen candidates are often administered without prior confirmation of immunogenicity. While administration of unconfirmed hits supports the shorter development time lines for personalized vaccines, it may enfeeble the efficacy of such vaccines.

The possible therapy escape and resistance mechanism are also not covered in this review. However, it is fair to assume they might be similar to the ones faced in CAR-T[110] and checkpoint inhibitor therapies.[111] Particularly, loss of antigen was shown to occur in relapse patients after CAR-T therapy. It is likely that patients may relapse after ACT due to loss of HLA expression or antigen presentation. The combination of ACT with therapies that counter the loss of antigen presentation may hold great promise. NK cells target cells that exhibit loss of HLA expression and therapies enhancing NK cell function might be good combination partners for ACT-based therapies. Another promising approach is the combination of ACT with approaches that induce epitope spreading and a robust endogenous response, which were shown to prevent the escape via antigen-loss.[112] Epitope spreading was, for example, observed when the APC growth factor Flt3L was introduced in adoptively transferred T cells.[113] Eventually, combinatorial therapies overcoming other resistance mechanisms such as PD-1 blockade[114] may also prove valuable in the ACT setting, and this is currently under investigation.

While we exclusively discussed autologous ACT approaches, adoptive transfers of T cells carry a risk of inducing autoimmune diseases in patients. Hence, it will be important to screen TCRs for off-target and on-target off-tumor specificities, particularly for affinity enhanced variants.[115] While this adds additional complexity and potential delays, safety is a non-disputable feature. Safety aspects of T cells therapies have also been discussed in great detail elsewhere.[116]

With more and more ACT therapies moving forward we will face to a greater extent the question how much to engineer the T cells before the adoptive transfer. A summary of possible

intervention points in signal one, two, and three of T cells to overcome current issues and to improve their utility was recently published.[117] We predict the arrival of a second generation of further engineered T cells building on the learnings from TIL and current ACT products and facilitated by the advances in gene editing. However, if the additional benefit of such designer T cells justifies the driven up cost in this already expensive personalized setting remains to be seen. Adoptive T cell therapy targeting neoantigens has the potential to contribute to the next wave of innovative interventions currently spearheaded by CAR-T therapies.

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Figures were generated with BioRender.com

Conflicts of interest

Florian Kast reports employment with Roche. Christian Klein, Pablo Umaña and Stephan Gasser report employment, stock ownership and patents with Roche. Alena Gros reports grants from Novartis, grants from VCNBiosciences, grants from Merck KGaA, personal fees from Roche "Speaker", personal fees from Achilles Therapeutics "Consultant", personal fees from Genentech "Consultant", and personal fees from Pact Pharma "Consultant" outside the submitted work; in addition, Alena Gros has a patent for E-059-2013/0 licensed and with royalties paid from Intima Bioscience Inc., Intellia Therapeutics, Inc., Tailored Therapeutics, LLC, Cellular Biomedicine Group, Inc., Geneius Biotechnology, Inc., a patent for E-085-2013/0 licensed and with royalties paid from Intima Bioscience Inc., Intellia Therapeutics, Inc., Geneius Biotechnology, Inc., and a patent for E-149-2015/0 licensed and with royalties paid from Intima Bioscience Inc., Intellia Therapeutics, Inc., Tailored Therapeutics, LLC.

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