HHS Public Access

Author manuscript

*Curr Opin Immunol*. Author manuscript; available in PMC 2016 April 01.

Published in final edited form as:

*Curr Opin Immunol*. 2015 April ; 33: 112–119. doi:10.1016/j.coi.2015.02.005.

# Targeting cancer-specific mutations by T cell receptor gene therapy

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## Abstract

The ease of sequencing the cancer genome, identifying all somatic mutations and grafting mutation-specific T cell receptor (TCR) genes into T cells for adoptive transfer allow, for the first time, a truly tumor-specific and effective therapy. Mutation-specific TCR gene therapy might achieve optimal efficacy with least possible toxicity. Recent clinical data confirm the long-standing evidence from experimental cancer models that antigens encoded by the tumor-specific somatic mutations are potentially the best targets for adoptive T cell therapy. Open questions are, how many somatic mutations create suitable epitopes, whether only individual-specific or also recurrent somatic mutations qualify as suitable epitopes and how neoantigen-specific TCRs are most efficiently obtained. Tumor heterogeneity needs to be considered; therefore, it will be important to identify immunogenic driver mutations that occurred early, are essential for cancer cell survival and present in all cancer cells.

## Introduction

In contrast to therapeutic vaccinations, adoptive T cell therapy (ATT) can very effectively eradicate large, long-established tumors in mice [1,2,3**,4] and induce sustained regression in humans [5**,6**]. The grafting of new antigen specificity onto patients' T cells by TCR gene transfer is widely applicable to target any antigen on the cancer cells. Current trials of ATT mainly target tumor-associated antigens, which are self-antigens also expressed on normal cells. Targeting of normal cells, simultaneously with the cancer cells, can be acceptable in selected situations where the normal cells are dispensable, e.g. B cells expressing CD19 [5**]. However, for many, if not most self-antigens, severe toxicity and/or lethality can be expected when ATT has significant cytocidal effects [7*]. Targeting self-

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antigens by ATT shares with many drugs the lack of specificity which led many medical doctors to the bold conclusion that there is “no efficacy without toxicity”. Compared to current immunological and non-immunological therapies, targeting somatically mutated antigens by ATT should dramatically improve the risk-benefit ratio because the targets are truly tumor-specific.

## Somatic mutations in cancer

All cancers bear somatic mutations, because they are the cause of cancer. Mutations are usually random but clustering of mutations can occur. The process of Darwinian evolution selects for mutations that confer a growth advantage, e.g. increase cell proliferation, inhibit apoptosis, impair DNA repair etc. Less than 1% of the mutations occurs in coding regions and can lead to altered proteins. Only somatic mutations can encode truly tumor-specific antigens (also referred to as “neoantigens”) and are considered here. In addition, we will consider tumor virus-associated antigens that are encoded by viral genes and can also represent non-self targets on cancer cells. Most adult solid tumors have acquired around 50 mutations at the time of primary diagnosis [8*]. Pediatric cancers and some leukemias have significantly less mutations (less than 10 on average). Cancers of carcinogen-exposed tissue, ultraviolet (UV)-associated melanoma of the skin and tobacco smoke-associated lung cancer, often carry 100–200 somatic mutations. Some cancers with genomic instability, e.g. colon cancer with microsatellite instability, can carry more than 1000 mutations [8*]. Most mutations leading to an altered protein are non-synonymous point mutations causing a single amino acid substitution [9] (Figure 1). Chromosomal translocations and frameshift mutations resulting from small deletions/inversions are less frequent, and result in fusion peptides. The vast majority of all of these mutations are individually specific. Some occur repeatedly in different cancers (recurrent mutation), and the likelihood that they are involved in malignant transformation is high (so-called cancer-driving mutation). Even when mutations affect the same codon of an oncogene, e.g. RAS $Gly^{12}$, different amino acids may replace the wild type amino acid and therefore lead to different mutant peptides. Cancer-driving mutations are probably present in all cancers, difficult to select against and can be individual-specific [10]. The number of cancer-driving mutations has been suggested to be ~140 [8*], but this may be an underestimate [11].

## Likelihood of creating a mutant epitope

Mutant peptides resulting from somatic tumor-specific mutation may or may not create a target on the cancer cell and/or induce an immune response in the host. Obviously, the probability of creating a targetable and/or immunogenic epitope increases with the number of mutations. Many factors like antigen expression level and processing, peptide trimming and transport, and binding of the peptide to MHC with sufficient affinity determine whether a mutant peptide is a suitable epitope. Because all these criteria have to be fulfilled, the probability for a non-synonymous point mutation or fusion peptide to create a tumor-specific antigen is low. A low probability appears to be true even when estimating the frequency of immunogenic epitopes encoded by vaccinia virus [12]. Of the ~100 vaccinia-encoded peptides that bound with an affinity $IC_{50}$ of 100 nM or lower to HLA-A2, 50% elicited CD8 T cell responses in HLA-A2 transgenic mice. Of these, only 15% were

naturally processed, and of these, 11% elicited CD8 T cell responses upon vaccinia virus infection; this means, only 1/14 of the predicted binders created an epitope. There is no reason to assume that point-mutant cancer peptides that differ less from self than vaccinia peptides would generate more frequently immunogenic epitopes. Based on these theoretical considerations, the number of mutant epitopes binding to a single MHC I allele with a $IC_{50}$ of 100 nM or less and eliciting a CD8 T cell response would be about four to five mutant epitopes for an average human cancer harboring around 50 mutations. This number increases to 20–25 because the outbred patient population has typically six different MHC I alleles. However, more epitopes may serve as a target than induce an immune response because many or most targetable epitopes are recessive, i.e. the host may not respond to such an epitope until the dominant epitope is lost [13,14]. On the other hand, some HLA alleles appear to be able to bind more epitopes than other HLA alleles, which makes calculation difficult [15]. Furthermore, frameshift mutations may increase the number of potential epitopes by causing long novel reading frames [16].

## Evidence for immunogenic mutant epitopes

From methylcholanthrene (MCA)- and UV-induced experimental cancer models, the existence of transplantation rejection antigens is long known [17,18]. These antigens were specific for each individual tumor and truly tumor-specific since they were caused by somatic mutations, first shown for UV-induced [9] and then for human [19,20] and finally MCA-induced cancers [21]. Despite this decade-long knowledge, immunotherapy remained focused on non-mutated self-antigens shared between cancers. However, with the already mentioned exception of CD19, targeting self-antigens (also called tumor-associated antigens) by immunotherapy, e.g. vaccines, has shown little if any success, because the power of self-tolerance mechanisms and the dangers of self-destruction have been underestimated [22*]. By contrast, the advent of fast and affordable cancer genome sequencing, the wealth of somatic mutations resulting from the cancer genome project and reliable algorithms to predict the binding affinity of mutant peptides to MHC I molecules has recently vitalized the interest in the potential immunogenicity of somatic mutations [6**, 23–26,27*,28,29*,30,31–33*,34]. Tumor-specific somatic mutations can only be firmly determined, if germline controls are included in exome sequencing, which is routine for human but not always the case for experimental cancer lines. For example, the Jackson Laboratory C57BL/6J mouse from which B16 arose spontaneously over half a century ago [35] will certainly differ significantly from a present-day C57BL/6J mouse. This may explain why this spontaneous cancer was reported to have almost 1000 “tumor-specific somatic“ mutations [25], which is very unusual for any spontaneous cancer. Thus, much of this “mutanome” was likely caused by genetic polymorphisms [36] or mutations acquired by the B16 cells during decades of *in vitro* culture or *in vivo* passage. Certainly, even syngeneic mice suffer from genetic drift and polymorphisms, and autologous, not just syngeneic DNA, is a *sine-qua-non* for a germline control.

Mutations can be located in the peptide anchor position to generate an immunogenic epitope by increasing the affinity for the MHC molecule [37]. In this case, the wild-type peptide would be presented on the cell surface less effectively. If the altered amino acid is a TCR-contact residue and wild-type and mutant peptide bind with comparable affinity to the MHC

[31*], then T cells responding to the wild-type self-peptide with high affinity will likely be deleted in the thymus. It has been known for decades that certain mutant peptides are shared between different cancers, and can be immunogenic (e.g. mutant RAS, BRAF or breakpoint regions of chromosomal translocations such as BCR-ABL or TEL-AML). Unfortunately, convincing experimental evidence that these conceptually attractive epitopes are efficient targets for rejection is still lacking and clinical data remain inconclusive. At least two reasons may account for the problem of identifying shared mutant epitopes that are efficient targets: peptides encoding these epitopes often have only intermediate or low affinity for MHC molecules; in addition, there often seems to be inefficient processing and transport of these peptides to be presented by MHC molecules on the cell surface [38].

## Intratumoral heterogeneity and the need for targeting ancestral or multiple mutations

The more stably and homogeneously a mutant epitope is expressed in the cancer cells, the more efficiently it is targetable. However, most cancers when clinically detected consist of several genetically diverse subpopulations of cancer cells. This diversification is the result of a clonal evolution from a single common precursor cell hit by the putative “initiating event”. It seems practical to distinguish between two types of mutations, those referred to as “ancestral” or “background” mutations, and those referred to as “foreground” or “polymorphic” mutations [39]. “Ancestral” (background) mutations are shared by all cancer cells in a given tumor. For example, p53 mutations can belong into this category [39,40]. These types of mutations often affect checkpoints of cellular communication and DNA repair [41] and “prime“ the cells to proliferate and acquire additional mutations. “Polymorphic” (foreground) mutations are shared only by subpopulations of cancer cells in a given tumor. Such mutations may be involved in the transition from the least proliferative to the more aggressively growing cells and affect gene expression for example by dysregulating miRNAs [37,39,42]. Which of these mutations should be considered driver and which passenger mutations should be immaterial to the main point: the vast majority of the cancer cells in a tumor should express the targeted antigen. At initial diagnosis, a 1 cm diameter tumor is the result of 30 doublings with only 10 more doublings remaining until death of the patient. Thus, most of the genetic diversification has already occurred when the original cancer is removed. As would then be expected, analysis *in vitro* of cancer cell clones derived from an autochthonous, never transplanted UV-induced cancer revealed that all shared the same ancestral mutant tumor-specific antigen defined by a T cell clone. Yet, different clones of the cancer expressed different tumor-specific antigens [37,40] that were the result of “foreground” (polymorphic) mutations. As would be expected, T cells induced in recipient mice by immunizing with the uncloned original tumor select for cancer subclones that lack the polymorphic antigens. This selection is not an indication of unstable expression of a given mutant antigen but intratumoral antigenic diversity. Targeting of multiple neoepitopes by ATT using different tumor-specific T cells at the same time should reduce the risk of selecting escape variants [14].[0] Together, “ancestral” and if possible multiple mutations should be targeted whether encoded by a passenger or driver mutation. Obviously, it would be ideal if these antigens were essential and could not be lost [10,43].

## Mutant epitopes can be retained in patients despite specific T cell responses

Non-destructive adaptive immune reactions, e.g. tumor-reactive IgG antibodies that imply CD4 T cell help, occur frequently in cancer patients [44]. Whether autochthonous non-virus-associated cancers spontaneously induce a destructive T cell response that can select for antigen-loss variants is still being debated [45,46]; since direct evidence from either mouse or human studies is lacking. It is long known that many UV-induced tumors that arose in immune-suppressed mice are rejected in naïve syngeneic mice and thus have a “regressor” phenotype [18]. However, regressor tumors can also develop in immunocompetent mice [47] with tumor-reactive CD8 T cells that can be induced to prevent tumor development [48]. Regressor tumors can even occur in mice with systemic tumor immunity in which SV40 large T antigen was induced by viral infection as oncogene/tumor antigen in the liver through Cre recombinase technology [49*]. The mice rejected subcutaneously injected SV40 large T-expressing cancer cells (concomitant immunity), while the autochthonous tumor progressed without losing its immunogenicity and antigenicity. Obviously, the process of cancer cell inoculation creates an artifact not reflecting autochthonous tumor growth. Thus, the T cells response to autochthonous tumors is fundamentally different from that of mice inoculated with cancer cells. The same case can be made for the Darwinian selection of cancer cells lacking the rejection antigen by a regressor tumor transplanted into normal immunocompetent mice. Such a selection occurs in many different transplant settings [2,26,37,50]. Similar to the above-mentioned sporadic cancer model, T cells did not impair tumor development in a transposon-based autochthonous cancer model [51]. Recent clinical case studies support the pre-clinical data that endogenous concomitant T cell immunity fails to select for cancer variants lacking the mutant epitope. In three individuals with ovarian cancer, the cancer genome was sequenced and 79 mutations were identified. Only a single one elicited a measurable CD8 T cell response at the time of first relapse (but not at the time of primary tumor diagnosis), but the response had disappeared at the time of second relapse even though the cancer cells had retained expression of the neoantigen [32*]. The data support the above consideration that measurable spontaneous T cell responses in the autochthonous cancer-bearing host are inefficient in selecting variants that lost immunogenic antigens. Similarly, tumor-infiltrating lymphocyte (TIL) therapy requires surgery which is performed with progressing, not regressing tumors. In some long-term responders following TIL therapy, the *in vitro*-expanded T cells contained a dominant population of mutation-specific T cells. Apparently, the TIL exerted their function only upon re-infusion into lymphopenic hosts, but did not spontaneously select against expression of the mutant antigen before surgery [6**]. In another melanoma patient experiencing a regression following anti-CTLA4 antibody treatment, mutation-specific CD8 T cells expanded upon therapy. Assuming that this T cell population had caused the tumor regression, the mutant antigen was not spontaneously lost before therapy [29*]. Loss of MHC I expression has been reported, rendering tumors resistant to ATT. However, in most cases MHC I expression can be upregulated by IFN-$\gamma$ [52]. It is not clear whether rare tumors that have irreversibly lost MHC I expression are the result of escape from T cell pressure during tumor development. Irreversible loss of MHC I expression is often due to loss of the $\beta_2$-microglobulin gene. This gene is located in proximity to a tumor suppressor

gene repeatedly deleted in cancer which may explain why MHC I-negative cancer cells have a proliferative advantage [52]. Together, there is good indication that at least before therapeutic intervention, cancers retain expression of immunogenic antigens. Partial responses after immunotherapy seem to be more associated with antigen-retention, whereas relapse after complete remissions are more associated with antigen loss as observed experimentally [53**] as well as clinically [33].

## High affinity of mutant peptides to MHC I required for cancer eradication

The retrospective analysis of melanoma patients responding to immunotherapy supports the data from experimental cancer models [2,53**]. A patient with a partial regression following anti-CTLA4 monoclonal antibody (mAb) treatment revealed a dominant CD8 T cell response against a mutant epitope [29*]. Similarly, the *in vitro*-expanded TILs of three patients that were successfully treated by the TILs contained mutation-specific CD8 T cells [6**,30]. In all cases, the mutant peptides bound with high affinity (low nM) to the respective MHC I molecules. Affinities in the 0.5 nM to 5 nM range have been shown to be critical to prevent tumor recurrence in experimental cancer models when targeting a single peptide [53**]. However, targeting a peptide that had a measured pMHCI $IC_{50}$ of 186 nM also showed efficacy and caused almost complete destruction of large long-established cancers even though the cancer eventually relapsed [53**]. We propose that a combination of TCRs targeting three or more mutant peptides with <50 nM pMHC affinity (or even <200 nM) may be needed but may also be sufficient to eradicate established cancers. These epitopes should be independent of each other, i.e. encoded by different chromosomes so they cannot easily be lost simultaneously. Such an approach would be analogous to that used for combination chemotherapy of childhood leukemia. By targeting the cancers with multiple independent drugs, relapse could be prevented and cancers eradicated [54].

## Source of T cell receptor

Accepting that neoepitopes are the best targets and in most cases retained on the cancer cells, the question is how to obtain TCRs for gene therapy. In principle, they can be isolated from humans or HLA-transgenic mice. In humans, TCRs can be isolated from TILs or PBLs of cancer patients (Figure 2). It may also be possible to isolate TCRs from healthy HLA-matched individuals by *in vitro* priming or after immunization with the mutant peptide, although these will not have been selected by the patient's thymus for lack of self-reactivity. Based on prioritized neoepitopes with predicted high affinity for a given MHC allele, pMHC tetramers may facilitate enrichment of specific T cells. Single cell PCR [55] appears to be a reliable method to find the correct TCR$\alpha\beta$ combination but alternative strategies have also been employed [56]. The advantage of obtaining the TCRs from the autologous host is that these TCRs have been screened for not binding self-antigens with a high affinity. However, an unresolved question is what determines the fate of neoepitope-specific T cells. In mouse models with surrogate antigens, T cell precursor frequency, TCR signaling strength, and the inflammatory milieu at the time of antigen encounter decided the T cell fate [57,58]. For example, depending on the amount of peripherally expressed antigen, the T cells were either deleted or became anergic [59]. It is not known under which immunological conditions T cells encounter mutant antigens in an evolving cancer. TCRs can be rescued from anergic T

cells if they persist in the individual with progressing tumor. TCRs could also be isolated from patients responding to checkpoint inhibitor (anti-PD-1 and/or anti-CTLA4 mAbs) therapy [60–63*]. Response rates are relatively high in melanoma and lung cancer patients, who have a high mutation load. The T cell repertoire appears to broaden following anti-CTLA4 mAb but not anti-PD-1 mAb therapy, compatible with the supposed mode of action of facilitating T cell priming versus unleashing existing T cell responses, respectively [64–66]. It will be important to elucidate how frequently neoepitope-specific T cells are induced/expanded following therapy against the background of self-reactivity (and associated autoimmunity). Many patients do not respond to checkpoint inhibitors, and we do not know whether the cancers of theses patients lack suitable neoepitopes or whether the patient fails to mount a T cell response. Consistent with the clinical experience, anti-PD-1 mAb treatment as monotherapy delayed tumor growth but could not achieve complete tumor rejection in models of large established tumors or autochthonous cancers with systemic immunity but local tolerance [49*,67*]. We also do not know whether vaccination with mutated peptides with or without checkpoint inhibitors [68–70] induce mutant-specific TCRs in individuals with advanced autochthonous cancer.

Neoepitope-specific TCRs can be isolated from HLA-transgenic mice, preferably those additionally expressing a human TCR repertoire because of issues related to immunogenicity of non-human TCRs [71]. These mice would be immunized with the mutant peptides. An advantage is that TCRs would come from a tumor-free host, thereby excluding the possibility of neoepitope-specific T cell deletion which could occur in tumor-bearing individuals. An uncertainty is whether some neoepitope-specific TCRs obtained in the mouse may cross-react with normal human proteins, because the TCRs generated by the mouse have not been negatively selected in a human thymus. There is also the bottleneck that transgenic mice are not available for all HLA alleles. Thus, it needs to be seen whether potent neoantigen-specific TCRs are easier obtained from humans or mice.

## How should TCRs be tested for efficacy *in vivo*?

Mutant epitopes as well as TCRs directed against them certainly differ in their power to support tumor rejection, therefore we consider pre-clinical testing as important to minimize the risk of clinical failure. *In vitro* assays do not necessarily predict efficacy *in vivo* [72]. Xenograft models with human cancer and T cells are afflicted with confounding factors (host species-specific factors necessary for T cell function), rendering human T cells notoriously ineffective in immune-deficient mice, as indicated by the absence of an acute lethal graft-versus-host disease. Furthermore, autologous T cells from the cancer patient are rarely available. Therefore, we suggest to establish syngeneic HLA-transgenic mouse cancer models for testing suitability of mutant epitopes and respective TCRs. The (mini)gene encoding the mutant epitope can be expressed in the murine HLA-expressing mouse cancer cells and T cells derived from the HLA-transgenic mice can serve as recipient for the TCR. In the clinic, TCR gene therapy is performed in individuals with established tumors, detectable at a minimal size of ~1 cm in diameter. Therefore, experimental cancers at the time of T cell transfer should be “established”. The term “established” should be clearly defined, because it is frequently used for a cancer cell inoculum few days after injection (e.g. [73–75]). In our opinion, an “established” tumor should have reached a clinically

relevant diameter of 1 cm and lack the acute inflammatory reaction which is usually the case ~14 days after the inoculation [76,77]. Such tumors are much more difficult to treat than early inflammatory lesions in mice, but come closer to the clinical situation. Mutant peptide vaccines with or without checkpoint inhibitors have been tested against early lesions in mice [68–70]. It will be interesting to analyze whether such vaccinations can be used for isolating mutant peptide-specific TCRs [70], once the tumor is "established", and whether vaccinations are therapeutically effective at that stage.

## Conclusion

Grafting mutation-specific TCR genes into T cells for adoptive therapy will be possible and holds the promise of a truly tumor-specific and effective therapy. For shared (cancer-driving) mutations, TCRs would exist "off-the-shelf", if such mutations create efficient targets for ATT. For targeting mutant peptides encoded by individual-specific somatic mutations, technological advances could significantly shorten the time interval currently needed to identify TCRs suitable for ATT.

## Acknowledgement

Work described here was supported by a Collaborative Research Grant of the Berlin Institute of Health, the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich TR36, the Deutsche Krebshilfe, the Einstein-Stiftung Berlin, the Wilhelm Sander-Stiftung, NIH grants P01-CA97296, R01-CA22677, R01-CA37156 and the Cancer Center at the University of Chicago.

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## Highlights

- T cell therapy can eradicate large tumors
- Cancer-specific mutations creating neoepitopes are potentially the best target
- Mutant epitopes can be retained in patients despite specific T cell responses
- TCR gene therapy of cancer is feasible

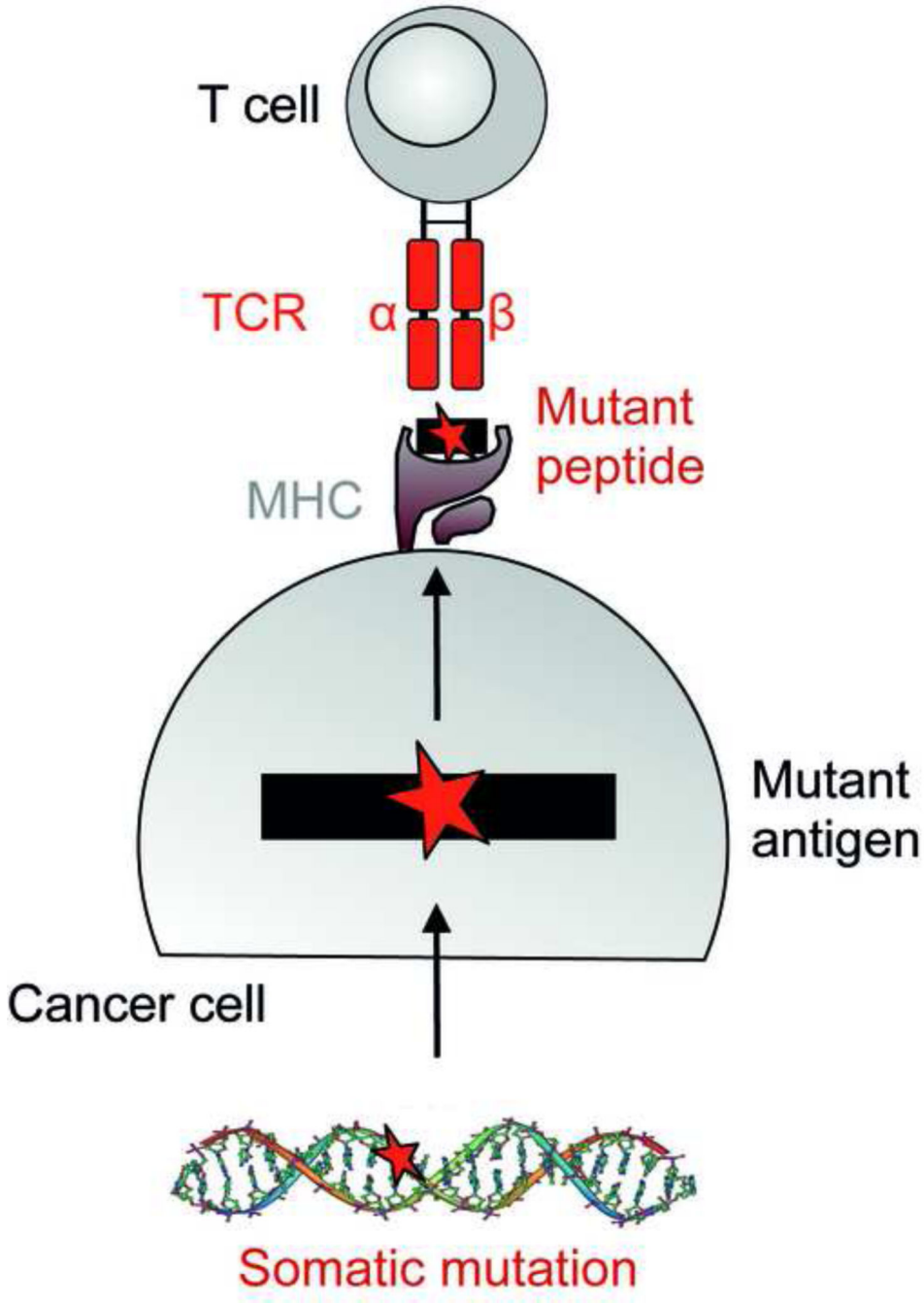


**Figure 1. Mutation-specific T cells**

Somatic mutations in cancer may create a neoepitope that can be recognized by T cells.

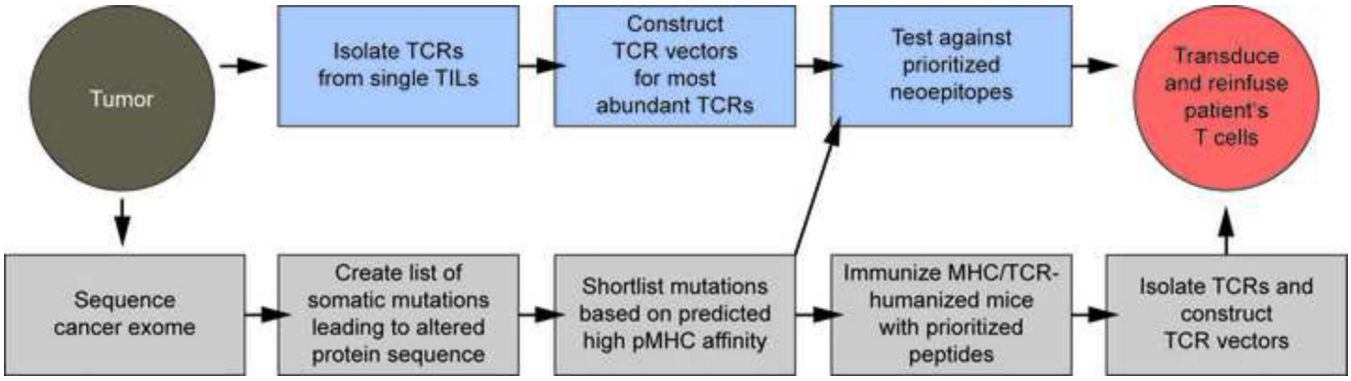


**Figure 2. Strategies to isolate mutation-specific TCRs**

TCRs can either be isolated from T cells of cancer-bearing humans (top) or cancer-free HLA-transgenic mice (bottom). The advantage of obtaining TILs from humans is that their TCRs are truly specific for the neoepitope. In contrast, TCRs from HLA-transgenic mice are derived from a tumor-free environment. Note that epitope/TCR analysis in an experimental cancer model is not included but in our opinion is important to exclude therapeutically inefficient epitopes and/or TCRs.