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Targeting neoantigens for cancer immunotherapy

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REVIEW

Abstract

Studies first carried out in the 1980s have demonstrated murine T cells can recognize mutated gene products, known as neoantigens, and that these T cells are capable of mediating tumor rejection. The first human tumor antigens isolated in the early 1990s were the products of non-mutated genes expressed in a tissue-specific manner; subsequent studies have indicated that tumor-infiltrating lymphocytes that are cultured *in vitro* frequently recognize mutated gene products. In addition, correlative studies indicate that clinical responses to therapies involving the use of antibodies directed against checkpoint inhibitors such as CTLA-4 and PD-1 may be associated with mutational burden, providing indirect evidence that these responses may primarily be mediated by neoantigen-reactive T cells. The importance of neoantigen-reactive T cells may be elucidated by the results of ongoing and future studies aimed at leveraging information gained from mutational profiling to enhance the potency of immunotherapies.

Keywords: cancer immunotherapy, mutation, neoantigen, tumor immunology

Introduction

Studies carried out over the last 25 years have provided important insights into the nature of antigens recognized by human tumor-reactive T cells. These antigens can be grouped into five general categories based upon their patterns of expression in both normal and tumor tissues of origin, as this provides a framework for evaluating therapeutic targets.

The discussion below first centers on the characteristics of such antigens identified as targets of tumor-reactive T cells, and the implications of these findings for clinical immunotherapy trials. A discussion of the results of clinical trials that deliberately targeted individual antigens is followed by a discussion of correlative studies analyzing the antigens that appear to represent the predominant targets of clinically effective bulk populations of adoptively transferred T cells. The discussion then centers on analysis of the results of studies in patients receiving antibodies directed against checkpoint inhibitors that, while still preliminary, have begun to provide clues as to the nature of antigens associated with tumor regression. The final section of the review contains a discussion of the implications of these findings for the development of future therapies.

Tumor antigens: broad categories defined by expression in tumors and normal tissues

Initial studies used to identify tumor antigens were primarily carried out by screening expression libraries using *in vitro* culture with tumor-infiltrating lymphocytes (TILs) or by *in vitro* sensitization of PBMCs against autologous tumor cells or autologous normal cells that were either pulsed with candidate T cell epitopes or transfected with genetic constructs encoding candidate antigens. The antigens identified using these approaches can be grouped into five general categories: antigens derived from gene products that are widely expressed in normal tissues at relatively low levels in comparison with malignant cells; differentiation antigens expressed at relatively high levels in a single tissue; antigens that are limited in their expression in adults to germ cells that lack MHC expression [cancer germline (CG) antigens]; viral antigens; and mutated antigens (Table 1). Although expression of the first two categories of antigens in normal cells may trigger central and peripheral tolerance mechanisms that lead to the selection of low avidity T cells, treatment of patients with a high avidity TCR that recognized the melanocyte differentiation antigen MART-1 resulted in severe skin, eye and ear toxicity, and resulted in durable responses in only a small percentage of patients (1).

CG antigens represent gene products whose expression in the adult is generally limited to germ cells that lack expression of MHC molecules and thus are not subject to attack by HLA class I-restricted or class II-restricted T cells, potentially allowing treatment with high avidity T cells while resulting in little or no on-target recognition of normal tissues. In clinical trials involving adoptive transfer of T cells targeting the CG antigen NY-ESO-1,

Table 1. Categories of tumor antigens

Category	Normal tissue expression	Expression levels in normal tissues	Examples	Advantages of targeting antigens	Disadvantages of targeting antigens
Over-expressed gene products	Broadly distributed	Low	PRAME	Expressed in a wide variety of cancer types	Potential for autoimmunity due to broad normal-tissue expression
Tissue-specific differentiation antigens	Narrow: single tissues	Generally high	MART-1, CEA	Many products expressed at high levels in one or limited number of tissues	Autoimmunity can limit the ability to develop potent therapies
Cancer germline antigens	Germ cells	Generally high	MAGE-A family, NY-ESO-1	Generally not expressed in normal adult tissues with the exception of germ cells	Some family members are expressed in adult normal tissues, leading to autoimmunity
Viral antigens	None	None	HPV E6, HPV E7	Not expressed in any normal tissues	The number of therapeutic targets may be limited by mechanisms of viral immune evasion
Mutated antigens	None	None	$KRAS^{G12D}$, $KRAS^{G12V}$	Not expressed in any normal tissues. Some hotspot mutations are present at relatively high frequencies in particular tumor types. Targeting hotspot mutations may help to obviate antigen loss	Many are limited to one, or a small number of, tumors

objective responses were observed in 61% of patients with synovial cell sarcoma, 55% of patients with melanoma (2) and 80% of patients with myeloma (3). High level expression of NY-ESO-1 has only been observed in a relatively small percentage of tumors, with the exception of synovial cell sarcoma, limiting treatments targeting this antigen to relatively small numbers of patients (4). Members of the MAGE-A CG gene family have been targeted in multiple trials; however, severe toxicities including four deaths that were attributed to the transferred T cells were seen in two trials targeting MAGE-A3 epitopes that were attributed to expression of the related MAGEA12 gene in rare cells present in normal brain tissue (5) or cross-reactivity of an affinity-enhanced TCR with a protein expressed in cardiomyocytes (6).

These observations led to studies aimed at targeting gene products that are not present in the normal human genome. Vaccination of patients against viral antigens such as the human papillomavirus (HPV) E6 and E7 oncogenes, gene products that are highly expressed in a limited number of tumor types such as cervical and head and neck cancers, appears to be effective at preventing disease progression in patients with premalignant disease (7) but not in patients with invasive cervical cancer (8). In a recent trial, durable complete responses were seen in two of nine metastatic cervical cancer patients receiving autologous TIL cultures that were selected on the basis of several characteristics that included responsiveness to libraries of overlapping peptides that encompassed the E6 and E7 proteins (9). The degree of reactivity against the E6 and E7 peptides appeared to be associated with clinical response to therapy, a potentially significant finding, particularly if it were to be replicated in a larger patient population.

Mutated genes encode targets that obviate issues arising from expression in normal tissue and that can potentially be recognized in all cancers, with the possible exception of some hematological malignancies that possess fewer than 10 non-synonymous somatic mutations (10). Recent studies indicating that that recognition of mutated tumor antigens (neoantigens) is associated with clinical responses in a variety of therapeutic settings have provided an impetus to develop novel therapies based upon analysis of the mutational landscape of tumors. The promises and difficulties with targeting neoantigens are discussed in the remaining sections of this review.

Correlative studies of neoantigen recognition and immunotherapy responses

Antigen cloning studies carried out over the past 25 years have led to the demonstration that T cells from patients with a variety of cancer types recognize mutated gene products (11); however, the conventional expression cloning methods used in these studies were time consuming and labor intensive, limiting the applicability of this approach for identifying targets for therapy. The advent of relatively inexpensive high throughput sequencing methods in the last few years, including whole genome sequencing and whole exome sequencing (WES) analysis of tumor and matched normal DNA and RNA-seq whole transcriptome (RNA-seq) analysis, have provided an opportunity to utilize a variety of reverse immunology approaches (i.e. predicting and identifying epitopes from nucleotide sequences) to identify neoepitopes recognized by tumor-reactive T cells.

In a recent study, WES of three metastatic melanomas, combined with expression analysis and use of the MHC class I-restricted binding peptide algorithm NetMHCpan, led to the identification of a total of seven neoepitopes recognized by three populations of human melanoma TILs (12).

Two neoepitopes were also identified as T cell targets in a patient with metastatic melanoma using an approach where WES and RNA-seq analysis were used in combination with the NetMHC class I peptide-binding algorithm to generate a library of MHC–peptide tetramers generated by an ultraviolet light-induced peptide exchange approach (13) that were screened for binding to the patient TIL sample (14).

Use of a screening approach based upon transient transfection of COS-7 or HEK293 cell lines with tandem minigene constructs (TMGs) consisting of 12 minigenes encoding mutated residues plus the 12 flanking normal amino acids plus constructs encoding autologous HLA gene products led to the identification of neoepitopes recognized by two polyclonal populations of melanoma TILs (15). Combining WES with the identification of peptides eluted from cell surface MHC molecules by mass spectrometry represents an example of another approach that has been used to identify neoepitopes recognized by T cells (16).

One question raised by these finding was whether or not this or a similar approach could be used to identify neoepitopes recognized by T cells in additional cancer types. Using a TMG screening method, combined with the pulsing of autologous antigen-presenting cells with relatively long peptides of 25 amino acids, one to three neoepitope targets were identified for 9 of the 10 gastrointestinal TILs that were evaluated in a recent report (17). All of the T cells evaluated in this study recognized neoepitopes that were unique to an individual tumor, with the exception of T cells from two patients that recognized an identical epitope containing a substitution of aspartic acid for glycine at position 12 of the KRAS oncogene ($KRAS^{G12D}$) in the context of HLA-C*08:02. Substitution of aspartic acid, valine and cysteine at this position in the KRAS protein, termed driver mutations, have been shown to stimulate cell growth through constitutive activation of the RAS/MAPK signaling pathway, and are commonly found in multiple tumor types including pancreatic, colon and lung cancers. These results provide support for the application of these methods to the identification of neoepitopes recognized in additional cancer types.

The ability to readily identify neoepitopes recognized by patient T cells has also provided the opportunity to evaluate the hypothesis that recognition of mutated antigens may play an important role in patient responses to cancer immunotherapies. In support of this hypothesis, durable tumor regressions have been observed in melanoma patients who received autologous TILs that appeared to predominantly recognize neoepitopes expressed by the patients' tumors (12, 18). Treatment of a patient with metastatic cholangiocarcinoma with an autologous TIL culture, over 95% of which consisted of $CD4^+$ T cells that recognized a single HLA class II-restricted neoepitope derived from the ERBB2IP protein, led to dramatic tumor regression (19).

In addition, adoptive transfer of a melanoma TIL population, 50% of which recognized a mutated HLA class I restricted PPP1R3B epitope, but that did not appear to recognize shared non-mutated antigens, was associated with a complete regression of all metastatic lesions that is ongoing beyond 10 years (18).

In another study, neoantigen-reactive $CD4^+$ T cells were identified within populations of tumor-reactive T cells from four of five metastatic melanoma patients that were screened for their ability to recognize panels of 31 amino acid synthetic peptides encompassing individual mutations (20). Approximately 4% of the $CD4^+$ T cells present within autologous TILs that were administered to one of the patients in this study, who was a partial responder to autologous TIL therapy, recognized a single neoepitope. In addition, a total of 13% of the $CD4^+$ T cells that were administered to a patient who exhibited a complete response to autologous T cells generated by *in vitro* stimulation with autologous tumor cells recognized three neoepitopes.

Studies in murine tumor model systems have also provided evidence that neoepitopes can serve as potent tumor rejection antigens. A study carried out using tumors derived from immunodeficient Rag2-knockout mice indicated that a mutated spectrin-β2 neoepitope represented the dominant tumor rejection antigen for the murine methylcholanthrene-induced sarcoma d42m1 (21). In another study, 11 of 50 mutated 27-mer peptides identified by WES of the B16F10 murine melanoma were found to induce immune responses preferentially recognizing the mutated epitopes (22). Immunization of tumor-bearing mice with an immunodominant peptide identified using this approach significantly slowed tumor growth and enhanced survival. Additional murine studies carried out by vaccination with mutated peptides (16) or synthetic RNA constructs encoding tandem arrays of epitopes, which appeared to predominantly induce MHC class II restricted responses in immunized mice, conferred disease control and survival benefit (23).

In a human vaccine study carried out in three patients with metastatic melanoma, vaccination with autologous dendritic cells that were pulsed with neoepitope peptides identified using WES and RNA-seq in combination with peptide–MHC binding algorithms led to *in vivo* expansion of peptide-reactive, and putative tumor-reactive, T cells (24). Although immunization did not appear to have a clinical impact on disease progression in these patients, these results demonstrated the feasibility of using this approach to identify neoepitope targets that could potentially be used for combination therapies that involve use of vaccines to boost responses to immune checkpoint inhibitors or adoptive immunotherapy.

Analysis of data from clinical trials involving treatments with antibodies directed against inhibitory molecules such as CTLA-4 and PD-1, termed checkpoint blockade therapies, has provided further evidence that neoepitope reactivity may play an important role in mediating responses to immunotherapy. Clinical benefit in metastatic melanoma patients treated with ipilimumab or tremelimumab, antibodies directed against the inhibitory ligand CTLA-4, was associated with mutational load and the presence of a tetrapeptide signature present on predicted neoepitopes in patients with long-term benefit but not present on predicted neoepitopes in patients with minimal or no clinical benefit (25); however, the lack of an independent validation set used to identify the tetrapeptide signature in this study has called into question the validity of this result (26, 27).

Results of a study involving treatment of metastatic melanoma patients with ipilimumab provided further evidence for an association between clinical benefit and overall mutational load or the overall load of predicted HLA class I-restricted neoepitopes, which, in contrast to results presented in the previous report, did not appear to contain a tetrapeptide signature (28). The results of a study evaluating the response of

melanoma patients to treatment with either pembrolizumab or nivolumab, two antibodies directed against the immune checkpoint inhibitor PD-1, indicated that neither total mutational load nor predicted HLA class I or class II neoepitope load was associated with response to therapy (29). Notably, however, patients in this study whose tumors were within the top third of the distribution of total non-synonymous somatic mutations survived longer than those whose tumors were within the bottom third of the distribution, indicating that multiple factors may influence response to anti-PD-1 therapy.

Mutational load was, however, associated with response to immune checkpoint blockade in a clinical trial evaluating responses to pembrolizumab in patients whose tumors were either mismatch repair-deficient or -proficient (30). In this trial, patients with mismatch repair-deficient carcinomas derived from either colon or additional tumor types that possessed relatively high mutational burdens exhibited objective clinical response rates of 40% and 71%, respectively, whereas none of the 18 patients bearing mismatch repair-proficient colorectal cancers responded to therapy.

Additional studies have also provided ana indication that mutational burden may be related to response to immune checkpoint blockade. Neoantigen burden was positively correlated with the clinical benefit and progression-free survival in patients with non-small cell lung cancer (NSCLC) receiving the anti-PD-1 antibody pembrolizumab (31). In a recent study examined the impact of intratumor heterogeneity and predicted neoantigen burden on response to immune checkpoint blockade in patients with metastatic melanoma or NSCLC (32), a high neoantigen burden was associated with longer overall survival in a predominantly early-stage cohort of patients with lung adenocarcinoma but not in early-stage patients with squamous cell carcinoma. A high predicted neoantigen burden was associated with an inflamed tumor signature, as defined by expression of CD8A, CD8B, and genes associated with antigen presentation and effector cell function, whereas increased intratumoral heterogeneity, as determined by evaluating the clonality of putative neoepitopes expressed by patient tumors, appeared to have a relatively small but significant negative influence on overall survival. Intratumoral heterogeneity also appeared to have a negative impact on progression free survival in patients with advanced NSCLC treated with pembrolizumab and on overall survival in metastatic melanoma patients treated with ipilimumab or tremelimumab.

Taken together, these results provide support for the hypothesis that tumor regression in response to adoptive immunotherapy and to immune checkpoint inhibitors may primarily be mediated by T cells that recognize neoepitopes expressed by patients' tumors (reviewed in (33)). Renal cell carcinomas respond to immune checkpoint blockade but possess relatively low mutation loads (34), however, indicating that additional factors, such as the expression levels of PD-1 ligand on tumors or normal tumor-infiltrating cells (reviewed in (35)), are likely to play important roles in modulating clinical responses to immune checkpoint blockade.

Summary and future perspectives

Overall, the results of adoptive immunotherapy and checkpoint blockade studies suggest that neoantigen-reactive T cells may play important role in mediating clinical responses to these therapies, as approximately one quarter of melanoma patients receiving adoptive immunotherapy have exhibited long-term durable complete responses (36) and long-term follow up has indicated that approximately one third of melanoma patients receiving nivolumab were alive after 5 years (37). Treatments combining anti-PD-1 and anti-CTLA-4 immune checkpoint inhibitors aimed at increasing response rates appeared in initial studies to result in higher response rates than treatment with either agent alone (38), but led to higher levels of severe autoimmunity than were seen in patients treated with either inhibitor alone. In addition, long-term follow-up of patients receiving combinations of immune checkpoint inhibitors have not been reported, but adjustments of the dosage and timing of infusions of the individual inhibitors are being evaluated to determine whether or not schedules that limit normal tissue toxicities while maximizing tumor regression can be identified.

Further progress in developing novel approaches to the treatment of patients for whom current immunotherapies are of limited or no clinical benefit may depend upon gaining a better understanding of the biological basis of these responses. At the present time it is not possible to determine the percentage of expressed mutated gene products that are capable of giving rise to naturally processed and presented neoepitopes; however, results of screening candidate epitopes identified using MHC class I peptide-binding algorithms indicate that 10% or less of the mutations in a given individual are likely to give rise to an immunogenic epitope. If true, it would be unlikely that T cells reactive with more than one or two neoepitopes will be present in patients whose tumors contain fewer than 100 non-synonymous somatic mutations, many of which will not be expressed or will be expressed at levels that are unlikely to elicit immune responses.

Tumor immunoediting may lead to the elimination of tumors that express particularly potent neoepitopes (39); however, the effectiveness of adoptive immunotherapy and immune checkpoint inhibitors at mediating regression of multiple cancer types, combined with the observation that many of the immunogenic neoepitopes identified to date appear to be recognized by high avidity T cells, indicates that this may not play a major role in shaping the neoepitope landscape in most tumors. Variations in the levels of inhibitory factors in the tumor microenvironment that may be influenced by intrinsic features of individual tumors may play a more important role in limiting responses to these therapies than the numbers or potency of tumor-reactive T cells present within some, and perhaps most, tumors.

Intratumoral and intertumoral heterogeneity of neoantigen expression of neoantigens targeted by immunotherapy may also significantly impact on the effectiveness of treatments targeting neoantigens. The ability of adoptive immunotherapy to mediate durable tumor regressions in some patients may depend in part upon the fact that many TIL populations contain T cells that recognize multiple neoantigens. Targeting single neoantigens derived from trunk mutations (40) or driver mutations that are essential for carcinogenesis or metastasis may represent the most effective strategy, although defining driver mutations for some tumors remains a challenge.

Common hotspot driver mutations, such as the $KRAS^{G12D}$ mutation that is expressed by approximately 45% of pancreatic adenocarcinoma (41) and 13% of colorectal

adenocarcinomas (42) and for which HLA-C*08:02-restricted T cells have been identified represent attractive targets for the development of TCR-based therapies. The HLA-C*08:02 allele is expressed by nearly 10% of patients bearing tumors commonly containing $KRAS^{G12D}$ mutations, which would allow treatment of a relatively small percentage of patients with most common malignancies; however, recent studies have led to the identification of TCRs that mediate recognition of $KRAS^{G12V}$ and $KRAS^{G12D}$ epitopes in the context of HLA-A*11:01 (43), an HLA class I allele expressed by approximately 14% of Caucasians. Further studies may lead to the identification of T cells that recognize additional neoepitopes encompassing hotspot driver mutations and HLA restriction elements capable of presenting hotspot mutations in genes such as TP53, IDH1 and PIK3CA.

Finally, it is likely that therapies involving some combination of immune checkpoint inhibitors, potent anti-tumor vaccines and adoptive immunotherapies will lead to enhanced clinical responses, although overlapping mechanisms of action may limit the degree of improvement in response rates observed with some combination therapies. Nevertheless, a continued focus on identifying potent neoantigen targets will hopefully lead to development of strategies that enhance responses to immunotherapy in patients with many cancer types, including epithelial cancers that are the leading cause of cancer-related deaths.

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