Masters of Immunology

Neopeitopes of Cancers: Looking Back, Looking Ahead

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Abstract

The search for specificity in cancers has been a holy grail in cancer immunology. Cancer geneticists have long known that cancers harbor transforming and other mutations. Immunologists have long known that inbred mice can be immunized against syngeneic cancers, indicating the existence of cancer-specific antigens. With the technological advances in high-throughput DNA sequencing and bioinformatics, the genetic and immunologic lines of inquiry are now converging to provide definitive evidence that human cancers are vastly different from normal tissues at the genetic level, and that some of these differences are recognized by the immune system. The very vastness of genetic changes in cancers now raises different question. Which of the many cancer-specific genetic (genomic) changes are actually recognized by the immune system, and why? New observations are now beginning to probe these vital issues with unprecedented resolution and are informing a new generation of studies in human cancer immunotherapy.

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Learning Objectives

Cancer cells are different from normal cells in ways that are exploitable by our immune systems. Understanding what specifically makes cancer cells unique informs the development of therapies that can more effectively manipulate our immune responses to tumors. This article focuses on the concept of neoepitopes, how they are identified, and ways in which this knowledge can be applied in the creation of cancer vaccines and therapies. Upon completion of this activity, the participant should gain insight into the generation of neoepitopes and the complexity of affinity in determining tumor immunogenicity and should understand the approaches necessary for better predictions of the neoepitopes that will elicit immune responses powerful enough to induce tumor rejection.

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Introduction

Understanding of the generation and recognition of specificity has delighted immunologists considerably over the past two centuries. Knowledge of specificity has also brought us arguably the greatest tool to enhance human (and animal) health in the form of vaccines against an array of infections. Immunologists have also therefore been keen to utilize the principle of specificity in treating cancer. For cancers of infectious (viral) etiology, the problem of specificity is straightforward and has led to spectacular results, as in the case of prophylactic vaccines against genital herpes. However, for cancers of apparently noninfectious etiology (i.e., the majority of human cancers), the issue of specificity becomes quite complicated quite quickly. What, if anything, is specific in these cancers?

This simple question has been surprisingly difficult to answer, perhaps until now. The first hints that specificity may exist came from the early experiments of Ludwik Gross (1), Prehn and Main (2), and most persuasively, George and Eva Klein (3). Using inbred syngeneic mice, and the tumors that can be induced in
them by methylcholanthrene, these early reports noted that mice could be immunized against syngeneic tumors and rendered resistant to them by such immunization. Remarkably, each individual tumor elicited immunity only against itself, and not against any other tumor of the same type, induced in the same fashion even when large panels of tumors were tested (Fig. 1; ref. 4). These early experiments were quickly replicated and expanded upon. Although the phenomenon was shown most easily in carcinogen-induced and UV-induced tumors, spontaneous tumors also elicited weak but detectable specific immunity (5). Such experiments ought not to be, and were not, contemplated in humans. Whether or not human cancers harbor some cancer-specific moieties has therefore remained an open question. Those who have been willing to extrapolate the observations from carcinogen-induced mouse tumors have been inclined to believe that human tumors are similarly immunogenic (i.e., that they harbor specificity); others have remained atheistic or agnostic. Not surprisingly, this tension between the “believers” and the atheists has echoed through the subsequent history of cancer immunity, and it is this tension that may now begin to be resolved.

A series of technologies have been brought to bear on the search for specificity in cancers over the past five to six decades. A large body of literature describes the use of electrophoresis of several kinds and dimensions to look for bands or spots that may be unique to cancers. Although these studies have been enormously productive in enhancing our understanding of the biochemical complexity of tissues, highlighting molecular species that may be overexpressed or underexpressed in cancers, they failed to turn up something that might be truly cancer specific. Serology, in its many incarnations from classic polyclonal sera to B-cell hybridomas to SEREX (serologic analysis of cDNA expression libraries) analyses, has been similarly unsuccessful in identifying a truly cancer-specific molecule (6, 7). Regardless, many antibodies whose targets are expressed selectively, or preferentially on cancer cells, but are not truly cancer specific, are in wide and productive clinical use today.

The study of genes provided the first unambiguous demonstration that cancers harbor truly cancer-specific changes. In a series of pathbreaking papers in 1983–84, Yuasa and colleagues showed that specific point mutations in the ras oncogenes were responsible for their transforming activity (see ref. 8 as an early example). These were the first demonstrations that cancers contained truly cancer-specific moieties. However, such oncogenic genetic changes (or mutations in driver genes in contemporary parlance) have not been shown to be responsible for eliciting cancer immunity, even though T-cell responses to mutant oncogenes have been shown to be immunopreventive (9). The following sections of this Masters of Immunology article address the necessarily intertwined (but not identical) questions about what is cancer specific and what elicits cancer immunity.

Search for a Shared Cancer Antigen

In the 1990s, it became possible to identify the targets of T cells of mouse and human cancers. Such studies, led by Thierry Boon,
showed that T cells from tumor-immunized mice and cancer patients often recognized completely unaltered, i.e., normal peptide sequences that were shared among cancers (10). These sequences were derived either from differentiation antigens shared between normal and cancer cells of the particular differentiated lineage or from cancer–testis antigens, which were shared among cancers and germinal tissues but not detectable on normal adult tissues. The realization that T cells of cancer patients easily recognized unmutated sequences gave rise to high enthusiasm to find vaccines against entire classes of cancers (such as melanomas or lung cancers). Important questions remained: What was the immunologic basis of immunizing against self-antigens without precipitating catastrophic autoimmunity? The unmutated sequences did not elicit significant tumor rejection in mouse models (11). What was the scientific and preclinical rationale for the human studies?

Regardless, the attention of the field shifted dramatically from the search for cancer specificity to trying to break tolerance against self-antigens that were expressed on cancers and some normal cells alike. Taking a page from the vast experience with cancer chemotherapy, a degree of autoimmunity began to be deemed an acceptable price to pay for a certain degree of anticancer activity. Although several early studies showed hints of clinical activity, two large randomized, well-controlled, and well-executed phase III clinical trials that tested the efficacy of immunization of melanoma and lung cancer patients with a cancer–testis antigen, MAGE, persuasively failed to show clinical activity (12, 13). Nonetheless, reasonable questions remain: for example, a randomized study looked for efficacy in all patients immunized rather than among patients who generated a CD8 T-cell response to MAGE. In support of the efficacy of T cells against shared antigens, infusion of autologous CD4 T cells against another cancer–testis antigen, NY-ESO1, was shown to mediate a durable clinical remission (14). In another study, Yuan and colleagues (15) showed strong correlation of clinical and immunologic data in metastatic melanoma patients with heterogeneous tumor responses to ipilimumab therapy. Clearly, the final word on the utility of shared cancer antigens in human cancer immunotherapy has not yet been written.

Search for Unique Cancer Antigens

Although the unmutated or shared antigens became the point of overwhelming focus among the antigens recognized by human and mouse T cells, those studies also revealed that T cells of some patients recognized mutated, cancer-specific peptide sequences as well (see ref. 16 for a comprehensive, well-annotated compilation). This cancer specificity came with the burden that, in an echo of the original observations of Klein and colleagues and Prehn and Main (3–5), these mutations were unique to individual cancers and not shared across multiple cancers. They could not therefore be realistically used for immunizing patients. Advent of new technologies will later change that perception as discussed later in this review.

However, the search for cancer specificity and cancer vaccination continued in parallel in other arenas. Building on the intrinsic immunogenicity of autologous cancer cells and on the observations that cancer cells transfected with granulocyte-macrophage colony-stimulating factor (GM-CSF) are far better immunogens than untransfected cells (17), a series of clinical trials are exploring the efficacy of therapeutic vaccination with GM-CSF–modified autologous colorectal or pancreatic cancer cells (18). This approach aims to harness the individually specific as well as any shared immunogenicity of cancers.

In addition, the original observations that mice can be immunized against syngeneic chemically induced sarcomas have been pursued biochemically as a means to identify the molecular basis of specific cancer immunity. These studies, carried out over about 20 years, pointed to a family of commonly expressed molecules, the heat-shock proteins (HSP; specifically hsp70 and hsp90 members), which could be used to immunize against cancers, provided they were isolated from the same cancer cells (19). HSPs isolated from normal tissues elicited detectable but only minor tumor resistance. Extensive analyses have uncovered the fact that immunogenicity was actually derived from short peptides that were noncovalently associated with the HSP molecules, and not from the HSP molecules per se. These observations were based on a number of viral and model antigen systems (20). The peptides were the immunogens, while the HSP molecules were the adjuvants, deriving their adjuvanticity from the fact that HSPs could target/deliver the peptides to dendritic cells (DC) through interaction with the HSP receptors on DCs (21). These observations solved an immediate problem from the perspective of autologous cancer vaccination. If the HSP preparations isolated from cancer cells were intrinsically associated with antigenic peptides, one could immunize cancer patients with HSP–peptide complexes isolated from surgically resected cancers of each patient. The isolation of good-manufacturing-practices (GMP) grade HSP material from individual cancers was logistically and economically feasible. Several early clinical studies showed substantial hints of clinical activity (22, 23). However, a randomized phase III trial in patients with nonmetastatic renal cell carcinoma failed to show convincing clinical activity, except in a large subset of patients that were identified post hoc (24). (The HSP-peptide vaccine OncoPhage was nonetheless approved for treatment in Russia in 2008 for this large subset of patients (N = 362), becoming the first cancer vaccine to be approved anywhere in the world.) Based on phase II data demonstrating impressive clinical activity (25), a large NIH/CTEP–supported randomized trial testing the efficacy of autologous tumor-derived HSP–peptide complexes in patients with glioblastoma multiforme is currently under way. The HSP–peptide approach aims to harness the individually specific, rather than a shared immunogenicity, of cancers. Inherent in these studies is the notion that immune responses against cancer-specific antigens are more likely to have antitumor activity than shared antigens, as originally determined in mouse models.

Molecular Definition of Cancer Specificity

Although approaches of vaccination with autologous cancer cells or autologous cancer-derived HSP–peptide complexes harness the individually specific immunogenicity of cancers, neither approach addresses the original question: What is it that is specific in cancers? It is useful to recall that each individual tumor elicits immunity only against itself, and not against its tumor type, suggesting the incomprehensible possibility that each time a new tumor arises, it carries its own unique antigen! Clearly, there is not sufficient space in the genome for such an unlimited antigenic potential. In order to resolve this paradox, it was suggested over 20 years ago (26) that there are no cancer-specific antigens but only cancer-specific epitopes, which arise.
because of random passenger mutations in dividing cells. These cancer-specific epitopes arise simply because the processes of DNA replication and repair do not happen with complete fidelity (Fig. 2), and because they are random—each new tumor has a unique set of mutations, and hence epitopes, or neoepitopes (Fig. 2). In this thinking, the immunologic individuality of cancers simply recapitulates the genetic individuality inherent in all cells, whether normal or cancerous.

At the time this suggestion was made (1993), the identification of random passenger mutations in individual cancers was not technologically testable. That changed with the advent of high-throughput DNA sequencing and bioinformatics capabilities, which now make it possible to identify mutations and predict neoepitopes on an unprecedented scale. When it rains, it pours. From a long era in which we could not begin to define what is immunologically specific in cancers, we have now come into the era in which the literature is replete with theoretical possibilities of vast numbers of neoepitopes in a large array of cancers. Thousands of human cancer genomes have now been compared with matching normal genomes, and thousands of potential neoepitopes have now been predicted (27–30). These findings are entirely consistent with earlier anecdotal observations: (i) that neoepitopes are present in human cancers and that (ii) the neoepitopes are unique to individual cancers.

Figure 2.

A mechanism of generation of antigenic heterogeneity and individuality in tumors. Random mutations (point mutations, translocations, insertions, or deletions) occur in normal cells as a result of unrepaird errors in DNA replication (see A). The mutations will include one or more oncogenic (or driver) mutation (not shown), as well as many passenger mutations (shown as alphabets). These mutations occur at every division cycle and mark the lineage of that mutant cell as long as the mutation does not disadvantage the cell's survival, or mutations in other cells do not give their bearers a survival advantage. Note how the tumor mass resulting from these divisions (and ongoing mutations) becomes highly heterogeneous with respect to mutations. The immunologic consequences of these events were hypothesized in 1993 (26) and are shown in B. Starting from two identical normal cells, which are transformed (step 1) by the same driver oncogenic mutation (denoted in red as G), the progeny of each of the two transformed cells begin to differentiate from each other as a result of passenger mutations (denoted by random letters of the alphabet in black), which are random and hence unique to each tumor (step 2). A subset of these mutations (missense mutations) alters the sequences of expressed transcripts that are translated into mutated proteins and peptides derived from such mutated proteins. Some tumors may have more mutations than others (57). Because of antigen processing (proteasomal activity, chaperoning of peptides by other molecules) and presentation, a subset of the mutated peptides are presented by the MHC I molecules of the tumor (step 3). A subset of the MHC I–mutant peptide complexes will be recognized by the host TCR. Peptide corresponding to an individual driver or passenger mutation may or may not be recognized by the immune system, but as a class, peptides encoded by passenger mutations are far more likely to be recognized by the immune system simply because passenger mutations are more numerous. Fig. 2B is reprinted from Adv Cancer Res, Vol. 62, Srivastava, Peptide-binding heat shock proteins in the endoplasmic reticulum: role in immune response to cancer and antigen presentation, pages 153–77, copyright 1993, with permission from Elsevier.

Search for the “True” Neoepitopes: Starting with Certain Assumptions

How many of the potential neoepitopes, predicted by DNA sequencing and bioinformatic analyses, are real, and are all neoepitopes equal? Earlier anecdotal studies with mutant neoepitopes of human cancers had already drawn the correlation

Cancer Immunol Res; 3(9) September 2015
between immune response to neoepitopes and clinical outcomes (31). More recently, Brown and colleagues (32) identified hundreds of predicted neoepitopes in a wide array of human cancers, and correlative data suggested that the mutational burden of cancers was indicative of higher infiltration of tumors by T cells and better clinical outcomes. Fritsch and colleagues (33) have similarly compiled detailed data that suggest that the presence of neoepitopes with a higher binding affinity to their cognate HLA alleles is associated with better clinical outcomes. Such correlations are useful in generating testable hypotheses but do not prove causality. Specifically, they cannot help us identify prospectively which of the many predicted neoepitopes shall elicit immunity that will protect the host from tumor growth. That burden must, for now, be borne by the murine models in which predictions may be tested.

A recent flurry of studies attempts to approach this problem. All of the studies use the same initial steps; with some important variations, they all compare the expressed sequences of tumors to the normal genomes and identify the single nucleotide variants (SNV). All of these studies predict neoepitopes based on algorithms that predict MHC I–peptide binding, and all test the peptides for their ability to elicit CD8 T cells and tumor rejection. All studies begin with the following key assumptions, which have a solid experimental and theoretical basis (34–36) primarily from our experience with viral epitopes of mouse and human MHC alleles:

1. MHC I–peptide affinity is a key predictor of a peptide’s ability to elicit CD8+ T-cell response and to confer protective immunity.
2. CD8 T-cell responses, as measured in vitro, are reliable surrogates for antitumor activity in vivo.

In early studies, Castle and colleagues (37) predicted several MHC I neoepitopes in a spontaneous mouse melanoma, observed that a good proportion of these neoepitopes elicited CD8 T cells upon immunization, and demonstrated that some predicted neoepitopes also altered the course of tumor growth. Using a broadly similar approach and assumptions, Matsushita and colleagues (38) showed that a single neoepitope is a target of immunologic editing in a chemically induced mouse sarcoma. The issue of predicting the true tumor rejection neoepitopes from among a large number of potential candidates became the sharp focus of three subsequent reports (39–41) that examined this question in syngeneic tumors in inbred mice, and, coincidentally, in tumors induced by the same carcinogen, 2-methylcholanthrene. All three studies use roughly the same initial steps and made roughly the same assumptions. Some of their conclusions were identical, and others widely divergent. It is instructive to discuss these three studies in some detail.

Duan and colleagues (39) use the NetMHC algorithm to predict the neoepitopes restricted by the Kd allele in two BALB/c tumors. They ranked the predicted neoepitopes in a descending order of NetMHC matrix scores (i.e., affinity to Kd), and tested tens of the highest scorers for their ability to elicit tumor rejection. Surprisingly, none of them did. They noticed that the NetMHC scores of the nonmutated counterparts of these high–NetMHC-scoring neoepitopes were also high, leading to the suggestion that the CD8 response to these wild-type sequences must be deleted or tolerated, and that the CD8 response to the mutant neoepitopes may too have been tolerated because of the similarity to germline. In this light, a genuine neoepitope would need to be sufficiently different from self, in addition to having a reasonable affinity for MHC I. Duan and colleagues gave a numerical definition of difference-from-self by subtracting the NetMHC matrix scores of the wild-type counterparts from those of potential neoepitopes and named it the differential agretopic index (DAI). Not surprisingly in hindsight, the highest DAI-ranking neoepitopes were the ones that had mutations in the anchor residues, because these residues contribute disproportionately to the affinity to MHC I. Ranking the predicted neoepitopes on the basis of DAI rather than NetMHC scores, Duan and colleagues identified that 15% to 30% of the predicted neoepitopes actually elicited tumor rejection. Most surprisingly, they observed that these rejection-eliciting neoepitopes had a poor affinity (IC50 far worse than 500 nM) for Kd or indeed DP or Dq, even though their activity was CD8 dependent. They concluded that mutations in anchor residues, rather than the T-cell receptor (TCR)–facing residues, create the most dissimilar-from-self neoepitopes and that the neoepitopes with low affinity to MHC I may still elicit protective antitumor immunity. Both conclusions challenge some aspects of our current beliefs about MHC I–peptide–TCR interactions.

In defining the immunome of a fibrosarcoma of the h haplotype, Gubin and colleagues (40) prioritized mutation-derived neoepitopes based on their predicted class I binding affinity, their likelihood of being proteasomally processed, and their equivalent or preferential binding to MHC I compared with the respective wild-type sequences. These analyses revealed that the top two (and only two) neoepitopes, each of which had a mutation in a non-MHC anchor residue, represented the major epitopes seen by CD8 T cells that developed in tumor-bearing mice either spontaneously or following treatment with checkpoint-blocking antibodies to CTLA-4 and/or PD-1. When used together, the two neoepitopes induced tumor rejection as efficiently as checkpoint blockade therapy. This study additionally demonstrated that blockade of different immune checkpoints leads to largely distinct alterations in tumor-specific CD8 T cells. Broadly speaking, the conclusions of this study are robustly consistent with our understanding of MHC I–peptide–TCR interactions and enhance our understanding of the molecular mechanisms underlying checkpoint blockade.

Yadav and colleagues (41) add another dimension to the analysis of neoepitopes primarily by adding mass spectrometry to their toolkit. They identified a large number of potential neoepitopes of a fibrosarcoma of the b haplotype by application of the NetMHC algorithm. They also identified the neoepitopes actually presented by the tumor using mass spectroscopy. Upon crosschecking the two databases, they found seven common neoepitopes. Based on affinity to Kd, and on the assumption that the relevant altered residues face the TCR, they predicted that two to seven neoepitopes would elicit tumor rejection. This prediction was experimentally confirmed; in addition, one neoepitope of the remaining five, which was not predicted to elicit immunity, and which results from an anchor-residue mutation, also elicited tumor rejection. The observation that individual neoepitopes arising out of passenger mutations can mediate T cell–mediated tumor immunity is a common thread among the three studies; these observations were indeed hypothesized more than 20 years ago (26). The authors of all three articles agree that only a tiny minority of predicted neoepitopes actually elicits protective tumor immunity. However, the discordance among the three is enlightening with
Affinity for MHC I: Key Predictor of Immune Response against Mutated Self?

The assumption that a neoepitope with a high to intermediate affinity ($IC_{50}$ of <500 nmol/L) for its cognate MHC I is very likely to be a good neoepitope, and the converse, that a neoepitope with a low affinity ($IC_{50}$ of >500 nmol/L) is a poor epitope, have achieved the status of a consensus based on solid experimental data, mainly from viral systems (in which epitopes do not have a self-counterpart; refs. 34, 35). Such quantitative measurements are immensely helpful in making predictions of immunogenicity. The study by Assarsson and colleagues (36) in the vaccinia virus system is a useful case in point: Starting from all possible 9- to 10- amino acid peptides, 2.5% are high-affinity binders to a given HLA allele. Of the actual high-affinity binders, half elicit a CD8$^+$ response. Of these, only 15% are naturally processed and presented. Thus, if one did not know the status of actual antigen presentation (and for the cancer sequencing data, there is no bioinformatic or experimental high-throughput method yet to make that determination), about 7.5% of the predicted high-affinity binding epitopes shall be found to be immunogenic. There is some similarity between this study and that of Van Rooij and colleagues (42), who used the combined criteria of sufficient expression, proteasomal processing, and MHC binding to predict good neoepitopes in a human melanoma; the analysis yielded 448 potential neoepitopes, which were systematically tested. Only two were found to be recognized by the patient’s T cells. The proportion of actual neoepitopes from among the total tested is about a log-scale lower than that in the Assarsson study. Regardless, the enormity of this difference between the two studies is not very significant considering the many differences among them. Both studies show that only a very small proportion of good binders are true epitopes. In contrast, Gubin and colleagues (40) and Yadav and colleagues (41) observed that the predictions fit perfectly, and they were able to correctly predict the neoepitope that would mediate tumor rejection with complete or near-complete accuracy. Duan and colleagues (39), on the other hand, found that the assumptions about the predictive power of affinity of neoepitopes with MHC I are insufficient, and they went on to develop an additional algorithm of difference-from-self. In terms of the frequency of true neoepitopes among the many predicted neoepitopes, the observations of Duan and colleagues are consistent with those of the Assarsson and (36) and Van Rooij groups (42).

One aspect of the observations by Duan and colleagues (39) is inconsistent with much of our understanding of immunogenicity of epitopes. They report that the most effective neoepitopes have a low affinity for their cognate (and other) MHC I allele. As mentioned earlier, there are strong data in support of the consensus that good neoepitopes have an intermediate to high affinity for their MHC I alleles (34, 35). It is worthwhile examining if that consensus should apply equally to the mutated-self epitopes (which do have a self-counterpart). In fact, Sette and colleagues discussed this possibility preciously in their landmark article (34): “It is also possible that self-derived antigenic peptides may be characterized by relatively low MHC binding affinity because of selective elimination by thymic education and/or T cell tolerance of T cells reactive against high affinity MHC binding peptides.” Because testable cancer neoepitopes have begun to be available only during the last 2 to 3 years, data testing this proposition shall soon emerge. For now, except for the Duan study (39), such low-affinity binders have been excluded from testing for biologic activity, and the proportion of good epitopes among them is therefore unknown. If a systematic examination of immunogenicity of poor MHC I binders confirms the antitumor immunogenicity of a significant proportion of these low binders, a vast unexplored repertoire of neoepitopes as cancer antigens shall be available for investigation.

While discussing MHC I–peptide affinity, it is useful to recognize how such affinity is measured and predicted. Because predictions are based on measurements, the latter is the key. They are generally measured by the ability of a given peptide to compete with another peptide for binding to a given MHC I allele. Such measurements have been amply validated through a very large number of studies and are immensely useful (34–36). It is important to bear in mind though that the methods of MHC I–peptide binding in vitro bear little resemblance to the highly choreographed process of such assembly in vivo (43). Indeed, peptides that are unable to bind cell-surface MHC I molecules, but can be presented by endogenously expressed protein, have occasionally been reported (44). It is conceivable that a binding assay in vitro, which recapitulates the biologic assembly of MHC I–peptide complexes in vivo more closely, will provide somewhat different estimates of MHC I–peptide binding, at least for the peptides that seem to bind poorly in the current assays.

Checkpoint Blockade and Neoepitopes

Checkpoint blockade has emerged as a game changer in human cancer immunotherapy, and it is certain that immunization of cancer patients with neoepitopes shall be tested in combination with checkpoint blockade (45). Three distinct strains of ideas, and supporting data, have emerged that connect these two modalities.

van Rooij and colleagues (42) analyzed the tumor exome of a melanoma patient who had responded to CTLA-4 blockade and report that T-cell response to a neoepitope had increased significantly after CTLA-4 blockade. They suggest that this observation might reflect “a more general bias toward recognition of neomarkers from highly expressed genes with a high predicted HLA binding affinity.” Interestingly in this regard, Gubin and colleagues (40), who identified two biologically active neoepitopes in a mouse sarcoma, observed, “when used together, the two neoepitopes induced tumor rejection as efficiently as checkpoint blockade therapy.” The prospect that checkpoint blockade may operate even partially through amplification of the T-cell responses to neoepitopes is enormously appealing for aficionados of cancer specificity.

Duan and colleagues (39), who identified several biologically active neoepitopes of mouse sarcomas, tested the activity of a neoepitope alone, CTLA-4 blockade alone, or both regimens together, and note that the combination was significantly more effective than either agent alone. This observation suggests that exploration of synergy between immunotherapy with neoepitopes and checkpoint blockade might be productive.

Snyder and colleagues (46) attempted to identify a genomic biomarker in the cancers of melanoma patients who responded to CTLA-4 blockade. They observed that patients whose tumor neoepitopes had a homology to viral and bacterial antigens (in the form of a conserved tetrapeptide motif) were far more likely to
respond clinically to CTLA-4 blockade than patients whose tumor neoepitopes did not have such homology. At first glance, this conclusion is difficult to comprehend. What is a possible mechanistic basis for homology between random passenger mutations in the mammalian genome and pathogens? It is conceivable that, in the case of a lack of a substantial population of naïve T cells in the adult human (as a consequence of thymic involution), most of the anti-neoepitope T-cell responses are indeed cross-reactive memory responses to pathogens past. If this interpretation of the results of Snyder and colleagues (46) is true, incorporations of lessons from viral immunology to cancer immunology shall be critical. However, another aspect of the hypothesis presented by Snyder and colleagues requires consideration. More recently, combined checkpoint blockade of PD-1 and CTLA-4 has been used in patients with metastatic melanoma; the dual checkpoint blockade results in a dramatic increment (more than 2-fold) in the proportion of patients who show clinical responses (47). This observation suggests that it is almost certain that a significant proportion of patients who do not have the tetrapeptide motif (46), and do not respond to CTLA-4 blockade alone, are responding to dual blockade.

**Neoepitopes Recognized by CD4+ T Cells**

The entire preceding discussion has focused exclusively on the MHC I–restricted neoepitopes. The neoepitopes presented by MHC II and recognized by CD4+ T cells also have a long past, and now are a focus of renewed attention. The MHC II–restricted neoepitopes have been reported previously in human (48) and mouse (49, 50) tumors. Their ability to mediate tumor rejection has also been shown clearly in mouse models. Using genomic and bioinformatic approaches, recent studies now demonstrate that a large proportion of mutations in human and mouse cancers can generate MHC II–restricted neoepitopes that can elicit CD4+ T-cell responses (51). In mouse models tested, such responses contribute to tumor regression, by themselves, or in collaboration with CD8+ T cells. Kreiter and colleagues (51) suggest that “the less stringent length and sequence requirements for peptide binding to MHC II” may be responsible for the high proportion of mutations that generate MHC II–restricted neoepitopes. The mechanism through which neoepitope-elicted CD4+ responses contribute to tumor rejection is currently only vaguely understood and shall, no doubt, be further scrutinized.

**The Path Ahead**

This Masters of Immunology article began with an attempt to define that which is specific in cancers. Recent discoveries, as elucidated here, have answered that question: We now know in surprising abundance what is specific in cancers. It may be reasonable to entertain the possibility that we shall be able to translate the knowledge of specificity into successful immunotherapy in the clinic. Learning from the past, it is useful to be humble in moments of high enthusiasm, and judging from the flood of opinions and
reviews on the subject (perhaps more in number than the primary papers), this indeed is a time of high enthusiasm for the neoepitopes. It has been nearly 25 years since the idea of random-passenger mutations as neoepitopes was first proposed (26); the time for its critical testing has arrived. How do we translate this idea into clinical studies? Certain steps in the process are straightforward, while others pose significant hurdles (Fig. 3). The rest of this article is devoted to identifying the hurdles. This list does not include the very obvious steps of combination of immunization with neoepitopes with one or more means of checkpoint blockade, inhibition of tumor-induced immune suppression, and other modalities of cancer therapy that are or will be on the horizon. This list focuses instead on the steps that are needed for us to obtain a more perfect prediction of the true tumor-rejecting neoepitopes from among the many that can now be easily identified.

Calling tumor-specific variants and characterizing tumor heterogeneity

It would seem that comparison of the tumor and normal sequences and identification of mutations would be a straightforward process. It is actually anything but, for a number of reasons. Unlike chemically induced cancer cell lines, spontaneous tumors exhibit a high degree of intratumor heterogeneity characterized by complex patterns of clonal and regional variation. Accurate identification of somatic variants within such heterogeneous samples remains a challenge. Although many somatic variant calling algorithms have been described in published reports, the low overlap between variant calls made from the same sequencing data (52, 53) underscores the lack of robustness and inherent biases of current methods. Recognizing this challenge, The Cancer Genome Atlas (TCGA) and the International Cancer Genomics Consortium (ICGC) have joined forces to launch the ICGC–TCGA DREAM Somatic Mutation Calling Challenge, a crowd-sourcing effort to identify the best analysis pipelines and accelerate adoption of best practices in the field (54). Accurate calling of mutations is a necessary foundation for accurate characterization of the clonal diversity of highly heterogeneous tumors. Comprehensive characterization of somatic variants by single-nucleus whole-genome and whole-exome sequencing has already been demonstrated, with the nuc-seq protocol described by Wang and colleagues (55) achieving a mean coverage breadth of over 90%. The relevant clonal structure can also be obtained using more cost-effective single-cell sequencing protocols targeted at predicted variants. These bioinformatics-intensive approaches will be essential for creating the necessary edifice for any and all approaches on identification of neoepitopes.

Predicting antigen presentation

Mass spectroscopy of MHC-eluted peptides is the only method available today to identify the neoepitopes that are actually presented (41). At the current level of sophistication, this is not a high-throughput approach. Taking vaccinia as an example, as few as 7.5% of all potential epitopes may actually be presented (36). Prediction of presentability by MHC molecules requires a bioinformatic strategy; it is conceivable that we may now have accumulated enough of a database of mouse and human epitopes that it may be possible to algorithmically predict the presented epitopes more accurately than before.

Defining dissimilarity from self

Because most of our learning of T-cell immunity has been based on viral or model antigens (where no corresponding “self” exists), the need to define a neoepitope’s dissimilarity from “self” is unique to cancer immunity. Duan and colleagues (39) have made an attempt to quantify this dissimilarity in the form of the DAI algorithm. However, that algorithm is obviously biased toward anchor residue mutations and is incomplete. Broader quantitative approaches to the definition of dissimilarity from self are sorely needed.

Measuring T-cell response

This basic immunologic tool has made great strides in sensitivity and ease from the chromium release assays to flow cytometric and ELISPOT assays. Although it is unambiguously clear that immune responses to cancer are largely T-cell mediated and driven, there has historically been and there still remains a gap between the antitumor activity as seen in vivo and the T-cell activity measured in vitro. Specifically in the current context, neoepitopes that elicit tumor rejection in a CD8-dependent manner may still not be able to elicit CD8 responses that can be measured in vitro. Improved high-throughput assays that measure T-cell activation to large numbers of antigens in a highly sensitive and multifactorial manner shall go a long way in neoepitope discovery. Assays based on sequencing of T-cell receptors (56) may be one such example.

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Cancer Immunol Res, 3(9) September 2015
977


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Neoeptopes of Cancers: Looking Back, Looking Ahead

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