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Cancer Neoantigens

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Abstract

Malignant transformation of cells depends on accumulation of DNA damage. Over the past years we have learned that the T cell–based immune system frequently responds to the neoantigens that arise as a consequence of this DNA damage. Furthermore, recognition of neoantigens appears an important driver of the clinical activity of both T cell checkpoint blockade and adoptive T cell therapy as cancer immunotherapies. Here we review the evidence for the relevance of cancer neoantigens in tumor control and the biological properties of these antigens. We discuss recent technological advances utilized to identify neoantigens, and the T cells that recognize them, in individual patients. Finally, we discuss strategies that can be employed to exploit cancer neoantigens in clinical interventions.

1. INTRODUCTION

The T cell–based immune system functions as an external modifier of cancer growth. Early preclinical evidence for a role of the adaptive immune system in tumor control came from work demonstrating that mice lacking the adaptive arm of the immune system (i.e., lacking B and T cells) were more likely to develop tumors in response to carcinogen exposure, or even spontaneously (1, 2). Early data in favor of a role of T cells in control of human cancers were, for instance, provided by the observation that IL-2 could induce regression of metastatic melanoma in a small fraction of patients (3). However, broader relevance of the T cell–based immune system in human cancer was called into question until the early 2000s. At that point in time, results from two independent lines of clinical research started to provide compelling evidence for a role of tumor-specific T cells in regression of human melanoma and also other human cancers. Specifically, Rosenberg's team at the US National Institutes of Health demonstrated how infusion of ex vivo–expanded autologous tumor-infiltrating lymphocytes (TILs) can induce clinically meaningful responses in melanoma patients (4). This therapeutic effect is at least partly mediated by cytotoxic T cells (5), but CD4 T cells in these products are also likely to contribute (6). Secondly, following the pioneering work of Allison and colleagues (7), antibodies that target the T cell checkpoint CTLA-4 were developed and shown to display clinical activity in patients with metastatic melanoma (8). Furthermore, the subsequent clinical development of antibodies that target the PD-1–PD-L1 axis revealed that the effects of cancer immunotherapy are not restricted to melanoma but can also be observed in cancers such as non-small-cell lung cancer (NSCLC), bladder cancer, and microsatellite-instable cancers (9–14). While the PD-1–PD-L1 axis may also influence the activity of other immune cells (15), it is plausible that the clinical activity of antibodies against these molecules primarily reflects their effects on T cells. Specifically, response to anti-PD-1 therapy in melanoma is predicted by the presence of CD8 T cell infiltrates (16). Furthermore, the predictive potential of pretreatment interferon (IFN) signatures (17) and the increase in such signatures in clinical responders (18) are also consistent with T cell reactivity as a driver of tumor regression.

Collectively, these data imply that in a substantial fraction of cancer patients, the available T cell repertoire can recognize epitopes that are presented in the tumor microenvironment in the context of major histocompatibility complexes (MHCs), the modus operandi of T cells. Understanding the nature of these tumor epitopes is of relevance for a number of reasons. First, it would allow one to understand whether human cancers differ in the number or type of tumor epitopes they express, and whether lack of a sufficient pool of epitopes could limit the activity of cancer immunotherapies for a subgroup of patients. Second, it would allow one to determine whether either spontaneous or therapy-induced immune pressure could alter the repertoire of tumor epitopes through Darwinian selection. Third, and most important, it could allow one to steer immune responses toward such determinants, offering the promise of potentially superior tumor control.

The nature of human cancer regression antigens has been a matter of significant debate over many years. However, work over the years has provided firm evidence that T cell epitopes that arise as a consequence of DNA alterations, so-called neoantigens, form a—perhaps *the*—prime target of tumor-specific T cells. Here we review the evidence for the relevance of neoantigens and the biological properties of these antigens. We discuss the technological advances that have been made to identify neoantigens, but also the T cells that recognize them, in individual patients. Finally, we discuss the ongoing development of strategies to exploit neoantigens, neoantigen-specific T cells, and neoantigen-specific T cell receptors (TCRs) in clinical interventions.

2. EVIDENCE THAT NEOANTIGENS MATTER

Following malignant transformation of cells, the repertoire of peptides that is displayed on the cell surface by MHC molecules is altered. As a consequence of oncogenic pathway activation and epigenetic changes, tumor cells frequently express proteins, such as the cancer-germline (C/G) antigens, for which expression in healthy tissues is restricted to immune-privileged sites (19). As a second process, the DNA alterations that tumor cells accumulate can lead to the formation of entirely novel stretches of amino acid sequences that—depending on their characteristics—can bind to MHC molecules. Contrary to, for instance, the C/G antigens that are genomically self, the genetic code for such neoantigens is entirely absent in healthy tissue. The fact that neoantigens are truly foreign to the body from an immunological perspective ensures that the quality of the available T cell repertoire reactive against them should not be affected by central T cell tolerance, which normally eliminates (high-affinity) T cells specific for self-antigens in the thymus. In addition, the tumor-restricted expression of neoantigens ensures that the (therapeutic) generation of T cell reactivity against these antigens will not be associated with on-target, off-tumor toxicity in normal tissues. In this review, we consider any peptide for which its generation is a direct consequence of somatically acquired genetic changes in tumor cells to be a potential neoantigen. These changes include single-nucleotide variants, insertions and deletions (indels) that lead to frameshifts, and structural variants. In addition, for virally associated cancers, any expressed open reading frames in the viral genome may also be considered potential sources of neoantigens. Next to these classes of peptides that are unambiguous direct consequences of genomic alterations, the term neoantigens has on occasion been used to describe peptides that are presumed to be fully tumor specific, such as certain phosphopeptides or peptides that arise as a consequence of aberrant RNA splicing. While it is possible that some phosphorylation or RNA-splicing events will be entirely specific to tumor cells, low-level occurrence of the same event in healthy tissue is very difficult to exclude, and it seems preferable to restrict the term neoantigens to those peptides for which the exclusive production by tumor cells is beyond doubt.

Over the years, the research community has obtained compelling evidence that neoantigens form important drivers of the tumor-specific T cell response in a number of malignancies. This evidence can be divided into three classes: (*a*) the occurrence of T cell reactivity against these antigens, (*b*) the relationship between mutational load and clinical outcome to T cell checkpoint blockade, and (*c*) the antitumor effects of therapeutic manipulation of neoantigen-specific T cell reactivity.

2.1. T Cell Recognition of Neoantigens

The first evidence for T cell recognition of mutant peptides in human cancer was provided in landmark studies by Wölfel et al. (20) and Coulie et al. (21), now over 20 years ago (see **Figure 1** for a historical timeline). In both these studies, cDNA libraries prepared from tumor cell lines were used to identify tumor-associated RNA transcripts that sensitized target cells for recognition by tumor-specific T cells. Over the next years, similar technology was used to identify neoantigens in additional patients, and an in-depth analysis of T cell reactivity in one melanoma patient led Lennerz et al. (22, p. 16013) to conclude, "These results document a high degree of individuality for the cellular antitumor response and support the need for individualizing the monitoring and therapeutic approaches to the primary targets of the autologous T cell response, which may finally lead to a more effective cancer immunotherapy." However, the concurrent identification of shared tumor antigens, such as the melanocyte differentiation antigens and C/G antigens (23),

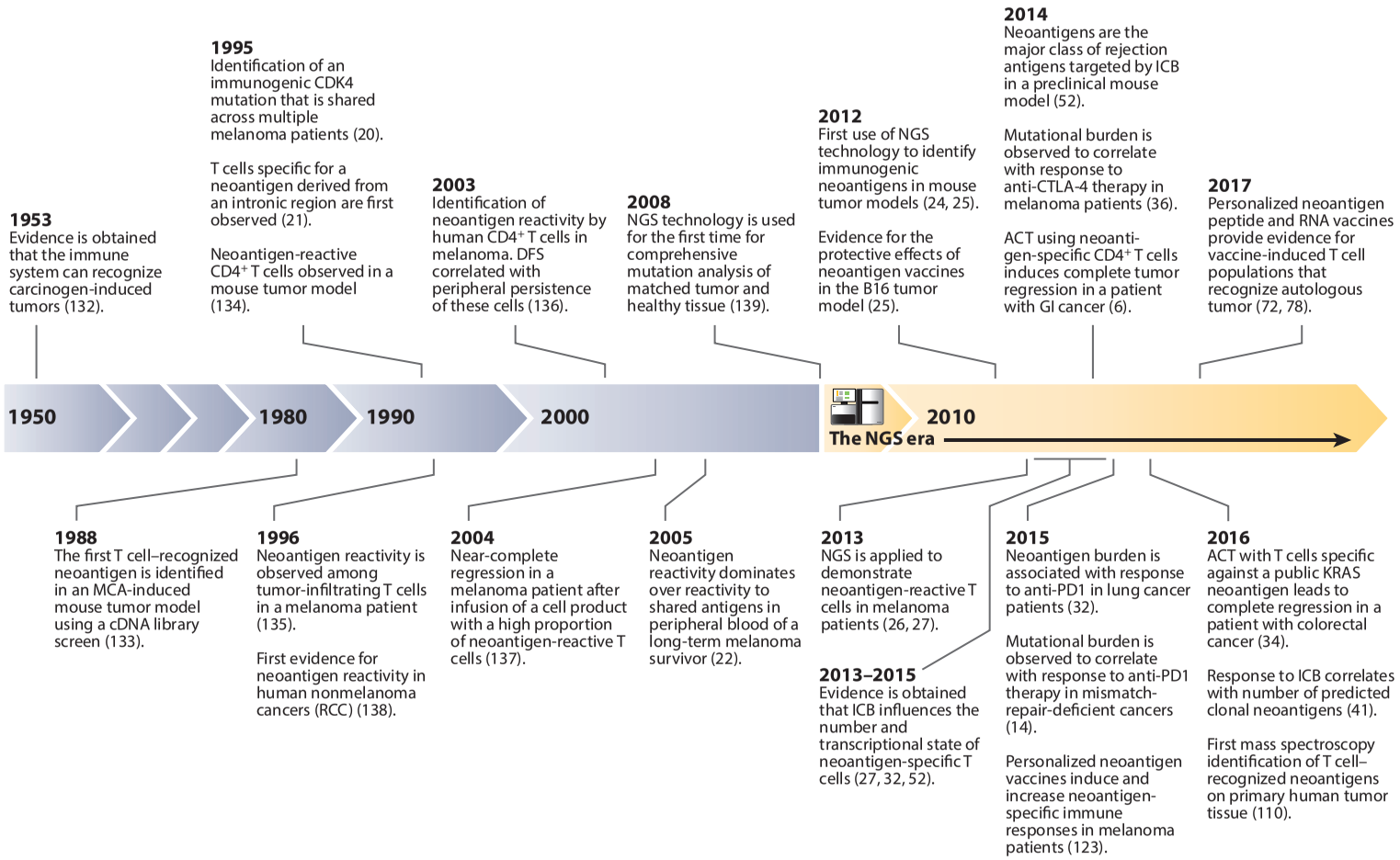

Figure 1

Historical overview of cancer neoantigens. Abbreviations: ACT, adoptive cell transfer; DFS, disease-free survival; GI, gastrointestinal; ICB, immune checkpoint blockade; MCA, 3-methylcholanthrene; NGS, next-generation sequencing; RCC, renal cell carcinoma.

that could potentially be used as targets in large patient groups, plus the technical complexity of dissecting T cell reactivity on a patient-specific basis, led to a waning interest in the role of cancer neoantigens. Now, some 20 years later, technological advances, in particular with respect to high-throughput exome sequencing and T cell–based assays, have made it feasible to revisit the occurrence and importance of T cell reactivity against mutant antigens (see Section 4 for a technical discussion of neoantigen identification pipelines).

The renaissance of the field of cancer neoantigens began with work by the Schreiber and Sahin groups, who utilized mouse models to demonstrate that cancer genome data can be used to identify sets of potential neoantigens that can then be assessed for T cell reactivity (24, 25). Shortly thereafter, the first data were published that exploited the same kind of methodology to assess T cell recognition of neoantigens in cancer patients (26, 27). These and subsequent studies revealed that both CD8 and CD4 T cell responses against mutated antigens are frequently observed within TIL products in melanoma patients (26–31). Neoantigen-specific T cell reactivity has subsequently also been demonstrated in a number of other human malignancies, including NSCLC, ovarian cancer, squamous cell carcinoma of the head and neck, cholangiocarcinoma, and colorectal cancer (6, 32–35). Importantly though, at this point in time it is not clear whether the fraction of patients with detectable neoantigen-specific T cell responses is comparable across these tumor types. In addition, differences in methodology, plus likely publication bias, complicate comparisons between these studies.

The frequency with which T cell reactivity against neoantigens occurs in tumors such as melanoma that show a high rate of clinical response to T cell checkpoint blockade and TIL therapy formed a first simple test of the possible role of cancer neoantigens in these therapies. As a second indirect test, a number of studies have examined whether T cell checkpoint blockade or TIL therapy can alter the magnitude of T cell responses against molecularly defined neoantigens. In case studies of melanoma and NSCLC, an approximately fivefold increase in neoantigen-specific T cell reactivity was detected upon anti-CTLA-4 therapy, and a neoantigen-specific T cell response became detectable after start of anti-PD-1 therapy (27, 32). By the same token, neoantigen-specific T cell responses were shown to increase approximately 100-fold and 1,000-fold following TIL therapy in case studies of head-and-neck squamous cell carcinoma (33) and melanoma (31), respectively. Collectively, these data demonstrate that, in at least some human cancers, T cell recognition of neoantigens is common and that the magnitude of neoantigen-specific T cell populations can increase upon immunotherapy.

2.2. Clinical Importance of Mutational Load

If neoantigens play a significant role in T cell–mediated control of cancer, tumors that display a large number of T cell–recognized neoantigens could be expected to be particularly sensitive to T cell attack. Ideally, one would like to be able to directly measure the number of MHC-presented neoantigens on tumor cells, and also the number of T cell responses against these antigens, in large cohorts of patients. However, technologies that allow this have not been fully developed. As a proxy for neoantigen formation, a number of clinical studies have examined whether tumor mutational burden (TMB) is correlated with clinical responsiveness to cancer immunotherapy. In patients with NSCLC, clinical benefit of PD-1 blockade was shown to be correlated with TMB (32). A similar, although weaker, correlation was observed for melanoma patients treated with anti-CTLA-4 therapy (36, 37). Consistent with these data, PD-1 blockade was shown to display high clinical activity in tumors with DNA mismatch-repair deficiencies across 12 tumor types (13, 14). Of note, a correlation between TMB and clinical response has also been observed for melanoma patients treated with TIL therapy (38) and may thus represent a shared property of

cancer immunotherapies that exploit the endogenous tumor-specific T cell response. Interestingly, progression-free survival of NSCLC with a low mutational burden appeared inferior in patients treated with anti-PD-1 as compared to patients receiving chemotherapy (39). On the basis of the above data, Jaffee and colleagues evaluated the relationship between mutational load and clinical responsiveness to anti-PD-1/PD-L1 therapy across 27 tumor types (40). This revealed that clinical response following PD-1/PD-L1 blockade is also correlated with TMB across different types of cancer.

While these studies provide clear evidence that the number of mutations in human tumors to some extent predicts response to cancer immunotherapies that exploit that natural T cell repertoire, a number of issues deserve further attention. First, while TMB can certainly be expected to correlate with neoantigen formation, it is possible that the amount of DNA damage is also associated with immune control through other mechanisms, for instance by causing cellular stress that may be picked up by innate sensing mechanisms. Mouse models that assess whether mutational burden influences tumor control in a setting of a fixed T cell repertoire (and in which additional mutations can therefore not lead to a broadening of T cell reactivity) may be useful to test this notion. Second, the correlation between TMB and response to immunotherapy is far from perfect, and this is explained by at least four mechanisms.

1. Of the mutations that lead to the generation of novel amino acid sequences in tumors, only a small fraction result in a T cell–recognized neoantigen. In other words, each mutational event merely represents one additional ticket in a neoantigen lottery. In this lottery there will be patients who, in spite of a low mutational burden, by chance have generated strong T cell antigens. Only the development of technology to more accurately predict or directly measure neoantigens on tumor cells and/or the T cells that recognize them can avoid this stochastic effect.
2. The clonal distribution of mutations in the tumor cell mass may influence their value as predictors of immunotherapy response. Specifically, work from Swanton and colleagues has demonstrated that the predictive value of the inferred neoantigen burden, as determined by in silico predictions of T cell epitopes, was improved when solely focusing on predicted neoantigens that were found to be clonally expressed by tumors in lung cancer patients treated with anti-PD-1 therapy (41). In addition, the authors observed neoantigen-specific T cell responses against clonal mutations but not mutations estimated to be expressed by only some of the tumor cells (i.e., subclonal antigens) in a small number of patients. With respect to these data, while it is certainly plausible that the clonality of neoantigens will contribute to their quality as tumor-rejection antigens (24), other factors may also explain or contribute to the observed association between intratumoral heterogeneity and therapy outcome. In particular, a number of escape pathways have now been described that allow tumors to evade T cell attack following immune checkpoint blockade (42–47), and as in any Darwinian selection process, the ability to select more fit tumor cell variants will scale with the genetic diversity present in the cell population that is under selective pressure.
3. As further discussed below, the emerging data on the role of mutation-derived neoantigens as tumor-rejection antigens do not exclude a contribution of other types of antigens, and tumors may vary in the expression of these nonmutant antigens. Response to PD-1/PD-L1 blockade in renal cell carcinoma and Merkel cell carcinoma is considerably greater than what would be expected on the basis of TMB, and this may be explained by a role of endogenous retroviruses (40) and Merkel cell polyomavirus as nonmutant antigens in these diseases.

4. Perhaps most importantly, while the presence of antigen is a requirement for T cell control of cancer, it is only one of many requirements. In particular, parameters such as general immunosuppression, ability to allow T cells at the tumor site, and sensitivity of tumor cells to T cell attack are additional restriction points. A joint analysis of the predictive value of (clonal) TMB together with other parameters of the cancer immunogram (48) should therefore be valuable. Indirect evidence for the importance of other parameters of the cancer immunogram is provided by the fact that clinical response to PD-1/PD-L1 blockade in, e.g., small-cell lung cancer and mismatch-repair-proficient colorectal cancer is considerably less than what would be expected on the basis of TMB. A working model to explain this deviation for colorectal cancer could, for instance, be the role of TGF-β as a T cell exclusion factor (49).

As an attempt to move beyond the analysis of TMB, a number of groups have aimed to infer the neoantigen burden of human tumors on the basis of in silico predictions of T cell epitopes using cancer exome data. While the effort is laudable, a number of concerns should be noted. First, of the predicted neoantigens, only a miniscule fraction can be shown to be recognized by T cells in patients (see below and 50). Thus, approximately 99% of the predicted epitopes can be assumed not to be under selective pressure by T cells, and with such an exceedingly high level of noise in these predictions, any advantage over the mere counting of mutational events appears unlikely. Second, all published efforts to infer neoantigen burdens lack simple analyses of specificity and precision of the epitope prediction pipelines. In future work that aims to establish neoantigen burden as a potentially superior predictive marker, it will be useful to, for instance, predict neoantigen burden using not only the nonsynonymous genetic changes but also the synonymous genetic changes in the same set of tumors. Only if predictive value is substantially higher when focusing on the nonsynonymous changes does it seem plausible that the analysis is a meaningful reflection of neoantigen load, rather than a convoluted way to express mutational burden. By the same token, it is useful to predict neoantigens not only for the HLA alleles carried by the patient but also for HLA alleles that have a distinct binding motif. In this case, superior predictive capacity when using the matched HLA alleles would be the desired result.

In summary, the correlation between DNA changes in human tumors and response to cancer immunotherapies forms reasonable—but still indirect—evidence for a role of neoantigens in tumor control. For the reasons outlined above, TMB will, however, always remain a biomarker with only modest clinical value when used in isolation.

2.3. Manipulation of Neoantigen-Specific T Cell Reactivity

The most direct way to determine the role of neoantigens in tumor control would be to assess the consequences of alterations in either the presence of neoantigens, or the presence/strength of neoantigen-specific T cell reactivity. Direct evidence for a role of neoantigen-specific T cells in tumor control in mouse models was provided by Schreiber and colleagues, in work in which they demonstrated restored tumor control of escape variants upon reintroduction of a T cell–recognized neoantigen (24). In case studies in human melanoma, the presence of T cell reactivity against neoantigens was followed by the subsequent outgrowth of lesions that lacked expression of these epitopes, consistent with T cell–based selection (29).

Evidence in favor of a therapeutic effect of manipulation of neoantigen-specific T cell reactivity comes from a number of studies. In mouse models, vaccination with neoantigens results in improved tumor control (25, 51, 52). Furthermore, work from Rosenberg has provided strong evidence for the value of neoantigen-specific T cells in the induction of tumor regression in the

human setting. In a first case study, treatment of a patient with cholangiocarcinoma with a TIL product in which >95% of infused cells were specific for a private neoantigen resulted in an objective clinical response (6). In a subsequent study, a colorectal cancer patient was treated with a TIL product with a high level of CD8 T cell reactivity (75% of total TILs) against a KRAS mutation, and a lesion that recurred following a nine-month partial response displayed loss of the HLA-C allele that formed the restriction element for this mutant KRAS peptide (34), consistent with T cell–mediated selection pressure. Finally, recent work demonstrated a complete remission of advanced breast cancer following infusion of TILs with high levels of neoantigen-specific T cell reactivity in combination with PD-1 blockade (53). Together, these case studies provide the most compelling evidence for a role of neoantigen-specific T cells in tumor regression in human cancer.

2.4. Role of Other Antigens in Tumor Control

Even though mutant antigens appear to form an important ingredient in T cell–mediated tumor control, as based on the data discussed above, it is important to consider the possibility that other antigens contribute as well. In particular for tumors with a clinical response rate that is greater than what would be expected based on mutational load, a contribution of nonmutant antigens seems possible. For virus-associated cancers such as Merkel cell carcinoma and Epstein-Barr virus- and human-papillomavirus-associated cancers, the viral antigens will likely contribute to clinical response, and conceptually these antigens can be seen as shared neoantigens. In addition, there is evidence for a role of certain self-antigens as targets in clinically effective tumor-specific T cell responses. In particular, the value of the NY-ESO-1 antigen (one of the cancer/germline antigens) has convincingly been demonstrated in a number of studies that tested infusion of NY-ESO-1-specific TCR–modified T cells in different malignancies (54–56). While the fraction of patients with sufficiently homogeneous NY-ESO-1 expression is small, these data do demonstrate that therapeutic targeting of a self-antigen can lead to tumor regression in the absence of detectable on-target toxicity. Whether similar effects may be obtained for other self-antigens remains to be established, but the human endogenous retroviruses that are expressed at high levels in, e.g., renal cell carcinoma (19) and that can be targeted by T cells (57, 58) may be interesting candidates.

3. BIOLOGICAL PROPERTIES OF NEOANTIGENS

Even though the role of neoantigens in the activity of clinically successful immunotherapies is now broadly accepted, a number of important questions remain, for instance regarding the size and quality of neoantigen repertoires across tumors, the determinants of neoantigens that dictate their capacity to induce T cell responses, and the plasticity of neoantigen repertoires in response to T cell pressure. Recent years have seen a rapid accumulation of data that provide some first insight into these questions.

3.1. The Neoantigen Space of Human Cancers

Analysis of large-scale sequencing data sets containing thousands of human tumor samples has revealed considerable variability in the number of somatic mutations in different cancer types, as well as within individual tumor types (59, 60). Numbers of mutations range from roughly 0.01 to 1 per megabase in childhood and hematological cancers to over 400 per megabase in cancers associated with exposure to external mutagens, such as melanoma (ultraviolet light) and lung cancer (tobacco smoke). While recurrent mutations in known oncogenes do exist [such as the CDK4 and KRAS mutations against which T cell responses have been observed (20, 34, 57)], most

tumor mutations are found in passenger genes (i.e., genes that do not confer a survival advantage on the cells in which they are mutated), and these mutations are not shared at appreciable frequency across the patient population (61).

As outlined in the prior section, for a number of cancer types there are now sufficient data to indicate the frequent occurrence of spontaneous neoantigen-specific T cell reactivity (melanoma) or a relationship between TMB and checkpoint blockade (a number of tumor types). With the availability of large mutational data sets covering numerous cancer types, it is appealing to use these combined data in an effort to predict in which tumor types neoantigen-specific T cell activity could be expected to be formed or induced, solely based on their mutational burdens. Using melanoma (of which most tumors contain more than ten mutations per megabase of coding sequence) as a reference tumor type in which neoantigen-specific T cell reactivity is observed in the majority of patients, a first rough prediction would be that frequent generation of neoantigen-specific T cell responses should also be expected in other cancers with mutational burdens close to ten mutations per megabase, such as lung, stomach, and colorectal cancer (62). In addition, occasional neoantigen-specific T cell responses may minimally be expected in tumors with approximately one somatic mutation per megabase, such as breast cancer and pancreatic cancer. For two partially related reasons, the above analysis is likely to underestimate the repertoire of neoantigens that are present on tumor cells. First, not all tumor-expressed neoantigens may lead to the induction of detectable T cell reactivity (63), for instance because of inefficient T cell priming, thereby leading to an underestimate of the available neoantigen repertoire. Second, in particular for tumors with a large number of mutations, competition between T cell responses may restrict the number of T cell responses that develop (64). In an attempt to benchmark the neoantigen repertoire of human cancers using an external reference, we recently compared the number of predicted neoantigens in different human malignancies to the number of predicted epitopes in viral proteins that are known to elicit clinically relevant T cell reactivity. Intriguingly, this external benchmarking effort suggests that up to 50% of tumors of 25 different tissue types can be considered more foreign than the lowest of these pathogen benchmarks (65). This begs the question why neoantigen-specific T cell reactivity has not been detected more frequently in other types. Possible explanations, discussed below, include the lack of efficient induction of T cell reactivity by cancer lesions and the editing of previously recognized neoantigens during tumor outgrowth.

3.2. Which of the Predicted Neoantigens Are Seen by T Cells?

Moving beyond the repertoire of potential neoantigens as determined by in silico predictions, what do we know about the neoantigens that are seen by the immune system? On the T cell side, it is clear that both CD4 and CD8 T cells can respond to neoantigens and contribute to immune surveillance. While most studies have focused on neoantigen reactivity of CD8 T cells (22, 27, 32, 66, 67)—because of their capacity to directly kill (tumor) cells and because many tumors lack expression of MHC class II molecules—it is now evident that CD4 T cell activity can also be elicited in response to tumor mutations. Naturally occurring CD4 T cell responses against neoantigens have been observed in melanoma (30), gastrointestinal cancer (68), and lymphoma (69), and evidence for the clinical relevance of CD4 T cells to tumor control comes from the observation that infusion of large numbers of neoantigen-specific CD4 T cells led to tumor regression in a patient with metastatic cholangiocarcinoma (6). At present it is unclear by which mechanism(s) neoantigen-specific CD4 T cells contribute to tumor control, but prior data in mouse models have demonstrated that CD4 T cell responses can induce control of MHC-II-negative tumors by the boosting of tumor-specific CD8 T cell responses (70). In addition, production of CD4

T cell cytokines such as IFN-γ at the tumor site may contribute to tumor control (71). While naturally occurring T cell responses against neoantigens do not show a profound bias towards the CD4 or CD8 T cell lineage, a recent clinical study using long-peptide-based vaccines targeting patient-specific tumor mutations observed the frequent induction of neoantigen-specific CD4 T cell responses, even though the vaccines had been designed to include predicted MHC class I epitopes (72) (see below). In addition, a bias toward CD4 T cell reactivity was previously observed in mouse models of neoantigen vaccination (51).

The molecular mechanism behind this "off-target" priming of CD4 T cells and its contribution to the clinical activity of neoantigen vaccines are currently unclear. As one possible explanation, priming of CD4 T cells by a growing tumor could be particularly inefficient, and analysis of the naturally occurring immune response would thereby substantially underestimate the pool of MHC-II-restricted neoantigens. As a second, much less optimistic, explanation, many of the CD4 T cell responses that are induced by neoantigen vaccines might be directed toward epitopes that simply are not presented at the tumor site and that are therefore intrinsically irrelevant to tumor control. A better understanding of the value of both natural and therapy-induced neoantigen-specific T cell responses is clearly required.

Regardless of the responding T cell subset (i.e., CD4 or CD8 T cells), an important observation that emerges from the currently available data is that the vast majority of nonsynonymous tumor mutations in human tumors do not lead to detectable T cell responses. Within the constraints of technical factors that may limit the detection of neoantigen-specific T cell populations—such as assay sensitivity, the precision of the neoantigen prediction pipelines that are used as a filter in many of these efforts, and the frequent use of cultured T cell populations, the composition of which may be skewed by in vitro expansion—several biological mechanisms are likely to contribute to this sparsity of neoantigen recognition by T cells. First, the priming of neoantigen-specific T cells may be relatively ineffective, for example due to a lack of proinflammatory signals within the tumor microenvironment (73). Second, T cell responses against the full breadth of predicted neoantigens may be limited by immunodominance, the process in which T cell reactivity is dominated by responses to only a small subset of potential epitopes. The mechanisms underlying immunodominance are poorly understood, but work in the context of antiviral immunity suggests that contributing factors include the stability of peptide-MHC complexes and their density on the cell surface of antigen-presenting cells, HLA genotype, and the functional avidity of the available cognate TCR repertoire (74–77). It will be interesting to explore to what extent these factors can be reliably predicted in silico, and whether this improves the identification of truly immunogenic neoantigens.

Evidence in favor of the notion that not all MHC-presented neoantigens induce detectable T cell reactivity was first obtained in work that used healthy donor lymphocytes to generate neoantigen-specific T cell responses in vitro (63). In addition, recent data from two personalized neoantigen vaccine trials indicate that robust neoantigen-specific T cell populations can be induced de novo from undetectable prevaccination numbers (72, 78). These three studies are consistent with inefficient T cell priming and immunodominance as factors that limit the breadth of the naturally occurring neoantigen-specific T cell response in cancer patients. As a third and more sobering explanation, the continuous interaction between the evolving tumor and the T cell–based immune system may result in selection, or immunoediting, of the neoantigen repertoire that is expressed by individual tumors. Compelling evidence for loss of a T cell–recognized neoantigen as a consequence of CD8 T cell pressure has been obtained in a sarcoma mouse model (24), and data in human melanoma likewise indicate that neoantigens that are recognized by CD8 T cells can be lost over time (29). However, whether editing results in the disappearance of a large part of the available neoantigen repertoire, or forms a less significant escape mechanism,

remains to be established. As a side note, if editing of neoantigens proves to be common, this may influence the relative value of MHC-I- and MHC-II-restricted neoantigens in therapeutic interventions. Specifically, as the effects of CD4 T cells on the tumor cell population will in most cases be indirect, the loss of an MHC-II-restricted neoantigen by a subpopulation of tumor cells is less likely to give a profound selective advantage to those cells. By the same token, while tumor mutations that are only expressed by a subset of tumor cells will lose a substantial part of their value as vaccine candidates, for CD4 T cell epitopes such subclonality may well matter less.

What are the further characteristics of the few neoepitopes that are truly immunogenic? From a clinical perspective, preferred neoantigens would be formed by epitopes encoded by mutations that are shared across patients and, to reduce the risk of immune escape, locate to driver genes that are essential for tumor survival. Indeed, T cell reactivity against such "public" driver mutations has been identified in a number of cases (20, 57, 68, 79, 80). However, looking at the entire pool of data on T cell–recognized neoantigens, such public neoantigens in driver genes appear to be the exception rather than the rule, and the majority of mutations that have been described to elicit T cell responses locate to passenger genes with no apparent role in tumorigenesis (62). Recent work suggests that this may in part be due to population-level immune editing, where the profile of immunogenic oncogene mutations that are eliminated early during tumorigenesis depends on the patients' HLA genotype (81). To test this model in a rigorous manner, it would be useful to determine the relative frequency of somatic $CDK4_{R24C}$ mutations in patients with or without the HLA-A0201 allele, or to perform similar analyses for other oncogene-derived neoantigens for which T cell recognition is not only predicted but has been experimentally validated. Regardless of whether the frequent detection of T cell reactivity against passenger mutations rather than driver mutations is a consequence of editing or simply a reflection of the greater preponderance of passenger mutations, the direct consequence of the above is that clinical strategies aimed at enhancing and monitoring T cell responses against tumor-specific mutations will need to be patient specific, in order to exploit the full repertoire of available neoantigens.

A number of recent studies have forwarded the concept that immunogenic neoantigens bear structural resemblance to pathogen-derived antigens. This concept of molecular mimicry has previously been implicated in the development of autoimmune diseases, where TCRs specific for microbial and environmental antigens have been found to cross-react to self-antigens (82, 83). A first study reported that pathogen-like sequence motifs in predicted neoantigens were common across patients responding to anti-CTLA-4 therapy, but not in nonresponding patients (36). However, the proposed pathogen-like peptide motifs were not observed in an independent analysis of a collection of neoantigens for which T cell reactivity had been experimentally validated (84), and a correction of the original paper suggested a lack of proper validation of the data (85). In addition, in a subsequent analysis of an independent melanoma cohort, van Allen and colleagues (37) did not observe any evidence in favor of such tetrapeptide motifs in responders to CTLA-4 blockade. Two recent studies reported that extension of neoantigen prediction algorithms with a measure of similarity to known pathogen-derived peptide sequences significantly improves the prediction of long-term survival of treatment-naive patients with pancreatic cancer and of melanoma and lung cancer patients treated with anti-CTLA-4 and anti-PD1 therapy, respectively (86, 87). As argued above, the low (and generally unknown) precision of in silico prediction pipelines would appear to make it difficult to observe any signal among the noise of false-positive predictions, suggesting that this issue requires further validation. On a more general note, the creation of large data sets of T cell–recognized neoantigens will likely be a crucial step to better define the determinants of neoantigen immunogenicity.

4. ABILITY TO PREDICT AND IDENTIFY NEOANTIGENS

As discussed above, only a minor fraction of the mutations in human tumors lead to the induction of T cell reactivity. To improve our understanding of the role of neoantigens in tumor control, and in particular to facilitate the exploitation of these antigens in therapeutic approaches, strategies that use the genomic information on human tumors to efficiently determine which epitopes may be recognized by either CD4 or CD8 T cells are required.

The current process of identifying candidate neoantigens generally starts with the mapping of tumor-specific genetic aberrations using whole-exome sequencing (**Figure 2**). In addition, RNA-sequencing data may be used to include information regarding alternative splicing events, to determine whether a mutant gene is expressed in the tumor, and to determine the relative frequency of expression of the mutant allele. The calling of clonal or nearly clonal single-nucleotide variants can be expected to be efficient. However, false negatives for indels and structural variants may still be more common. In addition, tumors with a high degree of genetic diversity can be expected to contain a large number of mutations that are only present in a minority of tumor cells, and for better or worse, these mutations are presently ignored. Following identification of genetic variants in the tumor of a patient, three options for downstream prediction or downstream wet lab identification of tumor-specific neoantigens exist: (*a*) in silico computational prediction, (*b*) mass spectrometry, and (*c*) T cell–based assays.

4.1. In Silico Computational Pipelines

The question of whether a mutation leads to a T cell–recognized neoantigen can be broken up into two smaller questions: Is a particular mutation likely to encode a peptide that can be presented by an MHC molecule of a given patient? If so, is this peptide-MHC complex likely to be recognized by the available TCR repertoire (**Figure 2**)? A number of parameters determine whether a mutation-containing peptide can be presented by MHC class I molecules. First, the number of potential peptides from a given protein scales with the number of molecules degraded per unit of time (and in steady state, therefore also with the number of molecules synthesized per unit of time). In line with this, the representation of proteins within the MHC-bound peptide repertoire has been shown to correlate with RNA expression levels (88). Each of the subsequent steps of protein degradation, transport into the lumen of the endoplasmic reticulum (ER), and binding to ER-resident MHC class I molecules constitutes an additional filter on the peptide repertoire that can ultimately be presented at the cell surface. Of these three steps in the antigen-processing pathway, the ligand preference of MHC molecules has the largest impact on the MHC-presented peptide pools (89). Leveraging prior efforts in MHC ligand prediction in the research fields of infectious diseases and autoimmunity, where in particular neuronal network tools for in silico prediction of binding affinity to a large number of MHC class I alleles have been developed (90), this part of the antigen-processing pathway can be modeled with a reasonable level of confidence. As an example, NetMHC (91) and NetMHCpan (92) allow predictions of epitopes of different lengths for >80 HLA-A, HLA-B, and HLA-C alleles, with robustness of the output to some extent depending on the size of the training data set for a given allele. Next to the prediction of MHC binding affinity, many computational neoantigen prediction pipelines incorporate information on the likelihood of correct proteasomal processing and peptide transport into the ER (Netchop, NetCTL) (93, 94). In addition, the stability of the interaction between peptide and MHC, which appears to correlate more strongly with immunogenicity than MHC binding affinity, may be predicted (95). While there is a fair level of agreement that these different parameters influence epitope density on MHC molecules, a major question is how to best combine these parameters. The use

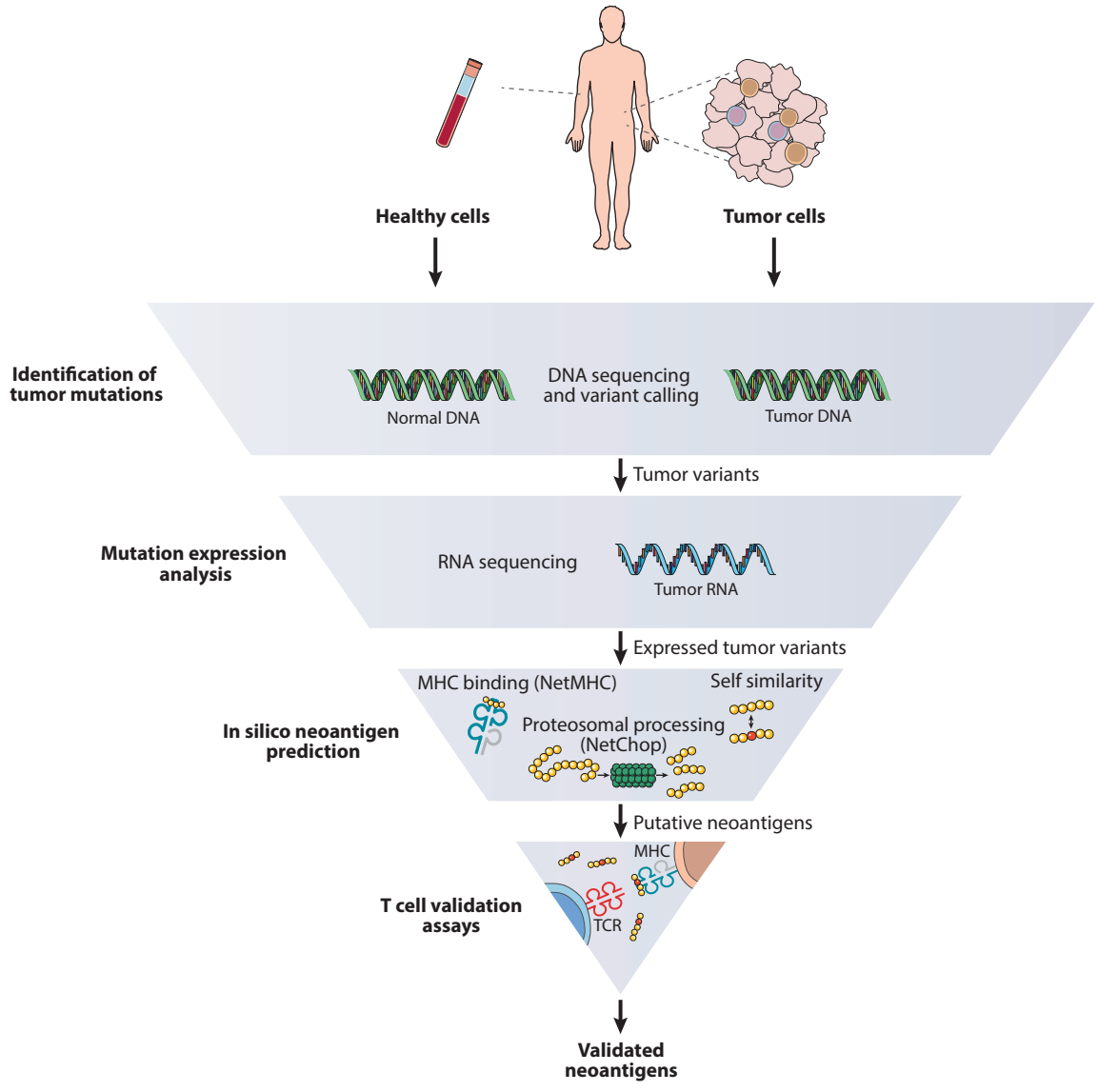

Figure 2

Workflow for neoantigen prediction and detection of T cell–recognized neoantigens. Exomes of normal and tumor DNA are sequenced to identify tumor-associated mutations, and expression of tumor variants is subsequently analyzed using RNA-sequencing data. Putative neoantigens may then be inferred in silico, using algorithms that predict binding of variant peptide sequences to the patient's MHC haplotype, predict the peptide cleavage products generated by the proteasome, and analyze the similarity of variant peptide sequences to self-peptides. Such filtering is optional but is of particular value in situations of high tumor mutational burden, where the available patient material may not suffice for the experimental validation of all identified tumor variants. In addition, in silico neoantigen predictions may be replaced by, or complemented with, mass spectrometry analysis of MHC-associated neoantigens.

of binary cut-offs for these different steps is straightforward but ignores the fact that, for instance, inefficient processing can be compensated by a high level of expression or HLA binding, or vice versa. To train models that integrate the score of potential epitopes with respect to the different steps in antigen presentation, mass spectrometric data of MHC ligandomes have been utilized. Furthermore, to avoid ambiguity in assignment of the detected peptides to a specific HLA allele, Rooney and colleagues (96) have utilized a set of monoallelic cell lines, thereby training a neural network that outperforms networks trained on data sets that solely covered MHC binding affinity. To further improve this approach, the development of strategies that can quantify the number of molecules of different peptide species that are bound by MHC, rather than simply measuring their presence or absence, may be of value.

The in silico prediction of MHC-II-restricted epitopes is a slightly more complex matter, due to the less stringent binding properties of the peptide-binding groove of these molecules. Efforts in the field of infectious diseases have resulted in the establishment of algorithms that are sufficiently robust to predict immunodominant CD4 T cell epitopes from viral pathogens with some level of confidence (97). A complicating matter with respect to MHC-II-restricted neoantigens is that, in the case of MHC-II-positive tumors, the CD4 T cells may recognize peptide-MHC complexes that were either created through processing of endogenous antigen by the tumor cells or acquired exogenously by antigen-presenting cells. For the latter pathway, the expression level of tumor antigens is considered of particular importance, to allow antigen-presenting cells to display a sufficient density of antigen. Building on this notion, Kreiter et al. (51) predicted potential CD4 T cell neoantigens in mouse tumor models based on a combination of predicted binding affinity of the epitope and RNA expression level of the encoding gene. Comparison of tumor control in mice that received peptide vaccines that were selected on the basis of either predicted affinity and antigen expression or solely antigen expression level revealed superior tumor control in the former group, consistent with expectations. A systematic comparison of tumor control in mice receiving vaccines that are equal with respect to binding affinity, but differ with respect to expression level and/or intracellular location of the donating protein, would be of particular value to better understand the parameters that determine MHC-II-restricted antigen presentation in the tumor microenvironment. In addition, to further our biological understanding of MHC class II epitopes, and to improve current prediction tools, mass spectrometry data of both exogenous and endogenous antigens presented by a series of MHC class II alleles would be highly desirable.

While presentation of a mutant peptide by MHC molecules is clearly a necessary condition, it does not guarantee the induction of T cell reactivity. Specifically, data obtained in the analysis of T cell responses against viral antigens in mouse models and human vaccines indicate that T cell reactivity is observed for only roughly 50% of MHC-presented viral epitopes. Furthermore, T cell responses against a small number of those viral epitopes that are being recognized dominate the pathogen-specific T cell response (89). Absence of T cell reactivity toward an MHC-presented epitope may occur either because a reactive TCR is difficult to generate or because reactive TCRs also respond to self-antigens and are therefore removed from the T cell repertoire. The latter reason for lack of T cell reactivity can be expected to be of additional importance in the context of neoantigen-specific T cell responses, because single-nucleotide variants, a dominant source of potential neoantigens, differ by only a single amino acid from the parental self-sequence. In prior work the impact of tolerance to a defined antigen on T cell reactivity toward related sequences has been assessed, revealing that in many cases a single amino acid change in MHC-presented self-peptides is sufficient to evade self-tolerance. Lack of T cell reactivity toward epitopes resembling self-peptides was in this data set biased both to charge-conserving single–amino acid substitutions and to single–amino acid substitutions of the N-terminal residue (98). Calis et al. (99) have initiated the development of models that may be used to assign a "foreignness value" to neoantigens, by

comparison of their sequences to the wild-type counterparts. Conceivably, this type of tool, and also tools that predict peptide immunogenicity irrespective of self-similarity (100), can help to increase the accuracy of neoantigen prediction. Unfortunately, the quality of such algorithms depends heavily on the size of the available wet lab data sets, and unlike data sets that describe MHC binding, data sets that describe immunogenicity of neoantigens are still very modest in size. With the small data sets that are presently available, it has been feasible to address the relative frequency of neoantigens formed by single-nucleotide variants that are predicted to result in either an improved MHC binding potential or an altered TCR exposed surface. In different data sets (31, 101), T cell responses have been observed against both neoantigen classes, and their relative occurrence appears roughly proportional to their proportion in the evaluated epitope sets (31).

As is the case for neoantigen presentation pipelines, no consensus exists on the incorporation of algorithms that predict immunogenicity. Furthermore, the possible role of other parameters that could conceivably influence immunogenicity—such as the capacity of different HLA alleles to cross-present antigens acquired by antigen-presenting cells (102), and differences in ER exit rate and cell surface expression level between HLA alleles (103)—is presently not understood.

A major consequence of our incomplete understanding of both epitope presentation and epitope immunogenicity is that different epitope prediction pipelines currently yield substantially different outputs. When T cell responses toward neoantigens are monitored, this is not a significant issue, provided that the monitoring technology used can handle a large enough number of putative neoantigens. However, when predicted neoantigens are exploited to design patient-specific vaccines, a low precision of epitope prediction pipelines will result in the exclusion of true neoantigens and may result in the suppression of the desired immune responses by T cell reactivity toward false-positive epitopes generated as output of the prediction pipeline. To improve our ability to predict T cell–recognized neoantigens, the Tumor Neoantigen Selection Alliance was recently formed, exploring the performance of the in silico computational pipelines established in individual laboratories. By providing a set of sequencing data to the ~30 participating groups from universities, nonprofit institutions, and biotechnology companies, the alliance can establish the (lack of) overlap in prediction output. Furthermore, to generate a gold standard in this comparison, the TESLA consortium aims to analyze T cell reactivity toward a set of the predicted neoantigens in patient material (104). Together with the increasingly large data sets of wet lab–validated MHC-presented neoantigens and T cell–recognized neoantigens that will come from other academic studies, this effort should increase our capacity to predict immunogenic neoantigens from genomic data.

4.2. Mass Spectrometry

To bypass limitations in our ability to predict neoantigens with high precision using in silico prediction pipelines, mass spectrometry can be used to directly analyze the MHC ligandome. Current mass spectrometry techniques make it feasible to identify thousands of MHC-presented peptide sequences from both cell lines and patient material (105). To identify neoantigens in the obtained mass spectra, these are compared to the expected spectra of all possible neoantigens, as based on cancer exome/RNA-sequencing data (i.e., compared to the list of all newly formed peptides resulting from DNA alterations that is also used as input for computational prediction pipelines). To increase the sensitivity of this approach, synthetic peptides of predicted neoantigens may be used as references. In addition, reduction of the search space by combination with in silico antigen prediction pipelines (106) may potentially be used to improve neoantigen identification.

Mass spectrometry–based analyses of MHC class I ligandomes have demonstrated that, as expected, only a tiny fraction of the obtained peptide sequences on mouse and human tumor lines

are formed by neoantigens (105). To date, the number of neoantigens identified on tumor cell lines and tumor samples (roughly between one and five per sample, with undoubtedly some publication bias toward successful identification efforts) is comparable to the number of neoantigens found to elicit T cell responses (see above). We, however, expect these to be only partially overlapping sets, with some MHC-displayed neoantigens not being seen by the T cell compartment, and some T cell–recognized neoantigens being missed by current mass spectrometry techniques. Of note, epitopes with a low predicted binding affinity have been found to be presented at sufficient levels to be detectable by mass spectrometry, perhaps explained by high expression of the antigen and/or high efficiency during other stages of the antigen-processing pathway. This observation underlines the potential value of strategies that yield an integrated output score of potential epitopes with respect to the full set of parameters that influence antigen presentation efficiency.

Several groups have demonstrated that the HLA ligandome includes the products of posttranslational modifications, such as phosphorylation, methylation, and glycosylation (105), and also peptide sequences generated by splicing of proteasomal degradation products (107). With respect to the latter class of noncanonical peptides, prior data have demonstrated the occurrence of T cell reactivity against spliced nonmutant tumor antigens (108, 109). To our knowledge, no neoantigens that contain posttranslational modifications or that are generated by peptide splicing have been reported to date. However, this may be due to the absence of the relevant sequences or modifications from the search space that is used. While we consider it highly likely that neoantigens with additional modifications will be identified in future work, the ultimate biomedical relevance of such peptides will in large part be determined by whether they constitute a substantial or a marginal part of the entire neoantigen repertoire.

A potential limitation in the use of mass spectrometry for neoantigen identification is that certain characteristics of epitopes may make them more difficult to detect, e.g., because of solubility issues, but the magnitude of this effect appears small (96). Another limitation in the use of mass spectrometry for identification of MHC-presented (neo)antigens in the clinical setting has been that traditional workflows have relied on the use of large numbers of cells from established tumor cell lines. However, Krackhardt and colleagues have recently demonstrated the feasibility of using primary patient tumor material for the identification of T cell–recognized neoantigens, using as little as 0.1 g of tumor material (110). Whether this technology already allows the detection of the bulk of the MHC-presented neoantigens is presently unclear. However, it does suggest that with further technological advances, the more widespread use of mass spectrometry for neoantigen identification in clinical settings may become feasible.

4.3. T Cell–Based Detection of Neoantigens

As an independent strategy to evaluate presentation of neoantigens by MHC molecules, T cells from either cancer patients (26, 27) or healthy individuals (63) may be used. Peptide input for such T cell–based detection systems may be the entire set of potential mutant peptides as identified by cancer exome/RNA sequencing (30, 111). Alternatively, winnowed-down sets of putative neoantigens, filtered through the use of computational pipelines, mass spectrometry, or a combination of both, may be exploited (27, 32, 41). As compared to the use of mass spectrometry, T cell–based assays have the advantage of directly testing whether an MHC-presented neoantigen has been picked up by the T cell repertoire of a patient. As a (related) downside, T cell–induction experiments using healthy donor material have demonstrated that MHC-presented neoantigens do exist that did not induce a measurable T cell response in the cancer patients in which this mutation was originally observed (63). In addition, the waxing and waning of neoantigen-specific T cell responses over time and as a consequence of therapy (29, 32) also imply that a T cell

Table 1 Advantages and disadvantages of different approaches for neoantigen identification

Approach	Capacity	Precision/sensitivity	Level of evidence obtained
In silico predictions	High throughput	Many false positives	No direct confirmation
Mass spectrometry	Low throughput	False negatives	Direct detection of MHC-presented neoantigens
T cell assay	Low throughput	False negatives	Direct detection of T cell–recognized neoantigens

analysis at a given point in time will miss part of the available neoantigen repertoire. T cell–based detection systems may rely on functional readouts such as IFN-γ production or CD137 expression. Alternatively, T cells may be detected using MHC reagents loaded with sets of neoantigens. When using fluorochrome coding (27, 112), lanthanide coding (113), or DNA bar coding (114) of peptides, one can test T cell reactivity against large numbers of putative epitopes with very high sensitivity and using small amounts of material. As a downside, these technologies do rely on epitope predictions, and the necessary MHC reagents can be generated with high efficiency for only part of the human MHC class I alleles (115). As a strategy to improve the sensitivity of functional assays, T cell populations may be enriched for phenotypic markers such as PD-1 (66, 116) and CD39 (113). However, broader analysis of the value of such enrichment strategies, in particular for the blood compartment in different human malignancies, is still required. As a more broadly applicable strategy, TCR sequencing of responding cell populations may be used to increase sensitivity of detection (117). Large data sets generated with these different methods, and preferably with methods that test for T cell reactivity in a fully unbiased manner, will be valuable to optimize computational strategies for epitope prediction, by providing a measure of what was potentially missed using different prediction strategies. In addition, comparison of large data sets of T cell–detected and mass spectrometry–identified neoantigens should help the field to better understand the determinants of neoantigen immunogenicity (**Table 1**).

4.4. A Need for MIANA

The processes to identify DNA alterations in cancer sequencing data and to predict potential epitopes from them rely on a number of software packages/algorithms that influence outcome. In addition, aspects of the DNA/RNA sequencing itself (fresh tumor or tumor line, tumor purity, read depth) influence the results. Given such a large number of variables, there is a significant risk that the pipelines used (including those used by us) are insufficiently transparent to allow their full understanding or replication by other groups in the field. For this reason, it appears important that the field establish a consensus framework for reporting on this type of work. Such "minimal information about neoantigen assays" (MIANA) should improve review of new work in the field, increase reproducibility, and increase our ability to combine data sets into meta-analyses.

Below we provide a (likely incomplete) list of factors that will potentially influence outcome, and for which standardized reporting will be beneficial:

1. **Sequencing data.** The source of DNA (fresh frozen tumor material versus FFPE tumor material versus cell line, tumor purity, time in disease history, site), as well as the sequencing protocol employed (sequencing depth, coverage, read length).
2. **Identification of genomic aberrations (variant calling).** The types of DNA alterations that are analyzed (single-nucleotide variants, insertions and deletions, gene fusions). The additional filters that are employed (such as cancer cell fraction, variant allele frequency, sensitivity to nonsense mediated decay, etc.). The use of RNA-sequencing data as an additional filter and if so with which cut-offs. The computational packages that are utilized in

all of the above and the settings that influence sensitivity versus precision that are used. The use of custom scripts, and the means to access such custom scripts.

3. **Prediction of potential neoantigens.** The computational tools (and versions of these tools) that are used and the cut-offs that have been employed.

A consensus framework that outlines how to report data in this growing research field, analogous to the previously developed MIATA (minimal information about T cell assays) framework (118), should facilitate progress in our ability to utilize genomic information to understand what is seen as nonself by the human T cell compartment.

5. THERAPEUTIC MANIPULATION OF NEOANTIGEN-SPECIFIC T CELL REACTIVITY

The most successful immunotherapeutic interventions to date have been the T cell checkpoint inhibitors that either broaden or allow improved function of the tumor-reactive effector T cell pool. However, T cell checkpoint blockade has no inherent tumor specificity, and dose-limiting autoimmune toxicity is observed, in particular for CTLA-4-blocking antibodies and for the combination of CTLA-4- and PD-1/PD-L1-blocking antibodies (119). Such toxicity is somewhat more acceptable for patients with late-stage disease and with a high likelihood of clinical benefit, but these toxicities will become an increasing concern in patient groups with a lower likelihood of clinical benefit, such as in those with tumor types with a lower TMB. In addition, with the increasing interest in the development of neoadjuvant immunotherapies in patients with a high risk of recurrence (120, 121), the development of effective immunotherapy regimens with a low level of toxicity will become increasingly important. As a second concern, the observed relationship between a preexisting T cell infiltrate and response to PD-1/PD-L1 blockade (16) suggests that in many tumors there may be too few neoantigen-specific T cells on which PD-1/PD-L1-blocking therapies can act.

For these reasons, it appears highly plausible that approaches that specifically increase the magnitude of the neoantigen-reactive effector T cell pool will synergize with T cell checkpoint–blocking therapies, with the potential to reduce treatment-related toxicity by lowering the extent of checkpoint inhibition, and the potential to extend the effects of current therapies to larger patient groups. Neoantigen-directed therapies can be divided into two broad classes: neoantigen vaccines that aim to increase the number of neoantigen-specific T cells in vivo, and neoantigen-directed cell therapies, in which neoantigen-specific T cells are provided to the patient to achieve this goal (**Figure 3**). A large number of academic groups and also biotech and pharmaceutical companies have initiated programs to develop such vaccine- and cell-based therapies, and over the next couple of years a substantial amount of data that evaluate the clinical potential of neoantigen-based therapies should come out. We note that the lack of consensus on aspects such as neoantigen prediction algorithms makes it quite possible that a (good) number of these studies will not show substantial clinical activity. At the same time, the very strong rationale behind this approach, plus the substantial evidence from mouse models and the more anecdotal data from human studies (see below), makes it plausible that effective neoantigen-based therapies can be developed.

5.1. Vaccine Strategies

Based on the accumulation of data demonstrating that only a fraction of potentially immunogenic neoantigens are recognized by T cells of, for example, melanoma patients (62) and that at least some of these neoantigens can be recognized by T cells (63), vaccines are a potential therapeutic intervention to broaden the neoantigen-specific T cell response. In addition, the magnitude of

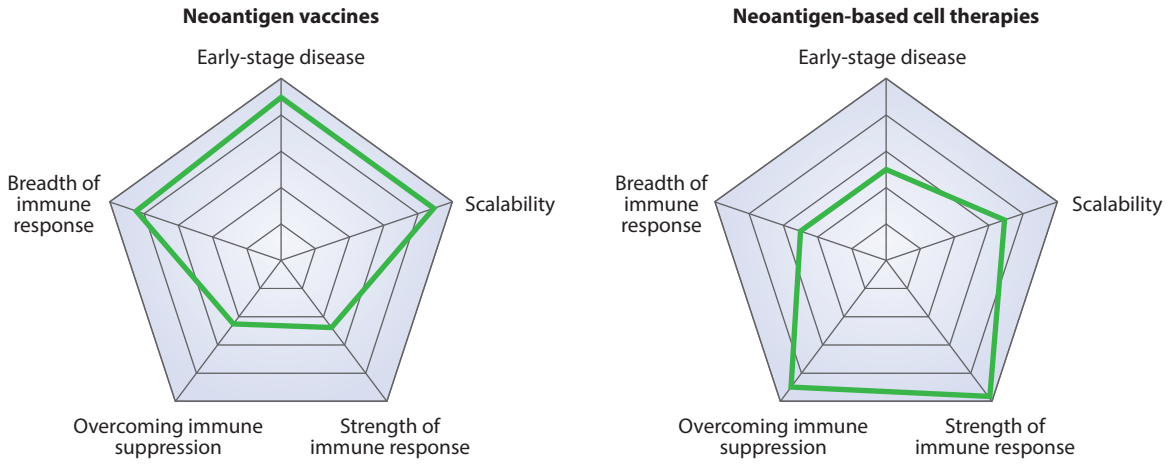

Figure 3

Potential value of neoantigen vaccines and neoantigen-based cell therapies. Spider plots depict relative strengths and weaknesses of neoantigen vaccines and neoantigen-based cell therapies with respect to their scalability, the strength of the immune responses they are expected to induce, their ability to overcome immune suppression, the breadth of the immune responses they are expected to induce, and the feasibility of development for early-stage disease, e.g., as neoadjuvant treatment.

neoantigen-specific T cell responses that have been observed in patient material, both in peripheral blood and at tumor sites, has generally been low (26, 27, 32), and boosting the magnitude of these T cell responses with neoantigen vaccines would appear useful. Cancer vaccines have obtained a particularly bad reputation over the last few decades, showing little if any clinical activity in a host of clinical studies, and a few reasons may be put forward to explain this. First, the vast majority of cancer vaccines tested to date have focused on inducing immunity against nonmutated tumor antigens, and the clinical impact of these vaccines may have been modest due to central (thymic) or peripheral tolerance mechanisms. As alternative explanations, our current vaccine strategies may not suffice to induce T cell responses of a magnitude that is sufficient to influence tumor control, or the magnitude of (neo)antigen-specific T cell responses is simply not a major determining factor in the efficiency of tumor control. The latter of these two explanations appears unlikely, in view of the fact that T cell priming by human tumors appears inefficient, and in view of the fact that the magnitude of vaccine-induced T cell responses in mouse models predicts tumor control reasonably well (25, 52). The former of these two explanations is at this point difficult to exclude, but the efficacy in premalignant lesions of the human papillomavirus vaccines developed by the Melief group (122) argues in favor of the potential of vaccines that induce T cell responses against immunologically foreign tumor antigens, at least in an earlier disease setting. In addition, work from the Schreiber and Sahin groups has provided evidence for the potential value of neoantigen vaccines in preclinical models. In the work by Sahin and colleagues, vaccination of mice with long peptides covering 50 mutations identified by exome sequencing resulted in T cell responses against one-third of the peptides used for vaccination, with an unexpected preponderance of CD4 T cell responses, and such vaccination could induce tumor control (25). In a follow-up study, tumor control was also achieved when using antigen-encoding RNA designed to only encode MHC class II epitopes (51). The data from Schreiber and colleagues demonstrated that vaccination with a synthetic long-peptide-based vaccine containing previously identified T cell–recognized MHC class I neoepitopes resulted in tumor control comparable to that observed after T cell checkpoint blockade (52).

A number of clinical trials using neoantigen-based cancer vaccines to treat cancer patients have been reported, and many are ongoing. In the first-in-human clinical trial conducted by Linette and colleagues, three stage III melanoma patients were vaccinated with an autologous dendritic cell–based vaccine loaded with seven in silico predicted HLA-A*02:01 restricted neoepitopes. Following vaccination, both a boosting of pre-existing CD8 T cell responses as well as appearance of previously undetectable T cell responses were observed (123). Whether these T cell populations were able to recognize neoantigen presented by the autologous tumor was not assessed in this study, and in view of the unknown or poor precision of many epitope prediction pipelines, such analyses should preferably form a core part of future neoantigen vaccine studies. Two subsequent clinical trials in melanoma patients tested the effects of neoantigen vaccines as adjuvant treatment following surgery. In the first trial, 6 stage III melanoma patients were vaccinated with long peptides covering up to 20 mutations together with POLY:ICLC as adjuvant. Four of the 6 patients had no recurrence of disease 25 months after vaccination, and the 2 patients who did have recurrence showed a complete clinical response following anti-PD-1 treatment (72). In the second trial, 13 patients were injected with RNA covering 10 mutations. Two of 5 patients with metastatic disease at the time of vaccination experienced objective clinical responses, and a third patient developed a complete clinical response upon combination with anti-PD-1 therapy (78). In both of these trials, vaccination increased the magnitude of the neoantigen-specific T cell response in all patients, as reflected by the appearance of previously undetectable T cell responses and the boosting of preexisting T cell responses. In addition, both studies demonstrated that part of the vaccine-induced T cell populations were able to respond to autologous tumor, consistent with a possible clinical relevance of these T cell populations. To test the feasibility of boosting neoantigen-specific T cell responses in ovarian cancer, Kandalaft and colleagues treated 25 patients with a dendritic cell–based vaccine loaded with autologous tumor cell lysate either as monotherapy or in combination with anti-VEGF-A +/− chemotherapy (124). In 6 patients, neoantigen-specific CD8 T cell responses were identified. While it is presently unknown whether these CD8 T cell responses could recognize endogenously processed antigen on autologous tumor cells, the fact that these T cell responses were induced with low densities of the individual antigens is encouraging, possibly suggesting the feasibility of neoantigen vaccines in tumors with a lower mutational burden.

At present one of the major unknowns in the design of neoantigen vaccines is whether it is best to primarily aim for CD8 T cell responses or CD4 T cell responses. CD8 T cell responses appear to show a greater association with response to T cell checkpoint blockade, but escape through epitope loss may be more likely. CD4 T cell responses appear more easy to induce, but whether such CD4 responses are primarily of value when a tumor-specific CD8 T cell response has also been induced is presently unclear. A second consideration is whether one should preferably aim to enhance preexisting immune responses or aim to induce novel T cell responses. Based on the emerging data on the epigenetic imprinting of T cell dysfunction it seems possible that expansion of preexisting T cell populations may result in cells that show reduced functional activity (125). In addition, a broadening of the tumor-specific T cell response by targeting multiple mutations may be important to reduce the likelihood of escape, in particular for tumors with a highly branched genetic structure. On the other hand, a focus on preexisting T cell responses ensures that a T cell repertoire is present, and that the epitope can lead to T cell activation in the context of the tumor microenvironment.

It is important to note that neoantigen vaccines address only one of the issues that can prevent T cell–mediated tumor control, and that successful induction of a neoantigen-specific T cell repertoire therefore does not necessarily equal tumor rejection. To further increase the likelihood of successful clinical development of these vaccines, combination with, e.g., STING agonists may be useful in case of tumors that show poor T cell infiltration (126), and combination with T cell

checkpoint inhibitors, to ensure functionality of the induced T cells at the tumor site, may well be critical.

As compared to neoantigen-based cell therapies, neoantigen-based vaccines may be preferable at an earlier disease stage because of the absence of noticeable therapy-related toxicity in the studies performed to date. In addition, the production of neoantigen vaccines is more scalable than the current cell therapy strategies, and neoantigen vaccines appear better suited to induce broad T cell responses. On the other hand, systemic immune suppression in late-stage disease may limit the activity of neoantigen vaccines in this subgroup of patients, and the magnitude of the vaccine-induced responses that can presently be induced is small as compared to what can be achieved with cell-based therapies (**Figure 3**).

5.2. Cell-Based Neoantigen Therapies

As an alternative to neoantigen vaccines, patients may be treated with T cell products that contain a substantial pool of neoantigen-reactive T cells. On the basis of the data that support an important role of neoantigen-specific T cells in the clinical activity of cancer immunotherapies, the Rosenberg group (see Section 2) has treated patients with both melanoma and other malignancies with TIL cultures that were selected for high reactivity against neoantigens. In a series of case reports, evidence was obtained for tumor control by infused neoantigen-specific CD4 and CD8 T cells in patients with metastatic cholangiocarcinoma (6), colorectal cancer (34), and breast cancer (53). While these data are indicative of the potential of neoantigen-specific T cells, other patients that were treated with similar neoantigen-specific TIL products did not have profound clinical responses (68). In addition, while the approaches that are currently used are of significant value to reveal the potential of neoantigen-directed T cell products, widespread application of TIL-based, neoantigen-specific adoptive T cell transfer is difficult to envision.

With the aim to move away from viable tumor material as a source of tumor-specific T cells, a number of approaches have been developed. First, peripheral blood T cells may be stimulated with neoantigens of individual patients to either boost preexisting neoantigen-specific T cell responses or induce novel responses from the naive repertoire. Proof of concept for this approach was obtained by the induction of neoantigen-specific T cell responses that recognized autologous tumor lines and that did show clinical activity in metastatic melanoma patients (127). In addition, development of technology to purify T cell populations that are enriched in neoantigen reactivity (66, 116) may be useful to increase the efficiency of such efforts. A second potential issue with the use of TIL-derived, neoantigen-specific T cell products is the fitness of the cells in the final cell product. Tumor-infiltrating T cells can acquire a dysfunctional state that may only be reverted temporarily or partially during the ex vivo culturing (125, 128). Furthermore, the replicative capacity of these cells following infusion may be modest. To avoid these issues, TCR sequences with a desired reactivity may be introduced into peripheral blood T cells (129). Ample data support the technical feasibility of TCR gene engineering using TCRs directed against shared self-antigens (54, 130). With the development of platforms for high-throughput identification of neoantigen-specific TCR sequences it becomes realistic to adapt this approach for the use of patient-specific TCRs (131). As an alternative to the use of patient-derived TCRs, TCRs obtained from neoantigen-specific T cells present in donor material may potentially also be utilized (63).

Even though the efforts to implement such cell-based therapies will be considerably more involved than the implementation of neoantigen vaccines, a number of potential advantages are also notable. First, the provision of cell products with a high proportion of neoantigen-reactive T cells is likely to induce T cell responses of a magnitude that cannot be matched by current-generation

vaccines. Second, because of the ex vivo growth phase of the T cells and the lymphodepleting conditioning of patients prior to adoptive cell transfer, activity of the cells may be less affected by immunosuppression in patients with late-stage disease, in particular when using peripheral blood–derived (TCR-modified) T cells. The development of efficient and scalable procedures to transplant the neoantigen-reactive TCR repertoire into more fit T cell pools will be an important step toward the generation of neoantigen-reactive T cell products in a standardized manner (**Figure 3**).

6. SUMMARY

The evidence supporting the relevance of neoantigens in clinically successful immunotherapies is compelling and provides a strong rationale for the therapeutic targeting of these antigens. In view of the suboptimal precision of many epitope prediction pipelines, and the fact that only a few neoantigens appear to be expressed on tumor cells, a main goal of initial clinical studies should be to use detailed immunomonitoring to better understand which mutational events yield superior cancer rejection antigens. Together with advances in vaccine design and in gene transfer technology, this should provide the field with the means to increase tumor-specific T cell reactivity in patients in a highly specific manner.

DISCLOSURE STATEMENT

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