Boosting Antitumor Immunity with an Expanded Neoepitope Landscape

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ABSTRACT

Immune-checkpoint blockade therapy has been successfully applied to many cancers, particularly tumors that harbor a high mutational burden and consequently express a high abundance of neoantigens. However, novel approaches are needed to improve the efficacy of immunotherapy for treating tumors that lack a high load of classic genetically derived neoantigens. Recent discoveries of broad classes of nongenetically encoded and inducible neoepitopes open up new avenues for therapeutic development to enhance sensitivity to immunotherapies. In this review, we discuss recent work on neoantigen discovery, with an emphasis on novel classes of noncanonical neoepitopes.

Introduction

Activation of proto-oncogenes, in concert with the inactivation of tumor suppressor genes, drive uncontrolled proliferation of cells and promote cancer development. Attempts to combat cancer by surgery, radiotherapy, chemotherapy, and targeted therapy have resulted in great clinical benefits. Such treatments, however, are frequently highly invasive and often associated with severe side effects, vouching for alternative forms of treatment that circumvent these problems. In addition, disease recurrence is commonly observed among treated patients, requiring therapies that can induce more durable responses. Over the recent decades, immunotherapy has made a breakthrough contribution in fighting cancer, and has yielded durable clinical responses, in many cases without major long-lasting side effects.

Immunotherapy is aimed to trigger a specific antitumor immune response in cancer patients. The successful application of immunotherapy is, among other things, highly dependent on the presence of cancer-specific antigens and T-cell lymphocytes that can specifically recognize them. T-cell activation occurs as a consequence of antigen recognition of specific nonself-antigens via their unique T-cell receptor (TCR) molecule. Costimulation of T cells via binding of other receptors can then further enhance T-cell activation that leads to the secretion of cytokines (1). One of these cytokines, interferon gamma (IFNγ), is the main effector molecule that can induce antiproliferative and proapoptotic pathways in the target cancer cells (**Fig. 1A**; ref. 2).

Resistance to immunotherapy is a major limiting factor for its successful and broad clinical application for cancer patients. This resistance can be the result of various distinct features, of which, several are stemming from alterations in tumor cells themselves. These tumor-specific features include insufficient immune recognition of cancer cells (**Fig. 1B**; refs. 3–6), a failure of immune cells being activated to their full extent (**Fig. 1C**), or an inefficient response of the cancer cells to immune targeting (**Fig. 1D**). When any of these features occur in the tumor cells, they are unlikely to trigger a strong immune response and would then be classified as "cold tumors" (7).

For a set of "cold tumors," immune-checkpoint inhibitors have proven to be effective, as this alleviates inhibitory signaling events exerted by some tumors. Currently, the main marker for the effective application of immune-checkpoint inhibitors is a high mutational burden, implying that the presence of neoantigens is the main determinant of a good response to this therapy (8). Unfortunately, a substantial fraction of "cold tumors" harbor a low mutational burden, which causes immune-checkpoint blockade to be ineffective in this group of cancers. Recent developments, however, have uncovered novel classes of neoantigens that are not derived from genetic mutations.

The identification of shared tumor-specific antigens as targets for immunotherapy has historically been focused on antigens that are derived from cancer-specific genetically hardwired alterations, which we here refer to as "classic neoantigens" (9). Initially, the search for tumor-associated classic neoantigens was concentrated in areas of cancer germline antigens, mutation-derived neoantigens, and antigens derived from proteins of oncogenic viruses (10). More recently, neoantigens derived from noncoding RNAs have been added to the classic neoantigen landscape as well. But, with the current discovery of several novel classes of nongenetically encoded and inducible tumor antigens, which we here collectively call "noncanonical neoepitopes," the landscape of the actionable targets for antitumor immunotherapy has been expanded significantly. These developments harbor the potential to significantly advance immunotherapy, especially against "cold tumors." Here, we review recent advances in the identification and potential utilization of both classic and noncanonical neoepitopes.

Cancer Germline Antigens

Because cellular dedifferentiation is a process commonly observed in tumors, one of the earliest approaches to identify cancer-specific antigens focused on germline genes that are reexpressed in cancer. As antigens in germ cells are excluded from immune surveillance, it was hypothesized that genes that are exclusively expressed in germ cells or during development, and which are specifically reactivated in cancer

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Cancer Res 2022;82:3637–49

doi: 10.1158/0008-5472.CAN-22-1525

cells, could serve as targets for immunotherapy (11). A significant advance to this hypothesis was driven by the identification of cancer/testis antigens (CTAG), the melanoma-associated antigens (MAGEA), and melanoma antigen recognized by T cells 1 (MART-1, also annotated as MLANA). All these genes were found to be expressed to high levels in many tumors, but lowly expressed in normal tissues (12–14). The list of such cancer germline antigens has expanded ever since, adding up to a total of over 200 unique epitopes (15). A significant body of research has indicated that these antigens can be successfully targeted and exploited for cancer cell eradication via an immune response. This was exemplified by autologous T-cell transfers with T cells engineered to express a TCR against New York esophageal squamous cell carcinoma 1 (NY-ESO-1), a peptide derived from CTAG1B. These autologous T-cell transfers elicited potent clinical responses and thereby cemented the role of cancer germline antigens in cancer immunotherapy (16, 17).

It has to be noted, though, that melanoma patients who received immunotherapy with T cells engineered against MAGEA3 or MART-1 antigens experienced serious adverse effects, in some cases even death as a result (18–20). These effects were attributed to the potential of these antigens to induce autoimmunity (19, 20). Multiple attempts have been undertaken in order to increase the specificity of targeting cancer germline antigens in immuno-oncological approaches. This was done by using either TCR-engineered T cells or chimeric antigen receptor (CAR)-T cells. In a recent study, it was attempted to circumvent autoimmunity by the use of CAR-T cells against a neuroblastoma-specific antigen, which were filtered by a counter selection on potential cross-reacting peptides (21). In this way, highly specific CAR-T cells were generated that exclusively recognized a peptide derived from a wild-type PHOX2B peptide, which were able to specifically eliminate PHOX2B-expressing neuroblastoma cells (21). It has to be noted that even though the PHOX2B transcriptional regulator is thought to be exclusively involved in the development of the peripheral nervous system and is a neuroblastoma-dependent gene, it has not been extensively studied whether the generated CAR-T cells recognize other somatic cells in any other human tissue (21, 22). Collectively, although the discovery of a great variety of cancer germline antigens and their immuno-oncological applications is promising, the progression into clinical settings still involves multiple unresolved challenges.

Oncogenic Missense Mutation–Derived Neoantigens

The term "tumor neoantigen" refers to a wide class of cancer-specific peptides that exhibit immunotherapeutic potential. As their discovery is practically challenging and involves complicated methodologies (Box 1), a guided effort for neoantigen detection was performed by narrowing down the search to peptides stemming from cancer-specific genetic mutations. Here, a clear distinction has to be made between driver mutations, which are causal to tumor establishment and maintenance, and passenger mutations, which do not alter the fitness of cancer cells (23). As passenger mutations are abundantly present in several cancer types, it is not surprising that the vast majority of identified neoepitopes originate from these mutations (24). Despite the high abundance of these passenger mutation-derived neoepitopes, they are not generally considered ideal candidates for therapeutic applications. First, these mutations are generally clonal. Targeting these neoepitopes by immunotherapy would cause a heterogeneous response and the consequential loss of these passenger mutations by immunoediting as the cancer cells do not rely on them for their

Box 1. Techniques and resources for antigen discovery.

The availability of tools and techniques for antigen discovery is a critical factor that limits endogenous cancer-specific neoantigen identification. Historically, antigen discovery involved protocols such as a series of molecular cloning, immune screening, and *in vitro* HLA-binding assays. With the advent of sequencing and proteomics technologies combined with computational data analysis, significant leverage for antigen discovery has been achieved (184). For example, proteogenomics protocols have been applied for cancer neoantigen discovery, which combines genomics-based mutation or splice-site identification and mass spectrometry–based peptide identification (88, 185, 186). Proteogenomics complemented with algorithms for the prediction of HLA binders significantly expanded the repertoire of cancer antigens (187–189). However, these methods were predictive in nature or relied on proteomics-based stable peptide detection, whereas the "immunopeptides" are highly likely to be degraded. A breakthrough solution was the development of an immunopeptidomics protocol to directly isolate HLA-bound antigens and detect them with mass spectrometry approaches (190). Immunopeptidomics also leverages the identification of noncanonical antigens such as the ones produced by aberrant translation and hence are nondetectable in genomics-based approaches. Immunopeptidomics combined with HLA-binding prediction significantly increases the specificity of antigen detection (191). The future challenges in the field of antigen discovery would be to develop protocols that not only recognize HLA-bound antigens but also predict the likelihood of recognition by TCR molecules.

With an increasing repertoire of identified cancer-specific neoantigens, new databases are developed to catalog them. For example, TANTIGEN 2.0 provides more than a thousand neoantigens along with supplementary information related to source and function (192). A more detailed resource, the Cancer Epitope Database and Analysis Resource (CEDAR), is currently being developed to expand the catalogs further (193). A breakthrough in the resource database was achieved with the development of The Cancer Genome Atlas, which provides high-throughput genomics studies of 20,000 primary cancer and matched normal samples spanning multiple tumor types (https://www.cancer.gov/tcga). This resource is very useful for antigen-discovery studies as it provides information on genetic mutations and splice sites, a major source of epitopes. Additionally, the Clinical Proteomic Tumor Analysis Consortium (CPTAC) has published a series of in-depth proteomics data sets of thousands of individual human tumors across multiple tumor types, thereby serving as a major source of antigen discovery using proteogenomics approaches (194). Similarly, an immunopeptidomics atlas of human cancer using immunopeptidomics protocols was recently developed, but further expansion of patients and tumor types is warranted (195, 196). Despite these efforts, antigens from noncanonical sources, such as aberrant translation, have not been cataloged. Additionally, a single resource that combines multiple studies identifying the multitude of antigens in variety of cancer types is yet to be achieved.

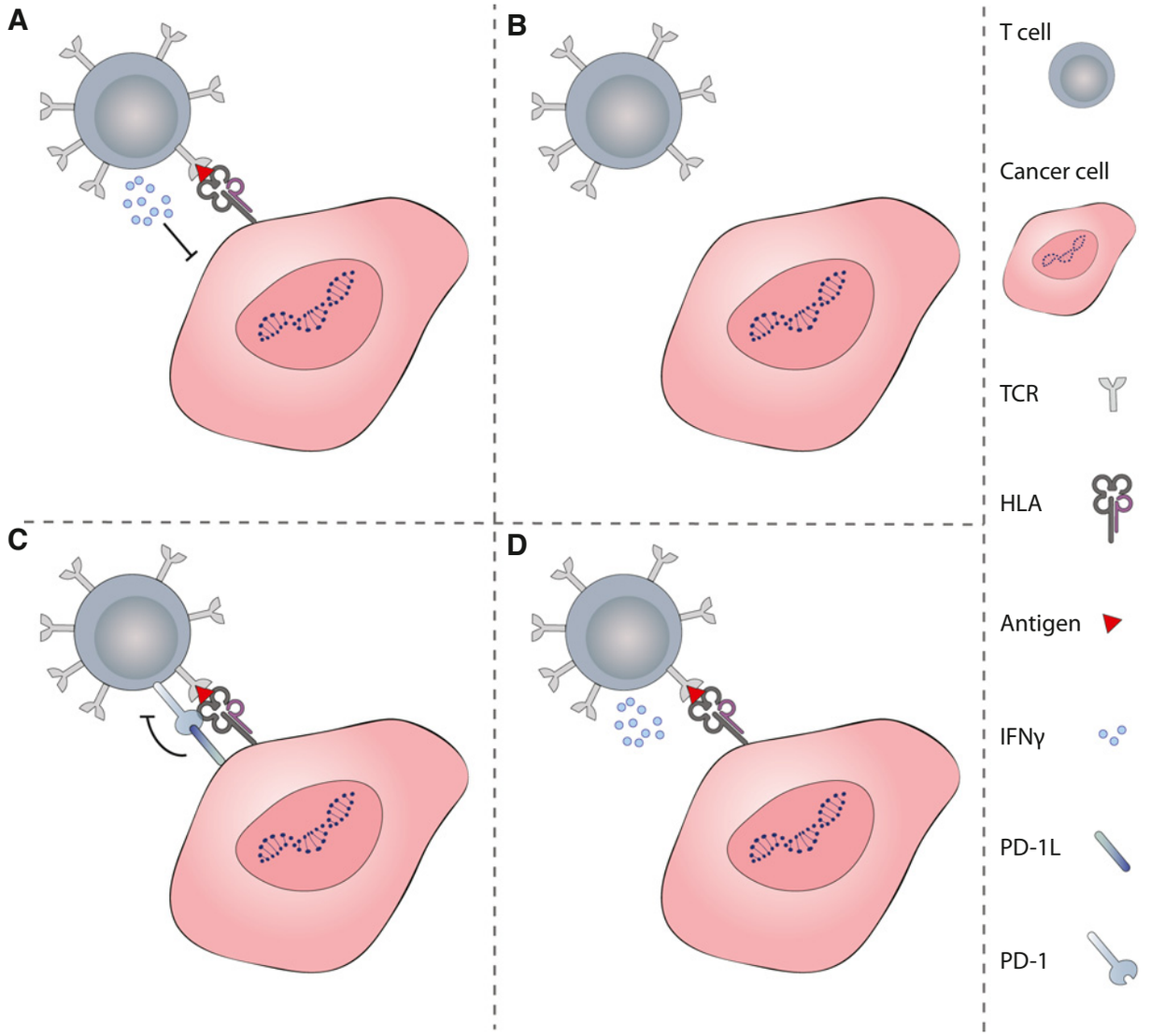

Figure 1.
Efficient processing and presentation of neoantigens lead to a T-cell response, but can be evaded by cancer cells. **A,** Processed peptides from intracellular or extracellular sources can be presented to the immune system on HLA class I or II molecules, respectively. Once a T cell expressing a TCR specific for a presented antigen recognizes the presented peptide, it gets activated and starts to secrete cytokines, among which, is IFNγ. IFNγ signaling in the target cancer cell will lead to the induction of antiproliferative and proapoptotic pathways. Tumor cells possess intrinsic and acquired resistance mechanisms that allow them to evade an immune attack (**B–D**). **B,** Cancer cells can escape immune recognition, either by an absence or low abundance of neoantigens, by the failure of the cells to process or present peptides, or by inefficient priming of T cells toward expressed neoantigens. **C,** Even in the case of efficient recognition of an HLA-bound neoantigen by a T cell, its activation can be inhibited, for example, by binding of PD-1L to PD-1. **D,** Due to mutations in proteins, the IFNγ signaling pathway, or other signaling pathways induced by activated T cells, cancer cells can become insensitive to the growth-inhibitory cytokine.

survival. Second, these mutations show a low recurrence between patients, which prevents the wide applicability of immune targeting of these neoepitopes.

By definition, oncogenic mutations are found only in cancers and not in normal tissues, and in addition, they are mostly clonal. Therefore, altered peptides stemming from messenger RNAs (mRNA) with these genetic mutations have been considered ideal targets for antitumor immunotherapy (25, 26). Indeed, the presence of neoantigen-specific tumor-infiltrating lymphocytes (TIL) and neoantigen-specific cells within populations of naïve peripheral blood monocytic cells (PBMC) from healthy donors underlined the high responsiveness of the immune system toward such neoantigens (27–29). For a broad application of immunotherapy, these neoantigens themselves and their immune presentation would need to be shared between as many cancer patients as possible. The neoantigen discovery protocols classically implement high-throughput genomic and proteogenomic analyses for this purpose and have identified some missense mutations that are shared between multiple cancer patients, so-called hotspot mutations. Examples of these hotspot mutations are found in the *TP53*

tumor suppressor gene and the *RAS* and *BRAF* oncogenes, which are found in a large proportion of all cancers (30–32).

Activating mutations in the *RAS* genes (*KRAS, NRAS*, and *HRAS*) have been established as the main driver of many cancers. Altogether, oncogenic mutations in *RAS* genes occur almost exclusively at codons 12, 13, or 61 and are found in around 30% of all cancers, generating one of the most attractive mutated targets to exploit for an immune attack (33). The identified missense mutations cause an alternative amino acid to be incorporated during mRNA translation at the mutated position. This gives rise to constitutively active RAS proteins, which result in growth factor independence of cancer cells that acquired these oncogenic mutations (33, 34). With respect to immunotherapy, *RAS* mutation–derived peptides would be ideal candidates for targeting by the immune system as they are cancer-specific and shared between many patients. Indeed, several studies showed promising initial results that these peptides are presented on HLA class I molecules and can elicit a T-cell response, thus demonstrating their immunotherapeutic potential (35–37). Moreover, an autologous transfer of T cells isolated from *KRAS*-mutated tumor infiltrates, was able to induce regression in multiple tumor metastases, albeit one metastatic lesion still progressed due to loss of associated HLA class I expression (38).

Similarly, a very specific oncogenic $BRAF^{V600E}$ mutation is found in the majority of malignant melanomas, and in smaller proportions of other cancers (39). Given the widespread development of resistance to a $BRAF^{V600E}$ inhibitor, vemurafenib, immunotherapy directed against this putative neoantigen is a promising alternative for a more durable treatment of *BRAF*-mutated cancers (40, 41). *BRAF* mutation–derived neoantigens are predicted to be both strong HLA class I and II binders and were able to elicit promising $CD8^+$ and $CD4^+$ T-cell responses, respectively (42, 43). It has to be noted, however, that mutated BRAF leads to diminished HLA class I–mediated antigen presentation, and intracellular peptides are normally not presented via the route of HLA class II, which possibly limits the clinical applicability of these findings (44, 45).

As mutations in the *TP53* gene lead to the loss of its tumor-suppressive function in the vast majority of all cancers, it has naturally been given a lot of attention as a putative target for therapy (30). Therapies aiming at restoring p53 function to induce senescence or apoptosis have for the most part not resulted in clinical benefit, hence paving the road for immunologic approaches (46). Importantly, mutant p53 proteins are expressed to high levels in cancer, whereas the wild-type counterpart is nearly undetectable in healthy tissues (47). So even though the largest part of mutated p53 proteins is still composed of the wild-type amino acid sequence, the entire protein was considered a cancer antigen because of its tumor-specific expression. Indeed, multiple wild-type p53-derived peptides were demonstrated to be potent neoantigens (48–50). No autoimmunity was detected in a transgenic mouse model that mimicked the presentation of p53 antigens by HLA-A*02:01 molecules, whereas p53-overexpressing cancer cells were selectively recognized by the raised p53-specific T cells (51, 52). There are, however, conflicting results on the presence of an association of high p53 expression with immune recognition, as some tumor cells with low levels of p53 protein were also recognized by these p53-specific T cells (50, 53, 54). This might be due to the fact that some mutant forms of p53 are not stabilized at the protein level, but are still subject to a high protein turnover, causing it to be considered a tumor-associated antigen. However, as wild-type p53 is expressed and continuously degraded in normal cells as well, it is doubtful whether antigens derived from this protein can serve as specific antitumor targets.

A guaranteed tumor-specific route for an immune attack on p53-derived antigens could be achieved via immune targeting of several well-characterized hotspot mutations in the protein that are shared between multiple cancer patients. The potency of this approach was exemplified by the identification of TCRs in populations of naïve PBMCs and TILs that specifically recognize these peptides (55–58). However, these hotspot mutation–derived peptides were poor HLA class I binders, which warranted a different approach to exploit them as immunogenic neoantigens. By screening of a phage library, a bispecific antibody was identified that, on the one hand, recognized the $p53^{R175H}$ hotspot mutation–derived antigen bound to HLA-A*02:01 and, on the other hand, was able to bind the T-cell receptor–CD3 complex (59). The binding of the bispecific antibody to both $p53^{R175H}$ antigen–bound HLA and the TCR–CD3 complex turned the inefficiently presented p53-derived neoantigen into a potent inducer of a T-cell response that even resulted in tumor regression of xenografted cancer cells (59). Importantly, this bispecific antibody was negatively selected for binding to the wild-type counterpart of the $p53^{R175H}$ mutation, thereby ensuring its specificity for the mutation and excluding binding to the wild-type antigen (59). This highly specific approach paves the way for the use of similar bispecific antibodies that can be used to target T cells to lowly abundant or lowly presented neoantigens more efficiently.

Anticancer immunotherapy directed against oncogenic missense mutation–derived neoantigens thus holds great promise. The risks of targeting a sole mutation, however, might lead to selective pressure for the induction of immune evasion, either by loss of the neoantigen itself or by downregulation of the HLA molecule it is presented by. The identification of potent and persistent T-cell responses induced by vaccines combining multiple personalized neoantigens (poly-neoantigens) are therefore encouraging (60–64). However, the challenges of labor-intensive identification of personalized neoantigens as well as limiting factors, such as a mutational load of tumors, hinder an easy application of such vaccines across multiple tumor types.

Frameshift Mutation–Derived Neoantigens

Some tumors harbor a plethora of mutations in the form of insertions and deletions (indels), which lead to frameshift mutations when they occur in translational open reading frames. These types of mutations are most commonly found in microsatellite-instable (MSI) tumors, which have deficiencies in DNA mismatch repair (65). Frameshift mutations in translational open reading frames most commonly result in the occurrence of premature stop codons, and thus ultimately lead to the production of chimeric truncated proteins that by definition are recognized as nonself by the immune system. Defective mRNAs that contain these premature stop codons, however, are detected by the cellular nonsense-mediated decay (NMD) pathway and are rapidly degraded, thereby preventing the mass production of faulty proteins (66, 67). Despite this limitation, a potent immune response is observed toward frameshift-derived neoantigens, as evidenced by high levels of immunoediting observed during the development of MSI tumors (68–71). This immunoediting causes these neoantigens to be selected against by immune surveillance. In addition, inhibition of immune-checkpoint blockade leads to potent immune responses toward these types of cancers (72–74). Therefore, the immunogenic nature of these antigens can be attributed either

to NMD escape or to efficient processing and presentation of the aberrant protein produced during the pioneering round of translation (75–79).

As one-base pair deletions are the dominant indels observed in MSI tumors, and these mutations frequently occur in the very same genes across these tumors, many frameshift mutation–derived neoantigens are also shared between cancers (69, 80). Hence, arguably, mutation-derived neoantigens are currently the most widely applicable neoantigens for antitumor immunotherapy in the clinical setting. However, the major limitation remains the dependency on the presence of indels, which causes this type of immuno-targeting to be inefficient for microsatellite-stable tumors as well as other types of tumors with a low mutational burden.

Splice Site–Derived Neoantigens

As a first step toward cellular protein synthesis, genes are transcribed as pre-mRNAs, which contain both introns and exons. During splicing, the spliceosome removes introns from this pre-mRNA by fusion of splice-donor and acceptor sites, thereby generating a mature mRNA. Only after the matured mRNA with only exons is formed, it is shuttled out of the nucleus to be translated into protein by cytoplasmic ribosomes (reviewed in ref. 81).

With the advent of large-scale RNA and exome-sequencing studies of cancer, it has become apparent that mutations in splice sites and mutations generating novel splice sites are abundant in tumors (82–85). Mechanistically, splice-site mutations can lead to either aberrant retention of introns in mRNAs or exclusion (skipping) of exons, mostly leading to loss of function of the encoded proteins (83). It should, therefore, not come as a surprise that splice-site mutations are often found in tumor suppressors genes, thereby driving oncogenic progression (83–85). Similar to frameshift mutations, splice-site mutations also induce NMD and the production of a truncated protein with a partly novel polypeptide sequence (86).

The high number of splice-site mutations found in cancer, and their associated changes in protein sequence output, potentially make them main contributors to the cancer immunopeptidome. Indeed, hundreds of putative mutated splice-site–derived neoantigens were identified, with some of them shared between many unique tumor samples, albeit only one of these peptides was confirmed in proteomics analysis (85, 87). However, a proteogenomic analysis of medulloblastoma samples showed that aberrant splice-site–derived neoantigens were the primary source of neoantigens in this cancer type, which were shown to harbor the capacity to provoke an HLA class II–mediated T-cell response (88).

A special subclass of splice-site–derived neoantigens stems from a distinct form of RNA, which is called circular RNA. Circular RNAs are RNA entities produced by back-splicing events and are dysregulated and distinctly expressed in multiple cancer types (89). Even though they lack a 5′ cap, they can be translated in a cap-independent manner; hence, the potential role of circular RNA in immunotherapy has recently been proposed (90, 91). It was shown that transfection of purified circular RNAs led to activation of RIGI, a nucleic acid sensor with the capacity of inducing an immune response (92). Furthermore, abnormal circular RNAs may be transported to immunocytes from tumor cells via exosomal transfer. All of these factors hint that circular RNAs can contribute to the immune recognition of cancer cells, but the actual demonstration of this hypothesis is unachieved and warrants further attention.

Gene Fusion–Derived Neoantigens

The term "gene fusion" refers to the formation of hybrid genes from two previously independent genes. These can be formed by several mechanisms, such as translocations or chromosomal anomalies. As many cancers have high levels of chromosomal instability, gene fusions are naturally detected in these tumors. Several gene fusion events were also shown to directly contribute to carcinogenesis, of which, EML4–ALK fusions and BCR–ABL fusions are the most renowned and well studied (93–95). In these specific cases, the generated gene fusions result in the production of proteins that have constitutive oncogenic kinase activity. This activity is acquired either by the loss of regulatory domains as a direct consequence of the gene fusion removing that domain or by differential regulation of transcription by promoter rearrangements (96–98). Even though these fusions are rather rare and are only lowly recurrent between tumors, the site of fusion is cancer-specific and can potentially produce neoantigens that are immunogenic (99–101). To make the approach of immune targeting of gene fusion–derived neoantigens worthwhile, it requires a widespread presence of a fusion gene with the same point of fusion, which in addition leads to the efficient immune presentation and recognition of the fusion-derived neoantigen. As gene fusions generally do not meet these high demands, it is not to be expected that gene fusion–derived neoantigens will be highly exploitable in the field of anticancer immunotherapy.

Cancer-Associated Virus-Derived Neoantigens

Infection by oncogenic viruses is one of the leading causes of human cancer. In fact, the first oncogene was identified in such a virus, the Rous sarcoma virus (102). Since then, several oncogenic viruses were discovered that contribute to the generation of cancer in humans, including Epstein–Barr virus (EBV) and human papilloma virus (HPV). Such viral infections can either directly induce oncogenic signaling by viral proteins or can indirectly cause cancer due to chronic inflammations. The oncogenic activity of HPV is well characterized and is attributed to the inactivation of the tumor suppressor p53 and the activation of the retinoblastoma protein pRb by the HPV-encoded E6 and E7 proteins, respectively (103). Given that virus-derived peptides are recognized by the immune system as nonself by definition, they have been studied extensively as targets for immunotherapy (104). Although oncogenic virus-derived antigens indeed proved to be highly immunogenic, virally induced tumors also displayed a high intrinsic capacity to evade the immune system (105–108). Encouragingly, in early-stage clinical trials with engineered T cells targeting the HPV E7 protein, HPV-associated tumor regression was observed in half of the tested patients, where in most cases even complete remission was seen (109). This spurs excitement on the possibility of generating pre-HLA–matched engineered T-cell therapies for oncogenic virus-derived antigens. But as anti-HPV vaccination programs for adolescent women are being implemented worldwide, it is the hope that this type of anticancer immunity will be able to prevent the development of cancer, rather than it being deployed with curative intent (110).

A more unexpected virally induced immune response was observed as a consequence of reexpression of endogenously encoded retroviral elements. Whereas in normal tissues such human endogenous retrovirus (HERVs, LINEs, SINEs, etc.) elements are not expressed, they were found to be expressed selectively in some tumor types (3, 111). Strikingly, antigens from such aberrantly expressed retroviral

remnants were presented to the immune system with high efficiency and were able to raise a potent $CD8^+$ T-cell response (112–114).

Surprisingly, there seems to be a different route via which retroviral elements can contribute to an immune response, without directly serving as antigens themselves. When specific endogenous retroviral elements were reactivated, either by activation of p53, inhibition of cyclin-dependent kinases CDK4/6, or by inhibition of DNA methylation, immune surveillance was induced in an unprecedented way (115–118). Directly or indirectly, all these manipulations led to the demethylation of silenced genomic regions and thereby the reversal of epigenetic inhibition of certain endogenous retroviral elements, causing their bidirectional transcription (115–119). Consequently, double-stranded RNA (dsRNA) was formed, which mimicked an endogenous viral infection and thereby triggered tumor cell–intrinsic interferon responses (115–122). Interestingly, this reactivation of retroviral elements led to both an overall enhanced HLA-mediated antigen presentation in cancer cells and their capacity to stimulate immune cells (116–119). This effect was also observed in melanoma where epigenetic silencing of retroelements promoted their immune evasion (123). Thus, the formation of dsRNA and the concomitant interferon response can boost a general antitumor immunity toward otherwise immune-evasive cancers, especially when combined with immune-checkpoint inhibitors (124–126). However, the difficulty of studying these elements by conventional methods due to their repetitive nature, as well as evidence of other kind of retroviral expression in noncancerous cells, leads to challenges in their immuno-oncological application.

Long Noncoding RNA–Derived Neoantigens

Similar to retroviral element-derived RNAs, long noncoding RNAs (lncRNA) were shown to enhance antigen presentation and immune surveillance. Because lncRNAs are thought not to be actively translated into polypeptides, the mechanisms behind this are linked to activation of networks for antigen processing and presentation by lncRNA-induced transcription (127). Recently, it was found that one such IFNγ-induced lncRNA activated the HLA class I machinery for antigen presentation, which is why this noncoding RNA was named lncRNA-induced MHC-I and immunogenicity of tumor (LIMIT; ref. 128). LIMIT-induced changes in antigen presentation potentiated antitumor immunity, especially in combination with immune-checkpoint inhibition (128).

Although lncRNAs were initially reported not to be translated, recent studies provide evidence for the contrary. Accumulating data have indicated that many, mostly very short, polypeptides are generated from noncoding RNAs (129–132). Even though most peptides derived from RNA sequences other than the canonical open reading frames have been suggested to have no cellular function and are short-lived, they do contribute to over 10% of the total immunopeptidome (133–136). These studies did not clearly delineate the tumor exclusivity of such generated peptides, but the evidence does indicate that these noncanonical peptides can be harnessed for an immune attack (119, 134, 135, 137). The most promising antigen from this class stems from the lncRNA *PVT1*, which is overexpressed in many cancers, most likely due to its coamplification with the *MYC* oncogene that resides in the same genomic locus (138, 139). In colorectal cancers, it was found that a noncanonical peptide originating from the *PVT1* lncRNA was a strong ligand for HLA-A*24 molecules, and a potent $CD8^+$ T-cell response against it was detected in immune infiltrates of several colorectal tumors (137). Although these are encouraging results, the possible availability of *PVT1*-derived antigens in normal tissues has not been completely excluded, warranting future studies to clarify potential issues with autoimmunity.

Noncanonical Neoepitopes

Beyond neoantigens, recent findings have expanded the landscape of the immunopeptidome with epitopes that are not encoded by the genome, which will be referred to as noncanonical neoepitopes. These neoepitopes are either the output of cellular processes that are specifically altered or induced in cancer and can give rise to a wide variety of neoantigens at the very same time. This is in stark contrast to genetically encoded classic neoantigens, which are restricted to antigens produced from one mutated gene at a time. This novel class of noncanonical neoepitopes could prove especially valuable in cancers characterized by a low mutational burden, as these cancers are thought to evade immune detection by means of a low availability of classic neoantigens (140–143).

Alternative Splicing–Derived Neoepitopes

Mounting analyses of tumor transcriptomes have led to the identification of a class of antigens that massively expand the immunopeptidomic landscape of cancer cells, namely, the alternative splicing–derived neoepitopes. It was already recognized that genetic mutations in the splicing factors U2AF1 and SF3B1 had a widespread impact on alternative mRNA splicing in cancer, due to their role in alternative splice-site usage (144–146). The potential impact of these alternative splice events on immune recognition of cancer, however, was uncovered only recently by a pan-cancer analysis of just under 9,000 tumor transcriptomes (147). This study revealed widespread alternative splicing events in cancer, dubbed neojunctions, which could be related to the presence of mutations in splicing factors (147). Proteomics analyses indicated that this alternative splicing gave rise to the translation of a whole array of novel cancer-specific peptides, including putative HLA class I binders (85, 147, 148). Underlining the commonality of these neoepitopes, the detected number of neojunction-derived peptides was almost 3-fold higher than the number of single-nucleotide mutation-derived peptides (147). These findings were extended with *in vivo* studies, showing that neojunction-derived neoepitopes can elicit a bona fide antitumor immune response (147). It has to be noted that the presence of these neojunction-derived neoepitopes in normal tissue has not been studied in depth, and therefore the presence of these aberrant epitopes in cells with nonmutated splicing factors cannot formally be excluded.

Pharmacologic modulation of the spliceosome was shown to lead to translation of mRNAs containing neojunctions and the subsequent production of highly immunogenic neoepitopes, but also a viral mimicry response owing to dsRNA formed from mis-spliced mRNAs (149, 150). Strikingly, these neoepitopes elicited a robust $CD8^+$ T-cell response and had a profound inhibitory effect on xenografted tumor growth, especially in combination with immune-checkpoint inhibition (149). A study on human tumor material showed that a large fraction of SF3B1-mutated uveal melanoma patients harbored TILs specific to neojunction-derived neoepitopes, underscoring their great potential as immunotherapeutic targets (151). This could prove to be an extremely valuable finding for the treatment of "cold tumors" that have a low mutational burden.

Posttranslational Modification–Derived Neoepitopes

Most proteins require posttranslational modifications, such as phosphorylation, glycosylation, acytelation, and amino acid conversions like citrullination, for their full function (152). As a result, polypeptides gain different molecular characteristics, leading not only to an alteration in protein function but potentially also to changes in the immunogenicity of antigens derived from them. Indeed, a wide range of posttranslationally modified peptides were shown to be presented on both classes of HLA molecules (153–155). Because modification of peptides has been linked to autoimmunity, it was realized quickly that the immune system can act on these posttranslationally modified antigens (156, 157). However, for efficient anticancer immunotherapy, it is a requirement that these modified target peptides would be tumor-specific. Encouragingly, levels of phosphorylated presented antigens differed between normal and cancerous cells (153, 158–160). And these could be used for specific targeting of cancer cells by T cells specific for these phosphorylated neoepitopes (153). However, as only a very limited number of cancer-specific modified peptides has been reported so far, the options to target this class of neoepitopes with immunotherapy seem to be limited for now.

A very surprising posttranslational modification was identified when immunity was observed against a chimeric peptide derived from the FGF5 protein (161). The antigen in this case was shown to be a fusion peptide from two fragments of the FGF5 protein that were originally separated by a stretch of 40 amino acids (161). The removal of this intermediate stretch of amino acids and the fusion of two distant peptides together was demonstrated to be a more general process executed by the proteasome, and hence this process was named proteasome-catalyzed peptide splicing (162–167). As the resulting peptides, called splicetopes, were presented on HLA molecules and were able to evoke CD8 T-cell responses, there could be a potential utility for these as targets for immunotherapy (161, 164, 165). However, it remains to be seen how widespread the occurrence of splicetopes is and whether these epitopes are cancer-specific, which warrants more studies to explore this potential.

RNA Editing–Derived Neoepitopes

Similar to alternative splicing, RNA editing was also shown to be highly dysregulated in various types of cancers (168). The most commonly dysregulated RNA-editing event is the posttranscriptional conversion of the nucleotide adenine to inosine by adenosine deaminases acting on RNA (ADAR; refs. 169 and 170). Because the translation machinery reads inosine as guanine, this editing leads to alternative decoding during mRNA translation, whereby distinct amino acids are included in the nascent polypeptide chain, and the protein sequence is ultimately altered. The widespread formation of these peptides across multiple cancer types and their HLA class I–mediated presentation was convincingly shown in different cancer types (171–174). But more importantly, a large abundance of $CD8^+$ T cells specifically recognizing these RNA editing–derived neoepitopes was seen in various tumors, indicating that these neoepitopes are highly immunogenic (173). Because RNA editing can also occur on transfer RNAs (tRNA), it is of great interest to determine the effect of dysregulated RNA editing on tRNA usage and decoding during translation, as this could potentially add a new layer to the already known RNA editing–derived neoepitopes.

Aberrant mRNA Translation–Derived Neoepitopes

Next to the posttranscriptionally derived neoepitopes, it has recently been discovered that inducible, aberrant translational events can lead to the production of cancer-specific neoepitopes as well. This specifically takes place in conditions of shortage of the essential amino acid tryptophan, where ribosomal stalling at tryptophan codons results in ribosomal-frameshifting events (175, 176). The aberrant polypeptides generated as a consequence of such frameshifts were found to be presented on HLA class I molecules, after which, they could efficiently be recognized by T cells (175). Interestingly, the induction of translational frameshift-derived neoepitopes was shown to be exclusive to cancer. It depends on oncogene-induced translational sloppiness, which was achieved by activation of the mitogen-activated protein kinase (MAPK) pathway (176). Importantly, in tumors with acquired resistance to MAPK-inhibitory–targeted therapy, such aberrant neoepitopes could still be induced and provoke T-cell recognition and attack (176, 177).

In addition to ribosomal-frameshifting, tryptophan depletion resulted in specific codon reassignments. Instead of tryptophan, phenylalanine was incorporated in conditions of tryptophan shortage, leading to substitutants—a novel type of aberrant peptides (178). Interestingly, this alternative translational decoding was enriched in cancers characterized by TILs and local IFNγ signaling, providing a rationale for the expression of substitutants in cancer cells. Indeed, substitutants were specifically enriched in human cancers, were detected in the immunopeptidome, and were found to elicit a T-cell response (178). The cancer specificity of these translation-derived neoepitopes underlines their potential utility in cancer immunotherapeutic applications of tumors with low levels of genetically encoded neoantigens. However, the transient nature of their expression may limit their effectiveness, warranting an *in vivo* proof-of-concept for their ability to elicit immune targeting of cancers. Encouragingly, a fasting-mimicking diet was shown to enhance antitumor immunity (179), which opens up new possibilities for diet-induced expression of neoepitopes. Alternatively, these neopeptides could be induced by IFNγ.

Concluding Remarks

The enormous repertoire of TCRs that is present within the immune system implicates that a vast variety of neoantigens could specifically be targeted for anticancer immunotherapy. The successful application of immune-checkpoint inhibitors for large numbers of cancer patients substantiates this hypothesis. A high tumor mutational burden is currently used as the main marker for predicting the efficacy of immune-checkpoint inhibitors, just because of the sheer fact that the presence of many neoantigens corresponds with a better chance of inducing a potent antitumor immune response. As not all cancer patients benefit equally well from this treatment, the search for cancer-specific neoantigens that are widely shared between patients, and the identification of potent TCRs against these epitopes, will likely expand future immunotherapeutic options for the treatment of tumors with a low mutational burden.

Recent discoveries of many novel noncanonical neoepitopes revealed new branches of the immunopeptidome relevant for anticancer immunotherapy (**Fig. 2**). Altogether, the cancer immunopeptidome is shaped by many different variables. First and foremost, the expression of classic neoantigens can elicit an immune response

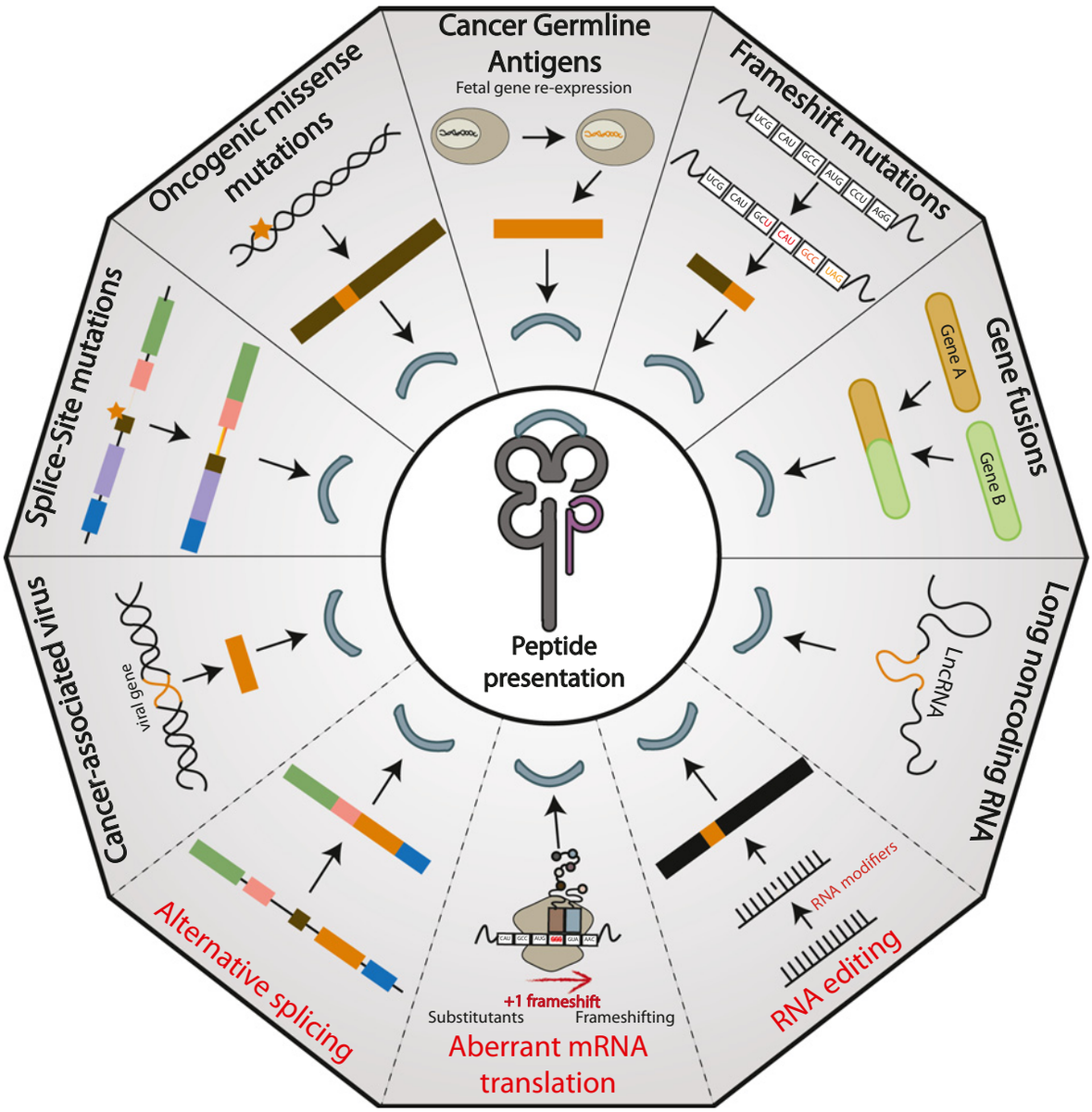

Figure 2.
Different classes of classic neoantigens and noncanonical neoepitopes are presented to the immune system on HLA molecules. Hardwired genetic cancer–associated alterations lead to the production classic neoantigens (processes with black names). The presence of these alterations can trigger immune activation, especially for tumors with a high mutational burden. For tumors with a low mutational load, the noncanonical neoepitopes (processes with red names) could prove to be a valuable alternative avenue to provoke an immune response.

already during carcinogenesis. However, the selective pressure induced by the immune system may either alter the immunopeptidome or suppress presentation, leading to immune evasion (180). This might also be the case for neojunction-derived neoepitopes, as in multiple myeloma a high level of these epitopes is associated with poor survival, possibly due to the coexpression of T-cell–inhibitory molecules and elevated interferon signaling (181). A second parameter influencing the outlook of the immunopeptidome is tumor heterogeneity and the capacity to acquire novel genetic mutations, which can lead to the loss of antigens within a tumor (182, 183). Through selection, immunotherapy-resistant tumors can emerge once all remaining cancer cells lost the main targeted antigens. Theoretically, all these limitations could be overcome by targeting inducible noncanonical neoepitopes, as they are cancer-specific, and can be simultaneously induced by aberrant translation in many proteins. However, whether their transient expression may limit applicability needs to be investigated. Additionally, the commonality of expression and presentation of noncanonical neoepitopes has not been explored in great detail so far. In addition, the immunogenicity of most of the neoepitopes from this novel class has not yet been studied extensively or has been tested

only with *in vitro* systems. These caveats warrant thorough analyses of immunopeptidomics data for the presence of noncanonical neoepitopes, as well as the validation of their immunogenicity using systems that represent their endogenous route of generation and immune presentation. Nevertheless, novel insights into the immunopeptidome provide new possibilities to combat cancer immune evasion by combining global checkpoint inhibition with specific targeting of noncanonical neoepitopes.

Authors' Disclosures

No disclosures were reported.

Acknowledgments

Funding for the present work was provided by the Dutch Cancer Society (KWF project 13647), the European Research Council (ERC-2018-ADG-GA 832844), and the AVL Foundation.

The publication costs of this article were defrayed in part by the payment of publication fees. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Received May 10, 2022; revised July 7, 2022; accepted July 21, 2022; published first July 29, 2022.

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