

Relevance of mutation-derived neoantigens and non-classical antigens for anticancer therapies

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ABSTRACT

Efficacy of cancer immunotherapies relies on correct recognition of tumor antigens by lymphocytes, eliciting thus functional responses capable of eliminating tumor cells. Therefore, important efforts have been carried out in antigen identification, with the aim of understanding mechanisms of response to immunotherapy and to design safer and more efficient strategies. In addition to classical tumor-associated antigens identified during the last decades, implementation of next-generation sequencing methodologies is enabling the identification of neoantigens (neoAgs) arising from mutations, leading to the development of new neoAg-directed therapies. Moreover, there are numerous non-classical tumor antigens originated from other sources and identified by new methodologies. Here, we review the relevance of neoAgs in different immunotherapies and the results obtained by applying neoAg-based strategies. In addition, the different types of non-classical tumor antigens and the best approaches for their identification are described. This will help to increase the spectrum of targetable molecules useful in cancer immunotherapies.

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Introduction

A main feature of the immune system is its ability to discriminate between self and non-self to protect the organism from exogenous microbial pathogens. This exquisite capacity relies on the presence of lymphocyte receptors that specifically recognize a wide array of antigen molecules. In the case of tumor cells, although it took many years, it was finally demonstrated that, despite being own cells, they express antigenic molecules than can be recognized by lymphocytes and that, in some cases, led to control of tumor growth.¹ Malignant transformation of tumor cells arises from a series of mutations, which ultimately lead to the generation of proteins with new sequences, changes in the expression of non-mutated proteins and even in post-translational modifications of some of these proteins. These changes in the repertoire of molecules expressed by tumor cells increase the antigenicity of tumor cells and allow the activation of lymphocytes capable of recognizing and destroying tumor cells.² B lymphocytes, by means of their surface immunoglobulins, recognize tumor antigens expressed on the cell membrane in their native conformation. On the other side, by using their antigen-specific T cell receptor (TCR), T cells recognize processed antigens presented as short peptides by MHC class I and class II molecules, leading to the activation of CD8 and CD4 T lymphocytes, respectively. Although B cell-mediated

immunity is important in the antitumor response, and antibodies and antibody-derivatives (e.g. CAR-T therapies) have been successfully used in tumor immunotherapy, and due to the relevance of cellular immunity, this review will focus on antigens presented by MHC molecules for T lymphocyte recognition. Lymphocyte recognition of tumor antigens determines the interactions between tumor cells and the immune system, modulates tumor growth, sculpting the tumor antigen landscape and shaping the effect of immune-based therapies.³ Therefore, identification of these antigen molecules expressed by tumor cells has been of paramount importance, not only to study and understand tumor/immune interactions, but to unravel mechanisms of response to therapies as well as to design new immunotherapies. For these reasons, during the last decades, with the implementation of new methodologies, a plethora of tumor antigens has been identified, and different studies have been carried out to characterize their expression pattern, considering differences between healthy vs tumor tissue, frequency of expression among individuals with a type of tumor, as well as their expression by different tumor types.^{4,5} These features may determine their immunogenicity, as expression in healthy cells promotes immune tolerance mechanisms and potential applicability. From initially identified antigens using cDNA libraries to the newly discovered antigens by means of next-generation

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sequencing (NGS) technologies and immunopeptidomics, a number of antigens with different expression patterns and immunogenicity levels have been described. They include overexpressed, differentiation and oncofetal antigens, among the different types of classical tumor-associated antigens,⁴ as well as those more tumor-specific antigens, such as antigens derived from viral sequences.⁶ Belonging to this last group of tumor-specific antigens, neoantigens (neoAgs) generated from mutations have emerged during the last years as a category of tumor antigens with important implications in cancer immunotherapy. These mutations or alterations, which result in new amino acid sequences or in post-translational modifications of original sequences, generate new epitopes recognizable by lymphocytes. In this review, we will first focus on mutated neoAgs and second, in a new category of non-classical antigens. As opposed to classical antigens, this last group usually includes proteins that have not been annotated in databases and can be added to the already available list of tumor antigens. Precise knowledge of these antigens, their expression, immunogenicity and how these features impact tumor growth and response to therapies, may help to develop better immunotherapies.

Neoantigens originated from mutations

Types of mutations responsible for neoantigen generation

NeoAgs are generated through different mechanisms, such as non-synonymous single-nucleotide variants (ns-SNVs), insertions and deletions (INDELs) and gene fusions (Figure 1a).⁷ Mutated peptides resulting from the processing of these new sequences must bind to MHC molecules and be presented on the cell surface, so that they can be finally recognized by those T cell receptors (TCR) that have escaped central tolerance mechanisms.^{7,8} Lack of tolerance against these sequences is a common event, since they are not expressed by healthy cells, with the exception of neoAgs generated from mutations originating familial cancers.

Ns-SNVs-derived NeoAgs

Among the different neoAgs, the most commonly studied are those originated from ns-SNVs, since in the majority of cases, and in different tumor types, the majority of neoAgs-specific T cells found recognized ns-SNVs.⁹ However, the identification of higher rates of ns-SNV-specific T cells may be biased, since methods for identification of ns-SNVs are usually more developed and reliable than those used for identification of

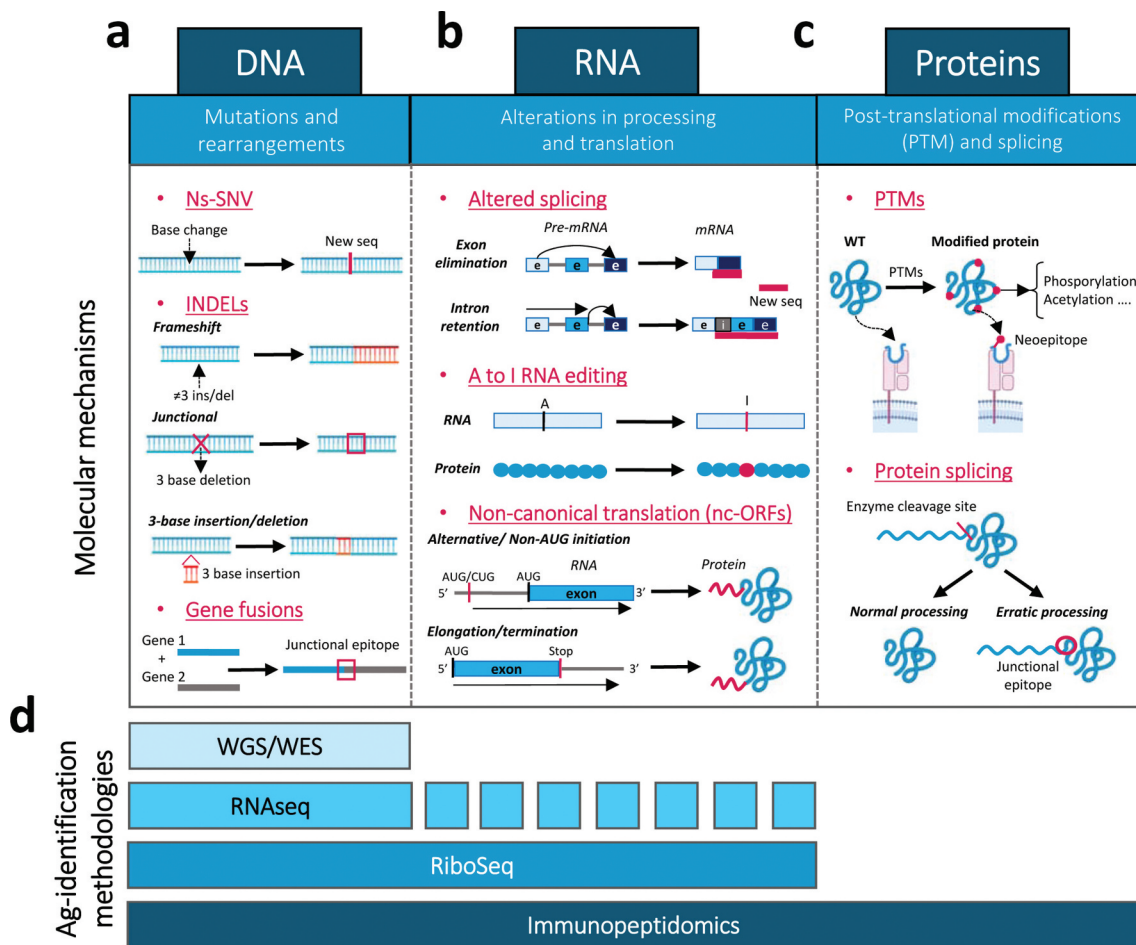


Figure 1. Molecular mechanisms responsible for the generation of tumor antigens at different cellular processes: methodologies to identify these antigens. New tumor antigens and epitopes are generated by different molecular mechanisms: (a) mutations and re-arrangements at the DNA level, including ns-SNVs, INDELs and gene fusions; (b) alterations in RNA processing and translation, such as altered splicing, A to I RNA editing and processes of non-canonical translation due to alternative initiation, elongation or termination; and (c) post-translational protein modification and splicing. (d) Several antigen identification methodologies can detect these new antigens or neoepitopes, including WGS/WES for DNA alterations, RiboSeq and RNAseq for DNA and RNA alterations (although not all RNA-associated alterations can be detected by RNAseq) and immunopeptidomics, which can detect any protein change originated from DNA, RNA or protein modification events.

INDELs or gene fusion antigens.^{7,10,11} Also, ns-SNVs are not submitted to non-sense mediated decay (NMD) mechanisms that eliminate transcripts with early stop codons usually generated after frameshifts.¹² Generation of neoAgs, including those derived from ns-SNVs, may occur by two different mechanisms: 1) by enhancing binding of the mutated peptide to MHC molecules; 2) by generating a different amino acid at TCR contact sites (Figure 2). By making visible a peptide that was not presented on its original sequence or by generating a new sequence to be recognized by a new set of TCR, these mutations play a significant role in tumor antigenicity, as they can induce highly tumor-specific T cell responses, with a lower risk of cross-reactivity with normal cells. Nevertheless, the number of immunogenic antigens derived from ns-SNVs is low compared to the total identified ns-SNVs, which indicates

that only a small proportion of mutated sequences are finally true neoAgs. Systematic studies analyzing immunogenicity of mutation-harboring peptides by using T cell stimulation assays or MHC-multimer technology have demonstrated that in different tumors (e.g. ovarian, gastrointestinal and melanoma cancers),¹³⁻¹⁵ only a small proportion of peptides are immunogenic and generate T cell responses. In some cases, this may be related to amino acid changes that do not fulfil one of the two criteria for effective recognition by T cells indicated above. However, in other cases, the immunosuppressive tumor microenvironment may preclude priming of neoAg-specific immunity despite the presence of peptide structural features compatible with immunogenicity.

Most ns-SNVs-derived neoAgs are patient-specific, although some shared neoAgs have been described among

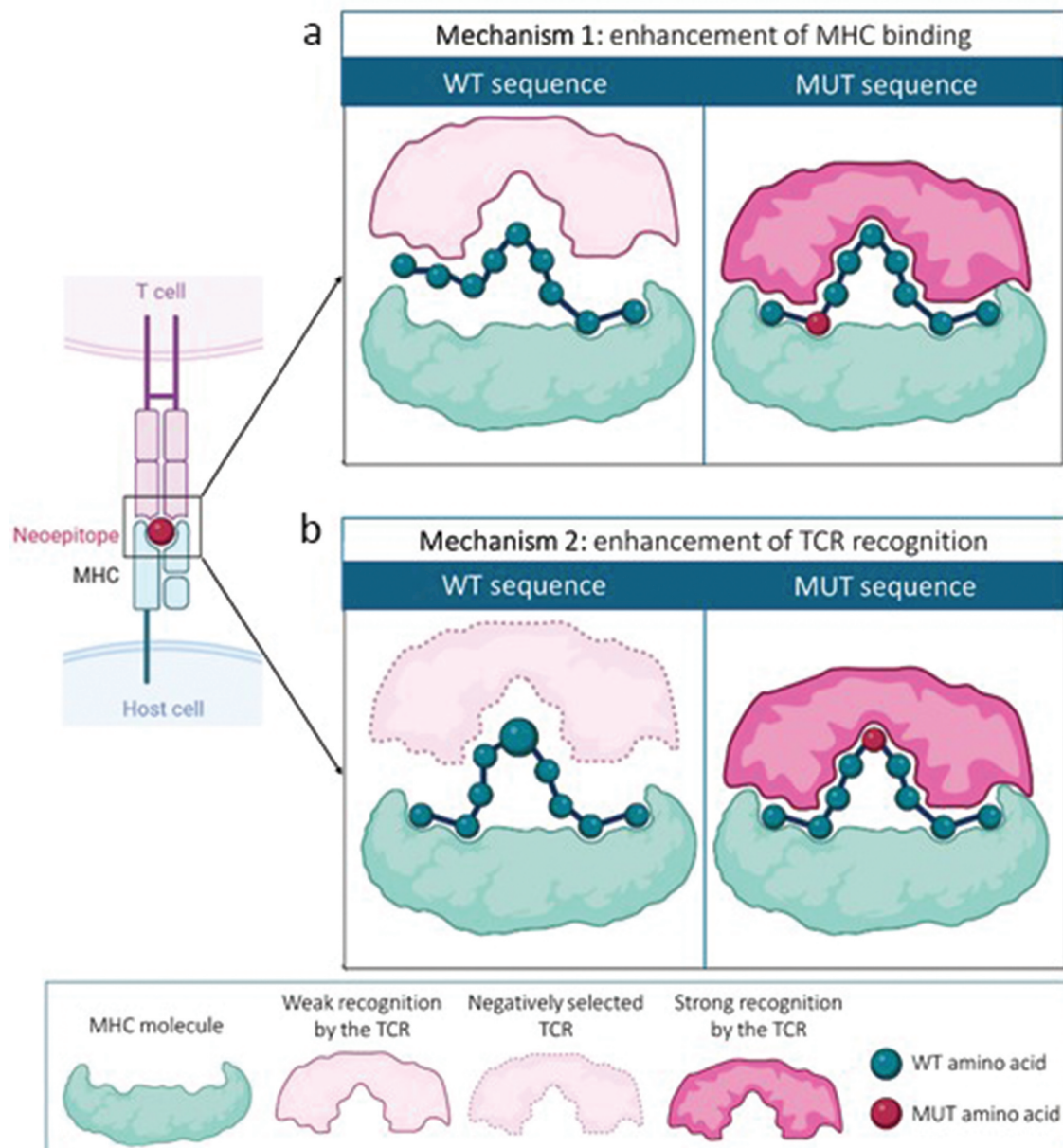


Figure 2. Neoantigen generation mechanisms. (a) neoantigens can be generated by a change of an amino acid located at an MHC-anchoring position, enhancing binding to MHC molecules and favoring a strong recognition by a TCR. (b) In other cases, the mutation occurs at an amino acid located at a TCR recognition site. The mutated amino acid has to differ sufficiently from the WT version to allow recognition of this neoepitope by a TCR that has escaped central tolerance mechanisms.

patients (e.g. KRASG12D in patients with pancreatic and colorectal cancer).^{16,17} Shared neoAg are considered an interesting target for “off-the-shelf” vaccination and other immunotherapeutic approaches, such as those based on transgenic TCRs. The challenge faced in this case is that shared neoAg are less prevalent with respect to the total neoAg load identified in each patient and importantly, neoAg are HLA-restricted, indicating that immunogenicity of a mutated peptide may depend on the HLA molecules expressed by the patient.^{18–20} Interestingly, a recent study using a high-throughput discovery platform has identified a considerable number of neoAg shared among patients and tumor types.²¹ On the other side, the fact that the majority of neoAg are private supposes a change of the cancer treatment paradigm into the development of personalized therapies and vaccines.¹⁸ For this matter, standardized neoAg identification protocols and vaccine production have to be established for large-scale applications.

INDEL- and gene fusion-derived NeoAg

NeoAg derived from INDELS and gene fusions may differ more from the original sequence than those generated by a single amino acid change, as is the case of ns-SNVs. These changes, especially those leading to frameshifted sequences, can generate new ORFs encoding sequences with a higher number of amino acid changes, increasing the possibility of generating neoAg and resulting in a higher immunogenicity. A pan-cancer study analyzing the load of ns-SNVs and INDELS and the possibility of neoAg generation from these mutations revealed different levels of INDELS across cancer types. Interestingly, despite a higher number of ns-SNVs and their corresponding putative neoAg, the number of neoAg per mutation was clearly higher for INDELS. With regard to the clinical significance of these mutations, the INDEL count was significantly associated with response to checkpoint inhibitor immunotherapy in several cohorts of melanoma patients.⁹ Similar results have also been reported in other patient groups,^{22,23} suggesting the relevance of the immunogenic potential of INDELS. In addition to these studies reporting the INDEL and the corresponding putative neoAg load, some initial studies also demonstrated T cell responses specific for INDELS corresponding to TGFBR2 or CDKN2A.^{24,25} Finally, in a more recent study carried out in patients with kidney cancer, the presence of INDEL-specific T cells was demonstrated by using the MHC multimer technology, which corresponded to the 21% of the recognized neoAg.²⁶

Similar to INDELS, fusion-derived neoAg have to be considered as an additional source of tumor antigens.²⁷ The high level of chromosomal instability observed in cancer results in chromosomal translocations or inversions that can cause gene fusions and the formation of hybrid proteins. Some of them, such as the BCR-ABL fusion protein, are oncogenic and result in specific epitopes from the fusion region that can be recognized by T cells.^{7,28} However, the number of well-described gene fusions is low, and the fusion region may vary between patients,²⁹ hindering clinical translation. Nevertheless, a pan-cancer study of fusion-derived neoAg revealed that these neoAg tend to have a higher immunogenic potential, as compared with neoAg derived from ns-SNVs or INDELS,

although the burden of these neoAg is not related with immunotherapy outcome.²⁹ Therefore, these mutated sequences can generate an additional set of potential neoAg with clinical relevance.¹⁹

Identification of mutation-derived NeoAg

Advances in NGS technologies and bioinformatic tools have enabled the identification of mutations and prediction of mutation-derived neoAg, allowing the development of different therapeutic strategies focused on neoAg as targets.^{30,31} For this purpose, different algorithms have been developed and are currently applied. Briefly, neoAg identification requires first whole genome/exome sequencing of tumor and healthy tissue, alignment to a reference genome and, after eliminating germline polymorphisms, identification of mutations by using variant caller tools (e.g. VARSCAN, SomaticSniper, Mutect2, etc).^{32–37} This results in a list of mutations that can be useful for tumor mutational burden (TMB) calculation, with potential applicability for clinical purposes. To predict neoAg within the list of mutations, most algorithms rely on HLA binding prediction algorithms (reviewed in Xie et al.¹⁹). Sequences of mutated peptides are assayed against the panel of HLA alleles expressed by the patient, previously identified from sequencing data. In addition to HLA binding, parameters such as dissimilarity with the self-proteome, similarity with epitopes present in pathogens or the presence of flanking amino acids favoring antigen processing, have also been considered by some algorithms.^{38–41} Together with these intrinsic immune-related properties of putative neoAg, other factors such as tumor heterogeneity play an important role. Indeed, differences between clonal and subclonal neoAg may determine their relevance in tumor rejection and edition, dictating the interactions between the tumor and immune system.⁴² Finally, if available and as an additional filter for neoAg identification, gene expression obtained by RNASeq can be used to identify those putatively expressed neoAg.³⁰ In this respect, it has been recently reported that many published mutated neoAg are also expressed (at the RNA level) in normal tissues and cannot be considered as true neoantigens, suggesting the need of using adequate tools to identify tumor-specific neoAg.⁴³ Overall, this strategy bears important limitations, such as the suboptimal prediction capacity of some algorithms (e.g. for less common HLA alleles), the inability to identify post-translational modifications that affect immunogenicity or the identification of peptides generated from splicing of proteins.⁴⁴

An alternative less used approach, not relying on gene sequencing, is immunopeptidomics, based on proteomic technologies. In this case, tumor tissue is lysed and peptide/HLA complexes are purified by using immunoprecipitation with anti-HLA antibodies, and after peptide elution, they are identified by mass spectrometry analysis, by matching peptide sequences with those found in databases or obtained from NGS data using patient tumor cells.^{45–48} The main advantage of this methodology is that it can identify peptides derived from genes with confirmed expression and translation. Since they are eluted from HLA molecules, identified peptides fulfil the requirements for antigen processing and binding to HLA molecules, not relying on prediction algorithms. On the other

side, the amount of biological material required for these studies is usually higher than that used for gene sequencing, although in recent years these methodologies are being improved.^{49,50}

However, in the absence of additional assays analyzing the presence of neoAg-specific T cells, none of the identification strategies mentioned above provide any information about neoAg immunogenicity. To gain insight on this, different assays have been carried out. A classical assay involves stimulation of patient T cells with peptides or tandem minigenes containing the whole set of predicted/identified neoAgs to detect T cell activation.⁵¹ In this context, besides to classical techniques measuring cytokine production or upregulation of activation markers, immunogenicity of neoAgs has been tested by using a fast, easy and very efficient method called MANAFEST, which detects changes in the frequencies of T cell clonotypes.⁵² Alternatively, fluorochrome-tagged HLA multimers loaded with the selected neoAgs are used to label T cells and identify those with neoAg specificity.⁵³ In this last case, any T cell specific for the neoAg of interest can be detected, irrespective of their functional capacity. Since in Ag-based stimulation assays, nonfunctional T cells are not detected, one cannot exclude that the lack of response is due to the poor/null immunogenicity of the neoAgs selected or to the immunosuppressive tumor microenvironment (TME) and highly differentiated and exhausted state of T cells observed in many patients. These methods have been used not only to confirm immunogenicity of neoAgs predicted by HLA binding algorithms or identified by immunopeptidomics but also in unbiased screening procedures, considering all peptides with mutations, irrespective of the HLA of the patient.⁵⁴ Besides the enhanced complexity of these technologies, availability of lymphocytes from the patients is necessary, making difficult in some cases the characterization of neoAg immunogenicity. Alternative approaches to overcome this include the use of blood from HLA-matched healthy donors (with a better lymphocyte availability and lacking tumor-induced immunosuppression) to perform in vitro priming assays,⁵⁵ and in vivo experiments using transgenic mice expressing the HLA alleles of interest.^{56,57} Taken as a whole, new methodologies have been developed to identify neoAgs generated by different types of mutations, helping to characterize their immunogenicity.

Impact of specific genomic alterations on the immune response to neoantigens

Several studies have found an association of high TMB with increased immune infiltration and response to immunotherapy.⁵⁸ However, the utility of TMB as a predictive biomarker has not been fully demonstrated across all cancers.^{58,59} Cancer types where TMB does not predict response generally show no relationship between neoAg load and T-cell infiltration. There are some genetic alterations, i.e. deletion and loss-of-function mutations in *HLA* and *B2M* genes, that impair antigen presentation,^{60–63} and T-cell recruitment into tumors. There are also some genetic variants that play an important role in the evasion of antitumor immunity. One of the first pathways to be related to poorly inflamed

tumors, despite containing a high number of mutations, was the WNT/ β -catenin signaling pathway.⁶⁴ Activation of this pathway inhibits the recruitment of dendritic cells of the Batf3 lineage, thus decreasing the infiltration of CD8+ T cells, through the negative regulation of CC chemokine ligand 4 (CCL4).⁶⁵ Various gene mutations are involved in the activation of the WNT/ β -catenin pathway. Thus, in addition to gene copy number changes, somatic mutations involving activation (exon 3) of CTNBN1 or loss of function of genes such as APC, APC2, AXIN1 and AXIN2, contribute to its activation.⁶⁶ Another example is mutations in the RNF43 gene, which are common in high microsatellite instability (MSI-H) tumors. It has been recently observed that frameshift mutations in RNF43 implying loss of functions are associated with non-inflamed TME by activating the WNT/ β -catenin signaling pathway.⁶⁷

Inactivating mutations in Janus kinases (JAK1 or JAK2) produce defective IFN γ signaling with multiple effects, including altered antigen presentation and failed recruitment of T cells into the tumor.^{68–70} On the other hand, loss-of-function mutations of the PTEN gene cause increased activation of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway and is also significantly associated with a non-inflamed tumor phenotype.⁷¹ Mutations in RHOA⁷² and hepatic serine/threonine kinase B1 (LKB1; also known as STK11)^{73,74} have also been associated with an immunosuppressive TME and reduction in tumor-infiltrating lymphocytes. Inactivating mutations of the Trp53 gene have also been associated with low immune infiltration of tumors.⁷⁵ Notably, TP53-mutated tumor cells lack the production of key chemokines necessary for the recruitment of NK cells and T cells to TME.⁷⁶ In glioma, mutations in the IDH1 and IDH2 isocitrate dehydrogenase genes that confer the ability to convert α -ketoglutarate into the oncometabolite R-2-hydroxyglutaramate⁷⁷ are also associated with low-infiltrated tumors.⁷⁸ Accumulation of R-2-hydroxyglutaramate limits intratumoral production of CXCL9 and CXCL10 chemokines, resulting in decreased T-cell recruitment.⁷⁸

Taking these findings into account, we must evaluate not only the number of neoantigenic mutations but also the presence of other genetic alterations that may play an important role in the evasion of antitumor immunity.

NeoAgs and efficacy of cancer immunotherapies

Since immune responses against tumor cells constitute the basis of immunotherapies, tumor antigens recognized by therapy-boosted cells play a prominent role.^{18,79,80} There are immunotherapeutic strategies that rely on antigen-agnostic mechanisms, including immune checkpoint inhibitors (ICI), IL-2 administration, adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs) or the use of oncolytic viruses. However, despite this, all of them stimulate T cells that comprise a pool of cells with specificity for tumor antigens. On the other side, strategies like vaccines and transfer of TCR- or CAR-transduced T cells fully depend on the knowledge of the antigen to be targeted. Therefore, either for immunotherapy design, to understand their efficacy or for patient selection, many studies have focused on the analyses of immunotherapy-

targeted antigens, characterizing their abundance, properties, specificity/similarity with self-proteins, etc. In this regard, neoAgs have also been considered as an important factor in the efficacy of some immunotherapies.

Immune checkpoint inhibitors and NeoAgs

Immune checkpoint inhibitors (ICI) have become the most common cancer immunotherapy used in the last decade. This therapy is based on the administration of antibodies blocking signaling pathways triggered by T lymphocyte negative receptors and denominated immune checkpoints.^{81,82} Although many molecules have been described in this group, so far most clinical applications involve blockade of PD-1/PD-L1 and CTLA-4-derived pathways. Inhibition of these negative signals rescues functional properties of tumor-specific T lymphocytes, rendering them capable of eliminating tumor cells. Since not all patients respond to ICI, important efforts have been carried out to identify biomarkers of response for patient selection.⁸³ Among them, TMB has emerged as an important feature. Initial studies carried out in highly mutated tumors like melanoma^{84,85} and lung cancer⁸⁶ demonstrated an association between TMB and response to ICI. Later, this was extended to other tumors, including those with MSI-H and/or mismatch repair deficiency (dMMR).^{87,88} By considering a set of patients with tumors bearing different TMB levels, Yarchoan et al. reported a positive correlation between TMB and response rate to PD-1/PD-L1 blockade,⁸⁹ and this sensitivity to ICI increases when focusing on clonal neoAgs.⁴² As indicated above, the mechanistic explanation postulates that tumors with a higher TMB would present increased neoAg levels, resulting in more “strange” or immunogenic tumors. Consequently, this would favor lymphocyte priming and tumor infiltration, deriving a higher possibility of being rescued by ICI. Although this correlation is true for heavily mutated tumors, where the high number of mutations may generate a sufficient number of neoAgs, it does not fit equally well for tumors with low or moderate TMB. Interestingly, for these tumors, neoAg load may behave as a better biomarker than TMB to predict response to ICI. In this regard, neoAg load, but not TMB, accurately predicts treatment efficacy in melanoma, lung cancer and gynecological tumors, whereas it has a poorer predictive capacity in urinary system and liver tumors.⁹⁰ Association between neoAg load and response to ICI can be observed not only when considering the classical mutations originated from SNV but also from frameshift mutations, known to generate a higher number of neoAgs per mutation.^{9,91}

However, most of these studies were inferred on associations between TMB or neoAg load with T cell immunity and concomitant response to ICI, without any experimental demonstration of the presumed molecular and cellular mechanisms. In a study across different cancers of patients where high TMB does not predict response to ICI, there is no association between neoAg load and CD8 T cell infiltration,⁹² indicating the relevance of these mechanisms mainly when infiltrating T cells are neoAg-specific. More recently, Puig-Saus et al. have demonstrated the existence of neoAg-specific T cells in patients responding to antiPD-1, at superior levels to those found in non-responder patients.⁹³ Interestingly, the

T cell responses to mutational neoAgs were not linearly correlated with the number of mutations. However, in another study in patients with metastatic urothelial carcinoma, early expansion of neoAg-specific T cells associated with the response to ICI.⁹⁴

Adoptive T-cell therapy and neoAgs

Treatment based on the passive administration of antitumor T cells is called adoptive T-cell therapy (ACT). ACT using TILs has been applied for years in different tumors, with pioneer studies in melanoma. In a randomized prospective study, TIL therapy demonstrated objective responses in 54% of melanoma patients, including 24% complete responses, which in most cases were durable.⁹⁵ The lack of toxicity to normal tissues in these trials suggested that the transferred TILs targeted molecules unique to cancer cells. Indeed, a series of retrospective studies revealed that infused TILs frequently targeted mutation-derived neoAgs.^{51–96–99}

Although TIL therapy has been shown to be very effective in treating melanoma, this has not been the case for other tumors. Interestingly, in tumors other than melanoma, the conventional procedure to expand TILs does not yield tumor-reactive TIL products with the same efficiency.¹⁰⁰ Among the possible reasons for this low reactivity are the lower number of mutations of these tumor types, and consequently, the lower frequency of mutation-reactive TILs. For some years now, a series of methods have been developed to detect somatic mutation-reactive autologous T cells and specifically increase their frequency in the infusion product.¹⁰¹ A series of clinical case reports provided the first evidence that enriching TILs in neoAg-specific T cells can mediate durable tumor regressions without causing toxicity in patients with non-melanoma epithelial cancers.^{17,102,103} An illustrative case is that of a patient with refractory hormone receptor-positive breast cancer who was treated with an autologous TIL product enriched in T cell clones recognizing four different neoAgs (SLC3A2, KIAA0368, CADPS2 and CTSE), representing the 23% of the infused product. Treatment with this TIL product in combination with pembrolizumab produced complete and long-lasting regression of multiple metastases.¹⁰³

However, the low presence of neoAg-specific TILs and their highly differentiation and exhaustion state in the tumor sample may make it difficult to obtain infusion products sufficiently enriched in neoAg-reactive cells to have therapeutic efficacy.¹⁰⁴ Further application of ACT led to the development of techniques to genetically modify normal peripheral blood T cells to express a receptor capable of recognizing the tumor, providing them with antitumor activity.¹⁰⁵ Genetic modification of normal peripheral blood T cells with an exogenous TCR specific for a neoAg (neoTCR) could be an interesting alternative to TILs targeting somatic mutations. The fact that neoAg are highly tumor-specific antigens would mitigate the danger of off-target toxicities observed with TCRs directed at self-antigens.¹⁰⁶

Among the different types of neoAgs, those derived from driver mutations are attractive targets for TCR therapy. This enables the development of off-the-shelf neoTCRs that could be used to treat a larger number of cancer patients who express the mutation and the adequate HLA restriction. This is the

case of KRAS, an oncogene that is frequently mutated in pancreatic and colorectal cancer. Recently, a patient with progressive metastatic pancreatic cancer showed objective response after treatment with a mixture of autologous T cells that had been genetically modified to express two HLA-C*08:02-restricted TCRs targeting the KRAS G12D driver mutation,¹⁷ expressed by the patient's tumors.¹⁰⁷ Additional TCRs targeting KRAS G12D and other hot-spot KRAS mutants restricted by different HLA molecules have been identified from TIL cultures^{108–110} that could be used to extend TCR therapy against mutant KRAS to a larger number of patients. TCR libraries against other driver mutations, such as TP53, BRAF, PIK3CA and EGFR, may be promising.

However, most neoAgs result from random passenger mutations, behaving as patient-specific or 'private' neoAgs. TILs specific to these mutations have been found in various types of cancer.^{51,96,97,102} Although private neoAgs may be subjected to clonal heterogeneity, an important mechanism of resistance to immunotherapy, TIL therapy directed against this type of neoAgs has been able to generate prolonged responses,^{102,103} deserving thus to be considered as a potential target of ACT. However, while private neoAgs are feasible targets for TIL therapy, they pose a significant challenge for TCR therapy because they require customization. Recently, a first-in-human Phase I clinical trial (NCT03970382) has demonstrated the feasibility of manufacturing multiple neoTCR engineered T cells using non-viral precision genome-editing. The safety of infusing up to three gene-edited neoTCR T cell products has also been demonstrated. In summary, the success of the first ACT trials was based on the presence of NeoAg-specific TILs and is now moving toward the genetic engineering of T cells with neoTCR. However, neoTCR identification remains challenging and the process must be tailored to clinical timelines.

In conclusion, currently used immunotherapies, including those based on ICI and adoptive cell transfer, have an important component associated with neoAg-specific responses, indicating the relevance of potentiating immunity against these antigenic targets.

Vaccines based on neoAgs

Upon identification of mutation-derived neoAgs in murine tumor models, immunization experiments using the mutation-containing peptides demonstrated not only the immunogenicity of these sequences but also their capacity to reject established tumors,^{111,112} suggesting the potential applicability of these antigens in vaccination strategies. There are several examples of neoAg-based vaccines carried out in murine models where the antitumor properties of these vaccines have been demonstrated.^{113–116} They include not only CD8 epitopes, initially considered as major rejection antigens, but also CD4 epitopes,¹¹⁷ which in addition to priming CD4 T cells with antitumor properties, may contribute to strengthen activation of CD8 responses.⁵⁶

Given the specificity and potency of neoAg-based vaccines in the preclinical setting, this prompted the development of vaccination clinical trials in cancer patients, becoming

vaccination the most common strategy based on neoAgs. To date, around 150 neoAg-based vaccine clinical trials have been initiated.¹¹⁸ Pioneer studies were carried out in melanoma patients, due to the high neoAg load present in these individuals. Carreno et al. used dendritic cells pulsed with CD8 epitope peptides to demonstrate induction of tumor-specific responses, where a broadening of antigenic breadth and clonal diversity was observed.¹¹⁹ Additional clinical trials have been reported (reviewed in Niemi et al.¹¹⁸), not only in melanoma,^{119–121} but also in glioblastoma,^{122,123} gastrointestinal cancer,¹²⁴ lung cancer,¹²⁵ pancreatic cancer¹²⁶ and hepatocellular carcinoma,¹²⁷ among others, indicating that vaccination is not only feasible in tumors with high TMB but also in those with a lower TMB. In general, the results of these studies have demonstrated safety in the vast majority of cases, and immunogenicity has also been described, resulting in the induction or boosting of CD8+ and CD4+ tumor-specific T cell responses. Indeed, in a pan-cancer clinical study administering neoAg peptide pools, similar safety and immunogenicity results were reported. Twenty out of 22 patients had no or mild adverse effects, 90% of them had measurable T cell activation and around 80% of peptides elicited immune responses.¹²⁸ Although most clinical trials are based on the administration of neoAgs derived from ns-SNVs, there are examples of vaccines containing immunogenic frameshift peptide neoAgs. As in other trials, colon cancer patients with MMR-deficient tumors included in this trial did not experience severe adverse effects, and humoral and cellular immune responses were observed in all patients.¹²⁹ Similarly, the Nous-209 "off-the-shelf" vaccine encoding frameshift peptides¹³⁰ has progressed to clinical phases, with a demonstrated immunogenicity in all vaccinated patients.¹³¹

In addition to safety and immunogenicity, interesting clinical responses have been observed in melanoma cohorts. As described by Ott et al.,¹²⁰ four out of six patients showed no recurrence for up to 25 months and two of the recurrent patients that were subsequently treated with anti-PD-1 presented complete tumor regressions. This was also described by Sahin et al.,¹²¹ whose metastatic melanoma cohort treated with a neoAg RNA-based vaccine showed two out of five patients with complete responses, and a third patient who was treated with anti-PD-1 therapy after vaccination also reached complete response. In a cohort of 29 patients with glioma vaccinated with the H3.3K27M shared neoAg, 39% demonstrated detectable T cell responses, associated with prolonged overall survival.¹³²

Despite these results demonstrating the feasibility, safety and immunogenicity of neoAg-based vaccines, just a few patients presented clinically relevant responses when vaccines were administered as monotherapy. Since vaccines boost the first step of the cancer-immunity cycle and the generated response can enhance the expression of immune checkpoints as an escape mechanism, the combination of neoAg-based vaccines with ICI appears as a better therapeutic strategy. In fact, some clinical trials based on the administration of vaccines + anti-PD-1/PD-L1 have demonstrated clinical efficacy, exemplified by the combination of the neoAg vaccine NEO PV 01 and nivolumab in melanoma, NSCLC and bladder cancer patients. In this clinical trial, considerable ORR rates were observed in the three cohorts (59%, 39% and 27%, respectively)

and longer progression-free survival.¹³³ These results have encouraged the development of vaccines in combination with ICI, which are currently being studied in different solid tumors with the inhibition of the PD-1/PD-L1 axis (NCT02287428, NCT03359239 and NCT04397003) or also in combination with anti-CTLA-4 monoclonal antibodies (NCT04117087, NCT03606967). Clinically relevant results have been achieved in tumors with a high TMB such as melanoma or lung cancer.^{119–121} This type of tumors also presents better clinical responses when receiving other therapies such as ICI, probably due to the presence of a high number of driver and clonal mutations that generate neoAgs and makes them more immunogenic. Nevertheless, as mentioned above, patients with tumors containing a lower TMB have also been enrolled in these trials. In line with combinatorial strategies, a recent work has been shared in the American Association for Cancer Research (AACR), where Khattak A. et al.¹³⁴ presented their phase II clinical trial results of the combination of mRNA-4157/V940 vaccine with pembrolizumab in patients with advanced melanoma with a high risk of recurrence after surgery. Patients receiving this combination presented 78.6% of recurrence-free survival, while the control arm that received pembrolizumab alone achieved a 62.2%. These data indicate a 44% reduction in the risk of recurrence and death in patients receiving the combination. When considering the risk of recurrence according to TMB (dividing their cohort using the threshold of 10 mutations per megabase (mut/Mb)) a similar reduction in the risk of recurrence or death was observed, presumably due to the high overall TMB values in both groups. In the same line, Rojas et al. have reported results of their neoAg RNA vaccine in 16 patients with pancreatic cancer, administered in combination with atezolizumab (anti-PD-L1) and chemotherapy in the adjuvant setting. A significantly longer median recurrence-free survival was observed in those patients with vaccine-induced T cell expansion.¹³⁵ In summary, all these results indicate the feasibility of neoAg vaccine preparation and administration to a variety of cancer patients, which in most cases are immunogenic and, when applied mainly in combination with ICI, yields promising clinical results. Notwithstanding, several challenges are ahead, and improvements in their design, generation and application are still needed.¹³⁶

Non-classical tumor antigens

Other than classical tumor-associated antigens and neoAgs generated by mutations described above,¹³⁷ novel tumor antigens can derive from additional alterations or changes in RNA maturation and translation or in protein processing (reviewed in Xie et al. and Nagel et al.^{19,138}) Antigens deriving from transposable elements have also been described, but they are not discussed in this review.¹³⁹

Tumor antigens derived from altered RNA processing or non-canonical translation

Several mechanisms related to RNA processing and RNA translation into proteins may suffer from aberrant events, originating new sequences (Figure 1b).

Antigens derived from altered splicing

Several cis-acting sequences and trans-acting factors are involved in the modification of newly transcribed RNA that eliminates large intronic sequences and joins neighboring exonic regions. This is a well-controlled and cell-type specific process known as splicing. Mutation or deregulation in splicing factors or genomic changes affecting cis-acting regions are abundant in tumors, resulting in elimination of exons (exon skipping) or intron retention, which is more prevalent in cancer.^{140–142} This altered splicing may result in novel cancer-specific proteins and tumor antigens, some of which are shared among patients.^{143,144} A well-known example of a tumor antigen derived from altered splicing is the EGFRvIII driver mutation mentioned above as a candidate for antigen-specific CAR-T therapies.^{145,146} In some cases, altered splicing results in transcripts with premature termination codons that are degraded by NMD. Thus, the antigens derived from these are poorly abundant (see below, altered RNA stability). However, some introns have coding potential and lack stop codons (exitrons). Retention of these introns results in transcripts that are invisible for the NMD machinery and high levels of tumor antigens.¹⁴⁷ Also, resistant to NMD are transcripts with skipped exons that keep the reading frame. Proteins translated from these transcripts produce tumor antigens at the novel exon–exon junctions or neojunctions, which are more likely to be shared among patients than those generated from SNV mutations.¹⁴⁸ In spite of this, additional work is required to ensure that neoAgs derived by altered splicing are indeed cancer-specific. Also, further experiments are required to evaluate the potential of drugs that affect splicing to increase the immunogenicity of cold tumors.^{149,150}

Antigens derived from altered RNA modification and stability

As indicated above, NMD decreases the stability of transcripts with premature termination codons (PTCs). Interestingly, there is a pioneer round of translation prior to NMD, and the protein resulting from this translation is preferentially degraded and transported to MHC molecules.¹⁵¹ However, the bulk of transcripts with PTCs, including those caused by frameshifting, is degraded by NMD, and therefore, they cannot produce antigens. Therefore, pharmacological inhibition of NMD has been shown to produce larger amounts of tumor antigens.¹⁵²

RNA stability is also controlled by polyadenylation. When this occurs at introns or non-canonical sites it can alter transcript stability but also nuclear export, cell localization and protein translation, leading to the production of truncated or novel proteins and tumor antigens.¹⁵³ RNA modification can also affect different steps in RNA processing and translation. RNA editing can change specific nucleotides in the RNA sequence, leading to novel antigens, and it is also altered in several cancers.¹⁵⁴ Most studied is adenosine-to-inosine (A-to-I) editing, which produces highly immunogenic neopeptides.¹⁵⁵ Further studies are required to demonstrate whether they are tumor specific.

Antigens derived from non-canonical translation

It has been established that only ~2% of the genome is transcribed and translated into canonical proteins.^{156,157}

Recently, this has been revisited, as many regions previously classified as “non-coding” have been shown to contain short or non-canonical open reading frames (ncORFs).¹⁵⁸ ncORFs can be translated to proteins shorter than 100 amino acids or microproteins or to proteins that do not follow the rules of canonical translation: they do not start with ATG or they are located upstream, downstream or overlapping with well-described coding sequences.¹⁵⁹ Most of them have been ignored by the scientific community until very recently, as most are not conserved and many are not stable.^{160–162} Interestingly, ncORFs have been described in cancer-specific transcripts expressed in several cancer patients and classified as long non-coding RNAs.^{163,164} As these ncORFs are predicted to contain disordered and hydrophobic regions, it is expected that they are more visible to the proteasome and with stronger potential to generate epitopes.^{162,165} ncORFs may contribute to over 10% of the total immunopeptidome.^{166–168} This number could be underestimated as some MS spectra originally assigned to canonical proteins could indeed correspond to non-canonical ones.¹⁶⁹ In addition, the total number of human ncORFs is yet unclear. While conservative estimates consider around 7,000, others find certain evidence for the existence for several hundred thousand.¹⁷⁰

Antigens derived from aberrant mRNA translation

Aberrant translation deserves a special chapter within non-canonical translation. Hypoxia and amino acid starvation caused by tumor growth, coupled with inefficient angiogenesis and insufficient blood flow, activate the integrated stress response (ISR).¹⁷¹ ISR signaling alters canonical translation initiation, resulting in a stress-associated proteome devoted to activate survival in the hostile environment or induce apoptosis. Such stress-associated proteome could comprise novel cancer-specific tumor antigens. Among all deficient amino acids, tryptophan has a special effect. Under low tryptophan, the ribosome is stalled at tryptophan codons resulting in frameshifting and codon reassignment to phenylalanine, leading to the generation of novel proteins that are a source of tumor antigens.^{172–174} Interestingly, this is cancer-specific.

Tumor antigens derived from altered protein modification and processing

In addition to DNA- and RNA-related alterations, proteins may also suffer different modifications that can render them as antigenic (Figure 1c).

Antigens derived from altered protein modifications

After translation, many proteins are modified by post-translational modifications (PTMs), resulting in specific epitopes. One example among several is phosphorylation. Aberrant phosphorylation can generate novel epitopes or can increase the efficacy of MHC binding compared to that of the non-phosphorylated form.¹⁷⁵ Some peptides have been described that are modified only in cancer cells that could lead, so far, to a limited number of tumor antigens.^{175–178}

Antigens derived from altered protein processing

Protein splicing can also be considered as a special modification. Similar to RNA, protein splicing allows the deletion of “intron” sequences while neighboring “exons” are fused. This has been well studied for FGF5, where the proteasome is the catalytic agent causing proteasome-catalyzed peptide splicing (PCPS).^{179–183} Epitopes from the junction region or splice-topes can bind HLA molecules and induce T cell responses, but it is yet unclear whether they are cancer-specific and how widespread is PCPS. While some estimates considered that 30% of the HLA ligandome could be originated from PCPS, more recent, conservative and cautious studies indicate that only around 2–6% could be explained as derived from spliced protein fragments, being unclear whether all originate from PCPS.^{184–186}

Methods to detect non-classical antigens

In addition to the methods described above for the identification of neoAgs based on whole genome/exome and RNA sequencing, novel technologies have been established that quantify canonical and non-canonical translation or that look for the peptides bound to the MHC molecules by immunopeptidomics (Figure 1d).¹⁸⁷ The finest method to evaluate translation is ribosome profiling, capable of detecting canonical and non-canonical translation but blind to further protein stability, processing or modification.¹⁸⁸ Instead, mass spectrometry (MS) should be capable of identifying any protein sequence but requires refining to detect less abundant and shorter proteins and a robust database with reliable information about the complete proteome. As we are far from the latter, the best method nowadays involves proteogenomic approaches where genomic, transcriptomic and ribosome profiling data are combined with immunopeptidomics.¹⁸⁹

Ribosome profiling

Ribosome profiling (RiboSeq) enables deep sequencing of ribosome-protected RNA fragments to determine regions of active translation.¹⁹⁰ The precision of the technology is such that it provides a fingerprint of ribosome function: it visualizes the codon shifts at base-pair resolution, allowing the identification of the complete translated ORFs. Given its high precision and unbiased acquisition, RiboSeq is capable of identifying ORFs shorter than 8 or 15 amino acids in length, which would be fundamentally challenging with other methods.^{160,191,192} However, depending on the quality of the data and the pipeline followed for analysis, many false positives can be retrieved. High-quality RiboSeq datasets should have a codon periodicity (percentage of reads 28–30 nucleotides long that identify known ORFs) over 70%, a good library complexity (meaning that most reads are unique and do not result from PCR duplicates) and allow the identification of 9–10 thousand canonical proteins in humans.¹⁷⁰ When less canonical proteins are detected, it is expected that also far less ncORFs will be identified. In a good RiboSeq library, 70–85% of the reads will be discarded and correspond to rRNA, tRNA, nuclear RNAs and even repetitive regions from retrotransposons or

pseudogenes, which are difficult to resolve. The remaining 15–30% of the reads will be mapped as ribosome footprints and 80% of them will correspond to canonical ORFs.¹⁹³ Thus, from a sequencing depth of 150 million reads, just around 5 million reads are left for ORF discovery.

Interestingly, this leaves enough space to identify ORFs that are not found in proteomic studies. The major reason for this is that, unlike with conventional Liquid Chromatography with tandem MS (LC-MS/MS), with RiboSeq data there is no penalty in the identification of short proteins, proteins enriched in lysines or arginines (generating peptides that are too small for MS after trypsin digestion) or proteins with low stability or low abundance, as sequencing methods have a PCR amplification step.¹⁶⁰ More importantly, databases comprising ncORFs predicted by RiboSeq (with more or less confidence) can be used to interpret spectra obtained by LC-MS/MS.

Immuno-peptidomics

As indicated above, identification of small non-canonical proteins that could serve as novel tumor antigens was inefficient following conventional tryptic or semi-tryptic (resulting from total or partial protein cleavage at the C-terminal side of a lysine or arginine) LC-MS/MS techniques.^{194–196} Protocols to enhance identification of non-canonical proteins included enrichment for short proteins with size exclusion chromatography or gel filtration and were not as successful as originally expected. This changed drastically with the advent of immuno-peptidomics, where peptides are dissociated from MHC molecules prior to peptide sequencing and quantitation via MS.^{45,46,187} Information about peptide mass (MS1 spectra) and mass-to-charge (m/z) ratio (MS2 or fragment spectra) are matched to those from reference proteomes and custom databases using protein inference algorithms. The use of robust non-canonical custom databases is mandatory, as more than 70% of MS-detected peptides cannot be assigned with high confidence when they are only matched to reference proteomes.¹⁹⁷ This already suggests that a relevant part of the immuno-peptidome may be derived from non-canonical sequences.

Different from trypsin-based conventional strategies, in immuno-peptidomics:

- The peptides are not trypsin products but the results of the natural degradation of the proteome by the proteasome or by cellular proteases, decreasing the bias over lysine or arginine residues. This also helps the identification of peptides derived from proteins with low stability or defective ribosome products, as the half-lives of HLA-binding motifs (hours) are in general longer than those of rapidly degraded proteins (minutes).¹⁹⁸
- The peptides are bound to MHC molecules after degradation in the cell. Reagents used for MHC immunoaffinity purification may involve native conditions instead of stringent lysis buffers. Peptide enrichment is obtained after high-affinity binding to MHC antibodies.¹⁹⁹
- The peptides that bind to class I and class II MHC molecules have specific characteristics that may serve as an additional quality control step to ensure proper

peptide identification. These include peptide size, of nine amino acids for HLA-I and 12–25 for HLA-II.^{200,201} In addition, specific HLA molecules have distinct binding motifs, and the affinity of a given peptide to the HLA of the sample under study can be predicted with computational approaches such as MHCflurry or NetMHCpan.^{202,203}

- The experimental HLA ligandome data define the landscape of peptides presented to T cells, excellent candidates for immunotherapy. This is a great advantage of immuno-peptidomics over other approaches to identify novel tumor antigens.

In spite of the outstanding performance of updated immuno-peptidomic protocols for antigen identification, novel pipelines should be developed to improve the confidence of individual candidates. Proteogenomic data for non-canonical proteins that lack RiboSeq validation has too high false-positive rates.²⁰⁴ Therefore, in addition to features related to specific HLAs (size, binding motives, etc.), the spectra and retention times can be validated with synthetic peptides, which can be not realistic for high-throughput screens, or predicted with specific machine-learning bioinformatic tools.^{205–207}

The Human Proteome Organization (HUPO) establishes that novel proteins should be detected by at least two independent uniquely-mapping peptides longer than eight amino acids with publicly available, high-quality peptide-spectrum matches (PSMs).^{170,208} However, this may be intolerable due to the short length of MHC-bound peptides and some non-canonical proteins. Additional strategies should be considered to define true non-canonical proteins. Deep learning tools can help novel peptide and protein discovery by MS. Dubious identifications can be filtered with deepLC and Prosit, which enable the validation of peptide retention time and fragment ion intensity.^{205,207} Combination of these tools with excellent immuno-peptidomic and RiboSeq data should increase the confidence of what has been described as “novel presented proteins,” which may lack a function but can be good antigens for immunotherapy.¹⁷⁰

Concluding remarks

Tumor antigens play a pivotal role in cancer development, by affecting the TME and the efficacy of most immunotherapies. The relevance of these molecules has led to important efforts for their discovery and classification, characterizing their main properties. These studies have demonstrated that ideal tumor antigens should have the highest specificity for the tumor, preferably in a clonal manner (such as many driver mutations), lacking thymic expression that would generate central tolerance mechanisms (dissimilarity with self-proteins) while appearing at sufficient levels in tumor cells for efficient recognition by TCRs. This enforced recognition should be favored by promiscuous presentation by different HLA alleles and analogy with microbial epitopes, leading in the best scenario to the activation of polyclonal T cell responses, avoiding escape mechanisms mediated by antigen or HLA loss, frequently observed in tumors.

Although some of the antigens identified so far do not fulfill all these properties, development of new combined methodologies is helping to broaden the antigen range, generating a new spectrum of targetable antigens useful for cancer immunotherapies.

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