

Facts and Hopes in Cancer Antigens Recognized by T Cells

Walther Brochier¹, Orian Bricard^{1,2}, and Pierre G. Coulie^{1,3}



ABSTRACT

T cells are key effectors of our immune response against tumors and exert their antitumor effects upon recognizing a variety of tumor-specific peptides presented by HLA molecules on the surface of tumor cells. The identification of the tumor-specific antigens of a given tumor is not required for immune checkpoint therapy (ICT), which mainly reactivates existing tumor-specific T cells together with T cells of unknown specificities. To decrease the activation of non-tumor-specific T cells, active or passive immunizations against

tumor-specific antigens are considered. These immunizations require the identification of at least some of the tumor-specific antigens displayed on the tumor cells of a patient. While this has become an easy task for tumors with a large number of mutations generating neoantigens, it remains difficult for the remainder. Here, we review some facts about human tumor-specific or tumor-associated antigens, as well as some hopes for their future use in cancer immunotherapy.

Introduction

In the 1990s, the molecular characterization of human tumor-specific antigens recognized by T lymphocytes (1) alerted oncologists and opened the door to clinical trials of cancer immunotherapy: the use of therapeutic vaccines with defined antigenic peptides and proteins. The hope surrounding their use was to turn to disappointment with the dismally small proportions of clinical responses obtained. With the exception of peptide-loaded dendritic cells, these first vaccines were in fact very poorly immunogenic, and there is hope that it will be different with mRNA or recombinant viruses. In the meantime, other strategies of cancer immunotherapy were explored and immune checkpoint therapy (ICT), namely immunostimulatory antibodies blocking the CTLA-4 or PD-1 inhibitory pathways in immune cells, turned out to be the long-awaited weapon that transformed cancer care (2).

Observations in preclinical models and in patients indicate that antitumor T lymphocytes present prior to treatment are key effectors of ICT. These T cells recognize tumor antigens, among which mutation-derived antigens (referred to as neoantigens) play a prominent role for several reasons: their perfect tumor specificity, the correlation between tumor mutation burden, a surrogate for the number of neoantigens, and better ICT outcomes (3, 4), their easy identification through DNA sequencing and bioinformatics routines, and the clinical efficacy of adoptive transfer of tumor-infiltrating lymphocytes that target them (reviewed in ref. 5). These results lead to at least two questions related to tumoral antigenicity and immunotherapy: Is cancer immunotherapy through T cells clinically efficacious only when the latter recognize neoantigens? Is there a future for cancer immunotherapy against tumors that harbor a low mutation burden?

Tumor Antigens: Processing and Presentation

Through their T-cell receptor, T lymphocytes recognize peptides presented by surface HLA molecules. HLA class I molecules present peptides derived from proteins synthesized within the cell to CD8⁺ T cells, while HLA class II molecules present peptides derived from proteins synthesized outside the cell that enter it through endophagosomes, to CD4⁺ T cells. Considering the importance of CD8⁺ cytolytic T cells (CTL) in destroying tumor cells, most of the work on tumor antigens has been focused on those presented by HLA class I molecules. Remarkably, a limited repertoire of proteins with distinctive features contribute to HLA class I-presented peptides (6). Some peptides derived from extracellular proteins can also be presented by HLA class I molecules through a pathway called cross-presentation.

HLA class I antigen processing deals with the production of antigenic peptides in the cytosol, their translocation into the endoplasmic reticulum by the peptide transporter TAP, their loading into HLA molecules and their subsequent transport to the cell surface for recognition by T cells. Antigen processing is complex. It varies according to cell type and environmental factors such as IFN γ . It includes proteolytic cleavages of the protein that contains the antigenic peptides. Several proteasome subtypes (standard, intermediate, and immunoproteasomes) are key in this process, with distinct cleavage specificities that generate distinct antigenic peptides. Thus, a given peptide might be produced by one proteasome subtype but destroyed by another (7, 8). The types of proteasomes present in a cell can vary: IFN γ transiently increases the proportion of immunoproteasomes.

After TAP-mediated transfer into the endoplasmic reticulum, peptides can bind to one of the HLA allomorphs encoded by the HLA alleles of the patient. Algorithms can predict the best HLA binders for most allomorphs (9, 10). Expression of the appropriate HLA allele by the tumor cells is another prerequisite, not a given as HLA or B2M (encoding β 2-microglobulin) mutations, or HLA allele or haplotype loss, are frequent in tumor cells.

Today, bioinformatic analyses of tumor DNA and RNA sequencing data are routinely used to predict which tumor-specific antigenic peptides can be recognized by T lymphocytes. However, the unknowns of antigen processing limit prediction accuracy and only a fraction of predicted antigenic peptides are actually displayed on tumor cells.

Chimeric receptor (CAR) T cells recognize tumor antigens produced by a completely different process than that described above

¹de Duve Institute, UCLouvain, Brussels, Belgium. ²Immunology Programme, The Babraham Institute, Cambridge, United Kingdom. ³Wallon Excellence in Life Sciences and Biotechnology (WELBIO), Wavre, Belgium.

Corresponding Author: Pierre G. Coulie, de Duve Institute, Université Catholique de Louvain, Avenue Hippocrate 74, Brussels B-1200, Belgium. Phone: 32-(0)2-764-7581. E-mail: pierre.coulie@uclouvain.be

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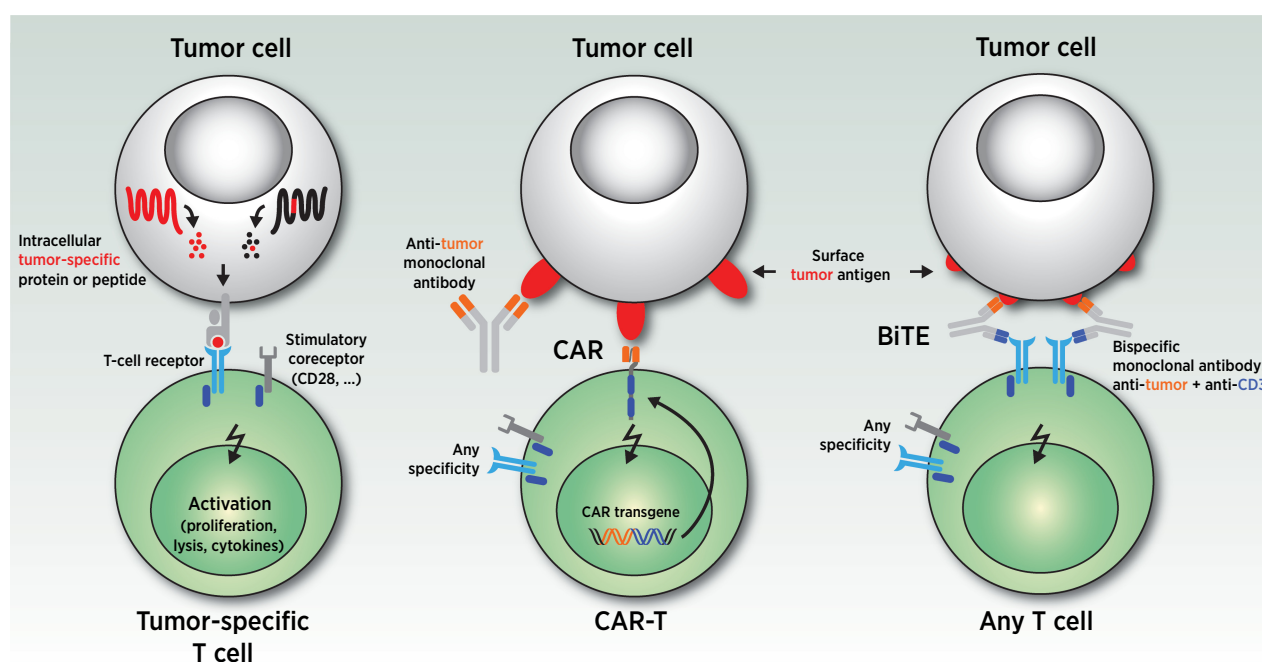


Figure 1.

Completely different modalities of antigen recognition by tumor-specific natural T cells, CAR T cells, and bispecific T-cell engagers (BiTE). Natural tumor-specific T cells recognize antigenic peptides presented on HLA class I molecules. The TCR recognizes the HLA-peptide complex. Any protein or polypeptide synthesized within the tumor cell can be the source of antigenic peptides. A given tumor-specific HLA-peptide complex can be recognized only by T cells carrying a TCR that is specific for this complex. The diversity of the TCR repertoire is very large, and in a patient with cancer, the frequency of T cells recognizing a given tumor-specific antigen is usually 10^{-7} to 10^{-5} of all T cells in the blood, with a possible 10- to 100-fold enrichment in tumors. CAR T cells recognize, with a small part of an antitumoral monoclonal antibody present in their CAR, surface tumor glycoproteins displayed on the surface of the tumor cell. Antigen processing and presentation by HLA are not required. The proportion of T cells that recognize the antigen can be very high, as large numbers of T cells can be transduced with the CAR construct irrespective of the specificity of their TCR, which plays no role in tumor recognition. The same principles are valid for BiTEs, which, like CARs, use a small part of an antitumoral monoclonal antibody for antigen recognition. Here too, all T cells can participate in tumor recognition. They do so if the CD3 part of their TCR is engaged by the bispecific (anti-CD3 and antitumor) BiTE monoclonal antibody. While the TCR of the T cells do participate in tumor cell recognition, it is not through their antigen-specific TCR α and TCR β chains but through their CD3 domain, thus irrespective of TCR specificity.

(Fig. 1). An important limitation to the use of CAR T cells is the paucity of tumor-specific glycoproteins displayed on the surface of tumor cells. As a consequence, currently used CAR T cells do not target tumor-specific antigens but differentiation antigens, leading to on-target off-tumor toxicity. It is however possible to generate tumor-specific CAR T cells by deriving the CAR from a TCR-like antibody selected for recognition of a given tumor-specific peptide-HLA complex (pHLA; ref. 11). There should then be no off-tumor toxicity, but typing the patient for antigen and HLA expression will be required.

Tumor-Specific Antigens and Tumor-Associated Antigens

In the 1990s and 2000s, many antigens recognized by CTL clones or lines derived from patients with cancer were identified (1). As these patients had no overt signs of autoimmunity, the fact that several of the identified antigens were not completely tumor-specific came as a surprise. It led to the distinction between tumor-specific and tumor-associated antigens. Four genetic mechanisms accounted for their high or low tumor specificity: mutations in the encoding gene (neoantigens); viral origin of the encoding gene (oncogenic viruses); DNA demethylation leading to tumor-selective gene expression but tumor-specific antigen expression (MAGE-type antigens); expression of the encoding gene only in tumoral and

normal cells of the same tissue (tissue differentiation antigens); tumoral overexpression of the encoding gene. A few other mechanisms leading to the expression of tumor-specific or tumor-associated antigens have been described.

Theoretically, DNA alterations in a tumor can lead to the expression of tumor-specific antigens through four mechanisms depending on whether the alteration is in the sequence encoding the antigen, or causes tumor-specific transcription, tumor-specific translation or tumor-specific antigen processing. With DNA alterations in the antigen-encoding sequence, tumor specificity is certain. With the other DNA alterations, tumor specificity is only a possibility.

Alteration in the DNA encoding the antigenic peptide

The simplest alteration is a nonsynonymous single nucleotide variation (nsSNV), with the resulting mutant amino acid residue present in the antigenic peptide and either recognized by the TCR, or anchoring the peptide in an HLA molecule. nsSNVs are the main source of neoantigens. Gene fusion following chromosomal translocations are another DNA alteration to consider, as they can lead to the production of chimeric tumor-specific antigenic peptides (12, 13). Another DNA alteration to consider is viral integration.

All of these alterations can lead to the expression of perfectly tumor-specific antigens that can be safely used in immunotherapy. Adoptive

transfer into patients of T cells recognizing neoantigens proved to be safe and to exert antitumor effects (5). Neoantigens and viral antigens are therefore the gold standard for cancer immunotherapy.

DNA alteration causing tumoral transcription of the sequence encoding the antigen

Except for antigens resulting from splice site mutations leading to the retention of introns that might not be seen in normal cells, none of the antigens resulting from such DNA alterations is necessarily tumor-specific: there might always be some transcription of the coding sequence in rare normal cells. Identification of DNA alterations that cause transcription in tumors is rare.

MAGE-type tumor antigens are encoded by cancer-germline genes such as *MAGEA3* or *CTAG1B* (NY-ESO-1; ref. 14). MAGE proteins assemble with E3 RING ubiquitin ligases to form MAGE-RING ligases involved in many cellular processes including cellular proliferation (14). The expression of *MAGE* genes is epigenetically restricted to tumors of various histologic types and to normal cells that do not express *HLA* genes such as germline or trophoblastic cells. Thymic medullary epithelial cells also express some *MAGE* genes, which could lead to some level of natural tolerance to *MAGE*-encoded antigens. That normal cells expressing *MAGE* genes do not express *HLA* genes indicates that *MAGE*-encoded antigens recognized by CD8⁺ T cells are tumor-specific even though *MAGE* gene expression is not restricted to tumor cells.

While no signs of toxicity were observed after vaccinations with peptides, proteins, or dendritic cells loaded with MAGE antigens, safety of anti-MAGE immunization was questioned in 2013 due to severe neurotoxicity in patients injected with autologous T cells engineered to express an anti-MAGEA3 TCR, derived from transgenic mice immunized with MAGEA3 peptide KVAELVHFL and modified to increase its affinity (A118T in the TCR α chain; (15, 16). The proposed explanation was cross-reactivity of the TCR towards MAGEA12 peptide KMAELVHFL, sharing 8/9 residues with the MAGEA3 peptide, associated with a very low frequency ($\pm 10^{-6}$) of *MAGEA12* transcripts detected in brain tissues (16). Another possible explanation recently emerged, with the observation of a strong cross-reaction of the clinical TCR against the peptide SAAELVHFL, encoded by gene *EPS8L2*, itself expressed in the brain at >100-fold higher levels than *MAGEA12* (17). In addition, a fatal cardiotoxicity was described with a modified TCR recognizing MAGEA3 peptide EVDPIGHLY. This TCR was subsequently shown to also recognize the peptide ESDPIVAQY from muscle protein titin (18). These toxicities are often presented as proof that MAGE antigens are not tumor-specific. However, they result from cross-reactions against antigenic peptides present in normal cells and encoded by non-MAGE genes. It is well established that each TCR can recognize a large variety of pHLAs. In the thymus, T cells whose TCR is activated by a self-pHLA presented on medullary epithelial cells undergo apoptosis. This negative selection is bypassed by adoptive transfer of T cells bearing a TCR whose sequence has been modified to increase TCR affinity. This makes cross-reactions of genetically modified anti-MAGE TCRs with self-antigens possible. It is possible that MAGE proteins in thymic epithelial cells lead to the elimination of high affinity anti-MAGE TCRs, so that clinically relevant TCRs need modifications to their natural sequence to increase their affinity. Cross-reactions are then always possible, but they are not inevitable. It might become feasible to select non-cross-reactive TCRs or TCR-like CARs for safe adoptive cell therapy against MAGE antigens. It is worth trying, as adoptive transfer of anti-MAGEA3 or anti-NYESO1 TCRs had antitumoral effects in patients (16, 18). Clinical efficacy was also observed with an

affinity-enhanced TCR against a NYESO1 peptide, without severe toxicity (19, 20).

While it is impossible to exclude *MAGE* expression in rare normal cells (as illustrated by *MAGEA12* expression in brain cells; ref. 17), it is noteworthy that the expression of some cancer-germline genes in thymic epithelial cells did not apparently lead to local toxicities in the patients treated with anti-MAGEA3 or anti-NYESO1 T cells.

Endogenous retroelements more highly expressed in tumors than in normal tissues are a potential source of tumor antigens that are not easy to identify. Human endogenous retroviruses (HERV) belong to a group of endogenous retroelements that contain two LTRs at either end of the proviral genome. A few thousand have retained open reading frames, thus coding potential (21). Using TCGA RNA sequencing (RNA-Seq) data, Rooney and colleagues quantified the cytolytic activity of immune infiltrates and identified the associated properties. In breast and clear cell renal carcinomas, expression of a small set of *HERV* was found to correlate with cytolytic activity (22). In 2018, a computational workflow identified more than 3,000 transcriptionally active *HERVs* in the TCGA RNA-Seq database, and clear cell renal carcinoma contained the greatest number of prognostic *HERVs*. One of these was further analyzed, with results suggesting the presence of intratumoral T cells recognizing HERV 4700 peptides presented by HLA-A*02:01 (23). A recent work focused on the identification of antigenic peptides encoded by *HERV* sequences specifically over-expressed in different cancers. A dedicated bioinformatics pipeline was developed, combining *HERV* sequence detection and immune cytolytic signatures in tumoral material compared with matched peritumoral tissues (24). *HERV* sequences overexpressed in breast carcinomas encoded peptides predicted to bind to HLA-A*02:01. Blood lymphocytes of normal donors primed *in vitro* against these peptides were able to lyse tumor cell lines expressing the relevant *HERV* sequences. HLA-peptide dextramers identified these *HERV*-specific T cells among tumor-infiltrating lymphocytes of HLA-A2 patients with breast cancer (24). Together, these results illustrate the immunogenic potential of *HERV* transcripts in common tumors with a low mutational burden. However, whether tumor-specific expression of certain *HERV* sequences occurs in some patients, is currently impossible to ascertain. More work is therefore needed to evaluate the clinical potential and safety of *HERV* antigens for cancer immunotherapy.

DNA alteration leading to tumor-specific translation of the sequence encoding the antigen

Insertion and deletion of nucleotides (indels) can generate translational frame shifts with novel open reading frames encoding potentially antigenic peptides. A pan-cancer analysis showed that renal cell carcinomas have the highest proportion of coding indel mutations (0.12 with a pan-cancer average of 0.05); in the same study, the number of indels was associated with response of melanoma patients to ICT (25). In another work, frameshift mutations were often found in common tumor driver genes, converging to a small set of 3' neo-open reading frame peptides that could possibly be used in personalized vaccines (26). Recurrent frame shift mutations are found in tumors with DNA mismatch repair deficiency and shared frame shift peptides have been used to immunize mice with a conditional knockout of *Msh2* in the intestinal tract, with a survival benefit observed when combined with naproxen (27). In DNA mismatch repair-deficient colorectal and endometrial tumors, frameshift mutation frequency was negatively correlated with the predicted immunogenicity of the resulting antigens, suggesting previous immunoselection against the most immunogenic frameshift antigens (28). Overall,

tumor-specific translation leads to tumor-specific neoantigens that may be used safely in immunotherapy.

DNA alteration leading to tumor-specific antigen processing

In 2006, van Hall and colleagues described a tumor-specific CTL clone that recognized a ubiquitous self-peptide, without alteration or higher transcription of the encoding DNA, but with lysis dependent on the absence of functional TAP in the tumor (29). In subsequent work, the group showed that the absence of TAP limits the repertoire of cytosolic peptides translocated into the endoplasmic reticulum, allowing for other peptides to be loaded into HLA molecules (30). These were termed T-cell epitopes associated with impaired peptide processing (TEIPP). As TAP deficiency and other alterations of the antigen

processing machinery are common in tumors, presumably as a result of immunoselection by CTLs, TEIPP are promising shared tumor-specific antigens.

Variety of Sources and Modifications of Peptides Presented by HLA Class I Molecules

From the preceding sections, it may seem straightforward to use bioinformatics tools on tumor DNA/RNA sequencing data to identify tumor-specific antigens for immune monitoring, active immunization or adoptive T-cell transfer, but such is not the case.

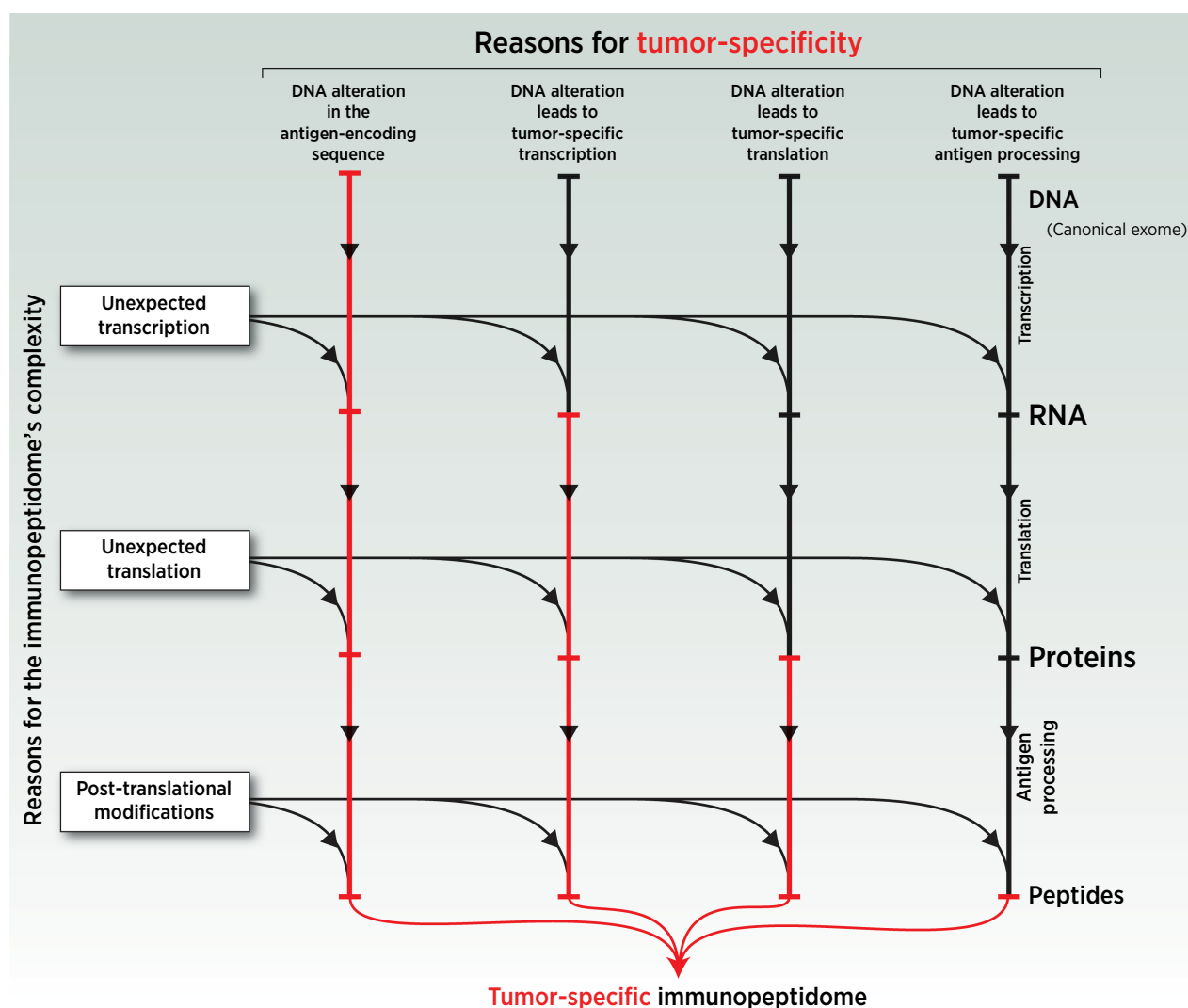


Figure 2.

Diversity and complexity of tumor-specific antigenic peptides recognized by T lymphocytes. For each of the four reasons for tumor specificity of tumor antigens (shown on top), the flux of genetic information is represented as a vertical line, from DNA to peptides. The line becomes red when the tumor-specific compound has been generated. At several steps, additional processes (shown on the left) can increase the diversity and complexity of the peptides that will eventually compose the immunopeptidome. Because of these processes, some tumor-specific peptides are excluded by classical workflows based on the canonical exome. Thus, unexpected transcription or translation, and posttranslational modifications, do not contribute to the tumor specificity of antigenic peptides but make them more difficult to identify with today's tools.

The set of peptides presented by HLA class I molecules on the surface of cells is called the immunopeptidome. Elution of these peptides followed by identification with mass spectrometry revealed their diversity and complexity, shaped by a number of processes such as unexpected transcription or translation, or posttranslational modifications. The resulting complexity of the immunopeptidome prevents the identification of many tumor-specific peptides by only analyzing the sequences of the classical exome and transcriptome. In Fig. 2, we represent the distinction between the reasons for tumor specificity and the reasons for complexity of the immunopeptidome of tumor cells.

Antigenic peptides presented by HLA class I molecules can be derived from genomic regions that are allegedly not transcribed or not translated. Several “aberrant”, or unexpected, antigenic peptides were discovered in cDNA libraries from tumors, such as an intronic transcript for the first human neoantigen (31), a reverse-strand transcript (32), alternate open reading frames (33–35), or lncRNA (36). Immunopeptidomics has allowed for the discovery of many more and shows that their proportion has been underestimated. The group of C. Perreault designed a dedicated proteogenomic workflow that showed that 90% of the total of 40 apparently tumor-specific antigens identified in two murine and 7 human tumors were transcribed or translated from “noncoding” regions (37). The mechanisms involved include transcription from 75% of the human genome instead of the 2% exome (38), dysregulation of the splicing machinery or splice site mutations leading to new splice variants or intron retention, changes in proteins implicated in translation (such as ribosomes, initiation factors, or elongation factors) leading to nonclassical translation events such as initiation at nonclassical start codons, translation of 5′ and 3′ UTR, frame shifting due to ribosome slippage or stop codon readthrough. Several of these noncanonical translated products are “DRiPs” (defective ribosomal products) that are, along with “old” proteins, the two main sources of peptides for HLA class I presentation (39).

Antigenic peptides can be shaped by posttranslational modifications. One such modification is peptide splicing, generating spliced antigenic peptides made up of sequences that are not contiguous in the parental protein (40–42). Peptide splicing takes place in the proteasome (41). The antigenic peptide can even result from splicing of noncontiguous peptides in the reverse order (43). Thus far, the six fully validated spliced antigenic peptides are *cis*-spliced, that is, with all sequences coming from the same protein. In these examples, it is not the splicing that is tumor-specific, but the presence of a nsSNV or the transcription of the encoding gene.

Splicing does not occur at random and some splicing rules have been proposed (44). The proportion of spliced peptides in the immunopeptidome is still a matter of debate. Some studies suggest that they account for 13% to 45% of the immunopeptidome and one-quarter of its diversity (45). Others have provided much lower estimations of 1% to 5% (46, 47), more in line with the observation that spliced peptides are quite rare among tumor-specific antigenic peptides identified through tumor-specific CTLs. There are clearly methodologic issues behind these discrepancies and validation guidelines have been recently proposed (45).

Other posttranslational modifications of tumor-specific antigenic peptides are phosphorylation (48, 49), glycosylation (50), and deglycosylation resulting in deamidation (51, 52). Several modifications can affect the same peptide: for example, a tyrosinase antigenic peptide produced by reverse splicing contained a double asparagine deamidation (51).

Perspectives

A promising avenue for cancer immunotherapy is immunization against defined tumor-specific antigens using vaccinations or transfers of genetically modified T cells. The latter have demonstrated their clinical efficacy but remain complex to implement; the former are easier to use but have yet to be proven clinically effective. For both immunization modalities, tumor specificity of the target antigens is a key element. Strict tumor specificity of the antigens, hence safe immunizations, is assured only with neoantigens but is not necessarily ruled out for others.

There will certainly be important progress made in the identification of neoantigens, by improvements in the prediction of those that are actually processed and displayed at the cell surface. Immunopeptidomics will help greatly by providing very large databases of processed and HLA-displayed peptides on which predictors can be trained. Another important step would be to rank the processed and displayed peptides by their immunogenicity to patients’ T cells. Today, we are only able to compare the sequences of the candidate neoantigens with those of all possible HLA-presented self-peptides, to which patient should be tolerized, and ignore candidate neoantigens similar to self-peptides. Even for tumors with a low mutational burden and poor responses to ICT, it might become possible to define the very few neoantigens against which immunization, combined with current or new modalities of ICT, may be clinically relevant.

Another important area of progress is the identification of tumor-specific antigens that are not neoantigens, that is, without alteration of the DNA sequence encoding the antigen. Their potential diversity is larger than anticipated but their tumor specificity is often difficult if not impossible to prove with today’s technologies. This does not mean that it is absent. Noteworthy, while safe adoptive transfer of large numbers of T cells against a tumor antigen requires strict tumor specificity, active immunizations through mRNA or recombinant viruses might tolerate some antigen expression in rare nontumoral cells. Immunizations against these antigens might have an antitumoral effect through T cells that recognize the vaccine antigens themselves, or through spreading the immune response towards neoantigens or other strictly tumor-specific antigens (53, 54). *MAGE* genes or *HERV* sequences remain attractive sources of shared tumor-specific antigens for immunizations against tumors with very few or no neoantigens, but caution will be required at all points along their clinical implementation.

Authors’ Disclosures

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