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# Tools to define the melanoma-associated immunopeptidome

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## Summary

**Immunotherapies have been traditionally applied in malignant melanoma, which represent one of the most immunogenic tumours. Recently, immune checkpoint modulation has shown high therapeutic efficacy and may provide long-term survival in a significant proportion of affected patients. T cells are the major players in tumour rejection and recognize tumour cells predominantly in an MHC-dependent way. The immunopeptidome comprises the peptide repertoire presented by MHC class I and II molecules on the surface of the body's cells including tumour cells. To understand characteristics of suitable rejection antigens as well as respective effective T-cell responses, determination of the immunopeptidome is of utmost importance. Suitable rejection antigens need to be further characterized and validated not only to systematically improve current therapeutic approaches, but also to develop individualized treatment options. In this review, we report on current tools to explore the immunopeptidome in human melanoma and discuss current understanding and future developments to specifically detect and select those antigens that may be most relevant and promising for effective tumour rejection.**

**Keywords:** immunogenicity; immunopeptidome; melanoma; T cells; tumor antigens; tumor rejection.

## Recognition of melanoma by the immune system

Malignant melanoma represents one of the most aggressive but also highly immunogenic tumours, which has traditionally been targeted by immunotherapies. High-risk resectable melanoma has long been treated by the immune-modulating cytokine interferon-α in an adjuvant setting following surgery.[1] Moreover, over a long period, a high number of clinical immunotherapeutic approaches have been tested in clinical studies aiming to specifically stimulate T cells by vaccination or to target a defined antigens by directed adoptive T-cell transfer.[2,3] However, the success of these approaches was either limited or associated with major side effects.[4,5] In contrast, high efficacy of adoptive T-cell transfer of tumour-infiltrating lymphocytes (TIL) with mainly unknown specificity was reported repeatedly over a long period of time although larger, phase three clinical trials were missing.[6] The discovery of immune checkpoints potentially exerting a major inhibitory effect on tumour-reactive T cells was an outstanding break-through in the emerging clinical application of novel immunotherapies.[7,8] Over the last decade, immune checkpoint modulators became available preventing exhaustion of endogenous T-cell populations with unknown specificity and improving tumour-cell recognition by supporting T-cell priming and depletion of regulatory T cells *in vivo*.[9] Immune-checkpoint-modulating antibodies targeting the programmed death-1(PD-1)/ programmed death ligand-1(PD-L1) axis meanwhile represent standard therapies in the treatment of diverse malignant diseases including melanoma, lung cancer, renal cell carcinoma, and head and neck squamous cell carcinoma, but also other malignant disease entities.[10] These therapeutic agents have proven exceptionally effective in patients with melanoma,[11] indicating that highly suitable target structures for tumour rejection are presented on melanoma cells. The identification and understanding of the nature of such target structures and the characterization of respective T-cell responses is of fundamental importance to improve current therapies not only in melanoma but also in other malignant diseases.

The immunopeptidome represents the sum of peptide ligands presented by the MHC class I and II complexes on the body's cells including tumour cells and therefore


 

**Box 1** Definition of the immunopeptidome

The immunopeptidome comprises the whole set of peptides represented on the cell surface in the context of MHC and recognizable by T cells. Parts of the immunopeptidome are presented on both, healthy and tumour cells. Tumour-specific peptide ligands representing neoepitopes seem to significantly contribute to the detectability of the tumour by the autologous immune system. However, other target structures may serve as rejection antigens depending on the source of T cells (autologous versus allogeneic/xenogeneic) recognizing respective MHC–peptide ligands presented by tumour cells.

in principle recognizable by T cells (Box 1). Recognition and elimination of target cells by T cells has been intensively studied for decades accompanied by immunological milestone discoveries such as MHC presenting antigenic peptides potentially representing epitopes to be recognized by T cells.[12,13] Until now, numerous approaches aimed at the mostly focused identification of immunogenic peptides suitable for further clinical application. In this regard, a prime example for MHC-restricted antigen recognition by T cells was the detection of foreign antigens.[13,14] Antigenic peptides derive from intracellularly degraded exogenous or endogenous proteins processed by the antigen-presenting machinery and presented by MHC class II and I molecules as peptide–MHC complex, respectively.[15] T cells recognize such presented peptide ligands by their T-cell receptor (TCR) and may subsequently eliminate target cells by a professional lytic machinery.[16–18] The precondition for this highly fine-tuned recognition is thought to base on the co-evolution of MHC genes and TCR genes.[19,20] Education of the immune cells in the thymus is an essential prerequisite for the critical distinction between foreign and self and therefore maintenance of self tolerance as recently reviewed.[21]

Although recognition of tumour cells by the patient's immune system has been long-term propagated,[22] routine recognition of tumour cells was questioned over the years and tolerance or ignorance of such transformed cells seemed to be dominant in the discourse of the immune system and cancer. Our understanding of the coherence of cancer and the immune system has improved and immune evasion has been recognized as one of the hallmarks of cancer.[23] Evolution of tumours under immune pressure and immune editing has been determined as a key element of immune evasion and subsequent tumour progression.[24] The tumour-related immunopeptidome is also affected by such immune editing[25,26] and characteristics of peptide ligands suitable for effective tumour rejection may depend on a number of factors, such as relevance of the source protein for cell survival, dependence of peptide presentation on the antigen presentation machinery and affinity of the peptide towards MHC.[27] Otherwise, target recognition is highly dependent on the relevant T-cell population and higher-affinity TCR towards MHC–peptide may be more effective in tumour cell rejection. Such TCR are generally observed against non-self antigens or in the context of a non-educated T-cell repertoire or mismatched MHC environment.[28–30] Hence, definition of the quality and antigenicity of peptide ligands presented by MHC is at least in part inevitably associated with a defined T-cell population displaying respective reactivity.

## Target structures presented on melanoma cells

Melanoma has been identified as a tumour entity that is potentially recognized by the immune system.[31] Extensive efforts of several groups have been aimed at the identification of exactly those peptides of the immunopeptidome rendering tumour cells immunogenic. The basis of these efforts was primarily the presence of T cells within peripheral blood mononuclear cells (PBMC) or TIL of melanoma patients correlating with anti-tumour activity *in vitro* or *in vivo*.[32,33] Potential antigen candidates can be assigned to two major classes, tumour-associated antigens (TAA) and tumour-specific antigens (TSA) (Table 1). TAA are antigens that might be dominantly presented by malignant cells but may also exist in normal cells. Differentiation antigens, as a subgroup of TAA, are representative for a defined cell type or tissue and eventually expressed in tumours originating from these cells. Peptide ligands derived from differentiation antigens in melanoma, such as gp100, MART1/Melan-A and tyrosinase,[34,35] may represent promising target structures inducing also spontaneous immune responses in patients with disease.[36] Responses against these antigens have been shown to be associated with vitiligo correlating with a good prognosis in patients with melanoma.[37] Another class of TAA are represented by cancer-germline antigens defined by exclusive expression in tumour and germline tissue and thereby representing an attractive means for targeting different tumour entities.[38] In melanoma, a number of cancer-germline antigens have been described, such as NY-ESO-1 or members of the Melanoma antigen encoding genes.[39] However, vaccination strategies targeting both classes of TAA have shown only limited activity so far.[4,40,41] Limited efficacy of vaccination studies targeting this class of antigens may be associated with the lack of high-avidity T-cell responses due to thymic negative depletion. However, both classes of TAA may still represent attractive target antigens in a non-self immune environment. Peptides derived from proteins that quantitatively have higher expression in tumour cells compared with normal cells represent another class of TAA used for targeting in melanoma and other tumour

**Table 1.** Classes of tumour antigens rendering melanoma cells immunogeneic

| Classes of tumour antigens | Description | Examples | Literature |
|---|---|---|---|
| **Tumour-associated antigens (TAA)** | | | |
| Differentiation antigens | Proteins mainly expressed in melanocytes and melanoma cells | MART1/MELAN-A, gp100, tyrosinase | 34, 121–123 |
| Cancer germline antigens (CGA) | Expression restricted to germline tissues and various tumours | MAGE family, NY-ESO-1 | 67,124 |
| Over-expressed antigens | Antigens more highly expressed in malignant cells in relation to healthy tissue | Survivin | 43 |
| **Tumour-specific antigens (TSA)** | | | |
| Peptides containing mutations | Peptide fragments exclusively expressed in tumour cells due to genomic alterations | SNV → aa exchange; In/Del → frame shifts; Chromosomal rearrangement → fused peptides | 49, 66, 125–127 |
| Peptides resulting from non-canonical translation | Changes on the transcriptional level | Alternative ORF; retained introns | 54,55 |
| Aberrantly spliced peptides | Tumour-specific distinct proteasomal processing | Fused peptides resulting from different protein regions | 45–48 |
| Peptides derived from TAP- and proteasome-independent pathways | Unconventional sources for peptides assembled in the pMHC complex | TAP-independent pathway of tyrosinase-derived signal sequence | 128 |
| Peptides containing tumour-specific post-translational modifications | Aa modification after ribosomal translation | Phosphorylation and deamidation | 107,129 |

aa, amino acid; In/Del, Insertions/Deletions of one or two bases; MAGE, melanoma antigen encoding genes; ORF, open reading frame; SNV, single nucleotide variant.

entities,[42] given Survivin as an example.[43] Problems may arise from proper quantification of gene or protein expression in different cell types, especially in proteins with highly dynamic expression as well as critical functions in normal cells rendering them difficult targets, especially in a non-self T-cell environment potentially selected to improve efficacy.[44] Another source of antigens is represented by proteasome-generated spliced peptides obviously representing a substantial part of the immunopeptidome.[45–48] However, cancer specificity and a potential role for targeting in malignant diseases has so far not been elucidated. The most attractive source of antigens is currently estimated to be represented by TSA or neoantigens not presented on normal cells (Table 1). Mutated peptide antigens resulting from non-synonymous point mutations are proposed to present an important source of such target antigens.[49] Mutations may affect binding of peptides to respective MHC by altered anchor residues or may lead to recognition of affected amino acids by mutation-specific T cells.[50] These neoantigens represent highly attractive targets when these peptides derive from public mutations,[26] although they may be also highly suitable for the design of personalized therapies. Moreover, mutations may result in non-canonical reading frames additionally representing an important source for neoantigens, as shown for autoimmune diseases and cancer.[51–56] In addition, post-translational modifications may produce aberrantly processed peptides potentially representing neoantigens.[57] So far, it is not clear to what extent these diverse alterations and modifications resulting in neoantigens may contribute to the immunopeptidome. However, the predominant role of neoantigens resulting from mutations became obvious in the context of data indicating a better response rate to checkpoint modulation for those patients with tumours with high mutational load, as observed in diverse disease settings.[58–60] In general, the high number of somatic mutations, as well as the limited tumour heterogeneity, in comparison to other malignant entities are most likely key issues in the high and prolonged response rates observed in patients with melanoma treated with immune checkpoint modulators.[61–63]

## Strategies for tumour antigen identification on melanoma

Although all the diverse classes of antigens have been described, it is currently unclear how widely they are presented. Moreover, their defined role as suitable rejection antigens needs to be further clarified. Depending on the origin of the antigen of interest, the focus is on the selection of the most potent rejection antigens in a personalized approach or to be applied in a defined patient collective. As described above, definition of suitable target antigens also needs to be regarded in the context of the T-cell repertoire that is therapeutically addressed. Early approaches mainly focused on highly selective target identification based on tumour-reactive T cells and a number


 

of diverse techniques have been applied to identify such antigens (Table 2). With respect to technical improvements and implementation of high-throughput technologies, new methods aim to detect the most relevant target antigens in a more personalized way and hence define more general determinants for tumour rejection.

### Direct immunology approach

For definition of target structures, TIL and PBMC from patients with a favourable course are a highly attractive source for subsequent identification of tumour antigens. For direct identification of tumour rejection antigens, isolated TIL or PBMC were co-cultured with tumour cells without any knowledge of the defined target structure. Lack of reactivity against autologous healthy control cells hinted at tumour-specific determinants. Great progress was made regarding the identification of specific target structures by the development of tum$^-$ variants in combination with the preparation of cDNA libraries and subsequent identification of immunogenic epitopes.[64,65] Subsequently, first TSA and TAA were detected in melanoma and characterized revealing marked tumour-directed T-cell responses.[66–68]

In concordance with the increasing number of identified targets, databases were established comprising identified relevant TAA.[69,70] In addition databases such as the immune epitope database (IEDB) were built, gathering published information about the origin of the identified epitopes and the results of functional analyses.[71]

### Reverse immunology approach

The so-called reverse immunology approach comprises all those methods identifying immunogenic tumour-derived epitopes by the implementation of three sequential steps: (i) the selection of antigen based on expression or tumour relevance, (ii) prediction of putative antigens, and (iii) experimental validation.[72] For step (i), putative antigen candidates are either selected by literature search of previously described over-expressed antigens or based on disease-specific expression profiles obtained from single patients or a compilation of different patient samples. As another possible starting point, proteins eliciting autologous humoral immune responses in cancer patients were identified by a recombinant expression cloning (SEREX) approach[73] and were then investigated with regard to their potential as a T-cell-mediated target.[29] As a prerequisite for (ii), public *in silico* binding prediction tools pave the way to broader application by the research community. In the early 1990s, a first structural approach for the provision of motifs was pursued for MHC class I,[74] followed by the identification of motifs for MHC class II binding peptides.[75] After proposals of several motifs in the following years, these efforts resulted in the

**Table 2.** Techniques used for identification of relevant tumour rejection antigens

| Technique | Examples | Approach | Antigen class | Presentation of candidate epitopes for testing | Interrogated TCR repertoire | Antigen validation |
|---|---|---|---|---|---|---|
| cDNA and peptide library of preselected target antigen | 64,65 | Direct immunology | TAA + TSA | • Transfection/Transduction with target antigen of interest<br>• Neoantigens and tandem minigene constructs<br>• Pulsing of longer or exact peptides | • Autologous TIL (patient derived)<br>• Autologous PBMC (patient derived)<br>• HLA-matched or mismatched healthy donors | • Functional analyses<br>• Phenotypic characterization including MHC multimers presenting exact peptide ligands |
| *In vitro* proteasomal digest of preselected target antigen | 130 | Direct immunology | TAA | | | |
| RNA/protein expression + *in silico* epitope prediction | 43 | Reverse immunology | TAA | | | |
| Exome sequencing + *in silico* epitope prediction | 92 | Reverse immunology | TSA | | | |
| Mass spectrometry | 98,99 | MS-based Immunopeptidomics | TAA | | | |
| Exome/RNA sequencing + Mass spectrometry | 104–107 | MS-based Immunopeptidomics | TAA + TSA | | | |

development of a first public database for peptide-binding motifs.[76] Currently, a constantly growing number of binding prediction algorithms is available for both human HLA and mouse MHC class I and II.[76–79] The importance of peptide ligand quality is strengthened by the fact that peptides with high affinity towards MHC have a higher probability of representing immunodominant peptides and being suitable for tumour rejection.[80,81] The prediction of the stability of the peptide–MHC complex is another important aspect for selecting the most attractive candidate epitopes.[82] In addition, the prediction of peptide-processing steps, such as proteasomal cleavage and transporter associated with antigen processing (TAP)-dependent transport to the endoplasmic reticulum, have found integration into more recent versions of prediction algorithms as recently reviewed.[83] Comparison and validation of large experimental data sets using different tools or combinations of them will foster an even more precise definition of rules for actual presentation.[84] Implementation of structural *in silico* analyses may provide deeper insights into conformational binding properties of potential HLA ligands.[85] One of the major drawbacks of prediction-based antigen selection is that not all epitope candidates with high predicted immunogenicity are actually presented as expected. For instances, $MART1_{27–35}$-specific T cells only recognized about half of the MART1-expressing HLA-matched melanoma cell lines.[86] Moreover, a distorted relationship between gene or protein expression and peptide presentation has also been reported.[87] Validation by a defined T-cell repertoire is essential for this approach and may be facilitated by the invention of novel technologies and further optimization of existing high-throughput technologies.[88]

Owing to the previous observation of immunogenic tumour-specific mutations and the generally high mutational load of melanomas,[61,66] systematic assessment of immunogenic tumour-restricted mutations was pursued by exome sequencing in combination with *in silico* epitope prediction.[25,89–92] This combinatorial approach was applied for instances on the analysis of tumour tissue from three melanoma patients followed by screening assays using 19-mer peptides encompassing the respective mutation.[92] In all patients, mutation-specific T cells were detectable and all three individuals responded to the transfer of autologous TIL products. The same approach was used for the identification of immunogenic MHC-II-restricted neoepitopes in a murine model showing tumour rejection upon vaccination with pre-screened candidates.[89] Recently, two early-phase clinical vaccination trials focusing on tumour-specific mutations reported promising efficacy in patients with melanoma. Ott and colleagues used mixtures of long peptides comprising patient-tailored single nucleotide variants for the vaccination of six patients and observed in four patients with stage III melanoma a stable clinical course with no reccurrence of disease up to 25 months.[93] Within the second study, 13 patients with advanced melanoma have been vaccinated with an RNA-based poly-neoepitope vaccine encoding multiple single nucleotide variants per construct and experienced a significant decrease of relapses compared with their pre-vaccination disease course.[94] The described observations are highly encouraging and demand larger clinical trials. In particular, the value of vaccination at defined disease stages compared with immune checkpoint modulation alone or in combination needs to be elucidated. Moreover, the characteristics of neoantigens representing most effective tumour rejection antigens as well as respective T-cell responses need to be investigated in detail to improve neoantigen-specific therapeutic strategies.

### Mass spectrometry-based immunopeptidomics

HLA-bound ligands that are presented on the cell surface can be directly identified as such. Since the identification of naturally presented ligands using the combination of high-performance liquid chromatography and tandem mass spectrometry,[95,96] this approach profited largely from recent developments and inventions for the optimization of mass spectrometers.

The ability to directly measure HLA-presented peptides by mass spectrometry rapidly broadened possibilities for epitope-specific (onco)immunological research and currently several strategies for the detection of HLA-bound peptides by MS are available, as has been reviewed recently.[97] Cell lines have been mostly used for antigen identification by MS due to the unlimited material that is available.[98] However, direct identification of ligands derived from primary tumour cells of patients reflects heterogeneity within one given sample and therefore may support the identification of those peptides that are well presented. In fact, primary patient tumour samples led to the designation of several novel and known immunogenic target structures with interesting potential for clinical applications.[99–101] In addition, the comparison of ligands identified in tumour material and patient sera may be attractive for biomarker development.[102,103]

The identification of neoantigens in solid tumours by MS immunopeptidomics among previously unassigned mass spectra was first shown by the analysis of murine tissues[104,105] and human cell lines.[106] Further development led to identification of naturally presented neoepitopes on cryopreserved human solid tumour tissue derived from melanoma by mass spectrometry.[107] Key issues of this analysis were the large number of peptides that were eluted, corresponding to a comparably deeper level of sensitivity in comparison with other studies. In addition, an optimized workflow for bioinformatics was developed using MaxQuant.[107]

Mass spectrometry-based immunopeptidomic neoantigen identification has been shown to be successful also in

  

lymphomas.[108] Several epitopes derived from the immunoglobulin constant region were presented on MHC I, but no epitope was detected mapping to the mutated variable region. In contrast, 14 epitopes presented on MHC II were detected that derived from the hypermutated variable region, therefore presenting true neoepitopes. Due to the authors' hypothesis, this rather unexpected distribution of naturally presented tumour-derived HLA ligands may derive from immunoediting of respective tumour cells. Hence, comparison of different modes of processing and epitope editing will help us to learn from analysed data sets and draw conclusions on similarities and differences of neoepitope presentation between different tumour entities.

There are a number of advantages of using mass spectrometry for the analysis of the immunopeptidome. The mass spectrometry-based immunopeptidomic approach represents an HLA-independent strategy for the identification of HLA ligands with direct proof of actual presentation. This is especially important as prediction analyses are highly limited to frequent HLA types for which a large data set is already available. Using a computational approach for the assignment of a data set of mass spectrometry-identified epitopes to its respective HLA restriction element, existing *in silico* prediction tools can be further improved in their accuracy if they are trained with data sets derived from mass spectrometry analyses.[109] In addition, measurement of an immunopeptidome that can be clearly assigned to one single HLA molecule leads to more input and a high-quality data set for adjustment of prediction algorithms, such as the detection of novel anchor residues.[84] Immunopeptidomes identified by mass spectrometry may therefore contribute to a further improvement of epitope prediction. Moreover, the unbiased search by mass spectrometry analysis enables the detection of ligands derived from post-translational modification, which might be missed by conventional epitope prediction.[107] Other examples are spliced peptide variants[46] and the so far less well described MHC II immunopeptidome,[110] although these peptides are currently also often difficult to detect by MS. In addition, so-called 'hot spots' of preferential proteasomal processing and presentation within highly expressed proteins may be identified by comparison of ligandomes derived from different samples and mapping of frequently detected regions in a protein sequence.[107,111] Processing of retrieved mass spectra represents one key component for the valid interpretation of the analysed data and requires integration of state-of-the-art bioinformatics and computational analyses.[112] Nonetheless, mass spectrometry-based immunopeptidomics currently inherits several limitations, such as the predominant dependence on those databases that are used for the assignment of analysed mass spectra. This bias may be overcome by the implementation of *de novo* sequencing.[113] Despite the application of stringent filters, the verification of true binders remains another objective that is currently faced throughout laborious validation procedures. Moreover, the reported sensitivity for mass spectrometry is still rather low, ranging between 0·5% and 3% yield of peptides captured by immunoprecipitation.[97] Further challenges are based on the robust identification of the actually presented HLA peptides, including the correct assignment of isobaric amino acids such as leucine versus isoleucine.[114] As another technical limitation, very hydrophobic or hydrophilic peptides are less well detected by current mass spectrometry technologies, leading to biased acquisition of the individual immunopeptidome.[115] In addition, as mentioned above, the integration of algorithms for the systematic detection of spliced peptides may contribute to a more comprehensive characterization of the melanoma immunopeptidome. With the high velocity of novel developments in the field, sensitivity in peptide ligand identification is expected to be substantially improved in the near future.

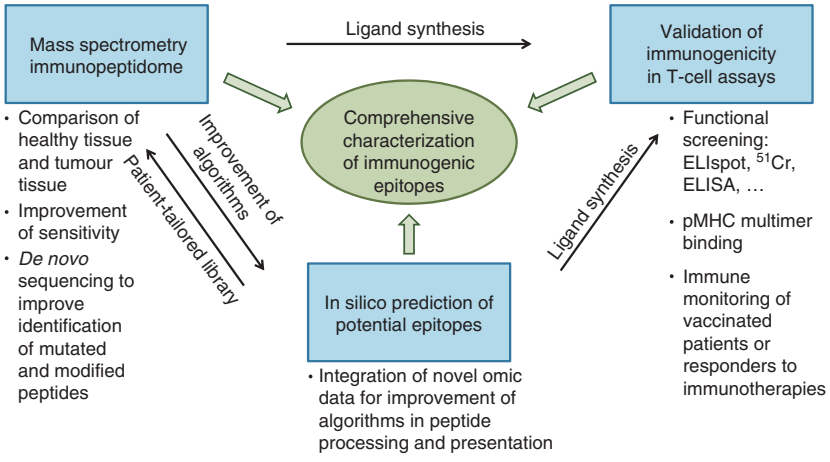


**Figure 1.** Schematic overview of relevant determinants for the comprehensive characterization of immunogenic epitopes.

## Clinical relevance and future challenges

As a result of the high efficacy of immune checkpoint modulation and T-cell-based therapies in melanoma we are currently able to learn much about suitable target antigens and respective T-cell responses in a broader patient population. This will be instrumental in obtaining better understanding of the efficacy and rejection capabilities of T-cell responses directed against differentiation antigens and/or mutated epitopes,[116,117] stressing the importance of thorough functional characterization of tumour-specific immune responses. Currently, we have no clear picture of which antigens are the most relevant for tumour rejection or what aspects may contribute to immunodominance, mainly characterized by the reactive T-cell population. Large-scale analyses may move the field forward, including a comprehensive description of immunogenic epitopes presented on an (individual) melanoma tissue and a combination of permanently improving techniques (Figure 1). However, detailed sequential functional T-cell analyses are essential.[107] There are central questions including a better understanding of the role of the whole antigen-presenting machinery, the role of CD8 versus CD4 responses and the contribution of each population to efficient tumour rejection. One of the biggest challenges is represented by tumour heterogeneity and immune evasion.[25,62,63] Analysis of the immunopeptidome of different metastatic lesions may therefore help to understand inter- and intraindividual heterogeneity and its impact on plasticity, immunogenicity, responsiveness to immunotherapy and immune escape. It will probably become more complex as soon as a better understanding exists of the impact of other systems, for example the respective microenvironment[118] and the microbiome,[119,120] on respective tumour-related immunopeptidomes and the outcome of immunotherapies encouraging an integrative view of systemic immunotherapies.

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The authors have no competing interests to declare.

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