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The impact of immunopeptidomics: From basic research to clinical implementation

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ABSTRACT

The immunopeptidome is the set of peptides presented by the major histocompatibility complex (MHC) molecules, in humans also known as the human leukocyte antigen (HLA), on the surface of cells that mediate T-cell immunosurveillance. The immunopeptidome is a sampling of the cellular proteome and hence it contains information about the health state of cells. The peptide repertoire is influenced by intra- and extra-cellular perturbations - such as in the case of drug exposure, infection, or oncogenic transformation. Immunopeptidomics is the bioanalytical method by which the presented peptides are extracted from biological samples and analyzed by high-performance liquid chromatography coupled to tandem mass spectrometry (MS), resulting in a deep qualitative and quantitative snapshot of the immunopeptidome. In this review, we discuss published immunopeptidomics studies from recent years, grouped into three main domains: i) basic, ii) pre-clinical and iii) clinical research and applications. We review selected fundamental immunopeptidomics studies on the antigen processing and presentation machinery, on HLA restriction and studies that advanced our understanding of various diseases, and how exploration of the antigenic landscape allowed immune targeting at the pre-clinical stage, paving the way to pioneering exploratory clinical trials where immunopeptidomics is directly implemented in the conception of innovative treatments for cancer patients.

1. Introduction

Immunopeptidomics has contributed immensely to our fundamental understanding of the biogenesis of antigenic repertoires bound to the human leukocyte antigen (HLA) molecules, also known as the major histocompatibility complex (MHC) in other animals. Perturbations affecting the activity or expression of key proteins of the antigen processing and presentation machinery (APPM) and of source proteins can lead to an altered immunopeptidome [1]. Consequently, even in the absence of infectious agents or cancerous transformation, the continuous yet dynamic process of antigen presentation informs the adaptive immune system about the health state of cells [2]. Therefore, detailed comparative analyses of the repertoire of naturally presented HLA bound peptides in controlled experimental settings, where the activity and or expression level of selected key proteins are modulated, can reveal how they contribute to the generation, translocation, editing, loading, and cell-surface presentation of the peptides.

The pioneering MS-based immunopeptidomic studies in the 1990′s and early 2000′s reported only tens of HLA bound peptides in a single analysis from samples in the range of billions of cells. These studies were transformative as they revealed the association of the peptides with the HLA binding groove, implying HLA binding specificities (also called binding motifs). Since then, the sensitivity of MS instruments has improved by orders of magnitude and tens of thousands of peptides can be routinely identified and quantified from as little as a few millions of cells. Such comprehensive measurements capture informative features of peptide populations, including sequence patterns, lengths and abundances, expanding our knowledge of HLA binding specifities and dynamics of the APPM that dictate the rules underlying the biogenesis of the HLA peptidome, including signatures of cleavage site specificity, influence of source protein expression, structure, turnover and cellular localization.

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2. Basic research

Immunopeptidomic basic research studies aim to answer key questions, for examples: what are the molecular structures of the complexes and what amino acid residues of the binding groove or the bound peptide influence peptide binding affinity and stability (Fig. 1 and Table 1). Do different cell types present a different repertoire due to differential activity of proteins involved in the presentation machinery or merely due to patterns of expression of source proteins? How does the expression of different catalytic subunits of the constitutive, thymic, and immuno-proteasomes rewire the repertoire? How do other proteases, peptidases and chaperones shape the repertoire? What is the limiting factor in the APPM – the supply of peptides funneled into the endoplasmic reticulum (ER) or the availability of newly synthesized HLA molecules?

2.1. How are HLA bound peptides processed, translocated, edited and loaded?

The cellular APPM ensures that the restrictive loading of peptides, derived from either intracellular or extracellular proteins, will take place in specialized cellular compartments [3]. There are two classes of immunopeptides and respective HLA proteins, with substantial differences their respective APPM and source protein populations. The class I APPM, degrades intracellular proteins into small peptides, mainly by cytosolic and nuclear proteasomes. These peptides are transported into the ER by the transporter associated with antigen processing (TAP) protein complex. In the ER peptides can be further processed by ER residence aminopeptidases (ERAP1, ERAP2) if they are not suitable for peptide loading yet. Peptides with a suitable sequence motif form a complex with the HLA class I and beta2-microglobulin (β2m). Peptides are loaded into HLA binding grooves with or without the help of chaperons of the antigen-loading complex, such as tapasin. After trafficking to the cell surface, class I complexes are scanned by CD8 T cells. In the class II pathway, peptides originating from the degradation of endocytosed extracellular proteins in the endosome-lysosome compartments are presented on HLA-II molecules for recognition by CD4 T cells [4]. Endogenous proteins can similarly be presented on HLA-II molecules when degraded through autophagy [5]. Additional steps are involved in class II presentation, such as HLA-DM mediated loading on HLA-II molecules and peptide exchange catalysis by HLA-DO and processing by other enzymes such as cathepsins [6]. Via cross-presentation, professional APCs can uptake exogenous antigens and present them on HLA-I, which is a crucial step in priming of naïve T cells [7].

Immunopeptidomic studies have greatly enhanced our understanding of APPM pathways and how they contribute to the presented peptidome. In 2013, Milner et al. attempted to define the relative contribution of the constitutive proteasome to the production of HLA-I

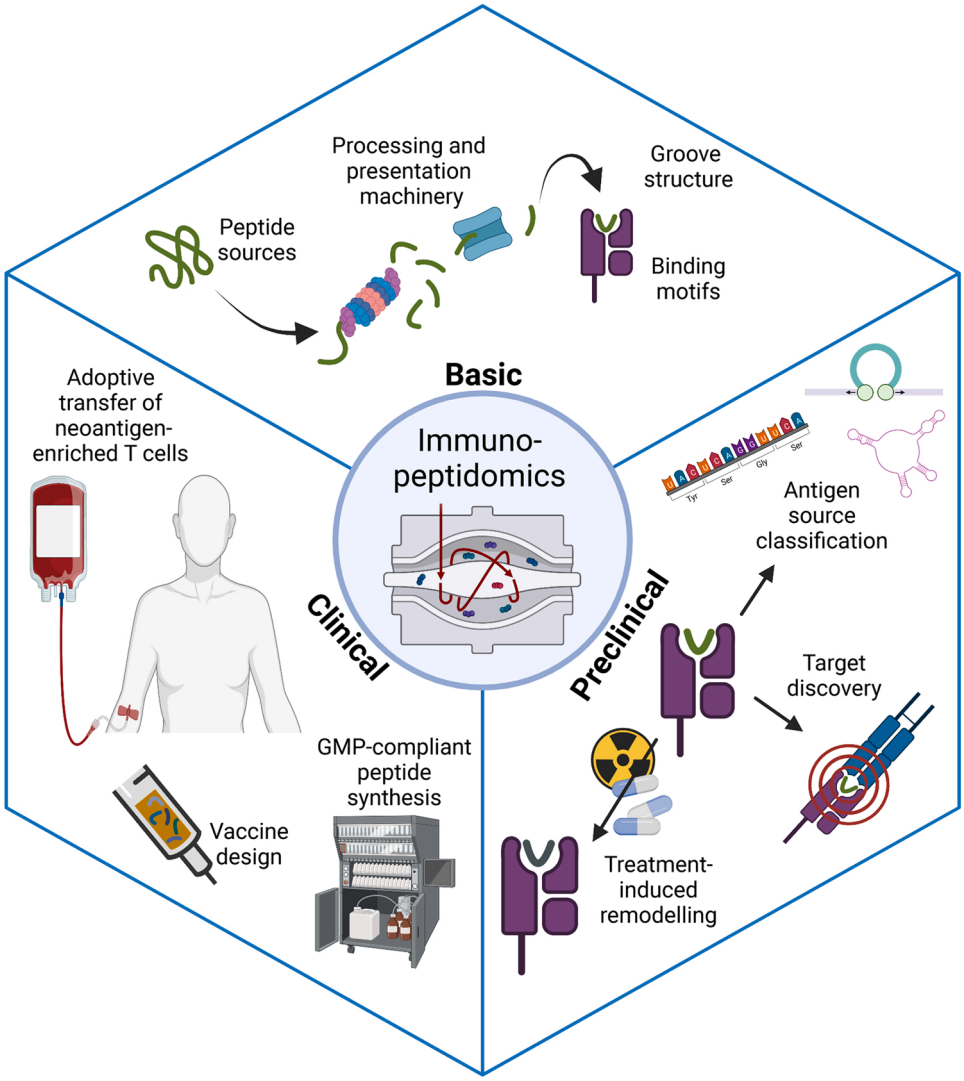

Fig. 1. Schematic overview of the three pillars of immunopeptidomics research domains: i) basic, ii) pre-clinical and iii) clinical application.

Table 1
A short list of selected key open question in the field of immunopeptidomics.

Selected open question in the field of immunopeptidomics	
Immunopeptidomics for basic research	– What properties make some proteins more presentable than others? – How tissue- and cell state- dependent is the immunopeptidome? – How is immunopeptidome homeostasis defined and maintained? – What rules govern the bottlenecks of peptide presentation? – How do alterations in the APPM impact the immunopeptidome? – What HLA binding groove characteristics regulate peptide binding restrictions?
Immunopeptidomics for pre-clinical research	– What makes a presented peptide clinically relevant? – How broadly immunologically relevant are non-canonical and post-translationally modified HLA-bound peptides? – What is the impact of drug- and environment-induced remodeling on the immunopeptidome? – How can we avoid adverse reactions or benefit from induced targets of interest? – How can multi-omics approaches further enhance antigen prioritization strategies?
Immunopeptidomics for clinical research	– What degree of personalization is necessary for selecting antigens for effective treatments? – For which interventions are warehouse approaches suitable? – How can the target identification, manufacturing, and delivery systems be optimally integrated?

peptides by following the effects of proteasome inhibitors, epoxomicin and bortezomib (Velcade), on the synthesis rates of both the HLA-I peptidome and the cellular proteome of the same cells. The proteasome inhibitors did not reduce the levels of HLA presentation, yet affected the rates of synthesis by mostly decreasing, but also increasing, some of the synthesis rates of the HLA peptides and the cellular proteins. Referencing these results, they discuss the possible involvement of other proteases and degradation pathways [8]. Immunopeptidomic analyses of murine dendritic cells expressing or not the immunoproteasome subunits MECL1 (β2i) and LMP7 (β5i) showed a substantial increase in the abundance and diversity of MHC bound peptides, attributed to changes in cleavage preferences [9]. Similar results were demonstrated by Kalaora et al. in human melanoma cells following overexpression of the immunoproteasome subunits PSMB8 (β5i) and PSMB9 (β1i), in agreement with changes that happen following treatment with IFNg [10]. Indeed, multiple studies reported that IFNg modulate the immunopeptidome by enhancing and diversifying the peptidome, especially through the upregulation of HLA-B allotypes [11–16].

Peptides generated by proteases in the cytosol must be translocated to ER lumen by the transporter associated with antigen processing (TAP) prior to their assembly with HLA-I. The important role of TAP makes it a vulnerable target for immune evasion. For example, to escape CD8+ T cell surveillance, dedicated proteins from viruses which cause chronic infections, such as CMV, HSV and EBV, inhibit the function of TAP during viral reactivation [17]. Malignant cells also frequently manipulate TAP expression, especially after increased immune pressure, resulting in immune escape and tumor progression [18]. Importantly, complete deficiency of TAP indeed significantly reduce surface levels of HLA-I, however, some peptides find their way to HLA-I molecules and were found to mediate T cell recognition [19]. With immunopeptidomics Lorente et al. identified TAP-independent ligands naturally presented by murine MHC class I molecules [20]. This ligandome showed increased peptide lengths, presence of multiple nested set peptides, and low theoretical MHC binding affinity. Peptides eluted from TAP-proficient cells are equally distributed in their parental proteins, whereas in TAP-deficient cells they are skewed towards the N-terminus and C-terminus of the parental proteins where peptides derived from signal peptides are frequently detected [21].

Additional studies in recent years focused on the processing of peptides taking place within the ER, mainly by the ERAAP, ERAP1 and ERAP2 aminopeptidases, due to their association with autoinflammatory disorders [22,23]. Nagarajan et al. found that peptides presented on ERAAP-deficient cells were derived from a different sources of proteins compared with wild type cells [24]. They identified an immunogenic peptide specifically presented by ERAAP-deficient cells from the *Fbxl19* gene that is associated with psoriasis, suggests a potential mechanism driving pathogenesis of this disease [24]. ERAP1 and ERAP2 were shown to alter peptide binding and presentation by particular alleles such as HLA-B*27 and HLA-B*40 that are associated with ankylosing spondylitis, thus demonstrating a potential pathogenic mechanism [25–27]. Furthermore, specific variants of ERAP1 with low enzymatic activity in epistasis with HLA-B*51 allotype have been shown to be associated with Behçet's disease, altering the antigen presenting specificity and destabilizing the complex through generating a peptidome of lower affinity [22]. ERAP1 and ERAP2 have distinct, but complementary and partially redundant effects on the B*51:01 peptidome, suggesting a differential association with Behçet's disease [28]. Interestingly ERAP1 inhibition altered the length distribution of peptides eluted from melanoma cells and was found to enhance the average predicted HLA-I binding affinity by reducing presentation of sub-optimal long peptides and increasing presentation of many high-affinity 9–12mers, suggesting that baseline ERAP1 activity is destructive for many potential epitopes [29]. Hence, chemical inhibition of ERAP1 may be a viable approach for manipulating the immunopeptidome of cancer.

Peptide loading onto HLA complexes is a critical step for peptide presentation. Within the APPM, the peptide loading complex (PLC) is responsible for peptide translocation into the ER and for placing suitable peptides into the HLA binding grooves [30]. Tapasin is a critical component of the PLC which performs peptide "editing" by promoting dissociation of low affinity peptides [31]. Tapasin-dependent HLA allotypes require the PLC for peptide loading, and they preferably present high affinity peptides while tapasin-independent allotypes do not require the PLC for peptide loading, resulting in greater promiscuity leading to presentation of low affinity peptides [32]. Therefore, expression level of tapasin-dependent and -independent HLAs can influence the breadth of the peptide repertoire and consequently to influence CD8+ T cell responses. Indeed, it was shown that tapasin-independent allotypes have greater breadth in HIV peptide presentation to T cells, making it more difficult for the virus to adapt to these HLA types, thereby explaining the protective effect of tapasin-independent HLA class I genotypes [33].

Similarly, various dependencies of HLA-II allotypes on peptide loading mediated by HLA-DM and HLA-DO have been reported [34]. HLA-DM opens the binding groove of HLA-II, allowing the exchange of the class II-associated invariant chain peptide (CLIP) with high affinity peptides in endo- and lysosomes. In the absence of HLA-DM, many HLA-II proteins do not exchange peptides and remain bound to CLIP, and DM-deficient cells are defective in class II antigen presentation. HLA-DO is suggested to act as a competitive inhibitor of HLA-DM that prevents peptide loading [35] and higher HLA-II-CLIP cell surface levels were found following over-expression of HLA-DO [36]. Nanaware et al. recently applied immunopeptidomics to characterize the repertoire of eluted HLA-II peptides from DO-sufficient and DO-deficient antigen presenting cells in vivo and in vitro [37]. They found that DO controlled the diversity of the presented peptide repertoire, with a subset of HLA-II peptides uniquely detected only when DO was expressed, which were found to be sensitive to DM-mediated exchange, suggesting that decreased DM editing was responsible for the broader repertoire. They concluded that regulated expression of HLA-DO might fine-tune the peptidome to enhance self-tolerance, while allowing focused

presentation of immunogenic epitopes during an immune response. Furthermore, the cysteinal lysosomal proteases, cathepsin L and cathepsin S, have been shown to mediate qualitative and quantitative differences in the HLA-II peptide repertoires [38,39]. The majority of HLA-II peptides were found to be presented regardless of the expression of these two proteases, although they alter the relative presentation levels of the peptides. Yet, the presentation of some relevant epitopes is critically regulated by cathepsin L or cathepsin S [38].

2.2. What are the sources of HLA peptides?

One of the main focuses of immunopeptidomics studies is the characterization of cellular source proteins that are processed and presented by the HLA presentation machinery. Beyond basic understanding of how proteins are sampled for presentation, knowing which proteins are likely to be presented in pathological conditions, for example when predicting the presentation of neoantigens derived from mutated proteins, is critical. Multiple research groups have shown that HLA-I presentation is associated with a higher expression levels of the source genes and proteins, and with a high turnover rate, which validates protein degradation as an important factor for HLA-I presentation [40–42]. Primary and secondary structure of proteins and particularly features linked to proteasomal degradation were found to have a strong influence on HLA-I peptide generation [9,42]. The source genes for HLA-I peptides are highly enriched in genes coding for intracellular proteins interacting with DNA, RNA, and other proteins, while HLA-II sources are enriched with transmembrane, endosomal, lysosomal and Golgi apparatus related proteins [43]. Immunopeptidomic studies demonstrated that HLA I bound peptides derive from selective regions of the human genome [42], and that the peptides are not randomly distributed along the proteins' sequences but are located within "hotspots" which fit proteasomal cleavage, peptide processing and HLA-binding rules [43].

HLA class I bound peptides can originate from newly synthesized, short lived proteins or defective ribosomal products (DRiPs) that get translated from canonical as well as non-canonical 'cryptic' translation [44–47]. Remarkably, HLA-bound peptides are protected from further degradation, and can remain on the cell surface for several hours [48]. Therefore, the immunopeptidome is not an accurate mirror of the proteome, but a compilation of the translation and degradation rates of the coding and the presumed non-coding transcripts and resulting proteins, and is further shaped by APPM dynamics. For example, Komov et al. treated cultured breast cancer cells with interferons and applied dynamic stable isotope labeling by amino acids in cell culture (SILAC) followed by MS analyses to follow proteins turnover and to distinguish between HLA-peptides derived from DRiPs or retirees by simultaneously analyzing the kinetics of synthesis of the cellular proteins and their derived degradation products as HLA-I peptides [15]. They found that DRiP HLA-peptides are derived from surplus subunits of the proteasome and ribosome. These surplus subunits are degraded as a result of the interferon-induced transition to immunoproteasomes and recomposed ribosomes that incorporate protein subunits that are induced by the interferons. The phenomenon of efficient presentation of excess unstable proteins is relevant for immune-detection of virus infected cells mediated by a rapid presentation of HLA-I peptides derived from excess canonical and cryptic viral proteins [15,49,50].

2.3. What is the limiting factor in HLA-I presentation?

While the HLA-I APPM has been thoroughly studied, it remained unclear whether the peptide supply in the endoplasmic reticulum (ER) or the availability of peptide-receptive HLA molecules limits presentation levels. In efforts to identify the main bottleneck for HLA presentation, Komov et al. modulated the HLA peptidomes with IFN-β or IFN-γ, and induced competition for peptide loading within the ER by expressing high levels of recombinant soluble HLA-A*02:01, in addition to the endogenously expressed membranal HLA-A*02:01 [14]. Both the membranal and soluble HLA peptidomes were identified and quantified by LC-MS/MS. The IFNs treatments increased the numbers of identified and quantified HLA-peptides, and importantly, the soluble HLA-A*02:01 overexpression did not alter the cell surface expression nor the numbers and intensities of membranal HLA-A*02:01 peptides presented by the transfected cells [14]. These results suggest that both membranal and soluble HLA draw peptides from the same peptide pool, that excessive amounts of peptides fit for loading exist in the ER, and that the membranal HLA presentation was not affected by the competing soluble HLA. Hence, the HLA peptidome is limited by the supply of peptide-receptive HLA molecules, rather than of high-affinity peptides in the ER. This has important implications: HLA downregulation was observed in various malignancies as a central mediator of tumor escape. Ultimately, restoration of HLA-expression and not of peptide processing could potentially overcome this barrier.

2.4. Defining HLA restriction

The first HLA-peptide elution and immunopeptidomics studies in the early 90′s with Edman degradation and right after with LC-MS/MS revealed the existence of the antigenic peptides and the commonality of specific anchoring residues that facilitate direct interactions and binding to the HLA complexes [51–56]. Throughout the years, different immunoprecipitation technologies have been developed, aiming to improve the purity and recovery of HLA-bound peptides to allow accurate definition of the binding motifs. Mild acid elution is an alternative technique enabling continuous stripping of peptides from cells in culture, revealing dynamics of peptide presentation in intact cells [57]. Tagged soluble HLA expression vectors of selected alleles were stably transduced into cultured cells and HLA complexes were collected and purified from liters of growth medium, minimizing interferences from the membrane-anchored endogenous HLAs [58]. When the sensitivity of MS improved, mono allelic cells lacking endogenous HLA expression were engineered to transiently or stably express HLA complexes of interest, reaching sufficient recovery from as little as few millions of cells [40,59,60]. PanHLA antibodies are commonly used for bulk HLA purifications from any given cell line or tissues, resulting in high peptide yields with very little bias against particular alleles [41]. However, in such datasets the definition of HLA binding restrictions require data post-processing to deconvolute the peptides into clusters with clear motifs [61–63] and to train machine learning tools to develop predictors of panHLA binding [60,64,65]. Such techniques resulted in defining binding specificities of phospho-HLA-I and HLA-II peptides across many HLA restrictions [66,67], as well as non-human MHCs, like bovine, canine, and murine MHCs [68–71]. When combined with additional sources of training datasets, such as of proteasomal processing or immune-recognition by T cells, advanced machine learning based tools offer improved performance in predicting peptides derived from any sources of proteins or polypeptides, that are likely to be processed, to bind the respective HLA, and to be recognized by T cells [40,72–74].

3. Pre-clinical discovery and applications

A strong motivation for conducting pre-clinical immunopeptidomics studies is reflected by the clinical relevance of HLA molecules for any disease or syndrome where cytotoxic T cells are involved, such as cancer, infection, and autoimmunity. They may mediate important risk factors or act as protective alleles in different diseases [75,76] and they have indispensable roles in the risk of acute graft-versus-host disease after allogeneic hematopoietic cell transplantation due to recipient mismatching of HLAs and the involvement of minor histocompatibility antigens [77,78]. Importantly, opportunities for therapies become attractive and immediate when the exact presented immunogenic peptides that play a role in these T cell responses are revealed.

Currently, MS based immunopeptidomics is the only methodology to comprehensively investigate the repertoire of HLA bound peptides

presented naturally in vivo [79]. The immunoaffinity purification of HLA complexes, in their native form, is often the method of choice. It allows the extraction of the peptides from any biological sample that can be immediately processed or freshly frozen. Any processing that would require extensive heating of the biological sample is detrimental for the native purification such as the removal of the paraffin in tissue preservation with formalin fixation (FFPE). Cell lines, tumors and healthy tissues [41,80,81], patient-derived xenograft tumors (PDX) models [82], body fluids such as plasma [83,84], pleural effusions [85], synovial tissue and synovial fluid, peripheral blood mononuclear cells [86], and enriched populations of specific cell types [87,88], have all been subjected to immunopeptidomics analyses that aimed at identifying antigens of interest among the endogenously presented peptides.

3.1. Type of antigenic sources

Naturally, all pathogens that lead to the presence of pathogenic proteins within host cells can be processed and presented to the immune system as either HLA-I or HLA-II antigens, with various efficiencies related to their mode of infection and cellular localization together with the host proteome. Kalaora et al. explored the presentation HLA bound peptides derived from bacterial sources that colonize in human melanoma tumors [89]. The HLA presentation of canonical antigens, which are encoded within the open reading frames (ORFs) of protein-coding genes, have been widely explored by immunopeptidomics. For example, cancer/testis antigens are commonly expressed in tumors across indications [90,91] and their immunotherapeutic potential has being tested in the clinic [92]. Furthermore, post-translational modifications (PTMs) such as phosphorylation, citrullination or glycosylation may also occur on presented antigens which have been reported to modulate antigen presentation and recognition [81,93–97]. In a recent study, Kacen er al. developed a Protein Modification Integrated Search Engine (PROMISE) that allows for combinatorial detection of multiple PTMs without prior biochemical enrichment [98]. Immunopeptidomics, combined with genomics and transcriptomics can support the identification of antigens derive from protein-coding genomic regions that have acquired somatic mutations, including nonsynonymous single-nucleotide variants (SNVs), nucleotide insertions or deletions and gene fusions [81,99]. Non-genetically encoded neoantigens can be produced due to tryptophan shortage, ribosome stalling, and tryptophan-to-phenylalanine codon reassignment [100,101]. HLA-bound peptides can derive from non-canonical antigenic sources [102], also called alternative, cryptic or dark-matter antigens, due to alterations in transcription, translation and in the cellular processing, leading to presentation of peptides from transposable elements, intronic regions, lncRNAs, circRNAs and new unannotated open reading frames (upstream, downstream, and overlapping with main annotated reading frames) [42,103–109]. There is great potential in targeting such non-canonical antigenic sources as was demonstrated by Wingarten-Gabbay et al., who explored with immunopeptidomics the antigenic landscape in SARS-CoV-2 infected cells and found peptides derived from canonical and non-canonical ORFs in spike and nucleaocapsid that were not represented in available vaccines. Some of the out-of-frame ORF-derived peptides elicited T-cell responses that were more potent than the known immunogenic canonical peptides [50].

3.2. Target identification at the steady state

For clinical translational, it is of great interest to discover the specific peptides that can trigger spontaneous immune responses in a particular disease setting (Fig. 1). For instance, a meta-analysis of acute myeloid leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia and multiple myeloma revealed discernable difference in the antigen landscape profile between each of those sub-types, reflecting cell-type specific biology [110]. For all four of these subtypes, cancer-specific immunogenic HLA ligands were detected from clinical samples [111–114] including a common cancer-specific antigen in at least 25 % of all examined cohorts [110]. Besides target identification, knowledge about the overall distribution and presentation profile of all peptides across healthy tissues, cell types, and pathological conditions is relevant to understand on-target as well as off-target toxicities. For example, targeting an antigen that is not absolutely tumor-specific imposes the risk of severe on-target, off-tumor toxicity. A known example for this is a study where patients who were treated with engineered T cells targeted to the MAGEA3 peptide suffered from lethal brain toxicity due to presentation of the antigenic peptide in particular brain cells [115]. In contrary, Kim et al. employed a population-scale immunopeptidomics across ~1500 tumor and normal tissues and identified an HLA-A* 02:01-restricted shared epitope derived from a cancer-specific alternative-spliced variant of the collagen type VI α-3 gene that is highly presented on tumor stroma across multiple solid cancers [116]. T cells transduced with affinity-matured TCRs against this epitope specifically eliminated tumors in vivo with no detectable off-target reactivity [116]. In autoimmunity, cross-reactivity of T-cell receptors and molecular mimicry of peptides in combination with presentation profiles of specific HLA alleles are playing important roles. In multiple sclerosis, a disease where the immune system degrades myelin sheaths of neurons, HLA-DR15 is a long established risk factor, accounting for up to 62 % of the disease's genetic aetiology [76]. B-cells and monocytes of multiple sclerosis patients with the DR15 haplotype present RASGRP2-derived self-antigens that can trigger CD4+ T-cell responses. T-cells triggered by RASGRP2$_{(78-87)}$ peptide showed cross-reactivity for several EBV and *Akkermansia muciniphila* antigens, suggesting that infection with these agents can trigger auto-immunity in vivo [117]. Similar observations were made for reactive arthritis, for which HLA-B27′s prevalence is estimated to lie between 30 % and 50 %. *Chlamydia trachomatis* infection is a consistent trigger of this arthritis [118] and Several *C. trachomatis*-derived epitopes with high homology to human epitopes and according conformational similarity in the HLA-B27 binding groove were discovered, implying their relevance for disease development [119]. The HLA Ligand Atlas, a human reference database of immunopeptidomes of 29 benign tissues and numerous HLA alleles is an important resource to support annotation of specific targets. Such efforts should be expanded to cover additional HLA allotypes across all the entire human population, as well as to cover particular cell types and tissues in context of common pathologies, and importantly, with an improved depth and coverage [120].

3.3. Treatment induced remodeling of the antigenic landscape

Cellular perturbations can alter the expression of the source proteins and of the cellular machinery; hence, they can have a drastic impact on the presented repertoire (Fig. 1). This impact may be beneficial, for example, by enhancing the presentation of neoantigens [121] and cancer associated antigens [122,123] to expand the pool of potential clinically relevant immunogenic targets, on the other hand it may also induce autoimmunity [124] or downregulation of neoantigen presentation [121]. In other instances, minimal alterations are beneficial. Nelde et al. performed a longitudinal study of the effect of lenalidomide, which is an immunomodulatory drug that can reinforce effector T-cell responses, in chronic lymphocytic leukemia. They demonstrated that lenalidomide exposure did not affect absolute HLA class I and II surface expression and with semi-quantitative immunopeptidomics analyses they found only minor qualitative and quantitative differences in HLA class I- and II-restricted peptide presentation. This study therefore validates the suitability of lenalidomide in combination with antigen-specific T-cell-based immunotherapies [125]. Immunopeptidomic studies also allowed the deep exploration of how standard of care drug treatments modulate the antigenic landscape, like decitabine [123,126], doxorubicin [127], palbociclib [122], and carfilzomib [128], as well as therapeutic immunotherapy agents like oncolytic viruses [129] and cytokines [100,130].

Immunopeptidomics can reveal unique mode of action for drug sensitivity. For example, Flucloxacillin (FLX), an antibiotic drug has been associated with severe immune-mediated drug-induced liver injury mediated by T cells that potentially recognize drug-haptenated peptides bound to HLA-B* 57:01 on liver cells. Puig et al. applied immunopeptidomics to cells expressing HLA-B* 57:01 and found a more diverse repertoire in treated cells that was enriched with peptides containing carboxy-terminal tryptophan and FLX-haptenated lysine residues on peptides [131]. They confirmed immune reactivity in vivo against FLX-modified peptides, hereby identifying a novel effect of antibiotics that may affect drug-induced inflammation.

Perturbations by radiation have also been explored. Wolf et al. employed immunopeptidomics to identify tumor-specific neoantigens in various UVB-irradiated single clone cell lines derived from a parental melanoma cell line B*29:05. They evaluated whether the clone cell lines' documented high immunogenicity and T cell infiltration was related to the presentation of common or shared neoantigens [132]. Tailor et al. gamma-irradiated CT26 colon carcinoma murine cells at doses of 10 Gy to explore alterations of the proteome and immunopeptidome [133]. They confirmed an induction of MHC-I and other key proteins of the presentation machinery in response to radiation, among them B2M, TAP1, ERAP1 and immunoproteasome subunits. Their analysis revealed a small increase in the total number of unique peptide sequences following treatment with an increase in overall peptide intensity 48 h post-irradiation, leading to the detection of mutated neoantigens under experimental conditions [133]. In humans, Löffler et al. applied immunopeptidomics to guide the selection of tumor antigens to assess the impact of radiofrequency ablation (RFA), on the induction of antigen-specific immune responses in patients [134]. RFA is an established treatment option for malignancies located in the liver that induces irreversible coagulation necrosis that releases danger signals and cellular content. As such, RFA may constitute an endogenous in situ tumor vaccination by stimulating antigen specific T cells. They found that RFA boosted or induced T cell responses specific for individual tumor antigens, however, they concluded that RFA induced modest immunological effects that may not be sufficient for the rejection of established tumors [134].

4. Driving the development of innovative immunotherapies

4.1. Informing the design of clinical trials

For clinical purposes, the selection and identification of targetable HLA-presented antigens should ideally combine several layers of evidence and not use a single technology to assure robustness. This was already highlighted in 2004, where the "Tübingen approach" was presented, describing key steps required for identification, selection and validation of peptides derived from tumor antigens [135], emphasizing the importance of HLA immunopeptidomics in obtaining a comprehensive view of the landscape of targetable antigens. Indeed, the innovative studies discussed above, as well as many others we could not cover in this review, immensely advanced the development of immunotherapies for cancer and other diseases. From supporting vaccines design against *M. tuberculosis* [136] to justifying testing TILs therapy in a pilot clinical trial in low tumor mutation burden ovarian cancers [137], immunopeptidomics has gradually became a key technology for clinical translation (Fig. 1).

Inclusion, or targeting of, multiple tumor-associated epitopes in a vaccine is considered essential in order to induce a broad and specific immune response against cancer cells. Therefore, immunopeptidomics-guided warehouses of shared tumor antigens, deeply characterized for tumor specificity, immunogenicity, and HLA restriction, were created. Such warehouses store peptides synthesized under Good Manufacturing Practice (GMP) standards for vaccination purposes, making such 'off-the-shelf' therapies accessible in a fast and reliable fashion. Nine HLA-A*02–restricted and one HLA-DR–restricted tumor-associated peptides, identified by MS and derived from highly overexpressed tumor antigens in tens of renal cell carcinoma primary tumors were formulated into the IMA901 multipeptide vaccine. The IMA901 vaccine was tested in HLA-A*02+ subjects with advanced renal cell carcinoma (RCC) in a phase 1, a randomized phase 2, and a phase 3 (n = 339, NCT01265901) trial. The initial clinical trials assessed the association of T cell responses to IMA901 with clinical benefits, and their results indeed suggested that therapeutic vaccinations with IMA901 induced specific immune responses that lead to improved outcomes [138]. However, in the phase 3 study, vaccination with IMA901 was combined with GM-CSF in addition to first-line sunitinib in patients with advanced, previously untreated metastatic RCC eventually. This treatment did not prolong overall survival compared with sunitinib alone and exploratory immunomonitoring analyses showed a significantly diminished magnitude of CD8-positive T-cell responses in this study compared with the phase 2 study [139]. Despite setbacks, the warehouse strategy was demonstrated to be clinically feasible in CLL [140], prostate cancer [141], glioblastoma [142,143] and malignant astrocytoma patients [144].

The prior identification of suitable target antigens with immunopeptidomics still remains central for the development of innovative immunotherapies [145] including antibodies directed against HLA–peptide complexes (such as T cell receptor (TCR)-mimic antibodies and bispecific antibodies) [146], chimeric antigen receptor (CAR) T cells targeting a cell-surface protein [147] and for directing the specificity of T cells by TCR-transduction [148]. Successful priming of peptide-centric CAR-T-cells by identifying targets with immunopeptidomics has already been demonstrated by targeting tumor-specific neuroblastoma antigens, inducing complete tumor regression in grafted NSG mice [149].

4.2. Immunopeptidomics integrated into the clinical manufacturing process

The validation of an LC-MS/MS antigen identification procedure [150] was an important step towards the direct implementation of immunopeptidomics in exploratory clinical trials. Mass spectrometry analysis was validated according to the principle of good laboratory practice (GLP), where accuracy, precision, specificity, limit of detection and robustness were validated according to the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) guidelines. Importantly, the validation process should be tailored to the available instruments in each laboratory, as described in a guidance document by Ghosh et al. [150].

The implementation of immunopeptidomics in real-time in vaccine manufacturing process (Table 2) was first published in 2019 by Hilf et al. where results from an innovative clinical study (NCT02149225) of actively personalized vaccination in newly diagnosed glioblastoma were described [143]. First, they established an immunopeptidomics-guided 'off-the-shelf' warehouse including multiple glioblastoma associated tumor antigens. They selected the relevant warehouse peptides for each of the patients based on the immunopeptidome and transcriptome of their individual tumors, as well as pre-vaccine T-cell reactivity against potential targets. This way, a patient-specific ranking of the warehouse peptides to select seven most suitable best-ranked HLA-class I peptides was performed. To these, two HLA-DR peptides and one viral control marker peptide were added to constitute the actively personalized vaccine 1 (APVAC1). In addition, another antigen selection procedure was tested in this study. For the APVAC2 vaccine, for each patient two additional peptides were selected for GMP manufacturing: either mutated neoantigens detected by immunopeptidomics in the patient's tumor or *in silico* generated neoantigens that were predicted to bind the patient HLA allotypes. If none of these options yielded targets, non-mutated peptides from tumor associated antigens found in the immunopeptidome analysis were included. In this clinical study, no mutated neoantigens were identified directly by MS, and eventually in silico predicted neoantigens and MS-identified TAA were specifically manufactured for the patients. The clinical trial was authorized in five

Table 2
Clinical trials that directly integrate MS-based immunopeptidomics in the decision-making process for personalized interventions.

ClinicalTrials.gov Identifier and related references	Description	Antigen-related treatment modality	Application of immunopeptidomics	Targeted antigens	Time allocated for antigen discovery
NCT02149225 [166]	GAPVAC Phase I Trial in Newly Diagnosed Glioblastoma Patients Completed	Multiple vaccination cycles with APCVAC1 peptide vaccine with Poly-ICLC and GM-CSF as adjuvants, followed by multiple vaccination cycles with APVAC2 peptide vaccine with Poly-ICLC and GM-CSF.	An off-the-shelf peptide warehouse consisting of 33 non-mutated HLA-presented antigens identified from 30 glioblastoma specimens using immunopeptidomics, the XPRESIDENT technology and T cell based immunogenicity assays. APVAC1: RNAseq and immunopeptidomics were applied to patient-derived tumor biopsy and analyzed to select peptides from the warehouse. Peptide candidates were additionally tested for immunogenicity with patient-derived PBMCs. APVAC1 consists of 5–10 HLA class I peptides from the warehouse. In addition, vaccines included two pan-DR HLA class II antigens and a viral marker peptide. APVAC2: Immunopeptidomics was applied to patient-derived tumor and healthy tissue biopsies, integrated with RNAseq and WES data from same samples, to discover presented mutations. Mutations confirmed by sanger sequencing were prioritized with HLA-peptide binding predictions, RNAseq and tissue distribution of wild-type gene expression. As no mutated neoantigens were eventually found by immunopeptidomics, APVAC2 consisted of either 1–2 predicted neoantigens (19 a.a. long peptides with mutation at position 10) or non-mutated glioblastoma-associated HLA class I ligands that were identified in the individual immunopeptidome and that were not part of the warehouse.	Neoantigens and tumor-specific antigens	APVAC1: 10 weeks APVAC2: 23 weeks
NCT04688385 [140]	Personalized Multi-peptide Vaccination in Chronic Lymphocytic Leukemia PatientsRecruiting	Multiple vaccination cycles with a multi-peptide vaccine cocktail and the adjuvant XS15.	An off-the-shelf peptide warehouse consisting of CLL-exclusive non-mutated 9 HLA class I and 5 HLA class II target antigens was constructed using immunopeptidomics and T cell based immunogenicity assays. HLA class I-restricted peptides for each patient are selected from the warehouse individually based on the patient-specific HLA allotypes and HLA immunopeptidome analysis of CLL cells while the 5 HLA class II peptides will be administered to each patient.	CLL-exclusive non-mutated antigens	N.A
NCT05195619 NCT04627246 [153]	Personalized Dendritic Cell Vaccines in Non Small Cell Lung CancerRecruiting Personalized Vaccine With Standard of Care Chemo Followed by Nivo in Pancreatic CancerRecruiting	Multiple vaccination cycles with autologous monocyte-derived dendritic cells pulsed with up to 10 long optimally design mutated peptides with a length of up to 25 amino acids.	Immunopeptidomics, WES and RNAseq applied to patient-derived tumor biopsy and WES on matched PBMCs, are analyzed together to detect, predict and prioritized expressed and likely immunogenic neoantigens, with priority given to neoantigens detected by immunopeptidomics. Up to 10 long optimally design mutated peptides with a length of up to 25 amino acids are prioritized.	Neoantigens	2–4 weeks
NCT04643574 [154]	NeoTIL in Advanced Solid TumorsRecruiting	Adoptive transfer of neoantigen-enriched, autologous T-cells.	Immunopeptidomics, WES and RNAseq applied to patient-derived tumor biopsy and WES on matched PBMCs, are analyzed to detect, predict and prioritized expressed and likely immunogenic neoantigens, with priority given to neoantigens and TSA detected by MS. Neoantigen or tumor-specific antigenic peptides and autologous CD40-activated B-cells engineered to expressed co-stimulatory molecules are added to the autologous tumor tissue fragments from which antigen-enriched TILs are expanded.	Neoantigens or tumor-specific antigens	2 weeks

European countries based on a standardized drug definition and GMP manufacturing process, leading to variable vaccine compositions for each patient. Fifteen patients received APVAC1 and eleven patients received APVAC2 vaccine. Favorable immune responses were observed for APVAC1, probably due to the fact that the neoantigens included in APVAC2 were selected without prior knowledge about their immunogenicity, arguing for the importance of T cell based immunogenicity assays as an additional selection criteria. In low mutational load tumors, like glioblastoma, exploitation of non-mutated antigen pool was justified, and the warehouse-based approach was shown to be suitable [143]. Similarly, the iVAC-XS15-CLL01 personalized multi-peptide vaccine in combination with the TLR1/2 ligand XS15 adjuvant in chronic lymphocytic leukemia (CLL) patients (NCT04688385) is based on the off-the-shelf peptide warehouse approach [140]. For each patients, based on the specific HLA allotypes and HLA immunopeptidome analysis of the CLL cancerous cells, patient-individual vaccine cocktail is selected from a CLL-exclusive warehouse.

Our group is also involved in the manufacturing of personalized cancer vaccines where immunopeptidomics, in combination with genomics, transcriptomics and in silico prediction algorithms, serve as the antigen discovery engine [151–153]. These are first-in-man phase 1 trials approved by the Swiss authorities where patients with difficult-to-treat tumors, such as non small cell lung cancer (NCT05195619), and pancreatic ductal adenocarcinoma (NCT04627246), are treated with therapeutic vaccination comprising autologous dendritic cells loaded with up to 10 long peptides (up to 25 mers). The long peptides are selected and designed specifically for each patient based on deep antigen discovery exploration that is performed in real time within 2 weeks from reception of surgically removed tissues. Such fast turnaround time is critical for clinical translation and is achieved ultimately due to dedicated sample processing, MS-analyses, genome and transcriptome sequencing, and integrative computational analysis processes that have been optimally designed for clinical application. Tumor associated antigens, mutated antigens (SNVs and indels), antigens derived from oncoviruses as well as from non-canonical sources (such as transposable elements, lncRNAs, novel ORFs) and post-translationally modified antigens are selected based on the genome, transcriptome and the HLA-I and –II immunopeptidome analysis of the individual tumors [102]. They are subsequently ranked based on annotations and defined rules set to prioritize the more likely naturally presented, tumor-specific, immunogenic, clonal antigens covering multiple HLA class I and II alloypes. The safety and immunogenicity of such personalized dendritic cell vaccine was demonstrated in a case study recently, where a complete oncological response was obtained with respect to both radiological assessment and the tumor marker CA-125 in a serous endometrial adenocarcinoma patient [152].

The relatively fast turnaround time allowing listing of personalized antigenic targets paved the way to personalization of other innovative treatment options such as the adoptive transfer of T cells. Expansion of antigen specific autologous tumor infiltrating T cells (TILs), in the presence of neoantigen-peptides and antigen presenting cells engineered to express co-stimulatory molecules, was demonstrated recently by Arnaud et al. as the NeoScreen approach [154]. The NeoScreen method enabled highly sensitive screening of tumor-associated and neoantigens and yielded a markedly broader repertoire of antigen-reactive TCRs than alternative methods by increasing the frequency of antigen-specific TCRs and by recruiting additional TCR clonotypes [154]. In addition, NeoScreen represents a valuable pipeline to expand tumor-reactive TCRs for personalized engineered T cell therapy of solid tumors. Currently, a phase 1 pilot study (NCT04643574) to assess the feasibility, safety and efficacy of adoptive transfer of autologous TILs enriched for tumor antigen specificity (NeoTIL) in advanced solid tumors is conducted, leveraging the integration of our clinical personalized neoantigen discovery pipeline and the NeoScreen approach.

5. Future and emerging technologies and directions

Innovative analytical developments improving the sensitivity of MS leading to the thriving of single cell proteomics studies are expected to have a similarly positive impact on the identification and quantification of HLA peptides in immunopeptidomics studies. Tandem mass tag labelling to barcode peptides can address both the issues of low abundance and low quality of MS/MS spectra [155–157], while recombinant heavy-isotope-coded peptide major histocompatibility complexes (hipMHCs) have been used as internal standards for normalization correction to enhance reproducibility of immunopeptidomics measurements [158]. Improvements in peptide identification rates and reproducibility also derive from miniature sample preparation [159], very low-flow-rate chromatography and modifications on the TIMS–ToF instrument combined with parallel accumulation serial fragmentation, leading to more than tenfold higher sensitivity [160], will have a clear impact on the analysis of HLA peptides. Deep learning approaches allowing more accurate MSMS scoring are already implemented in the new generation of search engine tools with significantly improved speed, accuracy and robustness, and many of these tools support the analysis of immunopeptidomics data across different acquisition methods [161–164]. Also, genetically engineered mouse models that enable the specific expression of tagged MHC molecules, in cancerous cells for example, would allow the purification and identification of antigenic peptides that are specifically expressed and presented on defined cell populations [165]. Such new developments are expected to significantly enhance our understanding about the antigenic repertoire and the functioning of the APPM with regard to cell origin, tissue specificity and about the cross-talk between cells in vivo, that enable immune recognition. It is therefore expected that major scientific advancements in all the domains we reviewed here will surely happen, considering the growing interest in implementing immunopeptidomics in academic research labs, core facilities, biotechnology and pharma companies, and will continue to support clinical translation to improve human health.

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