



The role of proteomics in the age of immunotherapies

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Abstract

The antigenic landscape of the adaptive immune response is determined by the peptides presented by immune cells. In recent years, a number of immune-based cancer therapies have been shown to induce remarkable clinical responses through the activation of the patient's immune system. As a result, there is a need to identify immune biomarkers capable of predicting clinical response. Recent advances in proteomics have led to considerable developments in the more comprehensive profiling of the immune response. “Immunoproteomics” utilises a rapidly increasing collection of technologies in order to identify and quantify antigenic peptides or proteins. This includes gel-based, array-based, mass spectrometry (MS), DNA-based, or computer-based (in silico) approaches. Immunoproteomics is yielding an understanding of disease and disease progression, vaccine candidates, and biomarkers to a depth not before understood. This review gives an overview of the emerging role of proteomics in improving personalisation of immunotherapy treatment.

Introduction

In recent years, it has become clear that immunotherapy, previously thought to be useful in only a few select malignancies, has significant clinical activity in a variety of cancers including melanoma, lung, bladder, head and neck, cervical, and most recently, solid cancers with mismatch repair-deficiency (reviewed in Dholaria et al. 2016; Sharma and Allison 2015; Syn et al. 2017). Advances in the area of immunotherapy for treatment of these cancers is based on our increased understanding of tumourigenesis and cancer progression, which involve accumulating mutations that result in a diverse set of antigens that the immune system can use to distinguish cancer cells from normal cells. This increased understanding of the immune system plus the development of immune modulation techniques have led to a

new era in cancer therapy, which harnesses our own immune system to treat cancer.

Under normal circumstances, to ensure that the immune system does not harm the host when reacting to a foreign antigen, humans have evolved immune checkpoint proteins (ICPs) to quickly stop an immune response. However, when cancer develops in a patient, multiple mechanisms of immune suppression activate to prevent effective anti-tumour immunity (Li et al. 2018). The ICPs are cell surface receptors expressed by immune cells that regulate the activation and effector functions of T lymphocytes, which are modulated by a set of co-stimulatory and co-inhibitory molecules (reviewed in detail in Haanen and Robert 2015; Pardoll 2012; Rowshanravan et al. 2018).

The best characterised ICPs—and the most actively exploited in the context of cancer immunotherapy—are cytotoxic T-lymphocyte protein 4 (CTLA-4) and the programmed cell death protein 1 pathway (PD-1 receptor/PD-L1 and PD-L2 ligands). The most well-prescribed cancer immunotherapies target those co-inhibitory T-cell checkpoint receptors using ipilimumab (CTLA-4) and nivolumab/pembrolizumab (PD-1) to reverse immune “exhaustion” and improve tumour responses. Other cancer immunotherapies have been approved for use in recent years, including preventive and therapeutic cancer vaccines, a bi-specific T-cell engager (Topp et al. 2011), and an oncolytic virus (Andtbacka et al. 2015). Of these, immune checkpoint antagonists that target the PD-1 pathway have generated the most

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interest, with response rates across tumour types that average 20–30% (Lipson et al. 2015). With many more immunotherapy drugs under development, a major challenge for the field is to identify which patients will benefit from immunotherapy treatment in order to personalise patient care.

Personalised (or precision) medicine is an approach that takes into account differences between individuals to guide treatment, with oncology being the most prominent field in this area. The emergence of the ‘-omics’ sciences (genomics, proteomics, metabolomics, transcriptomics, and interactomics) now provides us with workflows in order to gather detailed information into the immune response required for personalised medicine.

Specifically, proteomics provides information on protein expression, subcellular protein localisation, post-translational modifications (PTMs), and protein–protein interactions within an organism. As an extension of the proteomics field, the term “immunoproteomics” was first used in 2001 to focus on the profiling of proteins associated with the immune response (Jungblut 2001). The field of immunoproteomics is rapidly expanding and includes increasingly high-resolution and high-throughput techniques that have resulted in the identification of immune related proteins and peptides, presented due to invading pathogens, host cells, or immune signalling molecules. Recent advances in proteomics, including advanced mass spectrometry (MS) instrumentation, has provided a

detailed insight into tumourigenesis and progression, candidates for personalised cancer vaccines, and biomarkers of immunotherapy response. Here, this review will provide a broad overview on how immunoproteomics is being used to further understand the immune system and can be exploited to personalise immunotherapy for cancer treatment.

Common proteomic approaches for investigating the immunoproteome

The study of immune biomarkers or antigens using proteomics has been performed since the 1990s, with “classical” methods such as gel-based analysis, agglutination, enzyme-linked immunosorbent assay, or Western blotting used to study the immune response to cancer. However, in many of these traditional techniques, assay sensitivity and the low throughput nature of protein or peptide identification has often been the bottleneck in performing comprehensive immunoprofiling. Recent advances in proteomics, including improvements in MS protein profiling, have led to many of the rapid progress in our understanding of the immunoproteome. Common proteomics methods used to study the immunoproteome are compared in Fig. 1.

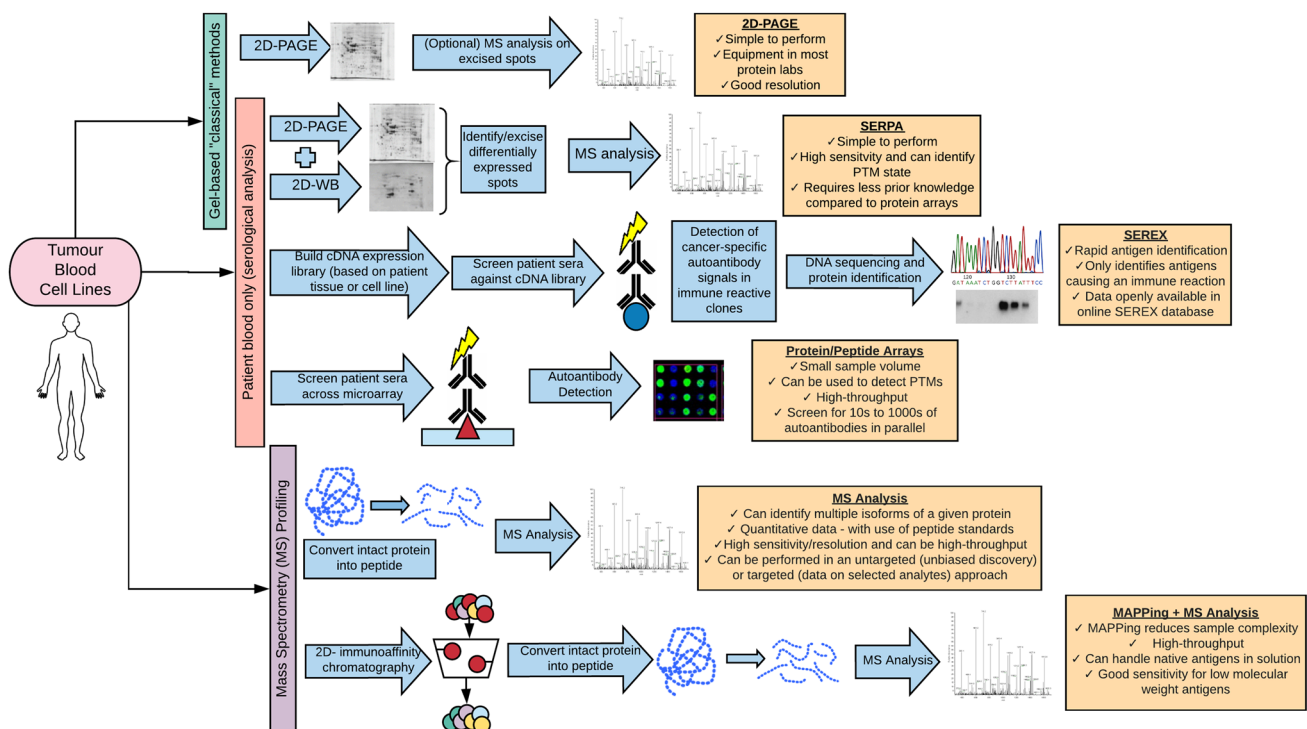


Fig. 1 Common proteomics methods used to study the human immunoproteome

“Classical” gel-based analysis of the immunoproteome

One of the most classical immunoproteomics approaches relies upon two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Overall, 2D-PAGE is a relatively simple method of analysis for immunoproteomics research. With the development of protein staining and image analysis software, it has become even more accessible to a general scientific audience. Even today, it remains one of the few techniques that allow high quality analysis of intact proteins on a proteome wide scale, including detection of protein PTMs. However, due to several key disadvantages, particularly difficulties in resolving very large, small, hydrophobic, or basic proteins (Fulton and Twine 2013), 2D-PAGE as a standalone immunoproteomics technique has been largely superseded by non-gel-based approaches.

Serological gel-based immunoproteome analysis

Several immunoproteomics approaches, such as Serologic Proteome Analysis (SERPA), Serological analysis of recombinant complementary DNA (cDNA) expression libraries (SEREX), and protein microarrays, are now being employed to identify tumour-associated antigens (TAAs) and their related antibodies in patient plasma/sera (Desmetz et al. 2009; Fulton and Twine 2013).

One of the most overlooked benefits of 2D-PAGE is the ease and efficiency with which it can be combined with other proteomic techniques to discover serological antigenic proteins and cancer biomarkers. When combined with Western blotting and MS-based protein identification (known as SERPA), 2D-PAGE provides a powerful approach for antigen identification. One major advantage to this technique is that it can be used to study a whole cell proteome, or sub-proteome (e.g. membrane fraction). Also, SERPA experiments allow for the identification of the PTM state of antigens, as well as providing the high sensitivity through use of the parallel immunoblots (Ganesan et al. 2016). Additionally, many gels can be run simultaneously to the blotting experiment, providing gels for reference maps and identification of immunoreactive proteins (Fulton and Twine 2013).

SEREX was first developed to discover tumour-specific antigens that elicit a high titre immunoglobulin G (IgG) antibody in sera from patients with multiple cancer types (Sahin et al. 1995). Although not entirely a proteomic technique, SEREX allows systematic and unbiased search for cancer-specific antigens, and immunogenic proteins based on their reactivity with an individual's serum. This approach is highly sensitive given the use of DNA detection, rather than solely protein detection methods (which can be limited by protein abundance). Unfortunately, a critical limitation of SEREX is that this method lacks the ability to differentiate

or detect PTMs in the identified cancer-specific antigens. Overall, the application of SERPA and SEREX technologies has been limited in the past due to assay specificity and the complexity of the assay preparation and procedure (Yuan et al. 2016).

Protein/peptide arrays

Some of the limitations of the classical 2D gel-based immunoproteomics methods have been overcome with the development of protein arrays to study the humoral immune response. In the last few years, affinity proteomics, represented mainly by antibody microarrays, have been developed and established as a key tool within proteomics, providing opportunities for multiplexed protein expression profiling. These protein/peptide chips have been generated with as few as 14 proteins to as many as roughly 17,000 proteins (Hu et al. 2012) that can be used to screen cancer patient sera for corresponding autoantibodies (Gnjatic et al. 2010; Hudson et al. 2007). An added benefit of protein arrays is that detection of PTMs can be incorporated as part of the array screening by using synthetic platforms such as a glycosylated peptide array (Blixt et al. 2010).

Like any blood-based assay, stringent adherence to guidelines on the sample collection and storage is required for protein microarray analyses to avoid inter- and intra-assay variation and improve result reproducibility. In addition, bioinformatics is critical for handling and processing the large datasets generated by these experiments. Current challenges of protein arrays in immunoproteomics, and how to address them, have been extensively reviewed by Delfani et al. (2016). The primary benefit of this method is the relatively low amount of patient sample required for analysis [2 µL for protein array vs. 50–100 µL for 2D-Western blot (2D-WB)], which means that pooled serum is not required and individual immunoproteome profiles can be characterised. Small sample size, combined with the high-throughput capacity of protein microarrays, makes it a powerful method of rapidly interrogating large numbers of patient sera samples.

“Modern” mass spectrometry (MS)-profiling

One of the biggest advances in characterising the immunome is the identification of HLA-bound ligands that are presented on the cell surface using high-performance liquid chromatography and tandem mass spectrometry (HPLC–MS/MS). This advance has rapidly broadened opportunities for high-throughput epitope-specific immunological analysis (methods reviewed in Caron et al. 2015), which had previously represented a bottleneck in immunopeptidome. Most modern MS proteomic methods use a “bottom up” approach, wherein the proteins are first digested into peptides. Subsequently, these resulting peptides are separated using liquid

chromatography (LC), then analysed by MS, followed by computational (in silico) data analysis.

This standard preparation procedure may be modified to use antibody-based immunocapture to enrich antigen proteins from cell lysates or sera, prior to MS analysis. Multiple Affinity Protein Profiling (MAPPING) is one application of this immunocapture-MS technique that has mostly been utilised to identify cancer related autoantigens (Hardouin et al. 2007a, b). The technique is based upon 2D-immunoaffinity chromatography, where antigens from tumour lysates are separated based upon their affinity for immunoglobulins from healthy controls and cancer patients in different chromatography steps, prior to enzymatic digestion of the isolated proteins, and identification by MS/MS.

Using proteomics for tumour antigen profiling for use in immunotherapy

The foundation of immunology is based on the ability of the host to perform self/non-self discrimination primarily to eliminate foreign pathogens. These pathogens usually contain molecular signatures that can be recognised as “non-self” by the host and trigger an immune response (Janeway Jr and Medzhitov 2002). Unfortunately, unlike pathogens, these molecular signatures are not generally expressed by tumour cells, making them more difficult to be distinguished from normal cells. However, T cells can recognise tumour antigens expressed on the surface of cancer cells. One class of tumour antigens, named tumour-associated antigens (TAA), are usually expressed in some normal tissues at low levels but are over-expressed in malignant cells. Many of these TAAs have been identified as the targets of tumour-reactive T cells, isolated from tumour infiltrating lymphocytes (TILs) found in lymph fluid or from blood (Cheever et al. 2009). Another class of tumour antigens are tumour-specific antigens (TSA or neoantigens), which are caused by mutations that alter amino acid coding sequences (non-synonymous somatic mutations) (Lu and Robbins 2016). These mutated peptides can be expressed, processed, and presented on the cell surface, and subsequently recognised by T cells. As normal tissues do not possess these somatic mutations, neoantigens appear to represent ideal targets for T-cell-based cancer immunotherapy.

The cell-mediated immunity arm of the adaptive immune response involves activation of immune cells (e.g. phagocytes or T cells) and can involve the release of other communicator molecules, such as cytokines and chemokines, in response to antigens. Specifically, T cells recognise short peptide antigens that are displayed on the surface of host cells in complexes known as the major histocompatibility complex (MHC). MHC-I molecules, termed Human Leukocyte Antigens (HLA) in humans (the preferred term used

throughout in this review), are highly polymorphic, particularly across the human population, with the bound peptides highly dependent on the patient’s HLA allotype. Therefore, profiling the MHC-I/HLA-I ligandome is of the utmost importance in understanding how the adaptive immune response is regulated in cancer.

In the presence of infected cells, mature T cells recognise HLA class I peptides from bacteria and viruses to trigger a “danger signal”, leading to infected cell removal. Transformed, pre-neoplastic and tumour cells also display atypical self-peptides from mutated or over-expressed TAAs. In immunotherapy, T cells can be directed against these cancer cells based on the pattern of cancer-altered HLA peptides. The characterisation of these antigenic peptides displayed on the surface of HLA molecules and specific T-cell epitopes has become essential for modern onco-immunological studies. This is because T-cell-based immunotherapeutic approaches require not only to identify TSAs, but also need to determine which of these TSAs are presented by the cancer cells of the individual patient. Moreover, peptides identified as being bound to HLA class I molecules may be able to be utilised in the development of therapeutic vaccines (discussed in “Using immunoproteomics for development of therapeutic vaccines” section and in detail in Haen and Rammensee 2013; Loffler et al. 2016) and for the development of T-cell receptor-engineered adoptive cell therapies (June et al. 2015).

In the last two decades of immunopeptidome research, antigens from various tumour cells have been identified, and their HLA class I-restricted epitopes have been predicted and confirmed using T-cell-based assays (Muller et al. 2003; Wang et al. 2006). Another classical method to identify immunogenic tumour-specific HLA class I-restricted epitopes relies on expression analysis of TAA followed by synthesis of predicted peptides and T-cell activation assays (Schultze and Vonderheide 2001; Weinschenk et al. 2002).

MS-based methods for profiling the HLA immunopeptidome

The most promising application using MS to study the immunome is the direct identification of ligands derived from primary tumour cells of patients, which can be used to examine HLA-presented peptide heterogeneity within one given sample. This method of analysis in patient tumour and plasma/serum could also lead to the identification of novel immunogenic target structures with potential for clinical applications (Berlin et al. 2015; Klar et al. 2014; Walz et al. 2015) and could be used in biomarker development (Bassani-Sternberg et al. 2010; Ritz et al. 2017). This MS-based immunopeptidomics approach can be also used to characterise neoantigens in solid tumours, with recent advances allowing for the identification of

naturally presented neoantigen epitopes on fresh frozen human melanoma tissue (Bassani-Sternberg et al. 2016) and in lymphoma tissue and cell lines (Khodadoust et al. 2017).

Currently, MS is the only unbiased methodology to comprehensively interrogate the *in vivo* endogenous HLA-bound peptide profile (Bassani-Sternberg and Coukos 2016), in human cell lines (Bassani-Sternberg et al. 2015; Singh-Jasuja et al. 2004; Weinschenk et al. 2002), patient tumour (Berlin et al. 2015; Dutoit et al. 2012; Walz et al. 2015), and biofluids such as plasma (Bassani-Sternberg et al. 2010). Most importantly, several studies have shown that MS technology can identify clinically relevant mutated antigens in humans (Bassani-Sternberg et al. 2016; Carreno et al. 2015; Kalaora et al. 2016; Khodadoust et al. 2017). However, MS immunopeptidomics analysis has been perceived as highly promising but not ready to translate into the clinic due to issues with robustness and a low-throughput sample capacity, involving many sample handling steps. To combat these issues, Chong et al. have created a high-throughput platform for sequential immunoaffinity purification of HLA-I and -II peptides samples that can be used in cancer cell lysates and, potentially, patient samples with limited tissue available (Chong et al. 2018).

Another emerging alternative approach is the direct capture and analysis of the endogenously presented peptides using MS profiling (Bassani-Sternberg et al. 2010; Berlin et al. 2015; Dutoit et al. 2012; Granados et al. 2014; Hassan et al. 2013; Mommen et al. 2014; Rozanov et al. 2018). This high-throughput workflow involves HLA immunoprecipitation, followed by elution of HLA-loaded peptides prior to sequence of both non-mutated and mutation-containing HLA class I peptides by MS. A recent publication has optimised this procedure to examine in HLA-DR immunopeptidome (peptides from exogenous proteins that are presented on HLA class II molecules and are recognised by CD4+ T cells) in scarce clinical samples (Heyder et al. 2016).

Although MS immunoprofiling has many benefits over prediction algorithms, it requires access to the pertinent patient sample and advanced MS instruments, as well as specialised bioinformatic software to interpret such complex datasets. Moreover, although identification of cancer-associated HLA peptides by MS could establish the *in vivo* existence of the peptide, it still does not confirm that it will elicit a potent T-cell response necessary for effective immunotherapeutics (Haen and Rammensee 2013).

Several recent studies have now exploited the benefits of both these approaches to combine MS profiling with prediction tools for characterising, scoring, and identifying peptides for therapeutic use (Bassani-Sternberg et al. 2016, 2015; Murphy et al. 2017; Rozanov et al. 2018). This combined strategy leads to greatly improved numbers of HLA class I peptide identifications from the human

immunopeptidome, but requires sophisticated (and often modification to existing) bioinformatics software to perform.

One publication demonstrating the power of a MS-based workflow in characterising the cancer peptidome identified over 10,000 unique HLA class I-bound peptides from five melanoma cell lines (Gloger et al. 2016). Another publication by Bassani-Sternberg et al. deepened the melanoma-associated immunopeptidome atlas to a depth of over 95,500 patient-presented peptides using high-resolution MS (Bassani-Sternberg et al. 2016). Creating in-depth atlases such as these are vital for molecular characterisation of T-cell specificities in patients (e.g. before commencing and post-immunotherapy) to address questions such as why only certain individuals respond to immunotherapy.

There are a number of key benefits of using MS technology for the analysis of the immunopeptidome. It represents the best HLA-independent method for the unbiased identification of ligands with direct proof of actual presentation. This is especially important as in the past prediction analyses were highly limited to frequent HLA types for which a lot of information is already available in the literature. Further, the unbiased MS immunoprofiling approach allows for the identification of ligands derived from PTMs and spliced peptide variants, which might be missed by conventional epitope prediction (Bassani-Sternberg and Coukos 2016).

However, MS-based immunoproteomics has several inherent limitations due to the nature of analyte detection. Very hydrophobic or hydrophilic peptides are harder to detect by current MS, which will result in the biased acquisition of the immunopeptidome (Olsen and Mann 2013). Due to the complexity and enormity of information generated in these experiments, specialised computational tools must be used for the meaningful analysis of immunopeptidomics data (computational methods discussed further in Alvarez et al. 2018). MS-based immunoproteomics is also dependent on the availability of information in online databases that are used for the assignment of experimental mass spectra, but this limitation can be overcome through the use of *de novo* sequencing (Gautam et al. 2007; Menschaert et al. 2010; Pitarch et al. 2016). Furthermore, the reported sensitivity for MS is still comparably low, ranging between 0.5 and 3% yield of peptides captured by immunoprecipitation (Caron et al. 2015), but this will be improved with the rapid advances in MS technology.

In silico approaches to profiling the HLA immunopeptidome

One strategy developed in the last few years is the prediction of HLA peptides of cancer proteins of interest using specialist “machine-learning” bioinformatic programs (Zhang et al. 2011). Several HLA peptide prediction tools are accessible online (Lundegaard et al. 2008;

Schubert et al. 2015; Vita et al. 2015), with most predictors trained using existing HLA-I binding assay data and output lists of potential HLA peptides from protein sequences (Lin et al. 2008). Previously, performing HLA predictions using this method was not possible due to the high degree of polymorphism of human alleles, but this is now changing as peptide-binding datasets are being collected on more allotypes (Andreatta and Nielsen 2016). These algorithms score sequences in their involvement in proteasomal cleavage and transport into the endoplasmic reticulum via antigen processing and HLA-I allele affinity (Bassani-Sternberg et al. 2015; Zhang et al. 2011). However, the precision success of these algorithms is uncertain, with a comparison of several computational tools reporting modest predictive performance and comparably lower sensitivity (Larsen et al. 2007).

Advances in detection of neoantigens

Several studies in recent years have suggested a significant role for neoantigens in cancer immunotherapy (reviewed in detail in Bobisse et al. 2016; Lu and Robbins 2016). Neoantigens have been identified predominantly in melanoma, but have also been identified in several other tumour types including lung and renal cancers (Lu and Robbins 2016). The majority of neoantigens are encoded by point-mutated gene products, although frameshift mutations have also been found to generate neoepitopes (Lu and Robbins 2016).

The classical direct characterisation of neoantigens by cDNA library screening is labour-intensive and low-throughput, which makes it less suitable for clinical translation (Bassani-Sternberg et al. 2016; Lu and Robbins 2016). With advances in MS analysis, several studies have reported direct identification of neoantigens by the analysis of the tumour ligandome using MS integrated with exome and transcriptome data (Gubin et al. 2014; Kalaora et al. 2016; Yadav et al. 2014). This workflow resulted in the direct identification of therapeutically relevant TSA in two murine models (Gubin et al. 2014; Yadav et al. 2014). However, reported mutated peptide ligands that have been identified by MS were derived from profiling monoclonal cell lines only (Gubin et al. 2014; Kalaora et al. 2016; Yadav et al. 2014), which does not represent the degree of heterogeneity seen in patient tumours. To date, direct identification of neoantigens from tumour samples has been hampered by limitations in instrument sensitivity and bioinformatics (Bassani-Sternberg et al. 2016; Lu and Robbins 2016). However, if this limitation could be overcome and this workflow translated to patient samples, this would represent a massive advance in neoantigen-directed immunotherapies.

Serological markers of immunotherapy response

The discovery of biomarkers that predict therapeutic response is of increasing importance in the development and application of effective immunotherapies for cancer. Predictive biomarkers for immunotherapy outcome will aid patient stratification, which will result in more cost-effective and rationalised patient care. The serum/plasma proteome provides a glimpse into ongoing disease processes with enormous advantages over standard proteomic-based tissue analysis. Traditional clinical tissue analysis, although requiring ever-decreasing sample volume with each technological advance, still requires highly invasive tissue biopsy, precluding its repeated use during the course of immunotherapy treatment. In comparison, liquid biopsies (of patient plasma/serum) can be drawn relatively non-invasively, safely and at repeated time-points during therapy. Additionally, singular tissue biopsies do not provide information regarding tumour heterogeneity, whereas the soluble immunopeptidome potentially samples a patient's complete proteome.

Using autoantibodies as predictive markers for immunotherapy outcome

Autoantibodies against TAAs are spontaneously produced in cancer patients as the secreted form of B-cell receptors. While the mechanism of autoantibody production is not fully clear, cancer patients do produce autoantibodies to proteins that are either mutated, misfolded, over-expressed or to proteins with PTMs. Recent studies support that these serum autoantibodies may be suitable biomarkers that can be used either alone or in combination with TAAs or other autoantibodies for cancer detection (Desmetz et al. 2011; Zaenker and Ziman 2013), but, more importantly, can provide important therapeutic guidance for future cancer immunotherapy.

Recent work by Jhaveri et al. used quantitative seroproteomics to identify antibody biomarkers in pancreatic cancer patients treated with an allogeneic pancreatic cancer vaccine (Jhaveri et al. 2016). Immunoprecipitation of isotope-labelled proteins with purified patient antibodies, coupled with high-resolution MS analysis, identified antibody targets (proteins) with fold changes in post- versus pre-vaccination patient sera in order to assess specific vaccine-induced antibody responses. In doing so, more than 150 different proteins that induce an antibody response after vaccination were identified, with three antibodies were identified as potential predictive markers for immunotherapy outcome.

Characterising the soluble immunoproteome for use as predictive markers for immunotherapy outcome

In addition to autoantibodies, secreted proteins circulating in peripheral blood could be potential predictive biomarkers for cancer immunotherapy outcome. It has been understood since the 1990s that the levels of the soluble HLA class I molecules (sHLA-I) are elevated in the serum of cancer patients (Nocito et al. 1997), but analysis of the sHLA peptidome only truly began in 2010 with the publication of a MS-based workflow developed by Bassani-Sternberg et al. (2010) and a recombinant antibody microarray method by Carlsson et al. (2010). In particular, the proof-of-concept study by Carlsson et al. generated the first large-scale protein expression profiles of the plasma immunoproteome for improved classification of glioblastoma multiforme, monitoring of immunotherapy associated effects as well as for selection of patients that will benefit from immunotherapy (Sahin et al. 1995).

Since then, research into the soluble immunoproteome has identified predictive markers to various immunotherapies. Serum protein ANGPT2 was found to be a predictive and prognostic biomarker of response to the inhibitors of immune checkpoints CTLA-4 and PD-1 in metastatic melanoma patients receiving immune checkpoint therapy (Wu et al. 2017). Similarly, advanced melanoma patients treated with an anti-CTLA-4 antibody (ipilimumab) with low baseline vascular endothelial growth factor (VEGF) levels experienced better clinical outcome (Yuan et al. 2014). In another example, serum HMGB1 is predictive and prognostic for oncolytic immunotherapy, which uses tumour lytic viruses to stimulate patients' own immune system against their cancer (Liikanen et al. 2015).

Following the recent advances in MS biofluid profiling, a serum protein signature associated with patient outcome after anti-PD-1 therapy in metastatic melanoma has just been published (Weber et al. 2018). To establish this signature, sera from 119 advanced melanoma patients collected prior to treatment with an PD-1 antibody (nivolumab) were analysed using matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) MS. Machine-learning algorithms were used to combine the clinical and MS data to identify a 209-protein/protein-fragment signature associated with outcome after anti-PD-1 treatment, which was validated across several relatively small validation cohorts. Further validation in much larger retrospective validation sets with long-term follow-up is needed, but this promising serum assay, if validated, could provide guidance in selecting patients for immunotherapy, and for furthering understanding of the mechanisms of sensitivity and resistance to PD-1 inhibitors.

One key issue in profiling the soluble immunopeptidome is establishing exactly how accurately sHLAs represent the peptide profile of membrane located HLA class I (mHLA-I)

molecules. Early work using sHLA showed a tight correlation between sHLA-I and mHLA-I peptide profiles (Hickman et al. 2000); however, the sHLA were isolated from cultured cells and not patient plasma. Bassani-Sternberg et al. (2010) provided more insight into this issue by comparing sHLA-I and mHLA-I peptide profiles from fresh plasma of multiple myeloma and leukaemia patients. Remarkably, in patients with advanced cancer, greater than 85% of plasma sHLA-I peptides were also present in mHLA-I profile, which suggests that this might be a promising approach for identifying promising targets and monitoring cancer immunotherapy.

Using immunoproteomics for development of therapeutic vaccines

Vaccines can be either prophylactic (used to avoid/reduce risk of infection by external pathogens) or therapeutic (aid in the treatment of disease). In recent years, therapeutic vaccines are now being developed to target an adaptive immune response against cancer in an alternative to conventional immunotherapy (reviewed in Adamczyk-Poplawska et al. 2011; Comber and Philip 2014; Galassie and Link 2015; Sahin and Türeci 2018; Ye et al. 2016). Often personalised vaccines are administered in cancer patients who are already resistant to previously administered lines of therapy. Therefore, research such as that conducted by Shetty et al., who employed an isobaric peptide-tagging MS method to characterise the HLA immunopeptidome in cisplatin-resistant ovarian cancer cells, will be vital in optimising personalised vaccines in the drug-resistant patient setting (Shetty et al. 2012).

Similar to conventional vaccines targeting viral components, vaccines containing specific host HLA epitopes have been a large focus of therapeutic vaccines to directly target the adaptive response to cancer cells (Adamczyk-Poplawska et al. 2011; Comber and Philip 2014; Vergati et al. 2010). These therapeutic vaccines can strengthen patients' anti-tumour immunity primarily due to the activation of tumour-specific CD8⁺ cytotoxic T cells to destroy tumour cells (Comber and Philip 2014; Guo et al. 2013). Ideally, in patients who respond to mutations immunologically, boosting the immune system with a personalised peptide vaccine containing antigenic targets may assist in eliminating the tumour.

Identification of novel neoantigens as targets for vaccine therapy

The personalised identification and validation of neoantigens, distinguishing cancer from healthy cells remains a major challenge in immunoproteomics (Comber and Philip

2014). Previous efforts to treat cancer using personalised peptide vaccines have not been generally efficacious, which may be because there is not a straightforward assay to select strong antigenic epitopes in each patient (Rosenberg et al. 2004). Personalised peptide microarrays, such as the array created by Qendro et al., may have future application for immunotherapy to overcome this issue, such as selecting optimal vaccine epitopes for cancer patients or used to monitor the immune response in patients during vaccine-based immunotherapy (Qendro et al. 2017).

Direct detection of low abundance target epitopes (viral or mutation-derived) is particularly difficult using conventional immunopeptidomics methods. One recently published study by Blatnik et al. created a novel method for the isolation and LC-MS³-based targeted detection of HLA-I-presented peptides from transformed cells, involving pre-selection of target antigen-derived peptides by *in silico* predictive software and *in vitro* binding assays (Blatnik et al. 2018). This targeted detection strategy allows for higher sensitivity necessary for low-abundant peptide detection which are usually missed with untargeted LC-MS/MS acquisition and will likely improve the design of therapeutic vaccines.

Immunomics research will also help advance anti-idiotypic vaccines, which use anti-idiotypic monoclonal antibodies as antigen surrogates (reviewed in Ladjemi 2012). Idiotypic vaccines induce a primarily CD4 and/or humoral response. Clinical trials of anti-idiotypic vaccine treatment specific to lymphoma immunoglobulin variable regions showed early promise, but several subsequent independent phase III trials of idiotype vaccine found either modest or no overall clinical benefit (Freedman et al. 2009; Levy et al. 2014; Schuster et al. 2011). However, in one of these trials, a subgroup of patients who generated strong immune responses were noted to derive substantial benefit (Levy et al. 2014). A possible reason for this variation in vaccine efficacy is that the patients who benefited from idiotype vaccination were those with active presentation of their immunoglobulin neoepitopes. This hypothesis is supported by Khodadoust et al. who report that HLA presentation of variable-region epitopes is not equivalent across all individual lymphomas (Khodadoust et al. 2017).

Tumour heterogeneity in the era of therapeutic vaccination

One major challenge in the development of a therapeutic cancer vaccine is the phenomenon of tumour heterogeneity. Even if tumour-specific immune markers can be identified for the development of a vaccine, these markers may not be present in all cancer cells, nor throughout the whole tumour (Iakovlev et al. 2007). In addition, even if a tumour is well known to express specific markers, such as melanoma-associated (MAGE) antigens in melanoma or Her2/neu in a

subpopulation of mammary carcinomas, not all cancer cells in a given tumour express these markers at all times (Jones et al. 2005).

The importance of this tumour heterogeneity was highlighted recently in work by Wittke et al. (2016), who attempted to identify a pattern of antigens/signal proteins in renal cell carcinoma patients being treated with an autologous tumour vaccine. In a panel of 36 known TAAs analysed in 133 tumour lysates, none of the antigens investigated were reproducibly identified across every patient sample. Elevated expression of danger signal proteins (HSP-60 and HSP-70) was found in nearly all tumour lysates analysed, and was shown to be necessary to induce an immune response in a related murine model. From this evidence, targeting multiple TAAs in a multi-epitope approach is required for the effective adjuvant vaccination of cancer patients to combat antigenic heterogeneity across the primary tumour and any metastases.

Using seroproteomics to aid cancer vaccine development

The high degree of variability in the beneficial effect of therapeutic vaccines among cancer patients and the level of tumour heterogeneity clearly outline the need for the precise sub-classification of tumours in order to develop effective anti-cancer treatment and to select appropriate patients for personalised therapy. Examining antibody responses in patient sera could potentially aid in the identification of T-cell antigens and T-cell responses that could be valuable as predictive markers for response to therapeutic vaccination.

In a recent study by Jhaveri et al., the authors coupled immunocapture enrichment and metabolic labelling to identify antibody targets only expressed in pancreatic cancer patient sera with improved disease-free survival after personalised cancer vaccine therapy (Jhaveri et al. 2016). The authors reported that three secreted proteins that were targets of post-vaccination antibodies in the patient sera could predict for survival. They suggested these could be exploited for the design of improved vaccines.

Overall, peptide-based therapeutic vaccines, despite their limited effectiveness to date, would be considered a very valuable personalised treatment in the clinic. Ideally, identifying novel and perhaps stronger immunogenic peptides through improvements in immunoproteomics, combined with a better understanding of adjuvant and cytokine therapy, should result in more clinically effective vaccine treatments.

Future directions and conclusions

The depth and sophistication of our ability to study the immunoproteome have increased dramatically in the past decade, largely due to advances in MS protein profiling and

bioinformatic computational tools. Challenges to the comprehensive profiling of the immunoproteome still remain, such as troublesome characterisation of low abundance T-cell epitopes, and detection of lower-abundance serum cytokines. Difficulties associated with resolving and sequencing peptides from a complex mixture is not unique to immunoproteomics—it is a common frustration in proteomics as well. As HLA-bound peptides usually have varied termini and the proteolytic specificities that generate them are reasonably diverse, peptide sequence assignment can be challenging and significantly reduces the yield of confident identifications overall. Finally, the sensitivity of immunomic profiling platforms and the level of sample consumption used in its analysis (moving towards proteomic analysis of biopsy samples) still have not fully realised its potential, which will limit its translation into clinical care in its current state.

With the rapid advances in ‘omics technologies, new opportunities for research in this area are emerging, including the integration of transcriptome/interactome and immunoproteome data to improve chimeric antigen receptor (CAR) immunotherapy (Perna et al. 2017), as well as further systems biology research into the immune response to cancer. However, as progress in ‘omics technologies will drive increasingly large-scale immunoproteomics research, standardised reporting of experimental procedures and deposition of data in dedicated repositories needs to be performed to ensure transparency and reproducibility in published data. To address this, a new initiative by the Human Proteome Organisation-Human Immuno-Peptidome Project (HUPO-HIPP) has recently published guidelines on the minimal information about an immunopeptidomics experiment (MIAIPE) required for publishing immunopeptidomics data (Lill et al. 2018).

With the increasing complexity of our understanding in the functional heterogeneity of immune cells and its value in clinical diagnostics and immunotherapy monitoring, researchers must continue to develop personalised proteomic tools, such as single-cell functional analysis, which could prove to be an integrative component in cancer immunotherapeutics. Single-cell proteomics analysis includes the variations of protein level and the variations of protein–protein correlation, most commonly performed using single-cell Western blotting (Qian et al. 2017) or using microchip-based toolkits (Lu et al. 2017). For capturing and characterising this functional heterogeneity, single-cell proteomics has previously been utilised by Ma et al. to monitor the activity of immune cells taken from melanoma patients participating in an adoptive cell transfer (ACT) trial that uses genetically engineered T cells expressing cancer-specific T-cell receptors (TCRs) (Ma and Fan 2013). By understanding the functional characteristics of their immune cells at a single-cell level,

thereby removing the issue of cell heterogeneity, patients may be more appropriately stratified pre-treatment for the best available immunotherapy strategy and their immune response can be monitored during therapy so that any intervention can be made in a timely manner.

Targeted MS may also prove to have a valuable role in characterising the immunoproteome. The quantitative assessment of proteins that regulate the tumour-immune interface is one of the most difficult analytical challenges in developing immunotherapeutics. One study recently published by Morales-Betanzos et al. (Morales-Betanzos et al. 2017) developed a targeted MS method using parallel reaction monitoring (PRM) to provide measurements of PD-1, PD-L1, and PD-L2 (and their N-glycosylated forms) at femtomole per microgram tissue protein levels in formalin-fixed, paraffin-embedded sections of human melanoma biopsies. PRM is emerging as a very powerful technique in the multiplexed analysis of 10–100 s of analytes with high specificity because the MS/MS data are acquired in high-resolution mode that can separate co-isolated background ions from the target peptide ions (Bourmaud et al. 2016; Rauniyar 2015). As it focuses MS/MS scans on only a small set of pre-determined and previously identified peptides, it avoids the imprecision and irreproducibility of data-dependent acquisition strategies. Stable isotope-labelled quantified peptide internal standards can also be added to perform absolute quantitation for PRM acquisition of the endogenous peptides, as well as improving data quality with lowered limits of detection and quantification (typically in the low attomole range) (Gallien et al. 2015). A highly sensitive, reproducible, and reasonably high-throughput assay that could measure of several proteins in the PD-1 blockade, including those modified by glycosylation, in the same experiment would be highly valued in determining if anti-PD-1 immunotherapy is suitable for the patient in a timely manner necessary for translation to the clinic.

One major opportunity for MS-based proteomics that is rapidly emerging may be the ability to investigate the complexity of signalling in T cells. New high-resolution MS platforms, namely Data Independent Acquisition (DIA) MS, now have the power and sensitivity to profile the immunome governing T-cell responses in a comprehensive and unbiased manner, which could provide information about combination approaches for targeting complex immunosuppressive mechanisms within the tumour microenvironment (Haura et al. 2015). DIA-MS/MS demonstrates better sensitivity, reproducibility, and dynamic range than any untargeted data-dependent acquisition (DDA-MS/MS) method, and allows consistent quantification of proteins spanning a wide range of concentrations (Anjo et al. 2017; Zhao and Brasier 2016). This highly sensitive and unbiased discovery approach could help researchers further understand the problem of rapid and adaptive changes in immune cells, such as T cells, induced

by numerous input signals, including T-cell receptors and the immunoinhibitory signalling events.

Finally, on an overall systems level, understanding the rapidly changing protein profile of the immune system at various stages of cancer and throughout immunotherapy treatment has the potential to provide immune markers of patient health, and predictive markers of the immune response, which may in the longer term contribute to the development of personalised medicine. By defining the immunoproteome in such detail, proteomics may move from simply an analytical technique to the driving force behind directing future immunology research.

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