

# Chapter 2

## Immunoproteomics: Current Technology and Applications

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### Abstract

The varied landscape of the adaptive immune response is determined by the peptides presented by immune cells, derived from viral or microbial pathogens or cancerous cells. The study of immune biomarkers or antigens is not new and classical methods such as agglutination, enzyme-linked immunosorbent assay, or Western blotting have been used for many years to study the immune response to vaccination or disease. However, in many of these traditional techniques, protein or peptide identification has often been the bottleneck. Recent advances in genomics and proteomics, has led to many of the rapid advances in proteomics approaches. Immunoproteomics describes a rapidly growing collection of approaches that have the common goal of identifying and measuring antigenic peptides or proteins. This includes gel based, array based, mass spectrometry, DNA based, or *in silico* approaches. Immunoproteomics is yielding an understanding of disease and disease progression, vaccine candidates, and biomarkers. This review gives an overview of immunoproteomics and closely related technologies that are used to define the full set of antigens targeted by the immune system during disease.

**Key words** Immunoproteomics, Mass spectrometry, Antibody, Antigen, Cancer, Infectious disease, SERPA, SEREX, MHC, Epitope

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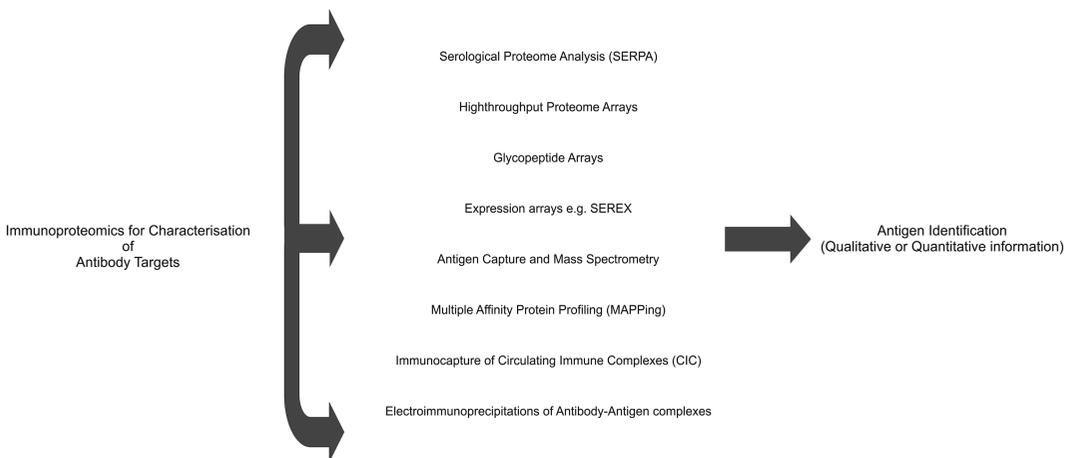
### 1 Introduction

The landscape of the immune system is constantly changing and is determined by the peptides presented by immune cells, whether from viral or microbial pathogens or cancerous cells. Detection and identification of these immune-active proteins or peptides can therefore be investigated using many of the approaches that have been developed for proteomics studies. As an extension of the proteomics field, the term “immunoproteomics” was first used in 2001 [1]. The field is rapidly expanding and includes increasingly varied techniques that result in the identification of immune related proteins and peptides, derived from invading pathogens, host cells, or immune signalling molecules. The study of immune biomarkers or antigens is not new and classical methods such as agglutination, enzyme-linked immunosorbent assay, or Western blotting have

been used for many years to study the immune response to vaccination or disease. However, in many of these traditional techniques, protein or peptide identification has often been the bottleneck. Recent advances in genomics and proteomics, including mass spectrometry instrumentation, has led to many of the rapid advances in immunoproteomics approaches. Immunoproteomics is yielding an understanding of disease and disease progression, vaccine candidates, and biomarkers. Herein, we focus upon providing a broad overview of immunoproteomics and closely related techniques that are used to study the immune response and their role in further disease diagnostics and vaccine development.

## 2 Immunoproteomics for Characterization of Antibody Targets

One of the two major arms of the adaptive immune system, also classically referred to as the humoral immune response, relies on activated B-cells secreting large amounts of highly specific antibodies, which bind to microbial or cellular targets, either neutralizing them or tagging them for elimination. Antibodies can be generated against microbial invaders, cancer antigens and sometimes misdirected against self-antigens, resulting in autoimmune disease. For a more complete overview of the antibody based immune response, readers are directed to a recent review [2]. Many methods have been developed in order to study the antigen targets of the humoral immune response and in the following section we provide an overview of the most commonly used. Fig. 1 shows a summary overview of these methods.



**Fig. 1** Overview of methods commonly used to interrogate antigenic targets of the humoral immune response

## **2.1 Classical Immunoproteomics: Serological Proteome Analysis**

One of the most commonly used immunoproteomics approaches relies upon 2D-PAGE, which separates proteins based upon orthogonal physical characteristics. When combined with Western blotting, the technique is commonly known as Serological Proteome Analysis (SERPA). The technique was originally developed in the 1970s and with some refinements popularized for use in biochemistry [3]. Early studies were hampered by challenges in protein identification, and instead used the gel maps to compare protein patterns under different cellular conditions. With many years of refinement, and rapid advances in mass spectrometry and genome sequencing, 2D-PAGE became the mainstay of comparative proteomics studies in the late 1990s and early 2000s. 2D-PAGE can be performed in most protein chemistry labs as a matter of routine, and advances in protein staining and image analysis software have made their use accessible to a broad scientific audience. With well-documented disadvantages, including difficulties in resolving very large, small, hydrophobic or basic proteins and the dynamic range of protein abundance, 2D-PAGE has been superseded by non-gel based proteomics approaches. However, 2D-PAGE has advantages and remains one of the few techniques that allow high quality analysis of intact proteins on a proteome wide scale, including detection of protein posttranslational modifications (PTMs). One of the most overlooked advantages is the ease and efficiency with which 2D-PAGE can interface with other biochemical techniques, such as Western blotting. When combined with Western blotting for detection of antigenic proteins, and mass spectrometry based identification of proteins from in-gel digests, 2D-PAGE provides a powerful approach for antigen identification. Combined, 2D-PAGE and Western blotting is commonly known as *serologic proteome analysis* (SERPA). The antigen used in these studies can be a whole cell proteome, or subproteome (e.g., membrane fraction). 2D-PAGE resolves the majority of proteins in a sample to a single protein spot, giving the potential to readily identify the antigenic proteins within the resolved proteome. Gels are then transferred to membranes and probed with sera from animal models or humans and developed as per any traditional Western blotting experiment. Many gels can be run in parallel to the blotting experiment, providing gels for reference maps and identification of immunoreactive proteins.

This now “classical” immunoproteomics approach is still widely used, and provides a robust way of screening the antibody reactivity profiles of serum in a variety of disease states, or post vaccination. Applications include discovery of antigenic proteins, biomarkers or correlates of protection, with many studies reporting bacterial diseases [4–29], cancers [30–40] and diseases such as multiple sclerosis [41]. Studies have included discovery of serodiagnostic markers for Q fever [42] and *Helicobacter pylori* [21, 25, 43, 44] as well as diagnostic markers of parasitic diseases, such as

Schistosomiasis [45]. Another report has used SERPA to discover proteins secreted *in vivo* by *Bacillus anthracis* [46]. SERPA has also been used to study the human serological response to vaccination with whole-cell pertussis vaccine [47], *Francisella tularensis* live vaccine strain [48] and human infection with *Francisella* [16, 48]. The latter studies focused upon discovering antibody based correlates of protection.

## **2.2 High Throughput Proteome Wide Screening of Antibody Targets: The Proteome Array**

Some of the limitations of 2D-gel based immunoproteomics have been overcome with the development of proteome or protein arrays to study the humoral immune response. Here, each open reading frame of interest in the genome is amplified by PCR, followed by cloning, protein expression and microarray printing [49, 50]. Bacterial proteomes are sufficiently small that the entire complement of proteins from the genome can be printed on a single array.

The chips are then treated in a manner similar to traditional Western blotting, probed with sera and reactivity detected after incubation with a secondary antibody with fluorescent conjugate. The chip based technology has the advantage of screening closer to equal amounts of antigens, interrogation of the entire theoretical proteome of the organism, and reduced volume of serum required for screening (2  $\mu$ L vs.  $\sim$ 50–100  $\mu$ L for large 2D-Western blot). The reduced requirement for serum means that pooling of sera from multiple animals or humans in a study is not required, and individual differences can be readily detected. These benefits, combined with the high throughput capacity of proteome microarrays, make it an attractive method of rapidly screening hundreds of sera. The use of advanced data handling algorithms is a requirement, as with DNA based microarrays, for meaningful data interpretation [51].

The complexity of protein purification and high throughput gene expression systems means that it can be challenging to produce proteome arrays that represent the entire proteome of an organism. In addition, the expressed proteins lack native PTMs, processing and correct protein folding is not guaranteed. Investigation into the use of yeast based protein expression systems may help address the issue of PTMs, however many bacteria elaborate a unique repertoire of glycoconjugates and glycoproteins that cannot be replicated by yeast based systems. Lack of non-protein antigens can be addressed by addition of native molecules to arrays, in order to gain a broader perspective of the humoral immune response. To date, there have been reported advances in array technology that address challenging protein antigens, such as membrane proteins [52], and non-protein antigens, such as carbohydrates [53].

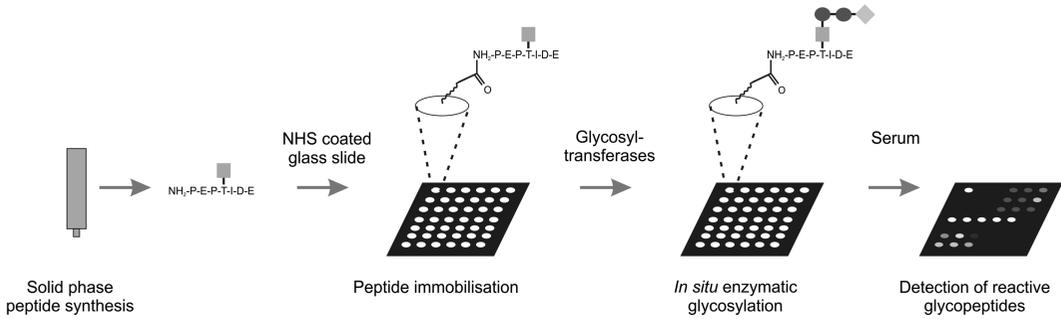
Proteome arrays have been used to study the humoral immune response of a wide range of pathogens, including smallpox vaccination [54, 55], Chlamydia infections [56, 57], Brucellosis [58, 59],

*Mycobacterium tuberculosis* infections [60, 61], salmonellosis [62], Herpes simplex virus [63, 64], *Plasmodium falciparum* [65–68], Q fever [42, 69, 70], toxoplasmosis [71], *Burkholderia pseudomallei* [72], *Borrelia burgdorferi* [73], *Francisella tularensis* [50, 74], and Epstein-Barr virus [75]. In the long term, this technology has the potential to aid development of improved serodiagnostic tests, vaccine development, epidemiological studies and shed light on the interaction of pathogens with the immune system.

### **2.3 Deciphering the Immune Response to Glycoprotein Antigens**

Carbohydrate moieties and glycoconjugates, including glycoproteins, are increasingly being shown to have roles in various diseases, including cancers and bacterial infections. Protein glycosylation is a highly abundant PTM and aberrant glycosylation of proteins has been shown to be associated with cancers [76] and autoimmune diseases [77, 78]. Truncated glycan moieties on glycoproteins are recognized as nonself and result in the generation of autoantibodies to glycopeptide epitopes [79, 80]. For example, *O*-glycosylation of mucin (MUC1) is particularly important in cancers, with patients reported to have autoantibodies to distinct epitopes on MUC1 that harbor truncated sugar moieties [81]. Of note, these autoantibodies recognize cancer specific epitopes, composed of the combined peptide sequence and the carbohydrate moiety [76, 82, 83]. It is, therefore, likely that there are other glycopeptide antigens in cancers. Investigation of glycan associated autoantibodies has been carried out using variations of chip based screening technologies. These have included a microarray display platform that allows the large scale screening of *O*-glycopeptide libraries for the investigation of disease associated autoantibodies [80, 84–87].

Recently, a high throughput chemoenzymatic synthesis and microarray display platform has been described that enables the production and screening of large *O*-glycopeptide libraries for disease associated autoantibodies. A combined synthetic and enzymatic approach allowed immobilization and generation of a glycopeptide epitope library on a microarray chip. As outlined in Fig. 2, *O*-linked GlcNAc containing peptides were synthesized by standard solid-phase peptide synthesis (SPPS) [84]. These glycopeptides were then immobilized on microarray plates coated with amine-reactive NHS-ester groups. This was followed by on-slide glycosylation with different polypeptide GalNAc-transferases and other elongating glycosyltransferases. In this way, a diverse library of synthetic *O*-glycosylated MUC1-peptides was generated *in situ*. This was used for serological screening and the results showed that the array was able to detect autoantibodies in the sera of patients with a confirmed diagnosis of breast cancer [84]. Rapidly synthesized libraries which represent the potential diversity of glycopeptide or glycoprotein epitopes pave the way to broader screening of glycan-epitopes and the elucidation of glycan epitopes within existing immunodominant peptides.



**Fig. 2** Uncovering glycopeptides epitope. Peptides are synthesized using solid phase peptide synthesis, including amino acid harboring an *N*-acetyl glucosamine residue. Peptides are then immobilized on glass slides, coated with NHS esters. This serves as a partial purification step. Addition of glycosyltransferases allow *in situ* addition of carbohydrate moieties to generate a library of glycopeptide epitopes. This is then screened with sera and reactive epitopes identified

The diversity of carbohydrate moieties across the domain of Bacteria is substantially greater than that of eukaryotes. Many monosaccharides are found exclusively within bacteria and are genus, species, or strain specific. Consequently, these unique sugars are often readily identified by the host immune system as foreign entities during infection. Frequently these sugars are part of a pathogen associated molecular pattern (PAMP), such as lipopolysaccharide (LPS) or peptidoglycan, that is recognized by host pattern recognition receptors (PRR) such as toll-like receptors (TLR) [88, 89] or nucleotide oligomerization domains (Nod) [90–93] as part of an innate immune response. However, it is increasingly being reported that bacterial glycoproteins also play a role in stimulating innate [94] and adaptive [95–98] host immune responses. Several of these pathogen glycoproteins, including the flagellin of *Campylobacter coli* and *Campylobacter jejuni*, are responsible for serospecific antibody responses [95, 98]. Additionally, the antibody response to anthrose, a unique sugar decorating the *Bacillus anthracis* exosporium glycoproteins (BclA and BclB), is currently being exploited for its potential use in detection and diagnosis of anthrax [99–102]. Despite a growing recognition of the importance of bacterial glycoprotein antigens, immunoproteomics methods directed specifically towards their identification are lacking. In fact, glycoprotein antigenicity is frequently discovered as a consequence of targeted glycoprotein characterization. Given the documented importance of bacterial glycoprotein antigens, methods designed for their global detection and identification would greatly benefit the field of immunoproteomics.

#### 2.4 Antigen Discovery Using Expression Arrays

Expression arrays are composed of bacterial, yeast, mammalian, or cell free cDNA expression libraries that are used to identify novel antigens. Known as serological analysis of recombinant cDNA

expression libraries (SEREX), these techniques have a large genetic component and have been termed by some as “reverse proteomics” [103]. SEREX was first developed for analysis of the humoral response to cancer in the 1990s [104], with the goal of identifying tumor specific antigens that elicit high titer immunoglobulin G (IgG) antibodies in patient sera. In this context, the technique permits the search for antibody responses and the molecular definition of immunogenic tumor proteins, based upon autologous patient sera (reviewed in ref. 103). Patient tumor mRNA is used to prepare prokaryotically expressed cDNA libraries which are then immunoscreened with absorbed and diluted patients’ sera for the detection of tumor antigens that have elicited a high-titer IgG humoral response. This approach has the advantage of being able to identify antigens expressed *in vivo*, and is unbiased, based only upon the reactivity of clones with autologous patient sera. A second phase of screening is also carried out, using sera from normal patients in order to define antigens that show cancer-restricted immune recognition [105, 106]. SEREX has been applied to the study of many cancer types, including renal [105, 107, 108], colon [109–111] and breast [106, 112–123] cancers leading to the identification of cancer specific antigens. One antigen, NY-ESO-1, was identified in esophageal squamous cell carcinoma and the gene expressed in normal testis and ovary, with aberrant expression in various types of malignant tumors [124]. NY-ESO-1 shows restricted expression patterns, elicits both cell mediated and humoral immune responses [125] and has been under development as a cancer vaccine target (reviewed in ref. 126).

Despite many advantages, SEREX presents some challenges, in that it is time consuming to construct cDNA libraries for each tumor sample. In addition, false positives are possible, either due to reactivity with prokaryotic expression components or lack of expression of PTM in prokaryotic expression systems. In particular, protein glycosylation of eukaryotic proteins can represent important antigenic epitopes, including disease associated changes in glycosylation. A few autoantibodies to PTM-protein epitopes have been reported, including those found in cancers [79, 80] and autoimmune diseases [77]. The use of eukaryotic expression systems can ensure that expressed proteins are glycosylated [127–130]. Tumor associated antigens identified from SEREX screening are updated in the Cancer Immunome database (ref. 131; <http://ludwig-sun5.unil.ch/CancerImmunomeDB/>). Over 2000 autoantigens are listed in this online database. An excellent review that discusses the classes of SEREX defined antigens and the wider impact of this technique upon cancer vaccine and diagnostic development can be found here [103].

## **2.5 Antigen Capture and Mass Spectrometry**

Immunocapture mass spectrometry aims to enrich antigen proteins from cell lysates, using mass spectrometry as the final means to identify captured proteins. There are many variants of

immunocapture and generally immunoglobulins from patient sera are immobilized on Protein A or Protein G, usually in column format. This is followed by the application of a cell or tissue lysate to the column, effectively enriching for antigenic proteins, i.e., those proteins to which there are antibodies in patient serum. Proteins are eluted from the column, enzymatically digested and subsequently identified by MS/MS [132].

#### 2.5.1 *Multiple Affinity Protein Profiling*

*Multiple Affinity Protein Profiling* (MAPPING) is an example of an immunocapture technique that has primarily been exploited to identify cancer related autoantigens [133, 134]. It is based upon two-dimensional immunoaffinity chromatography, whereby antigens from tumor lysates are separated based upon their affinity for immunoglobulins from healthy controls in the first dimension and immunoglobulins from cancer patients in the second. The first dimension removes autoantigens that are recognized by sera from healthy patients. Cancer restricted autoantigens then flow through to the second column, which then selectively binds them. The proteins eluted from the second chromatography step are therefore likely to be cancer specific and are identified by enzymatic digestion and MS/MS analyses [133, 134].

#### 2.5.2 *Capture and Identification of Circulating Immune Complexes*

Another variation of immunocapture targets circulating immune complexes (CIC). Immune complexes are formed from the non-covalent interaction between antigens and antibodies and are usually removed by mononuclear phagocytes through complement receptors and Fc-receptors [135]. This process constantly occurs in healthy individuals and ensures the rapid clearance of denatured proteins, antigens of gut bacteria or dead cells. Studies have shown that these antigen-antibody complexes can play a role in disease progression of human autoimmune diseases [136], cancer [137], or infectious diseases [138]. There is some discrepancy in the literature regarding the utility of CIC in disease diagnosis, treatment or as an indicator of disease severity [139–144]. Some have argued that identification of antigens incorporated into CICs may be of greater relevance than information regarding free antigens [144], and that antigens in CICs could provide information useful to understanding disease progression, and in developing diagnostic and treatment strategies.

CICs can be isolated from serum, as described in a recent report [144]. Patient serum was immobilized on a Protein A or G column and cell lysates passed over the column. Proteins that were bound to the immobilized patient sera were eluted and identified using tandem mass spectrometry of their tryptic digests [143–145]. A recent study identified CICs containing the proteins thrombospondin-1 and platelet factor 4 in the serum of 81 and 52 % of a sampling of rheumatoid arthritis patients, respectively [143]. This method is applicable to many other diseases for inventory of antigens within CICs.

### 2.5.3 *Electroimmuno-precipitation of Antigen–Antibody Complexes*

Electroimmunoprecipitation can exploit differences in electrophoretic mobility between an antibody and its corresponding antigen, resulting antigen–antibody complexes embedded in an agarose gel. Staining of the gels permits visualization of precipitated complexes. Elution of these complexes, plus enzymatic digestion, and subsequent mass spectrometry analysis can identify the unknown antigenic proteins of interest [146]. In rocket immunoelectrophoresis (RIE), a monoclonal antibody is used. However, crossed immunoelectrophoresis (CIE) involves two dimensions of separation [147, 148] and can therefore be used to identify antigenic proteins reacting with mixtures of monoclonal antibodies, polyclonal antibodies, or serum. Therefore, electroimmunoprecipitation can be used to capture antigens relevant to various disease states or contribute to validation of antigenic proteins. Electroimmunoprecipitation has the added advantage of being quantitative [147–149] and can therefore also be used to monitor the level of serum antibody response to a known antigen.

### 2.6 *Epitope Mapping*

Discovery of antigenic proteins is the first step in profiling the humoral immune response to disease. There is often a need to then further dissect the immune response and determine the region of the antigenic protein, or epitope, that stimulates the immune response; particularly in antibody design or epitope based vaccine design [150]. This can be carried out using a wide variety of techniques, a full description of which is beyond the scope of this review and we direct the reader to recent reviews [151, 152].

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## 3 Immunoproteomics in the Study of Major Histocompatibility Complex Peptides

The cell mediated immunity (CMI) arm of the adaptive immune response involves activation of cell populations such as phagocytes or T-cells and can include the release of communicator molecules, such as cytokines and chemokines in response to foreign invaders or antigens. T-cells recognize antigens that are displayed on the surface of host cells in complexes known as the major histocompatibility complex (MHC). The antigens found in complex with MHC molecules are short peptides that are derived from intracellular proteolysis of proteins. This antigen presentation and processing allows for the host recognition of foreign peptides from infected or transformed cells, by stimulating an immune response. In addition, there is constant surveillance of peptides derived from the host organism, and self-peptide presentation is involved in T-cell development in the thymus and regulation of self-tolerance.

There are two major subgroups of MHCs, denoted MHC I and MHC II, which are encoded by the human leukocyte antigen (HLA) gene clusters. These gene clusters are highly polymorphic, giving rise to hundreds of allelic forms, with only a subset present

in each individual. The polymorphism gives rise to differences in the MHC molecules, their binding pockets and affinity for particular peptide antigens, thereby influencing the repertoire of antigens presented to the immune system of an individual. The two major classes of MHC molecules (class I and II) are distinct in their three dimensional structure, pathways by which antigens are processed and the type of T-cell with which they interact. MHC class I gene cluster encodes the heterodimeric proteins that bind antigenic peptide from within cells, and are found on all nucleated cells types. MHC Class I molecules carrying peptide antigens complex with the CD8 co-receptor. This complex is primarily recognized by cytotoxic T-cells and leads to their activation and eventual death of the cell expressing the nonself antigen.

In comparison, MHC class II gene cluster encodes heterodimeric peptide-binding proteins and proteins that control peptides binding to the MHC heterodimers. Peptide loading onto MHC class II molecules occurs in the lysosomal pathway and MHC class II complexes are only found on specialized cell types, such as B-cells, neutrophils, and dendritic cells and can be induced on macrophages and human T cells. The CD4 T cell co-receptor recognizes MHC Class II antigen complexes, also resulting in T-cell activation. If the presented peptide is foreign, the T cells then proliferate, secrete cytokines, and differentiate into antigen-specific effector CD4 cells, which secrete cytokines and activate other cell types, such as B-cells. For both MHC class I and class II molecules, the antigens are peptide fragments which are recognized as nonself by T-cells, these antigens are known as T-cell epitopes. A detailed description of how these peptide fragments are generated is described in more detail [153–155].

### **3.1 MHC Peptide Enrichment**

The identification and characterization of peptides displayed by MHC molecules and specific T-cell epitopes has become essential for modern immunological studies, in many aspects of basic and applied research. For example, the development of vaccines with enhanced T-cell immune response [156–158]. A broad array of functional and biochemical approaches have been developed to identify peptide epitopes, including forward and reverse immunoproteomics, and mass spectrometry centric approaches (for example refs. 158–168). A recent review describes T-cell epitope mapping based upon screening of peptide libraries and screening for T-cell activation [169]. In the following sections, we review the contributions of mass spectrometry based immunoproteomics to MHC peptide binding and T-cell epitope identification and how this knowledge is furthering vaccine and diagnostic development.

### **3.2 Mass Spectrometry in MHC Peptide Discovery**

MHC class I and II proteins preferentially bind peptides of different lengths and general characteristics. Typically, MHC class I molecules have a binding cleft that accommodates peptides of

8–10 amino acids, whereas, MHC class II molecules bind peptides 8–30 amino acids in length. Peptides that bind the cleft of a MHC class II molecule are usually found to share a core sequence [170–175].

Over the past two decades, several methods of isolating MHC peptides have been developed. Early reports in the 1990s used acid treatment to elute peptides from the surface of cells [176]. Although simple to carry out, peptide elution was not specific to those bound to MHC complexes and difficulties arose when attempting to discriminate specific MHC peptides. Targeted immunoaffinity purification was also reported in the 1990s [177], in which monoclonal antibodies specific for an MHC class were used to enrich the MHC complexes. MHC bound peptides are then eluted by acid treatment and separated from proteins by size exclusion. Soluble MHC molecules, without a transmembrane domain, are secreted in transfected cells with MHC peptides bound. The secreted complexes are easily purified, for example with the use of immunoaffinity columns; this method is considered a facile method to isolate MHC peptides [178]. In all cases, it is assumed that peptides bound to MHC molecules are protected from proteolysis during sample preparation and that acid treatment is sufficient to dissociate peptides from their MHC binding partners. Immunoaffinity purification of MHC peptides has been applied in many areas, including the study of the central nervous system of multiple sclerosis patients [179, 180] and bronchoalveolar lavage cells isolated from patients with sarcoidosis [181]. Another study combines immunoaffinity enrichment with testing of subsequent fractions for biological reactivity, prior to peptide identification by mass spec [182] for the identification of tumor associated antigens. This approach has also been used for the successful identification of novel antigens in primary human breast cancer [183] and West Nile virus [184].

### **3.3 MHC Peptide Identification**

Purified MHC peptides were largely analyzed using Edman degradation. In particular, the shorter length of the MHC class I peptide ligands made them amenable to amino acid sequencing by Edman degradation. The use of Edman chemistry on a pool of MHC class I peptides revealed an increased signal for a particular conserved amino acid, or amino acid position [185], allowing progress towards identifying conserved residues or sequence motifs. MHC class II peptides are less amenable to this approach, due to their longer length and greater heterogeneity. However, other early biochemical studies established consensus binding motifs for both MHC class I and II peptide ligands [170–175].

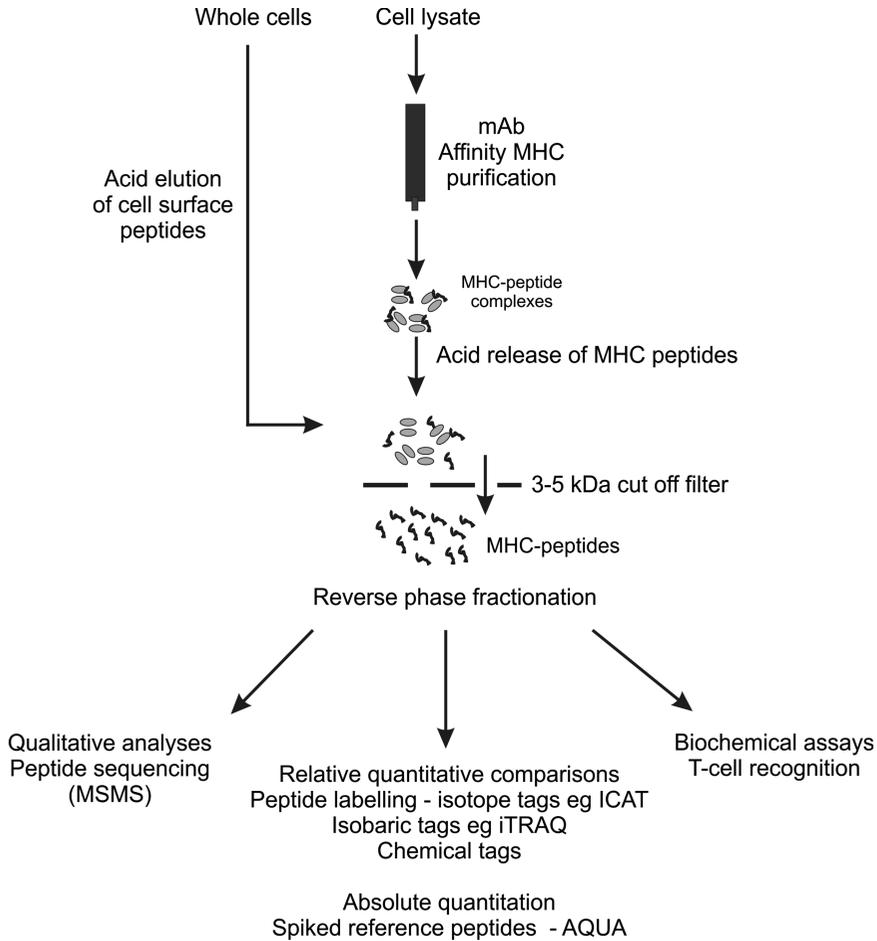
Due to the limitations in HPLC separation of peptides and Edman sequencing in early studies of MHC peptides, only short sequences of abundant peptides were determined. Pioneering studies in the early 1990s demonstrated the utility of the then

recently developed electrospray ionization mass spectrometry (ESI-MS), in combination with microcapillary HPLC to determine the length and sequence of peptides bound to HLA-A2.1 [159], one of the most widely distributed MHC class I molecules within the human population. Since this study, ESI-MS has been used extensively for the detection of peptides presented by major histocompatibility complex (MHC) molecules (for example refs. 159, 186, 187 and recently reviewed in ref. 188). Mass spectrometry affords the advantage of high resolution peptide mapping, allowing rapid identification of hundreds of MHC peptides in a single experiment.

Since the first report [189], rapid advances in mass spectrometry instrumentation, throughput and data handling mean that mass spectrometry is a widely used technique in the identification of T-cell epitopes. More recently, large scale proteomics methodologies have been used in comparative or quantitative studies of T-cell epitope identification. Studies have reported robust identification of epitopes, and refinements have been made to identify immunodominant epitopes and in distinguishing self and nonself MHC class I peptides. Precise splitting of the eluate from HPLC separation of MHC peptides, with a portion diverted to the mass spectrometer and the majority retained to assay T-cell activity, has allowed more precise correlation between MHC peptide identification and T-cell activation [190–192]. Other methods compared the LC-MS chromatograms of peptides eluted from MHC I complexes with those of reference cells. Mass spectrometry has been used to identify T-cell epitopes of *Plasmodium falciparum* [193], cancers [194, 195] and rheumatoid arthritis [196]. Others have employed novel approaches to hold antigen presenting cells in protein free medium, simplifying the repertoire of peptide antigens presented and reducing the background of peptides normally observed, allowing greater detection of exogenous MHC [197]. Fig. 3 gives an overview of the current workflow for MHC peptide isolation and identification.

### **3.4 Quantification of MHC Peptides Using Mass Spectrometry**

Qualitative studies provide an inventory of detected MHC peptides, and with the development of advanced proteomics technologies comes the opportunity to carry out quantitative studies. Quantification of MHC peptides allows for comparison of peptide repertoire and abundance with time, between tissues, self and non-self, or test and control and between individuals. Quantification can be relative or comparative, achieved using peptide labeling strategies such as the commercially available ICAT system [198], isobaric tags such as iTRAQ [199] or chemical tags (mass coded abundance tagging, MCAT) [200]. A recent study, for example, reported robust identification of over 100 MHC II peptides, and their relative quantification using stable isotope labeling [187].



**Fig. 3** Schematic overview of methods for MHC peptide purification and sequencing

Others have described the development of a selected reaction monitoring (SRM) method combined with absolute quantitation (AQUA) [201]. Selected reaction monitoring is a highly specific technique that targets specific peptides, with high sensitivity. When used in combination with a deuterated internal calibrant peptide, this permits the absolute quantification of target peptides [202, 203]. This approach was successful in quantifying the amount of a known ovalbumin peptide from the spleens of immunized mice after MHC affinity purification. Recently, the approach has been used to measure the presence and abundance of known MHC melanoma peptide antigens on the surface of several human melanoma cell lines [204]. SRM can be multiplexed for rapid and simultaneous identification and quantitation of hundreds of peptides, is robust and readily transferable between laboratories.

### **3.5 Characterization of Posttranslational Modifications of T-Cell Epitopes**

Other studies have combined mass spectrometry and functional assays for T-cell epitope identification [195]. The genetic polymorphism of the HLA alleles results in variation in the MHC complexes across the population, with differing binding affinities. It can, therefore, prove challenging to identify antigenic MHC I peptides presented by MHC class I molecules that are less frequently found across a population. This is important in the development of peptide-based vaccines for the therapeutic treatment of melanoma and other cancers, which requires the identification of antigenic peptides that will allow the majority of the population, regardless of their MHC encoded phenotype, to stimulate a T-cell response.

Glycosylation is a common PTM of proteins in eukaryotes and increasingly discovered in bacteria. Although largely ignored until recently, carbohydrates, glycolipids, and glycopeptides [205] are now recognized to modulate T-cell recognition [206, 207] having been shown to be presented by MHC complexes [208]. This has important implications in the immune response to pathogens, tumor cells, and self-tolerance. Several studies in the late 1990s provided evidence that naturally modified *O*-GlcNAc peptides were ligands for MHC class I molecules [208–211], and a crystal structure showed the glycan moiety to be exposed for recognition by CD8 T-cells [212]. After affinity enrichment of MHC complexes and elution of bound peptides, many of the techniques developed for the study of glycoproteomes could be applied to target and identify glycopeptide MHC peptides. Some approaches such as those using lectin enrichment have already been successfully employed for the enrichment of MHC bound glycopeptides [208]. Other approaches, such as hydrazide capture [213] and chromatographic enrichments, combined with advanced mass spectrometry approaches, such as precursor ion scanning of signature glycan ions, could lead to rapid and specific identification of MHC glycopeptides.

Similarly, it has been proposed that phosphopeptides may also be T-cell antigens [214], presented by class I MHC molecules on malignant cells [215] or MHC class II [216] and be attractive targets for cancer immunotherapy [217, 218]. Phosphopeptides associated with class I MHC molecules on the surface of tumor cells can be enriched by immunoaffinity purification of the MHC complexes, followed by elution and enrichment of phosphopeptides with immobilized metal-affinity chromatography (IMAC) [214, 216–219].

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## **4 Cytokines**

Cytokines are low molecular weight secreted proteins, ranging from 8 to 40 kDa [220, 221], with diverse roles in controlling growth, survival, differentiation and the effector function of cells and tissues (recently reviewed in ref. 220, 221). They are critical to an immune

response, and the secreted profiles of certain cells determine the nature of the response—Th1 versus Th2 and dictate whether the immune response is cell mediated or antibody based. Production of cytokines is tightly regulated, with an uncontrolled response potentially leading to septic shock. Therefore, controlled production of cytokines is key to many aspects of inflammation and immunity, including a balanced immune response. Therefore the types and levels of cytokines can serve as markers of disease progression.

The number of cytokines and closely related growth factors that have been identified has increased dramatically in recent years [222]. Unlike hormones, cytokines are active over short distances at sites of inflammation and can act in combination with other cytokines to give a variety of biological responses. Cytokine profiles can potentially be indicative of a particular disease state, so in order to correlate this, methods are required that can simultaneously measure levels of multiple cytokines. Although some cytokines are produced at ng/mL concentrations in body fluids, most are expressed at pg/mL levels and therefore, the most widely used current methods are based upon immunoassays, RT-PCR or bead based bioassays. Other methods for detecting cytokines or cytokine secreting cells include radioimmunoassay (RIA), immunoradiometric assays [223], cellular enzyme-linked immunosorbent assay (CELISA), cytometric bead array (CBA), radioreceptor assay (RRA), reverse hemolytic plaque assay (RHPA), cell blot assay, and cytokine flow cytometry. Identifying and quantifying the cytokines secreted in response to a disease state or pathogen are of interest in diagnostics and as vaccine correlates of protection. The cytokine quantification assays that have gained popularity have become increasingly high throughput, allowing an increase in the amount of information that can be collected about the roles of cytokines during disease or post vaccination. The use of bead based assays has allowed the multiplex measurement of multiple cytokines simultaneously [224–229]. These assays are robust, but they are inherently biased towards a predetermined panel of cytokines and provide only quantitative information. In addition, these methods provide no information regarding PTM of cytokines, which can be of importance in some cases. For example, IL-24 activity is dependent upon formation of a disulfide bond and glycosylation [230].

Several different immunoproteomics approaches have been reported that are able to detect and quantify cytokines and provide information regarding PTMs. A recently reported technique, known as immunoaffinity capillary electrophoresis (IACE), captures cytokines by immunoaffinity using specific antibodies, then separates the captured proteins using capillary electrophoresis. The resulting protein or peptide fractions are then analyzed by tandem mass spectrometry, providing cytokine identification [231–233]. This two dimensional separation also allows for differentiation between protein isoforms and identification of PTMs. Another

cytokine detection method also exploited an immunoaffinity capture step coupled then directly to analysis by mass spectrometry for protein detection and quantitation [234]. With both methods, the immunoaffinity capture step limits cytokine detection to a pre-determined panel. However, in the latter study, the authors' goal was to improve the speed of cytokine detection compared to current assay technologies (1–3 h) [234].

Other reports have focused upon unbiased detection of cytokines in serum, or *in vitro* secretion from immune cells, such as monocytes. Detection of cytokines in serum presents many challenges, characteristic of serum proteomics. Cytokines are typically a very small fraction of the low molecular mass proteome in serum. Although such proteins are amenable to detection using current mass spectrometry technologies, the challenge lies in their low abundance in relation to the high background of other serum proteins. In human serum, albumin and immunoglobulin G (IgG) make up 60–80 % of the total serum protein content [235], potentially masking the detection of low abundance proteins. The challenge of the dynamic range of proteins in serum is not new and there are many strategies for their depletion [236]. Additional concerns arise when albumin, known as the “tramp steamer” of the blood, interacts with many small molecules, fatty acids and proteins, acting as a transient carrier. Depletion of these transiently bound proteins, peptides and small molecules is possible and may distort the low abundance serum proteome. Methods have been reported for separation of low molecular weight serum proteins, using centrifugal ultrafiltration under solvent conditions that disrupt protein–protein interactions. Two dimensional liquid chromatography of tryptically digested proteins and identification using mass spectrometry facilitates the identification of the low MW proteome, including cytokines [237]. Others have also used ultracentrifugation, IEF [238] for identification of low MW serum proteome, while Groessl et al. [239] employed a label free MS based proteomics approach to characterize the human monocyte secretome, successfully identifying important proinflammatory proteins and cytokines. Advances in these mass spectrometry based methods pave the way for rapid, robust and unbiased serum cytokine detection, characterization and quantification during disease. This has the potential to contribute to understanding of disease progression, as well as revealing disease or post vaccination biomarkers.

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## 5 Immunoinformatics

*In silico* prediction of T or B cell epitopes has become a mainstay of immune related research. This is part of a growing field of immunoinformatics, or computational immunology, which describes the application of informatics technologies to problems of the immune

system. Several studies have used the term “immunomics” to describe the study of the detailed map of immune reactions of a given host interacting with a foreign antigen (the immunome). *In silico* methods have been developed in order to predict the sequence, structure and affinity of various epitopes of the humoral and cell mediated immune systems. As with many rapidly growing fields, the overlap or complementarity between closely related areas means the boundaries are less easily defined. For example, immunoinformatic studies of peptide epitopes is important in immunoproteomics and many studies combine epitope prediction with epitope sequencing. The various algorithms and bioinformatics techniques complement proteomics identification of peptide epitopes, and combined *in silico* and *in vitro* approaches bring more power to peptide identification or mapping. In the following section, we provide a high level overview of the key areas.

### **5.1 Immunoinformatics and B-Cell Epitopes**

B-cell epitopes are antigenic determinants from pathogens (or self) that interact with B-cell receptors [240]. The B-cell receptor contains a hydrophobic binding site composed of hypervariable loops that vary in length and amino acid composition. Epitopes that bind to the receptor are either continuous (linear) or discontinuous (conformational) [241]. According to accumulated knowledge, the majority of B-cell epitopes are discontinuous, with protein folding playing a large role in epitope formation. Prediction tools exist for prediction based upon amino acid sequence (for continuous epitopes) or structure based tools for discontinuous epitopes (for recent examples refs. 242–254). In the past, sequence based prediction tools have used amino acid hydrophobicity scales for epitope prediction. This approach is still used, for example BCIPEPT predicts continuous epitopes using propensity scale values, such as amino acid polarity, flexibility. The BCEPRED server [242] has been reported to predicted continuous B cell epitopes with an efficiency of 58.7 % [245]. Prediction of discontinuous epitopes is more challenging, with over 90 % of B-cell epitopes being discontinuous [255].

For both continuous and discontinuous epitopes, the current gold standard remains X-ray crystallography and observing the points of contact. From the accumulated structural data, several prediction methods have been developed, for example Discotope [243] and mapitope [246, 256]. Discotope combines amino acid statistics with protein spatial information and was trained on a dataset of X-ray crystal structures of antibody–antigen complexes. More detailed overviews of the current methods and databases are given [257, 258].

### **5.2 Immunoinformatic Prediction of T-Cell Epitopes**

In order to accelerate experimental approaches to MHC epitope prediction, computational methods or algorithms have been developed that can predict MHC-binding peptides and their binding

affinity [259]. These approaches fall into two areas, and are either sequence based or structure based [260]. Numerous algorithms are now available to carry out sequence based peptide epitope predictions (reviewed in ref. 261–263). These have the advantage of being fast, potentially screening whole genomes, but require large amounts of experimental data regarding the peptide binding preferences of the MHC molecule of interest. In comparison, structure based epitope modeling is slower, requiring the X-ray crystal structures of the MHC molecules but can be applied to all MHC types, including those that are uncharacterized. Advanced approaches include matrix-driven methods, finding structural binding motifs, a quantitative structure activity relationship (QSAR) analysis, homology modeling, protein threading, docking techniques and design of several machine-learning algorithms. Structure based predictions have the potential to discover non sequence based binders.

Both sequence and structure based computational approaches are based upon experimentally characterized peptides, but offer a more rapid indication of potential epitopes that could guide experimental studies. In both scenarios, experimental confirmation of peptide-MHC binding is still required.

In addition to sequence based or structure based predictions a number of computer algorithms have been developed that interrogate at the genome level for *in silico* prediction of T-cell antigens [264–267]. This has the potential to help in targeting low abundance T-cell epitopes in experimental studies. *In silico* methods, based upon various patterns in known MHC binding peptides, are cost effective and high throughput. They have the advantage of reducing the potential MHC binding peptide dataset, ruling out peptides that have no MHC binding potential. Even so, MHC binding is a prerequisite for T-cell activation, but does not guarantee it and experimental confirmation of T-cell activation is still required. There are also now epitope databases and web accessible tools for MHC binding prediction (for example <http://www.iedb.org/>). Other strategies have combined *in silico* prediction methods with mass spectrometry MHC peptide sequencing in order to increase the numbers of peptides identified [268–271]. This was exploited to target low abundance viral MHC peptides, synthesizing an *in silico* predicted MHC peptide as a calibrant, and using retention time and peptide mass–charge ratio in order to identify the corresponding experimental peptide [268, 269]. *In silico* prediction of MHC peptides is being demonstrated to be increasingly accurate when compared with experimental data [272, 273]. These approaches have the potential to increase the repertoire of detected MHC peptides. Moreover, sophisticated studies combining immunoproteomics and other approaches are beginning to decipher the origin and composition of the total repertoire of MHC peptides or “immunopeptidome” from a systems biology perspective [274].

The combined application of experimental studies and *in silico* based prediction will, in the long term impact upon vaccine development and personalized medicine. The information uncovers potential new antigens, which could be protein or peptide epitopes with the potential to stimulate protective immunity, i.e., to be part of a vaccine. The process is known as reverse vaccinology and has the potential to expedite the discovery and characterization of pathogen or disease epitopes. Reverse vaccinology identifies from whole genome sequences, antigenic extracellular proteins or peptides that are potential antigens. This approach has the potential to accelerate the sometimes slow and costly vaccine development pipeline. This was successfully pioneered for *Neisseria meningitidis*, causative agent of meningococcal meningitis and vaccines are now available for A, C, Y and W135 [275].

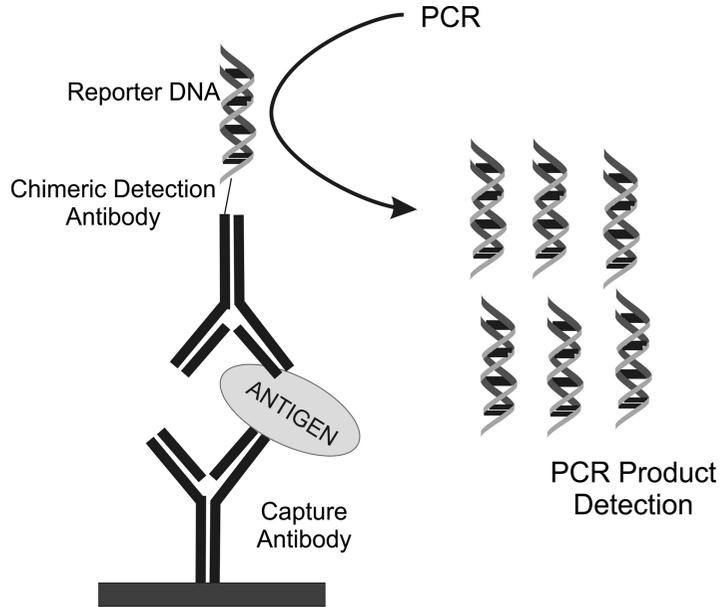
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## 6 Emerging Technologies and Applications

In the previous sections, we have provided a high level summary of the current, most widely used techniques loosely grouped under “immunoproteomics”. In the following subsections, we discuss emerging, or less widely used technologies that have potential to increase the breadth of immunoproteomics research.

### 6.1 Immuno-PCR

Immuno-PCR is a technique that was first reported in 1992 [276], and combines advantages of ELISA type assays, with the sensitivity of PCR and is aimed at detecting low abundance protein antigens. As outlined in Fig. 4, the antigen of interest is captured by a specific antibody and in a manner similar to traditional ELISA, a secondary antibody is used to detect binding. In this case, the secondary antibody is a chimeric antibody, with a DNA strand as the detection marker. The incorporation of a DNA tag allows amplification of the detection signal by PCR. This provides many of the advantages of PCR amplification, which are lacking in traditional ELISA assays. Immuno-PCR has been reported to have 10- to 1,000-fold increase in sensitivity compared to traditional antigen detection methods [276, 277], with high potential for the development of diagnostic assays. The technique has reported utility in detection of serological markers of ovarian cancer [278], CNS indicator proteins [279], detection and quantification of amyloid  $\beta$ -peptide in Alzheimer’s disease [280], early diagnosis of infectious disease [281], cytokine detection [282], and toxin detection [283–287]. In addition, this method is not aimed at discovery immunoproteomics, and has been developed for speed and sensitivity for use as a clinical laboratory tool [288]. Development of real time quantitative immune-PCR has added the ability to measure the amounts of antigen in a sample [289–291]. An excellent review provides more details on this approach [292].



**Fig. 4** Immuno-PCR. The setup of immuno PCR is similar to that of traditional antigen detection ELISA. A capture antibody immobilizes the antigen, and detection antibody added. Instead of the antibody–enzyme conjugate used for colorimetric detection in ELISA, the chimeric antibody with reporter DNA is used. Addition of primers, nucleotides and polymerase allows amplification of the signal. The linear amplification of PCR means that the number of PCR amplicons generated is proportional to the initial amount of antigen detected. This shows a simplified scheme, and many variations have been developed

## 6.2 MALDI-TOF for Immune Cell Surface Discrimination

MALDI-TOF is seeing increasingly widespread use in clinical microbiology laboratories for the routine identification of bacterial species (for example ref. 293–296). The approach is based upon protein signatures (without protein identification), and exploits not only the differences in cell surface proteins between cells types, but the dynamic change in those proteins under certain conditions. This has been demonstrated to be a robust, reproducible, rapid and potentially cost saving approach in medical diagnostics [297]. Recently, this approach has been successful in discriminating intact immune cells, including lymphocytes, monocytes and polymorphonuclear cells for the generation of an immune cell database [298]. The same approach was also able distinguish between stimulated and unstimulated macrophages [298]. Further to this, distinct differences in the MALDI-TOF protein fingerprints of the surface of macrophages were detected with the addition of M1 agonists, IFN- $\gamma$ , TNF, LPS, and LPS+IFN- $\gamma$ , and the M2 agonists, IL-4, TGF- $\beta$ 1, and IL-10. The differences in macrophage surface fingerprints were specific and readily

identifiable [299]. The method is rapid and reproducible and opens the door to an alternative method of immune cell analysis.

### **6.3 In Vivo Microbial Antigen Discovery**

*In vivo* microbial antigen discovery (InMAD) [300] was developed to identify circulating microbial antigens that are secreted or shed by bacteria, and detectable in sera. These circulating antigens can then be exploited for the development of rapid point of care immunoassays for bacterial diseases. The technique relies upon the humoral immune response to identify antigens that are circulating in sera. First carried out with the highly pathogenic bacteria, *Francisella tularensis* and *Burkholderia pseudomallei*, mice were infected with one or other organism and serum harvested [300]. The serum was filtered to remove whole bacteria, and termed InMAD serum. The filtered InMAD serum was then mixed with adjuvant and used to immunize mice. Bacterial proteins in the InMAD serum stimulate an immune response, which can then be monitored in order to determine the identity of the circulating bacterial proteins. Sera, collected from immunized mice was termed “InMAD immune serum” and was used in 2D Western blot or proteome array. In this way, the circulating bacterial proteins were identified [300] and have the potential to be rapidly translated into clinically relevant biomarkers for the disease diagnosis.

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## **7 Applications**

Immunoproteomics is still a relatively young field, with many academic reports, and a few being translated into clinical applications. However, there is huge potential for immunoproteomics-based assays to monitor or diagnose disease states or vaccine efficacy where antigens are involved. Bacterial and viral diseases are highly preventable through vaccination and an obvious application of immunoproteomics techniques is in antigen discovery for vaccine development. For example, efforts to develop a universal influenza vaccine with efficacy against all types of influenza need to be targeted against a conserved antibody or T-cell epitope. Mass spectrometry identification of influenza T-cell epitopes [301] is a step towards generating a vaccine that stimulates cross strain cell mediated immunity. A similar approach was used to identify conserved T-cell epitopes in dengue virus infected cells [302].

The remaining vaccine preventable diseases are challenging in terms of developing efficacious vaccines and discerning correlates of protection. Vaccinations against infectious disease are designed to stimulate a protective immune response. This immune response can be measured and correlated with the protection of the host against disease. In some cases, protective vaccination may only be established through detection of several immune parameters, such as immunodominant antibodies, cytokines etc. As immunoproteomics

studies advance in terms of sensitivity and throughput, this opens the door to rapid discovery of biomarkers of vaccine efficacy. Immunoproteomics approaches are being exploited to determine immune correlates of protection, which may then be used to monitor the protective status of the host. For example, proteome array studies have monitored the humoral immune response to smallpox and tularemia vaccines, and have noted a number of immunodominant proteins that have potential diagnostic applications [48, 54, 303–306]. These studies were extended further to investigate why smallpox vaccine fails to develop lesions in some individuals [307] and also comparing the antibody response to existing and next generation vaccinia virus vaccines [308].

Circulating antibodies represent important markers, reflecting the repertoire of nonself agents to which the immune system has been exposed. Antibodies amplify the signal of what may have been low abundance disease related proteins, have half lives of days to months and are stable to sample handling, so represent good biomarkers for diagnostic applications. As with all biomarker discoveries, validation and translation of immunoproteomic biomarkers to diagnostics is met with a number of challenges. Clinical diagnostic assays must be simple, robust, and sensitive, for example ELISA or antigen arrays.

Recombinant protein therapeutics are gaining popularity in a variety of applications. In addition to their desired therapeutic effects, they have the potential to stimulate an undesirable immune response against the recombinant protein. Protein therapeutics, such as recombinant IFN $\beta$  [309–311], IFN $\alpha$  [312, 313], and anti-TNF $\alpha$  antibodies [314, 315], are frequently observed to stimulate an undesirable immune response against the recombinant protein. The immune responses may be antibody or cell mediated and a combination of *in silico* prediction tools (reviewed in ref. 316) and *in vivo* validation by immunoproteomics methods could support prediction of immunogenicity for protein therapeutics, giving more rapid translation from discovery to clinic. Immunoproteomics approaches have the potential to have a high impact in this area, supporting the depletion of T-cell epitopes from protein therapeutics (reviewed in ref. 317).

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## 8 Future Perspective

The breadth and sophistication of the techniques developed to study the immunoproteome have increased dramatically in the past decade. The field has benefited greatly from advances in proteomics and immunoinformatics and will continue to develop. Challenges remain, such as characterization of low abundance T-cell epitopes, and detection of low level serum cytokines. However, new avenues of investigation are emerging, including application of

interactomics to immunoproteomics studies, and comprehensive systems biology studies of the immune response to disease. As our depth of knowledge of the immune response to infection, cancer or self-antigens (misdirected autoimmunity) increases, so do the opportunities for discovery of robust disease biomarkers for early diagnosis. Combined *in silico* and experimental studies promise to yield efficacious vaccine candidates and correlates of vaccine protection. On a systems level, understanding the rapidly changing protein landscape of the immune system at various stages of life has the potential to provide immune markers of vaccine health, and predictive markers of the immune response, which may in the longer term, contribute to the development of personalized medicine.

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