Review

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Immunoproteomics technologies in the discovery of autoantigens in autoimmune diseases

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Abstract: Proteomics technologies are often used for the identification of protein targets of the immune system. Here, we discuss the immunoproteomics technologies used for the discovery of autoantigens in autoimmune diseases where immune system dysregulation plays a central role in disease onset and progression. These autoantigens and associated autoantibodies can be used as potential biomarkers for disease diagnostics, prognostics and predicting/monitoring drug responsiveness (theranostics). Here, we compare a variety of methods such as mass spectrometry (MS)-based [serological proteome analysis (SERPA), antibody mediated identification of antigens (AMIDA), circulating immune complexome (CIC) analysis, surface enhanced laser desorption/ionization-time of flight (SELDI-TOF)], nucleic acid based serological analysis of antigens by recombinant cDNA expression cloning (SEREX), phage immunoprecipitation sequencing (PhIP-seq) and array-based immunoscreening (proteomic microarrays), luciferase immunoprecipitation systems (LIPS), nucleic acid programmable protein array (NAPPA) methods. We also review the relevance of immunoproteomic data generated in the last 10 years, with a focus on the aforementioned MS based methods.

Keywords: AMIDA; autoantibodies; autoantigens; autoimmune diseases; biomarkers; immunoproteomics; SERPA.

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Introduction

Immunoproteomics is the study of protein targets of the immune system via high-throughput proteomic technologies (1). The immune system ordinarily targets foreign-proteins to combat infection or prevent tumor development. However, under the influence of multiple factors such as environment, lifestyle and genetic pre-disposition, the immune system may lose self-tolerance and react against self-proteins (autoantigens), resulting in autoimmune disease. The adaptive immune response against such autoantigens causes cell death and inflammation – resulting in chronic symptoms characteristic of autoimmune disease. Autoantibodies and their cognate target antigens have been used as indicators of several autoimmune diseases. Typically, autoantibodies are used as biomarkers, rather than autoantigens – mainly because most autoantigens represent proteins that exist in normal/healthy people, while autoantibodies generally mark disease subsets with autoreactivity against these self-antigens (which may/may not be mutated or post-translationally modified).

Autoimmune diseases can either be localized to particular organs or be systemic, with effects in multiple organs of the body. We are in critical need of complex prognostic strategies to monitor and predict the course of systemic diseases in order to institute appropriate treatment modalities. Theranostic (drug responsiveness) biomarkers are believed to be very useful in predicting drug responsiveness and determining time/cost-effective treatment plans (2). Autoantibodies are a potent source of promising prognostic and theranostic biomarkers for systemic autoimmune diseases.

Whether autoantibodies are used as a diagnostic, prognostic or theranostic biomarkers, they are generally used in the clinical setting to probe for specific cognate autoantigens associated with particular disease states. This implies that if an autoantigen (and its corresponding autoantibody) has to be used as some kind of biomarker, it must be identified and defined. Thus, it is crucial to discover and identify autoantigen targets in

specific diseases, to develop effective diagnostic tools. Immunoproteomics includes a broad set of proteomics technologies that can be used for discovering autoantigens/autoantibodies which may serve as potential biomarkers.

The discovery of autoantigens/autoantibodies happens roughly in three phases: [1] screening for specific autoantibody/autoantigen combinations in patients, [2] molecular identification and characterization of the autoantigen, and [3] characterization of the candidate autoantigen's immunogenicity and the corresponding autoantibody signatures. A common theme in the screening phase is testing the autoreactivity of circulating antibodies within bodily fluids [such as serum, cerebrospinal fluid (CSF) or synovial fluid] from patient cohorts against proteomes sourced from primary cell culture, tissue/cell culture, tissue micro-dissection, or artificially generated peptide libraries/arrays. Proteins that test positively (putative autoantigens) must then be sequenced, identified and characterized using proteomics technologies. Here, we classify the common immunoproteomics methods used for autoantibody/autoantigen discovery into three categories, primarily based on the technology used for autoantigen screening or molecular identification: (i) mass spectrometry (MS)-based, (ii) nucleic acid-based proteomics and (iii) array-based immunoscreening technologies. The aim of this review is to analyze immunoproteomic data generated across these platforms and provide strategies for improving the autoantibody/autoantigen discovery process. Once an autoantigen is identified, its antigenicity has to be validated for it (or its corresponding autoantibody) to be used as a biomarker. This biomarker validation process is essential for US Food and Drug Administration (FDA) approval and for the successful translation of potential biomarkers from discovery phase to clinical applications. Readers are referred to a latest review on the biomarker validation process, as it is not in the scope of this review (3).

Mass spectrometry based technologies

State-of-the-art tandem MS technology is routinely used for identification of proteins in both academia and industry. Several MS-based technologies in the field of biomarker discovery have emerged over the last 20 years. Here we will look at the frequently used discovery approaches that employ MS for antigen peptide detection.

Serological proteome analysis (SERPA)/ PROTEOMEX

The most common method used for profiling autoreactivity of patient sera and identification of antigens in autoimmune diseases is referred to as serological proteome analysis (SERPA) (4) or PROTEOMEX (5). In this method, whole tissue/cell protein preparations containing potential autoantigens are run on 2-dimensional electrophoresis (2DE) gel in triplicate (Figure 1A). Two of these 2DE gels are used for immunoblotting: one against patient sera and the other against control sera from healthy donors. Unique protein spots that specifically react with patient sera, but not control sera, are detected on the immunoblots and are used as guides to excise gel plugs containing the corresponding protein spots from the third 2DE gel. The gel plugs are then treated with trypsin and resulting peptides are extracted for protein identification using LC-MS/MS or matrix assisted laser desorption/ionization (MALDI)-TOF. Inherent gel-to-gel variability, however, limits the accuracy of spot picking guided by immunoblot maps, which is especially true for low-abundance protein targets. A triangulation approach involving rigorous quality control steps has been suggested in order to accurately identify the protein (6). To confirm that the correct spots are selected, the 2DE gel from which plugs are excised is transferred to a nitrocellulose membrane and immunoblotted against patient sera. Because the diameter of the protein plug is typically smaller than the diameter of the protein spot, correctly selected spots should leave a halo of immunereactive material surrounding the hole created by the gel plug, thus confirming that the correct spot has been excised.

Recently, a modified SERPA adapted from difference gel electrophoresis (DIGE) has been described as a fluorescence-based bidimensional immunoproteomics (FBIP) approach (7). The protein mixture is labeled with Cy3 fluorescent dye and loaded on a 2DE gel. The proteins from this 2DE gel are transferred to a nitrocellulose membrane. In a co-hybridization scheme, the membrane is probed with patient sera to generate an antigenic map and with a range of monoclonal antibodies against standard proteins to generate a landmark map. The proteomic, antigenic and landmark maps are then overlaid and compared to identify potential antigenic spots on a second 2DE gel. This improvement enhances the accuracy relative to the previously described method of comparing different spot maps, and is helpful in selecting the correct protein spots across gels.

Many groups have modified SERPA by characterizing circulating antibodies from other bodily fluids such as

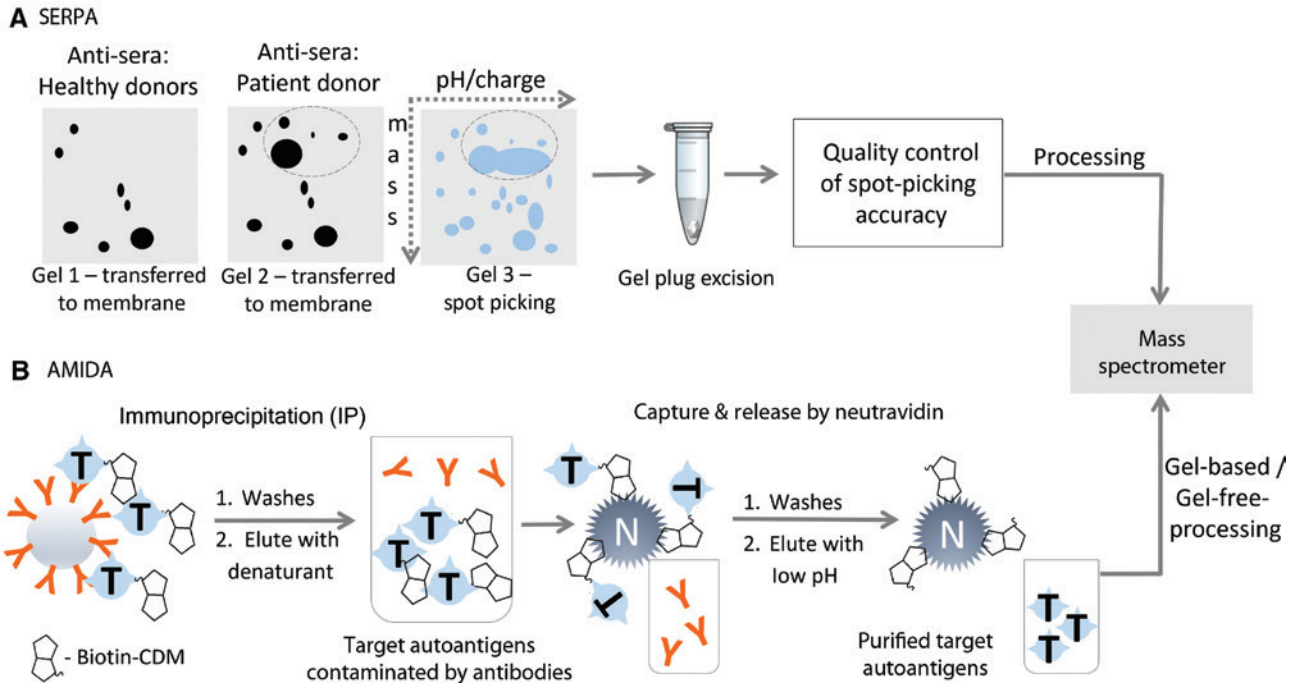

Figure 1: Mass spectrometry based immunoproteomics approaches.
(A) The experimental workflow of SERPA in identifying disease associated autoantigens. Control map, Antigenic map are compared and antigenic protein spots are selected and excised from the third 2DE gel. This gel plug is then processed for MS peptide sequencing. (B) The experimental workflow of AMIDA enhanced by biotin-CDM to remove contaminating antibodies and identify disease associated autoantigens; Y – antibodies from patients/healthy controls; T – target autoantigens from cell/tissue culture; N – neutravidin beads. Protein preparation containing potential target autoantigens are labeled with biotin-CDM and immunoprecipitation is done using patient antibody beads. The IP eluate is further purified via binding to neutravidin beads, washes followed by a low pH elution reversing the biotin-CDM-to-protein linkage. The purified potential target autoantigens can be processed for MS peptide sequencing.

CSF, bronchoalveolar lavage (BAL) fluid, synovial fluid etc. In some cases, other bodily fluids have also been used as a source for autoantigens (8). The challenge in this case is depleting high abundance proteins such as albumin from CSF, BAL or synovial fluid in order to resolve the low abundance proteins (9, 10).

The major advantages of SERPA are the identification of post translational modification (PTM) states of antigens and the high sensitivity afforded by immunoblots. The gel-to-gel variability in 2DE poses a number of challenges in accurately identifying potential antigenic protein spots for subsequent MS-based sequencing. Improvements to SERPA that we describe above could alleviate some of those challenges. However, the inability of 2DE to resolve hydrophobic, large, and/or basic proteins is also a concern, as a portion of the proteome cannot be screened using this approach.

Interestingly, several papers on autoimmune biomarker studies using SERPA describe putative autoantigens commonly found in multiple autoimmune diseases, including α-enolase, annexin II, and actin subunits. For example, α-enolase appeared as an autoantigen in 20% of 23 autoimmune studies (8, 11–33). Surprisingly, these same proteins also appeared as antibody targets in a control study of healthy individuals employing SERPA (34). This pattern of recurring autoantigens may be due to the common inflammatory nature of different autoimmune diseases, as well as possible autoimmune pre-disposition in seemingly healthy individuals. However, the repetitive results may also be an artifact of SERPA, again highlighting limitations that have fueled the development of alternative discovery methods.

Immuno-affinity capture technologies

Autoantibody mediated identification of antigens (AMIDA)

As opposed to using patient sera to probe 2DE gel blots, preparative-scale immunoprecipitation (IP) relies on patient/control sera to isolate and enrich autoantigens from soluble mixtures of potential target proteins. In

this approach, which is called autoantibody mediated identification of antigens (AMIDA) (35), patient/control immunoglobulins are first bound to Protein-A/Protein-G magnetic beads; relatively large amounts of whole cell/tissue protein lysates containing potential autoantigens are then co-incubated with the antibody-coated beads. Unbound proteins are washed away, allowing the bound, putative autoantigen proteins to be eluted. Eluted proteins are then resolved by gel electrophoresis or liquid chromatography (LC), processed, and sequenced via LC-MS/MS.

Preparative-scale IP enriches for reactive autoantigens relative to the rest of the proteome, which in principle should be more sensitive than SERPA. However, under the extreme denaturing conditions used for eluting proteins from the antibody beads, bound antibodies often leach from the beads, contaminating the eluted sample (36) and posing a challenge for MS-identification of protein targets. The antibodies being high abundance proteins mask the true peptide signals. This problem can be solved by a two-pronged approach: at the data level and/or at the physical level of the experiments. Typically the peptide sequences originating from antibodies, are removed from the MS data during analysis. This helps in the data analysis of true antigen peptides, however, the effectiveness of this analysis strategy is dependent on the dynamic range of the MS instrument used. One of the technical solutions to remove antibody peptides physically is, covalent cross-linking of antibodies to beads. This approach is helpful, but this process requires optimization and can become cumbersome when working with a large number of samples derived from patient cohorts. This experimental barrier likely explains why so few published studies have used AMIDA in the discovery phase of autoantibody/autoantigen biomarkers over the last 10 years (37–41).

Recently, we have addressed this limitation of AMIDA by synthesizing a novel pH sensitive reversible biotin tag called biotin-CDM, which can be used to tag target protein populations containing potential autoantigens (42). After IP elution, which contains both putative autoantigens and leached antibodies, candidate autoantigens are separated from patient/control antibodies using avidin beads (Figure 1B). Since the biotinylation reagent is completely reversible and does not leave any chemical groups on the proteins, the recovered autoantigens can either be run on a 2DE gel or processed using gel-free schemes for LC-MS/MS. Importantly, native or induced post-translational modifications (PTMs) of putative autoantigen targets can be characterized by using 2DE-LC-MS/MS, highlighting the versatility of this approach.

Circulating immune complexome (CIC) analysis

CICs are circulating protein complexes that contain potential autoantigens, antibodies, pro-inflammatory factors and other clotting factors that occur normally in healthy individuals, but are rapidly cleared by macrophages. However, in autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, and systemic lupus erythematosus, CICs accumulate in blood and can be analyzed to discover new autoantigens. Very similar to AMIDA, CIC's can be isolated from patient sera through binding to protein A/G beads. They are then eluted, trypsin digested, and directly subjected to LC-MS/MS for identification. The MS identification is expected to be obscured by peptides from immunoglobulins and various immune factors, necessitating 'subtractive' sequence analysis of non-immunoglobulin peptides. Overall, this approach – which relies on the dynamic range of protein/peptide detection in the MS instrumentation – is useful for identifying autoantigens in diseases where the presence of disease-specific CIC's is known (43, 44). Limitations include the requirement for sophisticated, often expensive, technologies such as multiplexing samples through Orbitrap.

Surface enhanced laser desorption/ionization – time of flight (SELDI-TOF)

SELDI-TOF is a simple proteomics approach where protein signatures are compared between multiple samples. Because the identity of proteins are not defined during this comparison, this technology cannot be used for the identification of antigens. However, some studies have used SELDI-TOF for fast screening of autoantigens in several autoimmune diseases, followed by additional MS for actual peptide identification (45–51). In this approach, antigen-antibody complexes are isolated from patient samples and immobilized on a SELDI chip prior to analysis of mass spectra. Protein peaks are semi-quantifiable and used to create protein signatures. However, a major limitation of this procedure is that only proteins <20 kDa size can be analyzed using this method. Moreover, antibodies can also dissociate from antigen-antibody complexes, significantly increasing the noise in the detection system.

Meta-analysis

In order to evaluate the quality of data generated using the above MS based approaches, we compiled a list of protein autoantigens discovered using these methods in the last

10 years. We used a protein abundance database to rank the integrated cellular abundance of each of these proteins expressed in a relative quantifying unit called 'parts per million (ppm)' (52). The unit ppm is used in order to extract, combine and normalize data from several studies using various experiments and technologies. In this meta-analysis, we plotted histograms of cellular abundances of protein autoantigens discovered using SERPA (Figure 2A) (8, 11–34) and immuno-affinity capture technologies such as AMIDA, CIC analysis and SELDI-TOF (Figure 2B) (37–41, 43, 45–51).

In this qualitative analysis, the shape of the abundance histogram of autoantigens discovered using SERPA appears to be biased towards high abundance proteins.

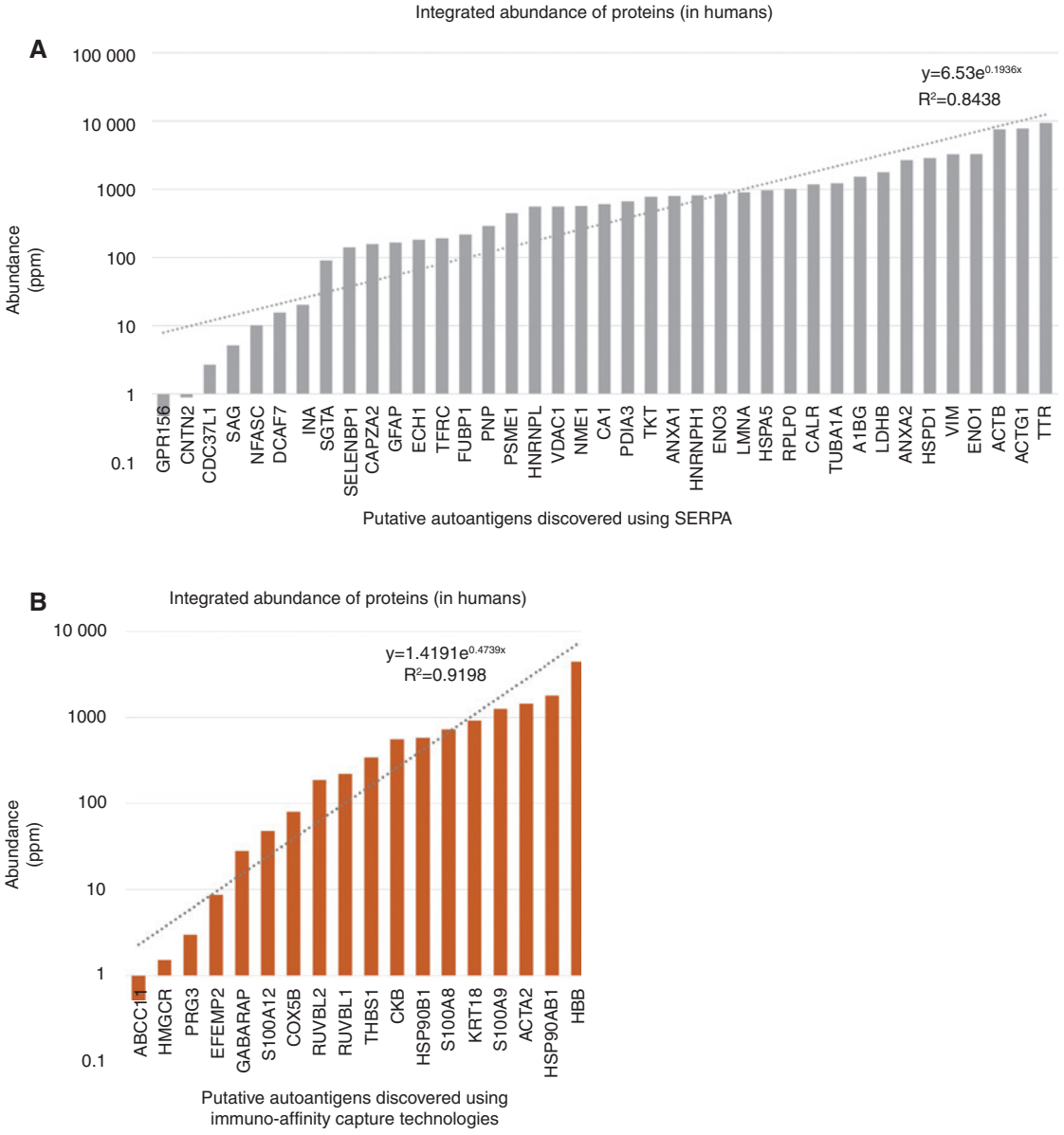

Figure 2: Meta-analysis of Immunoproteomic data generated using MS-based approaches.
The vertical axis represents the integrated protein abundance in ppm in a logarithmic scale. PPM (parts per million) is the unit of abundance that is used to quantify relative abundance within the proteome (35). (A) The horizontal axis represents each protein discovered using SERPA from 24 studies (8, 11–34). The histogram is clearly skewed towards high abundance proteins. Among these proteins, α-enolase, annexin II and actin appear as autoantigens in multiple autoimmune studies that employed SERPA. We notice that these notorious antigens are also on the higher side of the abundance histogram. This shows that SERPA maybe biased towards picking up high abundance proteins as putative autoantigens. (B) The horizontal axis represents each protein discovered using immuno-affinity capture technologies such as AMIDA, CIC and SELDI-TOF from 12 studies (38–42, 46–52). The vertical axis represents the integrated protein abundance in ppm (35) in a logarithmic scale.

Countering this problem requires that low abundance proteins be enriched by using either large-scale protein preparations (increasing the loading capacity of 2DE gels) or through alternative procedures that include fractionating bodily fluids, cells or organelles. In principle, immuno-affinity capture technologies are used for enriching autoantigens in biological samples and thus, these technologies should not be limited by protein abundance. When we compare the abundance histogram of protein autoantigens discovered using immuno-affinity capture technologies to that of SERPA, we see a marked difference between the shapes of the histogram. We have compared immunoproteomic data from 24 studies using SERPA (8, 11–34) and 13 studies using the other immuno-affinity capture technologies (37–41, 43, 45–51). We observe that the abundance histogram of autoantigens discovered using immuno-affinity technologies are more equally balanced between many high-abundance proteins and low-abundance proteins, thus the immuno-affinity technologies are not as biased as SERPA towards high abundance proteins. The immuno-affinity technologies could be better for identifying low abundance target autoantigens with further technical improvements.

Nucleic acid based proteomics

While gene expression libraries do not technically fall under the category of proteomics, the following methods have been quite successful in autoantigen identification and have unique advantages to offer to the field of Immunoproteomics.

Serological analysis of antigens by recombinant cDNA expression cloning (SEREX)

SEREX is one of the oldest methods used for the identification of autoantigens in several autoimmune diseases (53–64). Here a human cDNA library derived from an autoimmune patient is used to profile autoantibody repertoires from the same patient in a process called autologous typing. The proteins/epitopes that show autoreactivity are then identified through PCR-based sequencing of DNA from their respective clones. This approach is highly sensitive given the use of DNA-detection, rather than protein-detection methods (since the latter are limited by protein abundance). However, a crucial limitation of SEREX is that this method lacks the ability to differentiate or detect post-translational modifications (PTMs) that are likely to play a significant role in breaching immune tolerance in autoimmune diseases such as RA (65).

Phage immunoprecipitation sequencing (PhIP-Seq)

PhIP-Seq is used for profiling the autoantibody repertoires of individual patients, with the potential for 'personalized' diagnosis. In this method, a synthetic human peptidome library is screened against individual patient sera using phage display-based immunoscreening. The reactive phages are isolated and their DNA is sequenced in a high-throughput manner, allowing peptide identification after extrapolation from the phage DNA sequence (66). This technology has been applied to detect autoantigens in multiple autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (67). Again, however, the inability to screen for autoantibodies recognizing post-translationally modified proteins represents a significant limitation of this approach in identifying clinically useful biomarkers for various autoimmune diseases in which modified antigens are targeted.

Overall, nucleic acid based technologies are robust for screening autoantigens. In order to compensate for their limitation in characterizing the post-translation modifications, this kind of screening has to be always followed-up with an orthogonal MS based approaches to define the molecular characteristics of potential autoantigens.

Array-based immunoscreening technologies

As a relatively new technology, autoantigen microarrays have been successfully used to detect and characterize autoantibody profiles for several autoimmune diseases (68–75). These protein/peptide chips have been generated with as few as 14 proteins to as many as ~17 000 proteins (72) that can be used to screen patient sera for corresponding autoantibodies. Recently, plasmonic microarrays with fluorescent infrared enhancement have been shown to increase the dynamic range of antibody: antigen detection (76). Because the arrayed proteins/peptides are recombinant/purified, the protein concentration range is not as variable as physiological protein concentration ranges – overcoming the limitations posed by previously described proteomics methods that can be limited by protein abundance. Moreover, detection of PTMs can be incorporated in microarray screening by using synthetic platforms such as a glycosylated peptide array (77). An additional benefit of this technology includes

profiling autoantibody signatures during disease progression (78), as has been shown in a recent review describing the use of proteomic microarrays to study autoantibody profiles in systemic lupus erythematosus (79).

Two newer methods are NAPPA and LIPS. Nucleic acid programmable protein array (NAPPA) is an *in situ*, cell-free protein expression microarray technology that has been used in the discovery of autoantigens in type 1 diabetes and in the detection of multiple autoantibodies in ankylosing spondylitis (80). This technology is at the interface of nucleic acid-based proteomics and array-based technologies. The proteins are synthesized directly on the array along with a fusion tag and captured in place using an anti-tag that is fixed to the array. This is a promising screening platform for personalized diagnosis. Luciferase immunoprecipitation systems (LIPS) is a similar technology that detects antibody: antigen binding via luciferase enzyme and has been used to profile autoantibodies (81, 82). Purified candidates are attached to beads and using the luciferase detection system, the binding events of patient antibodies are detected. This technology is robust and has been used in the characterization of autoantibody signatures and validation of autoantigens.

Though these array-based technologies may be efficient in screening for autoantigens, these technologies are more useful in characterizing autoantibody signatures and study disease progression. When used for screening autoantigens, the candidate antigens have to be further characterized at the molecular level, using MS based technologies.

Current challenges in immunoproteomics

The proteomic search for biomarkers in the last 2 decades has resulted in a long list of candidate biomarkers for autoimmune diseases. Unfortunately, discovery efforts employing MS-based proteomics technologies have yet to yield any FDA-approved biomarkers (83). A number of issues may contribute to this shortcoming that is increasingly recognized in the field of proteomics (84, 85), including: [1] incomplete validation of biomarker candidates, [2] use of suboptimal statistical methods, and [3] technical or strategical limitations.

Validation of biomarker candidates

As previously discussed, a major concern is the overlap in detected autoantigens in multiple autoimmune diseases (see above section on SERPA). These common proteins are also known for their notorious repetitiveness in 2DE based proteomic studies (86). While autoantigen redundancy may be a general feature of systemic autoimmunity, the above 2DE meta-analysis raises concerns related to biases in the various discovery methods employed in different studies. It is possible that these proteins could carry different post-translational modifications or express different isoforms in the disease state that have not been deduced in the initial discovery stages – highlighting the need for further characterization. Furthermore, inter-individual differences introduce noise that may cloud interpretation of autoantibody/autoantigen data. If proteomic data are not validated in larger patient cohorts, then the discovered autoantigens may not ultimately translate into useful biomarkers.

Use of appropriate statistical methods

We compared the autoantigen proteomic data for multiple sclerosis and rheumatoid arthritis, across the methods described above. No common autoantigen proteins were identified for either multiple sclerosis or rheumatoid arthritis when comparing different discovery methods. Yet, this observation may be favorable, indicating that orthogonal approaches improve the likelihood of establishing a more diverse set disease of biomarkers. However, many groups have opted to run fewer proteomics experiments and rely upon ANOVA or other statistical methods to pre-filter their proteomic data before validating the biomarkers (87). This review suggests that patient cohorts of at least fifty should be used and that pre-filtering of the data should be avoided, in order to make meaningful progress in the identification and validation of protein biomarkers.

Technical and strategical considerations

As the biology of autoimmunity is very complex, distinguishing true from artifactual data is critical – and highly dependent on the use of appropriate controls. Furthermore, from our experience with proteomic techniques, variability in sample preparation and handling greatly affect the quality and reliability of proteomic data. Repeated freeze-thawing of both the patient fluid samples and protein extracts from cells/tissues should be avoided because this causes protein loss and inconsistency between samples. In comparative proteomics, label-free proteomics techniques such as LC-MS/MS might produce more artifacts relative to those

approaches that use intact proteins and fluorescence detection methods, such as DIGE. These considerations apply while working with any proteomics method used in biomarker discovery.

In terms of research strategies, a hypothesis-driven, targeted search may be better than an exploratory data-driven search at yielding disease-state relevant candidate biomarkers. For example, one could focus on particular PTMs implicated in a disease state during proteomics screening, profile autoreactive proteins in tissue biopsy, or use fractionated body fluids/organelles from patients as a source of autoantigens. When searching for prognostic or theranostic biomarkers, targeted immunoproteomics technologies such as glycosylated peptide array (77) or citrullination probe based MS technology (88) might be employed.

Conclusions

Autoimmunity is associated with self-directed, dysregulated immune responses that can negatively impact multiple organs depending on the particular disease entity. One potentially interesting use of the described immunoproteomics methods is to follow changes in autoantibody profiles or patterns of autoantigen recognition in longitudinal studies of disease progression. This type of analysis should provide a deeper understanding of autoimmune disease progression and, importantly, aid in developing novel treatment strategies.

While array-based screening technologies and nucleic acid-based proteomics offer high sensitivity and remove protein abundance bias, neither of these approaches are particularly useful for the detection of post-translational modifications. MS, on the other hand, is capable of detecting PTMs, but the use of this modality often requires targeted searches and significant amounts of patient sample. Despite these limitations, MS-based technologies are still invaluable in the protein identification phase of biomarker discovery. Of the MS-based technologies used in immunoproteomics, AMIDA seems to have the fewest limitations, suggesting that further refinement/development of automated AMIDA could expedite progress in identification and molecular characterization of autoantigens. Ultimately, these and other array-based methods will prove invaluable for characterizing autoantibody signatures and validating candidate biomarkers.

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List of abbreviations

IP	immunoprecipitation
CSF	cerebrospinal fluid
BAL	bronchoalveolar lavage
MS	mass spectrometry
LC	liquid chromatography
MALDI	matrix assisted laser desorption/ionization
SERPA	serological proteome analysis
AMIDA	antibody mediated identification of antigens
2DE	two-dimensional gel electrophoresis
TOF	time of flight
SELDI	surface enhanced laser desorption/ionization
FDA	US Food and Drug Administration
FBIP	fluorescence-based bidimensional immunoproteomics approach
DIGE	difference gel electrophoresis
PTM	post translational modification
CIC	circulating immune complexome
SEREX	serological analysis of antigens by recombinant cDNA expression cloning
PhIP-seq	phage immunoprecipitation sequencing
NAPPA	nucleic acid programmable protein array
LIPS	luciferase immunoprecipitation systems
ppm	parts per million

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