Applications of Functional Protein Arrays for Detection and Development of Autoantibody-based Diagnostics and Therapeutics

Autoantibodies Overview

The human immune system is a key line of defence against foreign pathogens. There are three main components in the defence mechanism, namely, discrimination of self from non-self, triggering response, and eradication of the foreign object from the host. Discrimination of self from non-self, or immunological tolerance, prevents the immune system from mounting an immune response to the body's own proteins and other host antigens. When this tolerance is lost, often due to a combination of genetic and environmental factors, the immune system will start attacking self-antigens or autoantigens, which will give rise to the production of autoantibodies (AAbs). AAb production is an important indicator of autoimmune diseases but is not exclusive to these conditions since AAbs are also present in many other diseases and have been increasingly reported to be detectable many years before the manifestation of symptoms. AAbs thus appear to be attractive biomarkers for the early diagnosis of disease.

Types of Autoantibodies

Having elevated levels of AAbs may reflect the overall condition of the immune response of an individual. There are five types of AAbs; natural, predictive, pathogenic, protective and neutral. Natural AAbs are present at birth and are mainly of IgM class; They are detectable in normal human serum in the absence of active immunisation and react with a wide range of self-antigens. These natural AAbs play an important role in maintaining immune homeostasis and remain highly conserved among individuals and through ageing[1].

Predictive AAbs are useful as biomarkers for early diagnosis of various diseases. Derksen et al. reported that the presence of anti-citrullinated protein antibodies (ACPA) AAb in the serum of rheumatoid arthritis patients can be detected four years before actual disease onset[2], after which the ACPA AAb levels start to increase as the disease develops. In cancer, tumour-associated AAbs were identified as strong candidates for a range of cancers. They are produced early in tumorigenesis and were reported to be measurable up to five years before the development of clinical symptoms[3]. AAbs were detected to be present for up to 18–20 years before the diagnosis of primary Sjogren's Syndrome, and researchers concluded that even earlier seropositivity cannot be excluded, as for most Sjogren's patients, the earliest sample analysed was positive[4].

Some AAbs are considered pathogenic as they contribute to malignancy of disease, for example in classic autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, Graves' disease, autoimmune hepatitis, multiple sclerosis, diabetes, and Sjogren's syndrome. These AAbs, although 'bad', may serve as important biomarkers that can be clinically utilised to aid prognosis and stratification of patients. They can also be used to determine disease stage and progression. Profiling and characterisation of these AAbs may help to shed light on previously unknown pathogenic mechanisms and pathways in certain diseases. Previous work by Nagele *et al.* has demonstrated the ubiquitous presence of AAbs in all mammals and has shown that their titer is influenced by a variety of factors, including age, gender, and disease state[5]. Han *et al.* demonstrated that pathogenic AAbs can be used to differentiate mild-moderate stage Alzheimer's and Parkinson's disease from nondemented control subjects[6]. In cancer-associated retinopathy (CAR), most disease-related AAbs are IgGs that are somatically mutated, suggesting that helper T cells drive the autoimmune B cell response, including anti-enolase AAbs in CAR patients[7]. AAbs against glycolytic enzymes were produced in response to their mutations, misfolding, degradation, overexpression in the cell, and the protein release from damaged tissue. Molecular mimicry has also been proposed as a mechanism of AAb formation and is a known contributor to the pathogenesis of some autoimmune conditions, such as rheumatic heart disease[7].

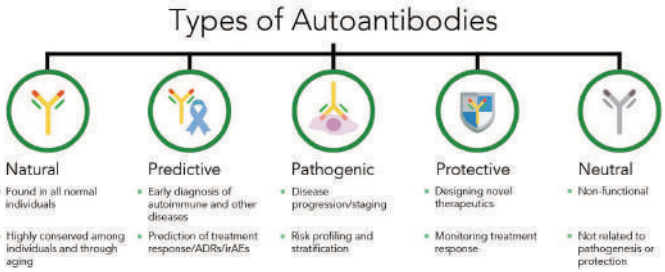

Figure 1: Types of autoantibodies

Protective AAbs, on the other hand, are 'good' AAbs that protect certain individuals from complications of disease. Due to their 'protective' effect, these AAbs may be targeted as therapeutics or even prophylaxis. Anti-pentraxin 3 (PTX3) AAbs were found to be associated with the absence of renal involvement in systemic lupus erythematosus (SLE). They were found to be less prevalent in active lupus nephritis patients compared with SLE without renal involvement and associated with less severe renal damage, especially with the combined evaluation of serum PTX3 levels[8]. Wilding *et al.* demonstrated neuroprotective potential of GFAP in neuroretinal cells of glaucoma patients. They found that the GFAP antibody is able to protect cells from oxidative stress, and detected a cross-reaction of the antibody to endoplasmic reticulum resident protein 57 on the cell membrane, which seems to lead to an improved signalling in the cells triggering the protective effects[9]. AAbs that have no apparent role in disease are referred to as neutral AAbs. These AAbs do not contribute to pathogenesis or protection and have very little value in prediction of disease.

Therapeutic Potential of Autoantibodies

Research on antibodies for therapeutics has grown enormously since the first development of hybridoma technology in 1975 by Georges Kohler and Cesar Milstein. Since natural antibodies typically each bind to a single antigen with high affinity and high specificity, this particular property of antibodies has attracted enormous interest for its potential utility in diagnosis and treatment of multiple diseases. Amongst the various antibody- or antibody-like scaffolds described in the literature, human and humanised antibodies appear to be superior therapeutic agents over other traditional moieties, including small molecules, as they have exhibited excellent safety and efficacy combined with minimal off-target effects. Chimeric and humanised mAbs, which have been the predominant mAbs entering clinical studies, were reported to have higher approval success rates than new chemical entities, especially in the field of oncology[10]. Furthermore, according to FDA labelling, the mAb half-lives are much longer than those of small-molecule agents[10], potentially increasing their efficacy.

Torchilin *et al.* found circulating antinuclear AAbs (ANAs), typically found in autoimmune conditions, which were also detected in cancer patients and in healthy elderly individuals, to have anti-tumour potential. Although the pathogenic role of ANAs in autoimmunity is well demonstrated, the researchers have identified that natural ANAs found in elderly individuals were actually capable of binding to the surface of a broad spectrum of cancer, but not normal cells[11]. They further explained that these AAbs identified in ageing animals and humans are significantly different from those identified in newborns and adults, with antinuclear specificities occurring more frequently in the aged, and therefore ANAs in the elderly could be an important component of the natural AAb repertoire and might participate in antitumour immunosurveillance[11]. They concluded that since circulating ANAs are found in approximately 30% of patients with cancer, and some of these patients even develop autoimmune syndromes as a result, the possible use of ANAs in cancer therapy via intentional induction of autoimmunity might be considered as an antineoplastic therapeutic strategy warranting further experiments and clinical trials.

Although one of the compelling advantages of antibody-based therapy is the specificity of binding by antibodies to target antigens, a large number of antibodies cannot penetrate cells to affect intracellular processes, mainly due to their size. Noble et al. found that select lupus AAbs are capable of penetrating into cell nuclei and therefore represent an attractive new concept in cancer therapy[12]. These cell-penetrating lupus AAbs have the capacity to disrupt key intracellular processes, including to precisely affect cancer cells by binding to their DNA and inhibiting the cell's DNA repair mechanism. It has, for example, been shown that the nuclear-localising lupus anti-DNA AAb 3E10 penetrates cells through an equilibrative nucleoside transporter (ENT2) that is expressed in nearly all cells and, once inside the nucleus, 3E10 inhibits DNA repair mechanisms. The degree to which 3E10 inhibits DNA repair pathways is not sufficient to kill normal cells, but cancer cells with pre-existing defects in their repair mechanism were found to be sensitive to 3E10[12], suggesting potential applications of the 3E10 lupus AAb in targeted cancer therapy, especially for tumours with defects in DNA repair mechanisms.

Bushey *et al.* isolated and characterised high-affinity human complement factor H (CFH) monoclonal antibodies (mAbs) derived from single B cells of patients with the AAb in an attempt to investigate why some patients with cancer never develop metastasis. They hypothesised that CFH AAbs that arise in lung cancer patients may promote anti-tumour cell activity and that CFH antibody administration might provide a unique way to stimulate a long-term immune response and treat cancer. These authors have previously reported an association between AAbs against CFH and early-stage lung cancer where they observed that CFH prevents complement-mediated cytotoxicity (CDC) by inhibiting the formation of cell-lytic membrane attack complexes on self-surfaces[13].

CFH is a regulatory protein that helps regulate part of the body's immune response known as the complement system, which is involved in eliminating foreign pathogens, triggering an inflammatory response and removing debris from cells and tissues. CFH, together with several related proteins, protects healthy cells by preventing the complement system from being overactivated. In order to develop a therapy that will closely mimic the native human immune response, researchers identified patients with anti-CFH AAbs and isolated their B cells for characterisation of anti-CFH autoantibodies, claiming in the process that cloning and expressing antibody genes derived from selected B cells is significantly more efficient than producing mAbs in mice by immunisation, followed by 'humanisation' of the murine Abs.

By treating cancer cell lines with the isolated CFH monoclonal antibodies, researchers further demonstrated that anti-CFH mAb7968 killed a variety of tumour cell lines *in vitro*, including NSCLC (three histological types), small cell lung cancer, gastric cancer and breast cancer. Furthermore, *in vivo* growth of both a subcutaneous brain tumour in a nude mouse model and a subcutaneous lung tumour in a syngeneic mouse model were substantially inhibited by this antibody without observable adverse reactions[13].

Functional Protein Arrays for Detection of Autoantibodies

A protein microarray is a highly-multiplexed, miniaturised platform used to assay thousands ofproteinsspotted on a solid support in a high-throughput, quantitative manner. The three most common types of protein microarrays are analytical, functional and reverse-phase. In the last decade, applications of functional protein microarrays in particular have gained substantial popularity, due to the great variety of analytes that can be screened using the functional protein array platform, including proteins, DNA, RNA, small molecules, lipids, enzymes as well as peptides. Functional protein microarrays are particularly well suited to the component-resolved identification of novel autoantigen-autoantibody pairs in what amount to highly multiplexed, miniaturised ELISAs across thousands of immobilised antigens.

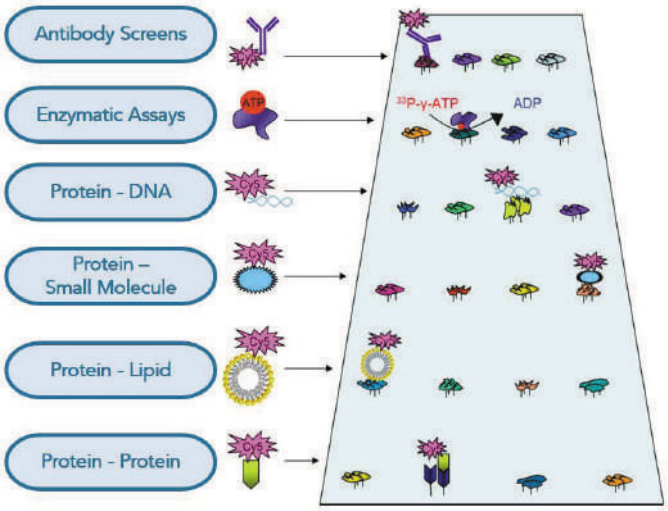

Figure 2: Functional protein array technology provides a versatile platform for autoantibody-based discovery.

There are different types of functional protein array platforms. Some utilise peptides or fragments of proteins while the others use purified proteins immobilised on glass slides. Peptides or fragments of proteins only make available a few epitopes per protein and therefore typically miss both discontinuous and numerous linear epitopes, as well as lacking post-translationally-modified epitopes. Purified proteins, on the other hand, can be denatured due to aberrant interactions with the array surface, impairing the availability of linear epitopes and disrupting conformational epitopes of the proteins. In this regard, in order to maximise the interaction between autoantibodies and proteins on the array, full-length, native proteins are ideal to be immobilised on glass slides where all the epitopes will be present for binding with autoantibodies.

One key element that determines the quality of a protein array is the surface chemistry. An ideal surface chemistry should resist non-specific adsorption and provide an environment that allows the immobilised protein to retain its native three-dimensional structure, stability and functionality. In addition, the method used to immobilise the proteins also plays an important role. While there are different approaches to immobilising proteins on solid support, affinity capture is an advantageous method as the structure and function of the protein is preserved, and the orientation of the protein is precisely controlled. Affinity tags like biotin can be either chemically or biochemically fused to protein domains and used to mediate immobilisation onto streptavidin-coated glass slides. For example, the Sengenics KREX technology uses a compact, folded, biotinylated, ~80 amino acid residue domain derived from the *E. coli* biotin carboxyl carrier protein (BCCP) which offers two significant advantages in protein production: First, the BCCP domain is efficiently biotinylated in a wide variety of expression systems without the need to co-express the *E. coli* biotin ligase; second, the N- and C-terminal of BCCP are physically separated from the site of biotinylation, thus the BCCP domain can be thought of as a stalk which presents the recombinant proteins away from the slide surface, thereby minimising any deleterious effects due to immobilisation[14]. BCCP also acts as protein folding marker and protein solubility enhancer, thereby ensuring that only correctly folded proteins end up bound to the array surface, which in turn greatly minimises the false-positives that otherwise arise from non-specific binding of AAbs to unfolded antigens on protein microarrays. Such use of appropriate surface chemistries in conjunction with arrays of folded, functional full-length antigens thus enables the overall process of discovering novel, biologically-meaningful diagnostic and therapeutic AAbs to be massively streamlined. The KREX technology has been successfully utilised in AAb biomarker discovery and has facilitated the identification of biologically meaningful, novel targets in malaria[15], neurodegenerative disease[16] and cancer[17].

Conclusion

AAbs are invaluable targets that are useful not only in the diagnosis of disease but also in development of new therapeutic interventions. In particular, development of AAb-based therapeutics has shown promising effect in influencing the course of not only autoimmune diseases but also cancer and other diseases. However, the right technology is required in order to detect true AAb signatures which are translatable into clinical use. Functional protein arrays have the advantage of a high signal-to-noise-ratio, low sample consumption and the option of multiplexing, which is an essential feature for clinical utility. In the field of personalised medicine and companion diagnostics, functional protein arrays have great potential as a tool to improve pharmacotherapy, especially for the pharmaceutical and biotechnology industry. Functional protein arrays thus have the capacity to accelerate and transform AAb-based research, thereby creating a new paradigm for the discovery, validation and development of novel diagnostic biomarkers and antibody-based therapeutics.

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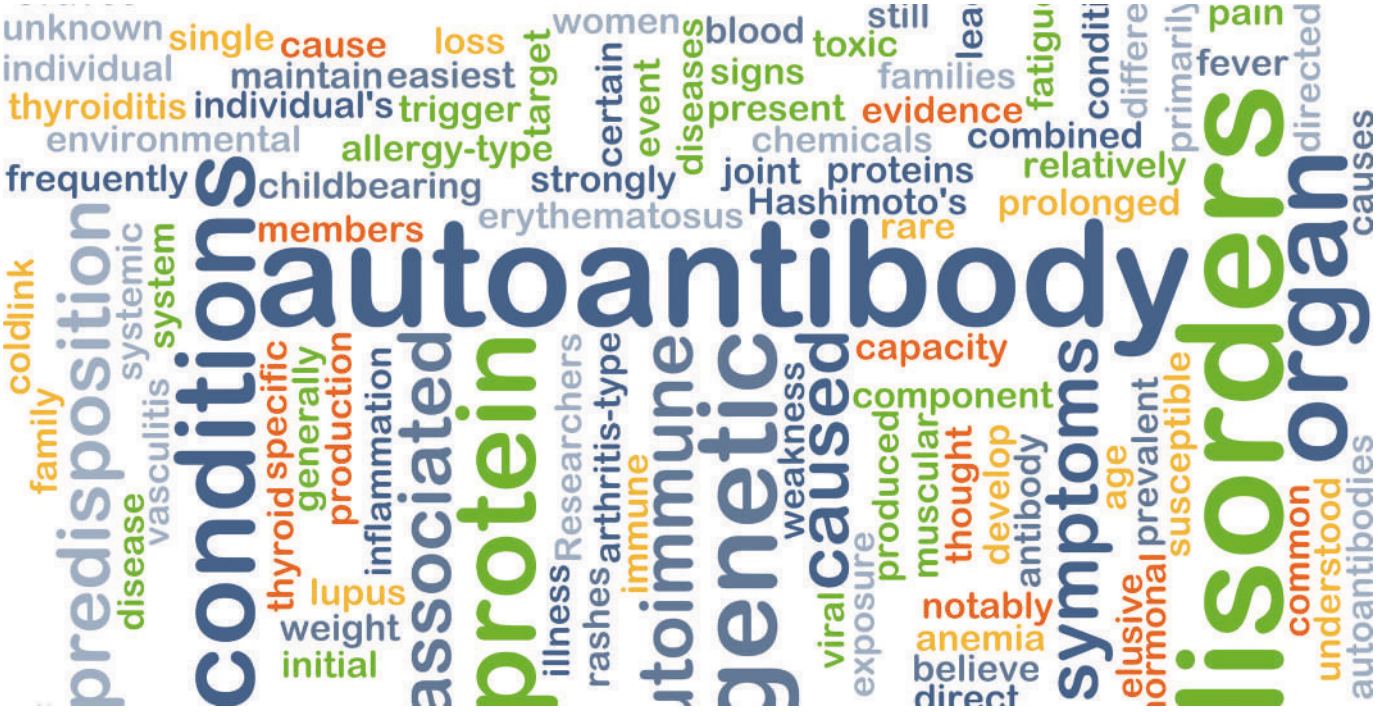

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