

Antigen microarrays: descriptive chemistry or functional immunomics?

József Prechl¹, Krisztián Papp² and Anna Erdei^{1,2}

¹Research Group of Immunology of the Hungarian Academy of Sciences, Eötvös Loránd University, Pázmány Péter sétány 1/C, Budapest 1117, Hungary

²Department of Immunology, Eötvös Loránd University, Pázmány P.s. 1C, 1117, Budapest, Hungary

Advances in protein microarray technology allow the generation of high content, reliable information about complex, multilevel protein interaction networks. Yet antigen arrays are used mostly only as devices for parallel immune assays describing multitudes of individual binding events. We propose here that the huge amount of immunological information hidden in the plasma of an individual could be better revealed by combining the characterization of antibody binding to target epitopes with improved estimation of effector functions triggered by these binding events. Furthermore, we could generate functional immune profiles characterizing general immune responsiveness of the individual by designing arrays incorporating epitope collections from diverse subsets of antibody targets.

Antigen arrays, antibody profiles

The utilization of DNA microarrays has contributed significantly to understanding gene function on a systemic level. However, in order to understand phenotype and physiology, genomic information must be interpreted by analyzing variations in the transcriptome, the proteome, and metabolites, all of which are factors influenced by the environment. The immune system in particular is strongly shaped by the environment; encounters with antigens are recorded in immune memory and are reflected in the antigen receptor repertoire of lymphocytes. By studying responses to past encounters on an immunomic scale, such data can be integrated into networks aiming to predict the outcome of future reactions related to immune function.

Protein microarrays share some methodological characteristics with DNA arrays, such as robotic printing of probes or targets in an indexed fashion, 'hybridizing' the array with the sample of interest, detection of interaction events by high-resolution scanning and image analysis. This technology developed into an important proteomics tool [1] and several applications based on antigen–antibody interactions have been described, these being essentially multiplex variants of the long-since described microspot test [2]. In one application, circulating antibodies with given specificities are identified by exposing serum to collections of arrayed antigens, called antigen microarrays [3].

Generation of these binding patterns, also called antibody profiles, helps discover novel autoantigens [4], identify antigenic epitopes [5], diagnose disease [6,7],

characterize allergic responses [8,9], or pinpoint vaccine targets [10]. These approaches utilize antigen panels focusing on particular subsets of the circulating antibody repertoire and usually detect a selected class or a few classes of immunoglobulins. In strong contrast to DNA microarrays, where generation of signals is governed by base-pairing and ideally just one complementary sequence binds to the probe, interactions on antigen arrays are far more complicated. Recognition of the antigen is often dependent on its conformation, presenting a technological hurdle in array production. Complex antigens can display several different epitopes, allowing binding of several antibodies with distinct fine specificity. Any given epitope can bind antibodies with different idiotopes, different affinities, different isotypes, and glycoforms. Thus, an immune complex is generated on the surface of the array. If the goal of our measurement is the correct understanding of how the humoral immune system is prepared to deal with that antigen, the composition of the immune complex with regard to the above properties should be determined. Compared to DNA microarrays, which quantify a single binding partner, antigen arrays can be used to determine both the quantity and quality of more than one binding partner. In fact, other consequences of antigen recognition, such as complement activation, can be monitored by mimicking physiological conditions during the incubation of an antigen microarray with serum, [11].

Progress in protein microarray technology, especially the accumulation of immunomics data, provides grounds for better exploiting this method. We argue that by using a relatively limited set of selected antigens and by improved monitoring of antibody quality we would be able to generate functional antibody profiles with the purpose of predicting disease and personalizing medical treatment.

Gauging antibody diversity with antigen microarrays

Adaptive immunity is specialized in generating specific antigen receptors with a high level of affinity in response to antigenic stimulus. This is achieved by the constitutive production of a vastly diverse repertoire of glycoproteins,

Glossary

Microarray feature: An element of an array, a micrometer-sized spot where interaction between the printed antigen and serum proteins takes place in the case of antibody profiling microarrays.

Immunomics: Comprehensive analysis of the collection of potential epitopes and their interactions with the immune system.

Corresponding author: Prechl, J. (jprechl@iif.hu).

the B-cell and the T-cell antigen receptors, and the appropriate tuning of production by positive and negative selection steps. As a result of clonal expansions and contractions, certain specificities are enriched while others are diluted out. The diversity that these receptors represent, and the short- and long-term dynamics of the changes in this repertoire have always intrigued immunologists, as this knowledge is key to understanding the mechanism of immunity on a systemic level.

In terms of tailoring their antigen receptor to a particular antigen, B cells surpass T cells in at least two ways; the affinity of the B-cell antigen receptor can be increased by somatic hypermutation and selection in germinal centers, along with shuffling of the heavy chain constant domains, known as isotype switching. Additionally, B cells, and especially their progeny, secrete a soluble version of the B cell receptor in the form of antibodies. This production is so sizeable that antibodies constitute close to 25% (w/w) of plasma proteins. This is an inhomogeneous pool comprised of antibodies of different clonal origin, whose diversity surpasses that of all other plasma proteins, even if we take into account the leakage of different tissue proteins into blood [12].

Estimates of antibody clonal variability that consider the theoretical diversity of variable regions, B-cell output, and immunoglobulin half-life, suggest that there exist millions to thousands of millions of different antibody specificities at any given point in time. The goal of antibody profiling is the identification of immunoglobulins secreted by primed B cells. Although the large number of naïve B cells contribute to the plasma IgM and IgD pool, these antibodies are predominantly low-affinity binders and are therefore usually undetectable by antigen arrays. Recognizing that fluorescent signal-based antigen microarray technology can detect around 10 attomoles (~ 1.5 pg) of bound IgG [6] and assuming that various clonal forms are evenly distributed and have sufficiently high levels of affinity to remain array-bound, we can estimate the maxi-

mum number of theoretically detectable clonal antibody forms (Figure 1a), based on the concentrations of various immunoglobulins in serum [13–17]. This is a simplistic approach because the latter two assumptions are not true and the spatial constraints of a microarray chip are ignored. These numbers are theoretical upper limits, which nevertheless suggest that a few million antigens would be sufficient to cover the examinable part of the antibody repertoire.

Current protein microarrays can hold several thousand antigens on a slide and can therefore detect the presence of an equal number of antibody specificities. This technology, as compared to sequencing variable regions of B cells producing the bound antibodies, will usually underestimate clonal diversity because several clones can bind to the same antigen, or even compete for the same epitopes. Also in strong contrast to sequence-based approaches for assessing clonal diversity [18], is the introduction of a definite bias for antibodies specific for antigens printed on any particular array. Thus, in order to capture each specificity, and thereby the real diversity of immunoglobulins in plasma, we would need to generate and screen the whole universe of antigens and print the selected subset on our array, which is obviously not feasible. Antigen microarrays are used for mapping certain subsets of the repertoire and some commonly used formats are shown in Figure 1b. Clearly, these arrays examine a rather limited number of targets and usually focus on certain antibody classes and so neglect subclass differences.

What to print? Selection of optimal epitope content for functional profiling

We can define the examinable part of the antibody repertoire as binders of the complete set of antigenic epitopes that promoted positive selection of the parental B cells. These epitopes are either of self or of non-self origin, with some shared epitopes represented by the intersection (Figure 2). The antigen microarrays in current use contain

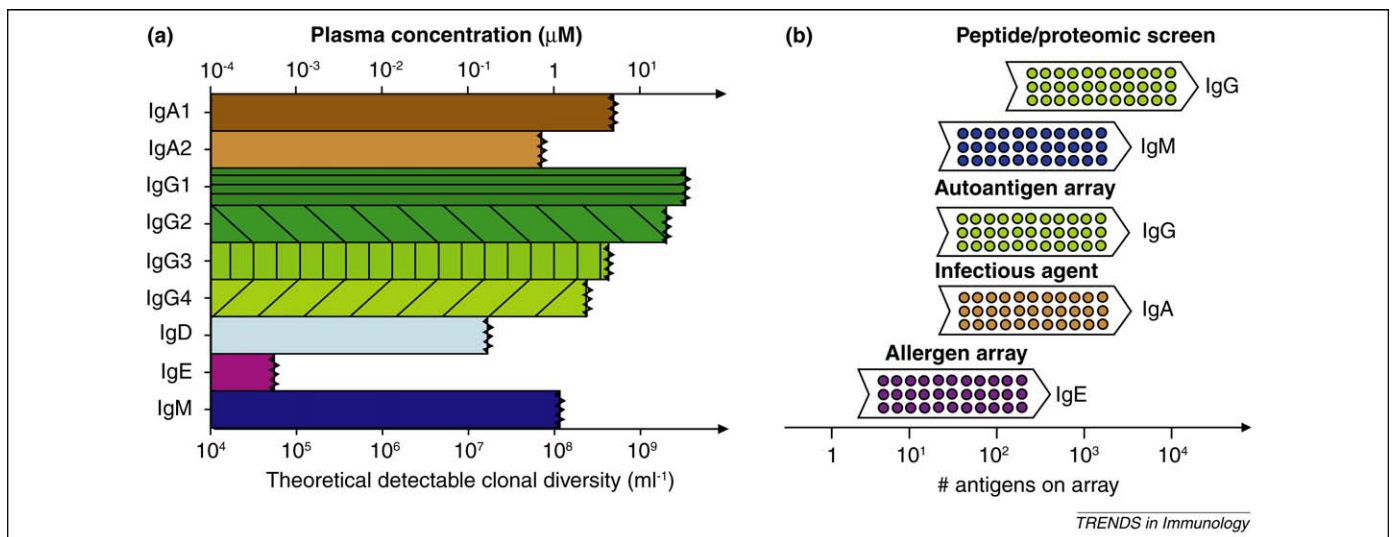


Figure 1. Immunoglobulins in the serum and current profiling strategies on antigen arrays. (a) Median molar concentrations of the various antibody isotypes in adult serum are shown; individuals can have significant alterations from these values even within the normal ranges. Theoretical diversity is proportional to, and limited by, the number of molecules of a certain isotype in a given volume. Maximum detectable diversity on an ideal array containing all targets was estimated by a simple algorithm, calculating with 10 attomolar sensitivity and assuming evenly distributed binding of all antibodies from 1 ml of serum. (b) Current strategies focus on particular antigen subsets, using antigens in the indicated range to detect only selected antibody classes.

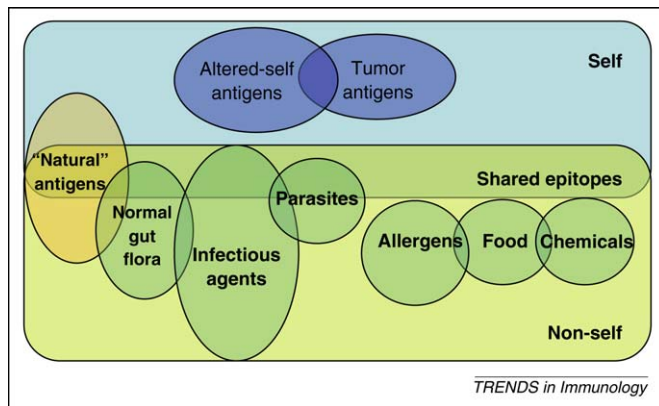


Figure 2. The complete set of epitopes targeted by the antibody repertoire. The complete set of epitopes is divided into various subsets based on the origin of the molecule; different medical disciplines focus on these subsets. Some epitopes can be shared by any two subsets; only those considered significant, between neighboring subsets, are indicated. For simplicity, the targets of natural antibodies are referred to as natural antigens.

epitope collections from one particular subset, selected for defined screening purposes, often with the primary purpose of disease diagnosis.

Natural antibodies are already rearranged and develop before birth [19]. At this early stage, however, specificity is mostly governed by molecular recombination events rather than antigenic stimuli [20]. Once born, one is quickly populated with billions of microbes and is continuously exposed to antigens in the environment, resulting in the diversification of the antibody repertoire. Commensal microbes seem to stimulate non-specific, polyclonal expansion of B cells [21] and a compartmentalized antigen-specific IgA response that is involved in maintaining gut homeostasis [22]. However, systemic responses are observed only when the mucosal barrier is compromised due to trauma, lack of effective innate immune protection, or to inflammation [23]. Exposure to infectious agents usually leaves a long-lasting imprint on the immune system. Proteins and peptide epitopes with discriminative potential for a particular disease have been identified by screening the whole bacterial or viral proteome [24–26] or selected proteins from parasites [27] and bacteria [5,28]. Using such strategies, immunodominant proteins and peptides can be identified in any pathogen. It is important to point out that only a small percentage of the microbial proteome represents the main targets of antibodies [25,28], though antibodies against these targets might cross-react with different species. Circulating antibodies against such targets can be detected in both exposed individuals and those who have recovered from an infectious disease [24].

Another heavily investigated area on our map is that of autoantigens. Autoimmunity is often associated with the appearance of high-affinity antibodies against self-epitopes, and antigen arrays have been used extensively for the characterization of autoantibody profiles in rheumatoid arthritis [29], systemic lupus erythematosus [30], alopecia areata [31], multiple sclerosis [4], and experimental diabetes [32]. Abnormal expression of self- or quasi-self antigens, as in the case of tumor-associated antigens, can also lead to changes of autoantibody profiles [7,33,34]. Finally, antigens that induce the development of IgE are used for diagnosing and typing allergic patients

[35,8]. Our knowledge about the elements of the complete epitope set is growing continuously and databases are devoted to collecting and analyzing these data (e.g. www.immuneepitope.org) [36].

Could we design arrays that reflect cumulative immunological experience instead of ongoing pathological events? How should we select epitopes from this complete set? We are most likely to have an immunological memory of pathogens that circulate in the population, and to show some degree of individual reactivity to self-antigens. Selected epitopes from these subsets should dominate on an antigen array if we intend to increase the frequency of binding events with the purpose of paying more attention to antibody function.

What to detect? Characterizing antibody function on microarrays

Mapping antibody binding on our epitope collection is of limited value unless we associate it with some functional information, which most often means definition of the bound immunoglobulin class. Autoreactive IgM is common and not generally associated with pathology, whereas autoreactive IgG is often associated with autoimmune disease. IgG recognizing food components has no clinical relevance, whereas the presence of IgE implies allergy. We identify array-bound immunoglobulins by secondary reagents, and make the assumption that the information obtained relates to the biological events triggered by the antibodies (Figure 1b). This is generally true when we distinguish immunoglobulin classes, but it can be misleading when detecting IgG or IgA. Subclasses of these immunoglobulins show variable effects regarding affinity for different Fc receptors and the ability to activate complement. Detection by either monoclonal or polyclonal class-specific secondary reagents does not necessarily reflect these differences. Antibody subclass-specific reagents have been applied successfully to microarrays [37,38]. Importantly, the data obtained reflect the cytokine environment during germinal center reactions that lead to antibody production and class switching [28]. Using reagents that better reveal the biological function of the bound antibodies therefore provides a more realistic picture of the nature of immune responsiveness of the individual.

In an attempt to further characterize antibody function on antigen arrays, we developed the technology of on-chip complement activation [39]. By mimicking the conditions of *in vivo* interactions between antigen and serum components on the microarray, we can generate immune complexes containing complement components. Complement C3 is at the convergence point of the three activation pathways and it is cleaved to generate a reactive C3b fragment that binds covalently to nearby molecules. By quantifying the deposited C3 fragments on the array, one can measure the net outcome of antigen–antibody interactions; i.e. the ratio of the different bound isotypes with distinct complement-activating abilities. Measurement of complement deposition can correctly distinguish serum samples after immunizations with pro- or anti-inflammatory bias; combined with IgG detection it could therefore replace subclass determinations [40]. Antibody-induced complement activation, in addition to isotype, is influenced

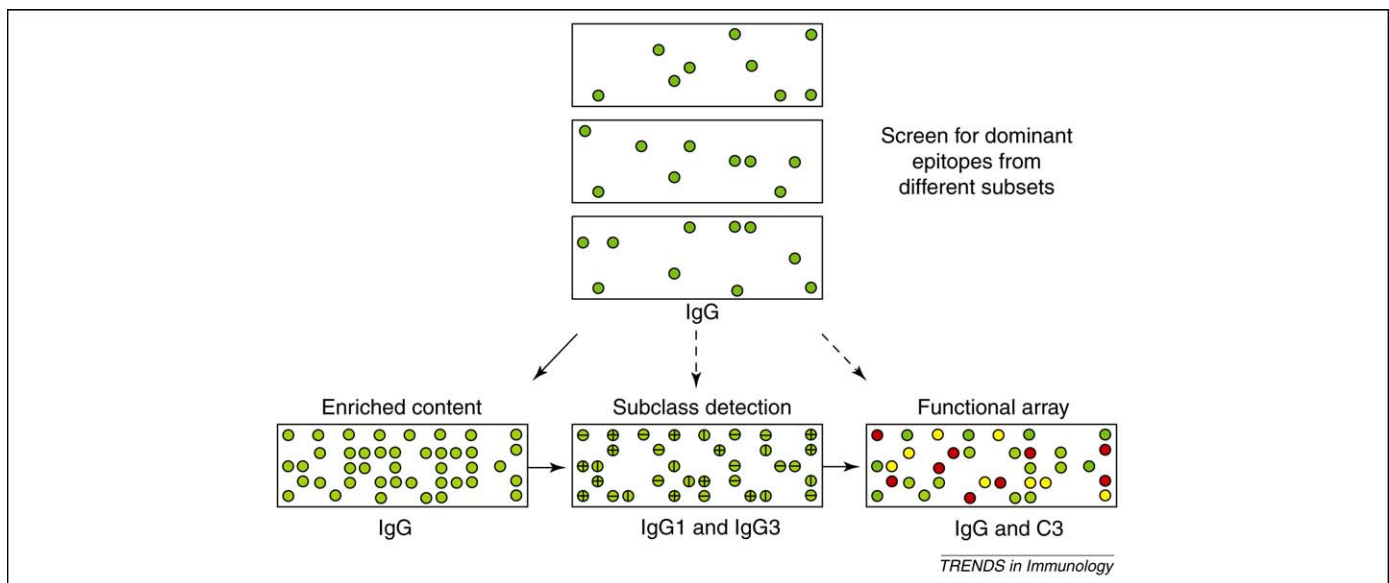


Figure 3. Strategies for improved generation of functional immune profiles. Arrays with selected universal B cell epitopes ensure an increased hit:content ratio; this is achieved by using dominant epitopes from relevant epitope subsets, such as the proteome of endemic pathogens. Measurement of immunoglobulin subclasses and/or complement activation on the features provides a more function-related view of the immune response. Functional tests can be incorporated into the screening phase (broken-line arrows). Colored circles represent binding events, with the following symbols: horizontal bar, IgG1; vertical bar, IgG3; cross, both IgG1 and IgG3 signals; green, IgG; red, C3; yellow, both IgG and C3 signals.

by antibody affinity [41] and glycosylation [42], characteristics that are not reflected by secondary reagents. Other components of the complement system, such as C1q and C4, can also be detected readily on microarrays and serve as indicators of the pathway that initiates the complement cascade.

Taken together, current detection strategies are suitable for screening purposes but need to be improved with regard to describing antibody function. The use of subclass-specific secondary reagents and functional assays, such as complement activation, preferably in a multiplex format, is expected to integrate more biology into antibody profiling on antigen microarrays.

Conclusions

Our circulating antibodies tell the story of what our immune system has seen and how it responded. To extract some of this information using antigen microarrays we need to decide which epitopes we use for probing and how to detect the binders. While the quality of the features determines which subset of the repertoire we are probing, the number of features on an antigen array limits the diversity it can measure.

If we dichotomize values, that is classify results as positive or negative, n antigens will permit categorization into 2^n sets. Introducing another independent measurement parameter can drastically improve this already high level of theoretical diversity. For m measured parameters 2^m values can be assigned to a feature, resulting in 2^{mn} diversity.

Measuring more than one type of binder can therefore exponentially increase the amount of information extracted from a given feature. If these different binders mirror different functional aspects of the immune complex, we can classify the tested sera into functional groups.

To generate a functional profile in any one individual we can select epitopes from the growing, verified database of B

cell epitopes in a manner that will increase the chance of positivity and the hits:content ratio of the array (Figure 3). Autoantigens and antigens from infectious agents endemic in the tested population are the best candidates to meet this requirement. Simultaneous measurement of up to four types of binders, immunoglobulin isotypes, complement components, is technically feasible using commercially available fluorescent detection. On the basis of the formula given above, we assume that determining the profile of binding for three molecules, e.g. IgM, IgG and C3, on an array containing about 100 features would be suitable for identifying characteristic functional antibody profiles. By shifting the focus from what antibodies bind to, to what kind of antibodies bind, we think we could generate functional antibody profiles with more general predictive powers. It has been experimentally proven that antibody profiles generated by antigen microarrays can help predict future immunological responses [37]. By extending these findings, we expect the development of antibody profiling arrays that supplement genomic data with information on how an individual's immune system interacts with the environment. In summary, even though the results obtained from antigen microarrays depend on biochemically defined interactions, the information these arrays provide is shaping our views about the antibody repertoire and has the potential of developing into a functional tool of immunomics.

Acknowledgements

Research on antigen microarrays is supported by the Hungarian Academy of Sciences, grants KMOP 3.1.1 from the National Agency for Development, NKTH-OTKA K68617, OTKA K72026 from the Hungarian Scientific Research Fund and Cell-Kom RET 2006/06. K. P. is a Zoltán Magyary fellow and is supported by EEA and Norway grants.

Conflict of interest

J.P. is employed by the Hungarian Academy of Sciences and Diagnosticum Ltd. The Academy has a patent application pending on the use of micro-

arrays for complement activation measurement; Diagnosticum Ltd is licensing intellectual property rights covered by this patent.

References

- Cahill, D.J. and Nordhoff, E. (2003) Protein arrays and their role in proteomics. *Adv. Biochem. Eng. Biotechnol.* 83, 177–187
- Feinberg, J.G. (1961) A 'microspot' test for antigens and antibodies. *Nature* 192, 985–986
- Robinson, W.H. (2006) Antigen arrays for antibody profiling. *Curr. Opin. Chem. Biol.* 10, 67–72
- Kanter, J.L. *et al.* (2006) Lipid microarrays identify key mediators of autoimmune brain inflammation. *Nat. Med.* 12, 138–143
- Gaseitsiwe, S. *et al.* (2008) Pattern recognition in pulmonary tuberculosis defined by high content peptide microarray chip analysis representing 61 proteins from *M. tuberculosis*. *PLoS. One.* 3, e3840
- Mezzasoma, L. *et al.* (2002) Antigen microarrays for serodiagnosis of infectious diseases. *Clin. Chem.* 48, 121–130
- Casiano, C.A. *et al.* (2006) Tumor-associated antigen arrays for the serological diagnosis of cancer. *Mol. Cell. Proteomics* 5, 1745–1759
- Deinhofer, K. *et al.* (2004) Microarrayed allergens for IgE profiling. *Methods* 32, 249–254
- Hiller, R. *et al.* (2002) Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J.* 16, 414–416
- Davies, D.H. *et al.* (2005) Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc. Natl. Acad. Sci. U. S. A.* 102, 547–552
- Papp, K. *et al.* (2007) On-chip complement activation adds an extra dimension to antigen microarrays. *Mol. Cell. Proteomics* 6, 133–140
- Anderson, N.L. and Anderson, N.G. (2002) The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* 1, 845–867
- Schauer, U. *et al.* (2003) IgG subclass concentrations in certified reference material 470 and reference values for children and adults determined with the binding site reagents. *Clin. Chem.* 49, 1924–1929
- Schauer, U. *et al.* (2003) Establishment of age-dependent reference values for IgA subclasses. *Clin. Chim. Acta* 328, 129–133
- Vladutiu, A.O. (2000) Immunoglobulin D: properties, measurement, and clinical relevance. *Clin. Diagn. Lab. Immunol.* 7, 131–140
- Polmar, S.H. *et al.* (1972) IgE in immunodeficiency. *Am. J. Pathol.* 69, 499–512
- Lucey, D.R. *et al.* (1992) Comparison by race of total serum IgG, IgA, and IgM with CD4+ T-cell counts in North American persons infected with the human immunodeficiency virus type 1. *J. Acquir. Immune. Defic. Syndr.* 5, 325–332
- Meijer, P.J. *et al.* (2006) Isolation of human antibody repertoires with preservation of the natural heavy and light chain pairing. *J. Mol. Biol.* 358, 764–772
- Merbl, Y. *et al.* (2007) Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. *J. Clin. Invest* 117, 712–718
- Meffre, E. and Salmon, J.E. (2007) Autoantibody selection and production in early human life. *J. Clin. Invest* 117, 598–601
- Lanning, D.K. *et al.* (2005) Intestinal bacteria and development of the B-lymphocyte repertoire. *Trends Immunol.* 26, 419–425
- Macpherson, A.J. and Uhr, T. (2004) Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. *Ann. N. Y. Acad. Sci.* 1029, 36–43
- Manukyan, G.P. *et al.* (2008) Elevated systemic antibodies towards commensal gut microbiota in autoinflammatory condition. *PLoS. One.* 3, e3172
- Gieffing, C. *et al.* (2008) Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. *J. Exp. Med.* 205, 117–131
- Keasey, S.L. *et al.* (2009) Extensive antibody cross-reactivity among infectious gram-negative bacteria revealed by proteome microarray analysis. *Mol. Cell. Proteomics* 8, 924–935
- Davies, D.H. *et al.* (2007) Proteome-wide analysis of the serological response to vaccinia and smallpox. *Proteomics* 7, 1678–1686
- Gray, J.C. *et al.* (2007) Profiling the antibody immune response against blood stage malaria vaccine candidates. *Clin. Chem.* 53, 1244–1253
- Eyles, J.E. *et al.* (2007) Immunodominant *Francisella tularensis* antigens identified using proteome microarray. Crown Copyright 2007 Dstl. *Proteomics* 7, 2172–2183
- Hueber, W. *et al.* (2005) Antigen microarray profiling of autoantibodies in rheumatoid arthritis. *Arthritis Rheum.* 52, 2645–2655
- Graham, K.L. *et al.* (2004) High-throughput methods for measuring autoantibodies in systemic lupus erythematosus and other autoimmune diseases. *Autoimmunity* 37, 269–272
- Lueking, A. *et al.* (2005) Profiling of alopecia areata autoantigens based on protein microarray technology. *Mol. Cell. Proteomics* 4, 1382–1390
- Quintana, F.J. *et al.* (2004) Functional immunomics: microarray analysis of IgG autoantibody repertoires predicts the future response of mice to induced diabetes. *Proc. Natl. Acad. Sci. U. S. A.* 101, 14615–14621
- Madoz-Gurpide, J. *et al.* (2008) Integral protein microarrays for the identification of lung cancer antigens in sera that induce a humoral immune response. *Mol. Cell. Proteomics* 7, 268–281
- Taylor, B.S. *et al.* (2008) Humoral response profiling reveals pathways to prostate cancer progression. *Mol. Cell. Proteomics* 7, 600–611
- Bacarese-Hamilton, T. *et al.* (2002) Detection of allergen-specific IgE on microarrays by use of signal amplification techniques. *Clin. Chem.* 48, 1367–1370
- Vita, R. *et al.* (2010) The Immune Epitope Database 2.0. *Nucleic Acids Res.* 38, D854–D862
- Graham, K.L. *et al.* (2006) Autoantigen arrays for multiplex analysis of antibody isotypes. *Proteomics* 6, 5720–5724
- EL-Baky, N.A. *et al.* (2008) Efficacy comparison of gel-based, membrane and glass array techniques to detect human antibodies isotypes among the Egyptian HCV-patients. *Hum. Antibodies* 17, 63–71
- Papp, K. *et al.* (2007) On-chip complement activation adds an extra dimension to antigen microarrays. *Mol. Cell. Proteomics* 6, 133–140
- Papp, K. *et al.* (2008) Two-dimensional immune profiles improve antigen microarray-based characterization of humoral immunity. *Proteomics* 8, 2840–2848
- Peterfy, F. *et al.* (1983) Affinity requirements for antibody assays mapped by monoclonal antibodies. *J. Immunol.* 130, 1809–1813
- Arnold, J.N. *et al.* (2006) Mannan binding lectin and its interaction with immunoglobulins in health and in disease. *Immunol. Lett.* 106, 103–110