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RESEARCH ARTICLE

Two-dimensional immune profiles improve antigen microarray-based characterization of humoral immunity

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Antigen arrays are becoming widely used tools for the characterization of the complexity of humoral immune responses. Current antibody profiling techniques provide modest and indirect information about the effector functions of the antibodies that bind to particular antigens. Here we introduce an antigen array-based approach for obtaining immune profiles reflecting antibody functionality. This technology relies on the parallel measurement of antibody binding and complement activation by features of the array. By comparing sera from animals immunized against the same antigen under different conditions, we show that identifying the position of an antigen in a 2-D space, derived from antibody binding and complement deposition, permits distinction between immune profiles characterized by diverse antibody isotype distributions. Additionally, the technology provides a biologically interpretable graphical representation of the relationship between antigen and host. Our data suggest that 2-D immune profiling could enrich the data obtained from proteomic scale serum profiling studies.

Keywords:
Antibody profiling / Antigen microarray / Complement / Humoral immunity / Protein array

1 Introduction

In depth analysis of humoral immunity requires detailed characterization of the antibodies that are produced in response to immunogens. This involves, and is often restricted to, the determination of the amount and ratio of antibody isotypes and depends on the measurement of several classes and subclasses of antigen-specific antibodies. Characterization of the contribution of antibodies with diverse isotypes to an immune response helps determining the nature of the response with respect to its duration, T-helper cell bias, protectiveness, or pathogenicity. Class switching is regulated by the stimuli and costimuli delivered by the immunogen and the cytokine milieu of the germinal center. Humans have five antibody classes (IgD, IgM, IgG, IgA, IgE) with IgG further subdivided into four subclasses (IgG1 to IgG4) as determined by the heavy chain gene usage. The same Ig classes are observed in the mouse, whose IgG subclasses (IgG1, IgG2a/c, IgG2b, and IgG3) also are diversified. Importantly, IgG subclasses have different affinities for IgG Fc receptors (receptor for the crystallizable fragment of IgG (FcγR) [1] and dissimilar abilities to activate the complement system [2, 3], necessitating the need to determine their relative contribution to an immune response. Effector functions are also considerably influenced by the avidity [4, 5] and glycosylation [6, 7] of antibodies, but as these properties are more cumbersome to measure they are tested less frequently.

Incubation of an array of indexed antigens with serum allows the identification of a large number of specific antibodies in the circulation, a method called antibody profiling [8]. Though antigen arrays are becoming the tools of choice

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Abbreviations: anti-C3, goat anti-mouse C3; **anti-IgG,** goat anti-mouse IgG; **CFA,** complete Freund's adjuvant; **IL-4,** interleukin 4; **KLH,** keyhole limpet hemocyanin; **KO,** knock out; **pLA,** protein LA; **RBC,** red blood cell; **TD,** thymus dependent; **TI,** thymus independent; **TNP,** 2,4,6-trinitrophenol

for serum antibody profiling, current microarray instrumentation generally does not allow more than three parallel measurements in distinct fluorescence channels. This excludes simultaneous detection of all IgG subclasses, not speaking of other Ig classes. In an attempt to give a better view of *in vivo* immune complex formation and to functionally characterize array-bound antibodies we have modified experimental conditions so as to allow complement activation on the antigen arrays [9]. Complement is an innate system of detector, regulator, and effector proteins, which is activated either directly by antigens or indirectly *via* antibodies bound to antigens, and has significant influence on the development of adaptive immunity [10, 11]. Some antibodies are particularly potent while others are ineffective at activating complement, depending on their isotype, affinity, and glycosylation [2, 3, 7]. Antigens that come into contact with blood plasma are thus wrapped in varying mixtures of recognition molecules including antibodies and complement activation products. The composition of these immune complexes both reflects earlier immunological experience and crucially influences all later steps of an immune response. Gaining insight into the nature and function of antibodies bound to a particular target on an antigen microarray would therefore extend the use of such arrays.

Using immunization protocols that induce characteristic immunity with distinct antibody isotype dominance patterns, we show that concurrent measurement of Ig binding and complement deposition on antigen microarrays is suitable for discriminating and identifying such immune responses.

2 Materials and methods

2.1 Materials

All materials were from Sigma–Aldrich (Hungary) unless otherwise indicated. Conjugates of 2,4,6-trinitrophenol (TNP) were generated by treating keyhole limpet hemocyanin (KLH) or BSA with trinitro-sulfobenzoic acid according to standard protocol. BSA conjugates with varying degrees of TNP content were produced by using 0.1, 0.01, and 0.001% of trinitro-sulfobenzoic acid. TNP conjugation efficiency was determined by spectrophotometry. TNP was conjugated to sheep red blood cells (RBC) using 0.1% TNBA, cells were washed afterwards and used fresh. Interleukin 4 (IL-4) containing supernatant [12] was produced in our laboratory; IL-4 concentration was measured by ELISA. Printed capture antibodies were heavy chain-specific (μ and γ) goat anti-mouse $F(ab')_2$ fragments from Southern Biotech. Alexa-647-conjugated goat anti-mouse IgG (anti-IgG), γ heavy chain- and light chain-specific (γ+L) (Southern Biotech, AL, USA) and FITC-conjugated goat anti-mouse C3 (anti-C3) (MP Biomedicals, OH, USA) were used for fluorescent detection. TNP-specific mAb H5, D10, 2.15, F4, GORK, Sp6, Hy1.2, M12 were a kind gift of Birgitta Heyman, Uppsala University. A polyclonal conjugate reacting with both κ and λ light-chains was created by mixing commercially available light-chain antibodies (Southern Biotech) and conjugating with Alexa-647 (Invitrogen, CA, USA).

2.2 Mouse protocol

Male C57/B6 mice (6–8 wk old), five *per* group, were used for immunizations. All animal experiments were in accordance with national regulations and were authorized by the ethical committee of the institute. Serum from Ig knock out (IgKO) [13] and C3 deficient (C3KO) [14] animals were a kind gift from Matyas Sandor, University of Wisconsin-Madison. TNP–Ficoll (Biosearch Technologies, CA, USA) was administered intraperitoneally at a dose of 50 μg/mouse. Rigid, highly repetitive structures, such as carbohydrate polymers (Ficoll), induce thymus independent (TI) responses, characterized by the dominance of IgM antibodies [15]. We used TNP conjugated to a massive carrier protein, KLH, to evoke thymus dependent (TD) immune response. TNP–KLH, at 100 μg/mouse dosage, was injected subcutaneously and intraperitoneally alone or emulsified in complete Freund's adjuvant (CFA) or injected intravenously along with 2 μg recombinant, intraperitoneally administered IL-4. CFA, containing mycobacterial extract, induces strong inflammation. In contrast, administration of the antigen *via* the intravenous route and in the presence of an anti-inflammatory cytokine (IL-4) is rather tolerogenic. TNP conjugated to sheep RBC (TNP–RBC) represents particulate types of antigen, with both TD and independent mechanisms involved in the immune response. For the immunization 4×10^7 cells/mouse were injected intravenously.

For TD responses we gave booster immunizations 21 days after the primary injection, using the same formulation, except for replacing complete Freund's with incomplete adjuvant. Sera were collected at the height of the immune response, that is, 7 and 21 days following the last immunization for TI and TD responses, respectively. Isotype distribution of TNP-specific antibodies was determined by ELISA and enzyme-linked immunospot assay (data not shown), using isotype-specific HRP-conjugated goat antibodies (Southern Biotech). For the radar chart representation optical densities derived from 1:500 serum dilutions were normalized for comparability by expressing optical densities as the percentage of the highest readings.

One individual in the TNP–KLH + IL-4 group had statistically extreme ELISA values for TNP-specific IgG and therefore did not meet our inclusion criteria. Microarray results of two animals (one from the TNP–Ficoll group; one from TNP–KLH + IL-4) were not reliable and were therefore excluded from further analysis.

2.3 Antigen array data

Antigen arrays contained TNP conjugated to bovine albumin at three different ratios, with an average of 12, 2, or 0.4 TNP molecules *per* bovine albumin, providing various epitope

densities. These conjugates were diluted in PBS containing 1 mg/mL BSA to the indicated concentrations of 1.3, 0.25, and 0.05 mg/mL. Thus, all TNP carrying features contained BSA and only the concentration of TNP was varied. Additionally, the following reference materials were printed on the slide: anti-C3 (MP Biomedicals), anti-IgG, goat anti-mouse IgM (anti-IgM) (Southern Biotech), KLH, lysozyme, BSA, protein LA (pLA), mannan, and whole murine serum. We printed these solutions in three different concentrations (1, 0.2, 0.04 mg/mL) in triplicates, using Calligrapher mini-arrayer (BioRad), onto home-made NC coated glass slides. The generation of microarray data is described elsewhere in detail [9].

Briefly, dried arrays were rinsed for 15 min in PBS just before use, then incubated with undiluted sera in a humidified chamber at 37°C degrees for 60 min. The reaction was terminated by washing the array with PBS. The mixture of the detecting antibodies diluted 1:5000 in 5% skimmed milk powder in PBS were added to the arrays, which were then incubated with gentle agitation for 30 min at room temperature in the dark. For the comparison of mAb, shown in Fig. 2, the basic method was slightly modified. Antibody concentrations were adjusted based on pilot experiments, so as to achieve antibody binding to TNP_{12}–BSA in a similar range, as assessed by pan-light chain detection. Assuming an average antibody concentration of 10 μg/mL in the hybridoma supernatants and taking into account that two to ten-fold dilutions were used, the estimated concentration of the mAb was in the 1–5 μg/mL range. In this experiment we wanted to compare complement activating abilities of different classes and subclasses, therefore neither IgM nor IgG detection was suitable. By measuring the antibody light chains we can assume that identical fluorescence intensities imply the presence of identical numbers of antibodies. Thus, complement activation by similar numbers of antibodies could be compared. Before treating with naive serum, arrays were incubated in appropriately diluted supernatant containing anti-TNP mAb for 30 min. The dilution was carried out in 5% BSA, 0.05% Tween 20 containing PBS. As discussed above, instead of the anti-Ig antibody, a κ + λ-specific fluorescent conjugate was used to eliminate isotype bias.

Slides were scanned on a Typhoon Trio + Imager (Amersham Bioscience) following standard protocols. Laser intensity was set to provide optimal signal intensity with minimal background and without saturated pixels. Data were analyzed with ImageQuantTL (Amersham Bioscience) software. Signal intensities were calculated by subtracting background from medians of signal intensity in a spreadsheet program (Microsoft Excel).

Fluorescence intensity data were normalized, both for IgG and C3, to yield identical pLA derived values, assuming that antibody binding and consequent complement activation on this fusion protein of bacterial superantigens is not influenced by the immunization schemes. Correcting inter-assay fluorescence intensities using values obtained from capture reagent readings (anti-IgG, anti-C3), instead of that of pLA, did not essentially change the results (data not shown). All results were within the dynamic range of the measurement. We created overlays of false color microarray images by ImageQuantTL (GE Healthcare). 2-D profiles depict Ig and C3 signals from the three concentrations of the indicated antigen.

2.4 Statistical analysis

Data are expressed as mean ± SD. Correlations and principal components were calculated with Statistica AGA software (StatSoft).

3 Results

3.1 Complement deposition on the array reflects properties of antigen-specific antibodies

Taking advantage of two-channel fluorescent detection and multiplexicity of microarray format we measured antigen-bound C3 fragments and antibodies in parallel. To confirm specificity of the technique we compared combined C3 and Ig profiles of wild type, C3 deficient, Ig deficient naive mice and mice immunized with a model antigen, TNP. An array containing TNP–BSA conjugates with different densities of TNP moieties *per* BSA molecule and different concentrations of these conjugates, as well as various reference proteins, was designed for addressing TNP-specific immunity (Fig. 1A, panel layout). Sera from immunologically naive wild type animals contain natural antibodies – mostly IgM – that can bind to high density epitopes with adequate avidity to induce moderate complement activation (Fig. 1A, panel naive). Absence of complement C3 completely abolishes (Fig. 1A, panel C3KO) while lack of antibodies diminishes this signal (Fig. 1A, panel IgKO). Thus, high densities of this antigen can initiate complement activation in an antibody independent manner. Immunization resulted in the appearance of higher affinity antibodies against TNP, as reflected by the appearance of IgG and C3 signals at lower conjugation ratios of TNP *per* BSA, and dilutions of these conjugates.

Next, we compared a pair of TNP-specific mAb, one carrying a mutation that impairs C1q binding [16, 17], using our assay (Fig. 1B). The mutant version was less efficient with respect to complement activation, validating the assay for semiquantitative measurements. We also tested complement-activating ability of several other TNP-specific mAb (Fig. 2). By adjusting their concentrations to give similar Ig binding signals on the array, we compared C3 deposition at identical Ig values, the results being in agreement with the isotype dependence of complement activation generally [18,19]. Notably, natural antibodies present in the naive serum that was used as a complement source, were avidly binding and initiating complement activation at the highest antigen concentration but disappeared at the lowest antigen concentration (Fig. 2A). An mAb with IgG2a isotype (F4) was

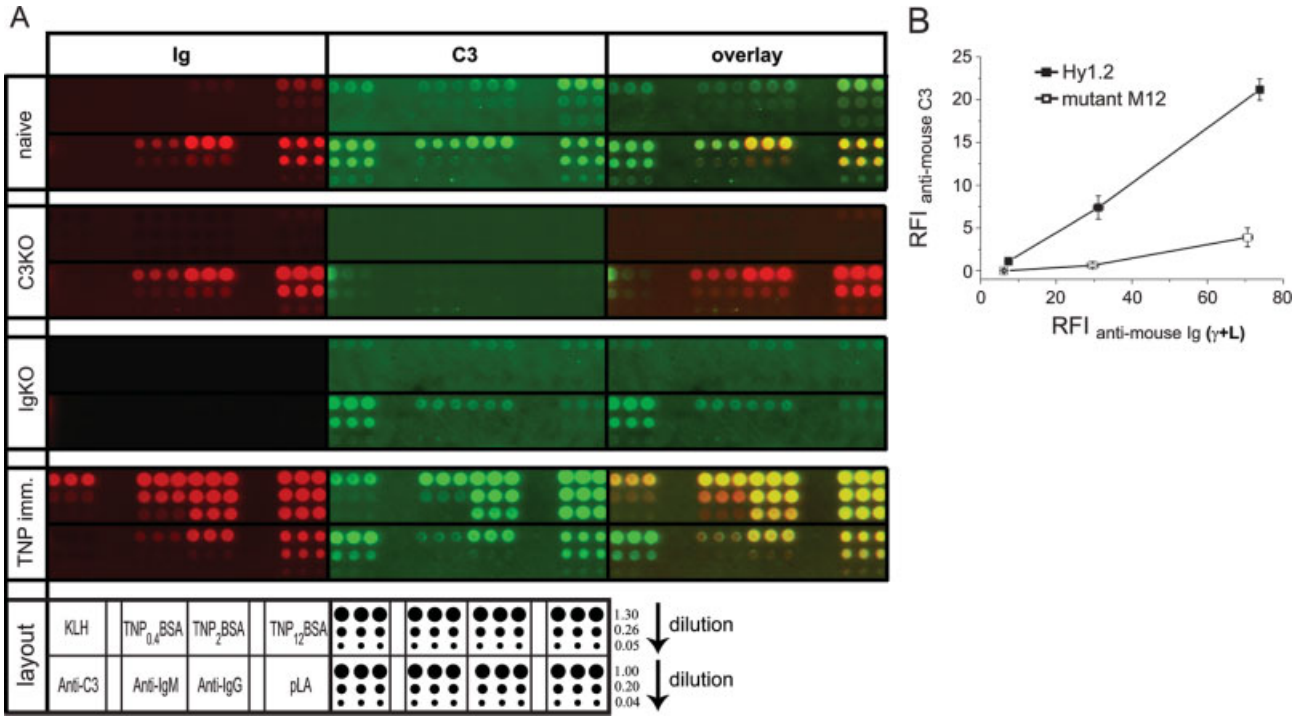

Figure 1. Parallel detection of antibody binding and complement C3 fragment deposition. (A) Representative false-color images of antigen microarrays incubated in sera from naïve wild type (naive), C3-deficient (C3KO), Ig deficient (IgKO) animals and an immunized mouse (TNP imm.) are shown, along with the layout of the subarray. We printed solutions of three different concentrations of every antigen and reference material. Additionally, three conjugates containing an average of 0.4, 2, or 12 TNP moieties *per* BSA were used. Polyclonal capture antibodies for mouse C3, IgM and IgG and a fusion protein of bacterial superantigens, pLA, were used for reference (see Section 2). All features are in triplicates. After incubating the arrays in sera, deposited C3 fragments and bound Ig were detected by fluorescently labeled mouse C3 and Ig γ heavy- and light chain-specific secondary reagents, respectively. (B) Comparison of wild type Hy1.2 and mutant C1q binder mAb M12 with respect to antibody binding and complement activation. Three data points in each curve indicate fluorescence intensities of C3 (anti-mouse C3) and Ig (anti-mouse Ig, γ + L) at three different concentrations of TNP_2–BSA.

the most potent complement activator, with IgG1 isotypes showing intermediate to low activity. Ig binding to TNP_{12}–BSA is a function of avidity, because every BSA molecule carries an average of 12 TNP labels. At a lower conjugation ratio, such as TNP_2–BSA, binding of both of the arms of Ig molecules is not assured and affinity becomes a limiting factor. This results in significant drops in Ig binding and also in C3 deposition when the concentration of the conjugate is lowered. It is important to note that monoclonal F4, unlike all the others, bound to TNP_{12}–BSA and TNP_2–BSA equally well (compare Figs. 2A and B), implying it had the highest affinity for TNP. IgG1 antibodies can initiate the alternative pathway of complement activation in addition to the classical pathway [18]. The contribution of two pathways may account for the nonlinear nature of the curve representing complement activation by monoclonal D10 (Fig. 2A). The fact that the particular clone of IgM we tested was only moderately effective is partly attributable to the detection of light chains which therefore compares antibodies on a monomeric basis. Taken together, these data suggested that our assay was suitable for the characterization of the biological activities of mAb.

3.2 2-D antibody profiling

Next, we immunized mice using immunization schemes (see Section 2) which result in characteristic distribution of antibody isotypes against the model antigen TNP. This distribution was first characterized by measuring TNP-specific IgM and various IgG isotypes by ELISA (Fig. 3A). The radar chart readily reflects the diverse patterns of antigen-specific antibodies achieved by the immunizations. These sera were then applied to the above described antigen array. Different patterns of Ig and C3 fragment binding were observed at different TNP conjugates (see Fig. 1 of Supporting Information), yet none of these measurements distinguished the immunization groups reliably alone. Chip-based Ig measurements showed positive correlations with all TNP-specific IgG isotype levels, as determined by ELISA (Table 1). In a similar way, C3 values, with the exception of those measured at the lowest TNP densities, were positively correlated to ELISA results for IgG levels. Significant positive correlation between C3 deposition on the array and the relative amount of ELISA derived antigen-specific IgM values was only observed at the lowest concentration of $TNP_{0.4}$–BSA. C1

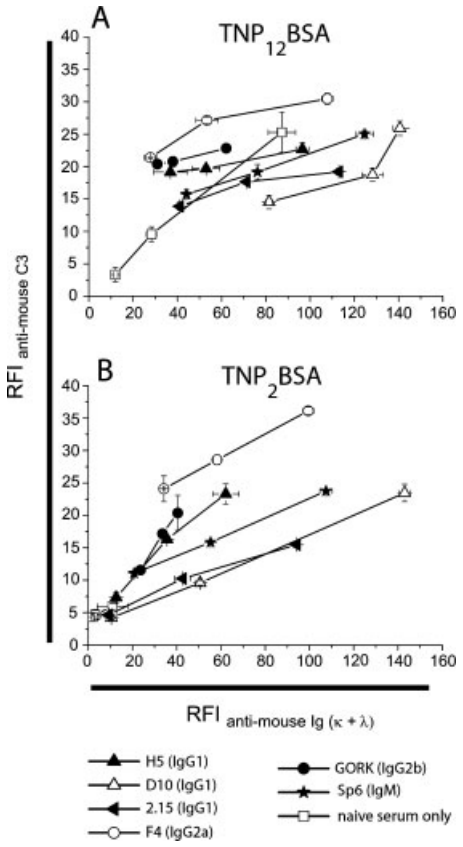

Figure 2. Comparison of the complement-activating abilities of TNP-specific mAb. Six clones of mAb were applied to the TNP arrays at dilutions that were previously determined to give comparable antibody binding, as determined by pan-light chain-specific detection (anti-mouse Ig, κλ). Fresh serum of naive mice was applied as a complement source. Results stand for (A) TNP_{12}–BSA and (B) TNP_2–BSA binding data at three different concentrations and are representative of at least three independent experiments.

Table 1. Correlation matrix[a] of array and ELISA measurements

Concentration[b]	ELISA				
	IgM	IgG1	IgG2b	IgG2c	IgG3
Antigen array					
Ig (γ+L)					
TNP_{12}					
1.3	−0.22[c]	0.38	0.23	0.33	**0.55****
0.26	−0.24	**0.44***	0.29	0.40	**0.60****
0.05	−0.26	**0.48***	0.33	**0.44***	**0.61****
TNP_2					
1.3	−0.27	**0.59****	**0.45***	**0.51***	**0.67****
0.26	−0.29	**0.73*****	**0.62****	**0.67*****	**0.75*****
0.05	−0.28	**0.77*****	**0.69*****	**0.70*****	**0.76*****
$TNP_{0.4}$					
1.3	−0.25	**0.84*****	**0.80*****	**0.78*****	**0.68*****
0.26	−0.16	**0.78*****	**0.79*****	**0.70*****	**0.51***
0.05	−0.07	**0.51*****	**0.51*****	0.41	0.20
Antigen array					
C3					
TNP_{12}					
1.3	−0.01	**0.61****	**0.50***	**0.57****	**0.67****
0.26	0.08	**0.70*****	**0.64****	**0.67****	**0.62****
0.05	0.04	**0.74*****	**0.68*****	**0.70*****	**0.57****
TNP_2					
1.3	0.06	**0.70*****	**0.55****	**0.59****	**0.67*****
0.26	−0.06	**0.89*****	**0.85*****	**0.8*****	**0.62****
0.05	−0.06	**0.74*****	**0.80*****	**0.71*****	**0.42***
$TNP_{0.4}$					
1.3	0.00	**0.69*****	**0.71*****	**0.62****	**0.56****
0.26	0.28	−0.12	−0.13	−0.22	0.06
0.05	**0.42***	−0.31	−0.30	−0.39	−0.15

a) Pearson's correlation coefficients are shown, unpaired measurements were omitted ($n = 26$).
b) Concentration of the conjugate solution printed on the slide.
c) Statistically significant r values are shown in bold font; $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

activation requires at least two IgG molecules whereas one IgM is still sufficient. Here, scarcely placed monomeric IgG molecules are presumably no longer able to bind C1q and cannot initiate complement activation, while C1q binding to the pentameric IgM is still potent.

By displaying the immune responses in a 2-D space generated from Ig and C3 fragment binding data, we achieved to separate immunization groups in a biologically meaningful fashion (Fig. 3B). This space reflects both innate (complement C3) and adaptive (Ig) elements of a humoral response against a given antigen. Using these coordinates an antibody response that favors complement activation results in an upward shift, nonactivating antibodies in the serum shift signals downward (Fig. 3C). In the case of naive and TNP–Ficoll injected mice, IgM dominated immunity appears as potent complement activation with weak Ig binding. TNP was conjugated to KLH for the induction of T-cell dependent responses and was used either alone or in combination with immunomodulatory agents. CFA is a highly powerful inflammation-inducing agent, which skews immunity toward cellular responses and promotes the appearance antibody isotypes with strong complement activating potential. This is reflected by higher C3 values of this group (TNP–KLH + CFA), as compared to those animals immunized without adjuvant (Figs. 3B–D). To simulate tolerogenic antigen encounter we injected TNP–KLH intravenously, in the presence of IL-4. This regimen indeed resulted in a response lacking the inflammatory antibody isotype IgG2c (Fig. 3A) and an overall IgG response with poor complement activating properties (Figs. 3B–D). Intravenous administration of TNP in particulate form, conjugated to sheep RBC, also showed poorer complement activating properties (Figs. 3B–D). Displaying our results as the loga-

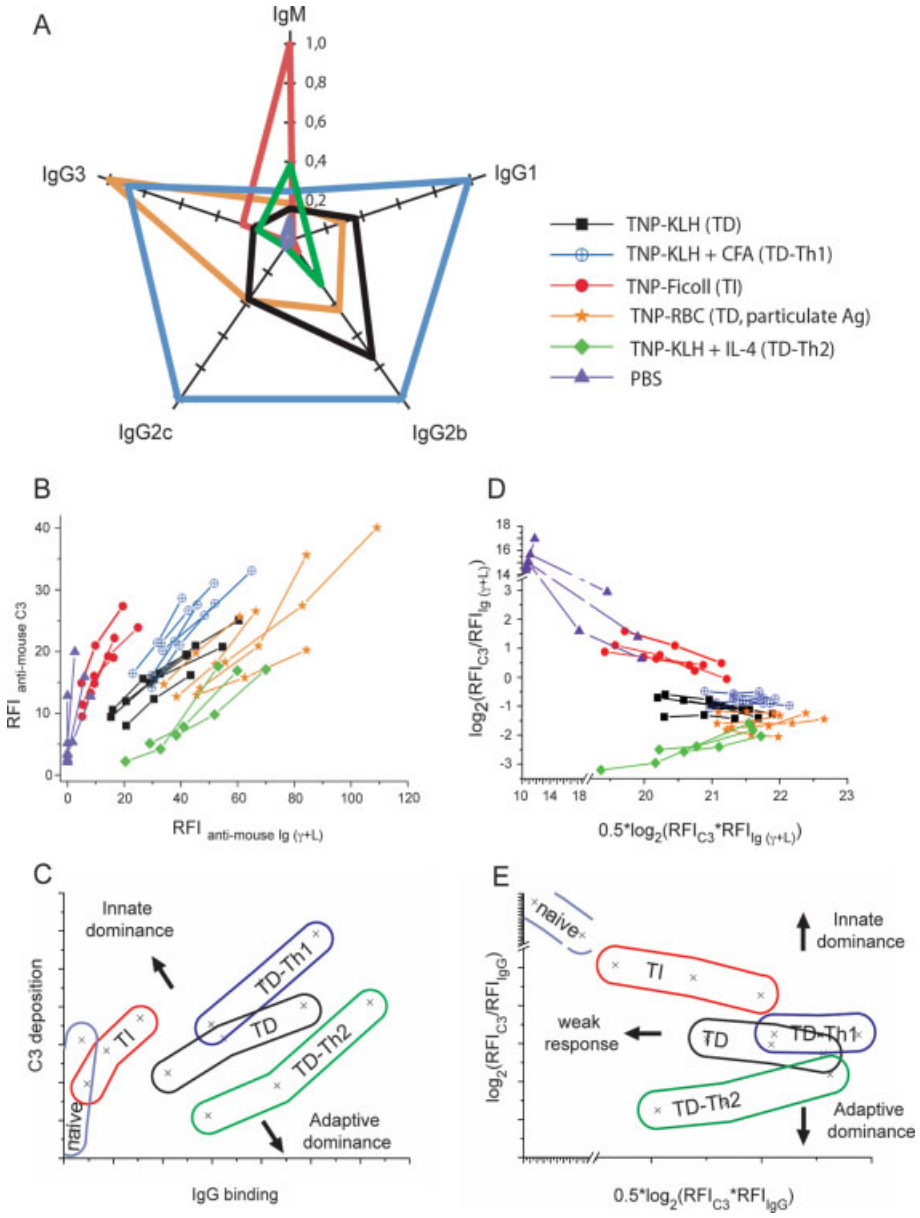

Figure 3. Isotype distribution profile of TNP-specific antibodies in the immunization groups. Six groups of mice were immunized using schemes that are known to result in characteristic responses (see Section 2). TI responses were induced by TNP–Ficoll, while TD responses were elicited by either soluble or particulate immunogens: TNP conjugated to a protein carrier, KLH (TNP–KLH) or to sheep RBC (TNP–RBC), respectively. TD responses were further skewed toward inflammatory reactions by CFA or toward anti-inflammatory conditions by IL-4. Control animals received PBS solution. (A) Levels of TNP-specific antibodies of the indicated isotypes were characterized by ELISA. Means of optical densities, expressed as percentage of the highest obtained values, of the respective immunization groups are shown in a radar chart. (B) Arrays, described in Fig. 1, were incubated with sera of animals of the above immunization groups. IgG and C3 binding data at three different concentrations of TNP_{12}-BSA conjugates from individual sera are shown in a 2-D representation. Using the coordinates defined by Ig and C3 relative fluorescence intensity (RFI) values, we can simultaneously depict antibody binding and its effect on complement activation. (C) Immune responses biased toward inflammation are characterized by the appearance of antibody isotypes and glycoforms with good complement-activating properties, while tolerance and Th2 cytokines enhance the production of antibodies with poor complement activating properties. Thus, innate or adaptive dominance in the recognition of an antigen theoretically appears as an upward or downward shift, respectively, when bound C3 products and IgG define the coordinates. Enclosed areas correspond to experimental data: TI = TNP–Ficoll, TD = TNP–KLH, TD1 = TNP–KLH + CFA, TD2 = TNP–KLH + IL-4. (D, E) Graphical representation of the logarithm of RFI ratios versus average logarithmic intensities for individuals of the six immunization groups highlights the essentially different characters of natural and adaptive humoral immunity. Convergence of the curves implies that large amounts of antibody will inevitably cause complement deposition when antigen is present at high concentration, yet segregation of different immunization schemes can still be observed. Data points were derived from binding values shown in (B), from measurements of individual sera on TNP_{12}–BSA conjugates.

rithmic ratio ($M = \log_2(C3/IgG)$) against average logarithmic intensity ($A = {}^1/_2\log_2(C3 \times IgG)$) of the measured parameters, as used for representing two-channel microarray data, further emphasizes the contrast between innate dominated and adaptive, noninflammatory immune responses (Fig. 3E).

3.3 Discriminative properties of immune profiling methods

Representation of characteristic immune responses in this 2-D scale shows that simultaneous measurement of IgG and C3 is both suitable and sufficient for identifying these distinct immune profiles. In order to confirm the discriminatory potential of our assay and compare it with the ELISA results we calculated principal components from the two sets of data. Results of end-point measurements from a single, optimal serum dilution were used for the comparison both for ELISA and array measurements. This analysis revealed that determination of Ig binding and C3 fragment deposition at three different concentrations of TNP_{12}–BSA on the array yielded factors which were as suitable for discriminating the immunization groups in a 2-D scale as determination of the five different isotypes by ELISA (Fig. 4). The first two principal components deduced from the array data account for 96% variance of the data, while 85% variance is covered by the first two components of the ELISA measurements (Figs. 4A and B). Using these components as coordinates individuals segregated into groups according to the immunization schemes in both cases (Figs. 4C and D).

4 Discussion

In this paper we introduce the representation of antigen–serum interactions in the dimensions of bound Igs and deposited complement C3 products, a simple and powerful solution for detailed immune profile determination on antigen arrays. Antigens attain a position in this 2-D space depending on their ability to bind Ig and activate the complement system. We validated this assay using an mAb, Hy1.2, and its mutant form that is deficient in C1q binding (Fig. 1B). We also compared a set of mAb with different isotypes and confirmed that murine IgG2a antibodies are efficient and generally better activators of complement than IgG1 (Figs. 2A and B). It is important to stress, though, that factors other than isotype, such as affinity and glycosylation, are also known to influence complement activation. Antigen-specific antibodies appear in the serum of immunologically experienced individuals, as a result of germinal center reactions that yield antibodies with increased affinity for the antigen and switched isotypes for optimal effector functions. ELISA measurements allow the precise quantitative determination of antigen-specific antibodies of various isotypes but only indirectly predict functional effects. We measured total Ig in combination with C3 products to generate a functional view of the immune reactions against the antigen. During an immune response, antibodies with different immunological properties are produced against the antigen. All these antibodies can bind to the antigen, forming immune complexes with different compositions and effector

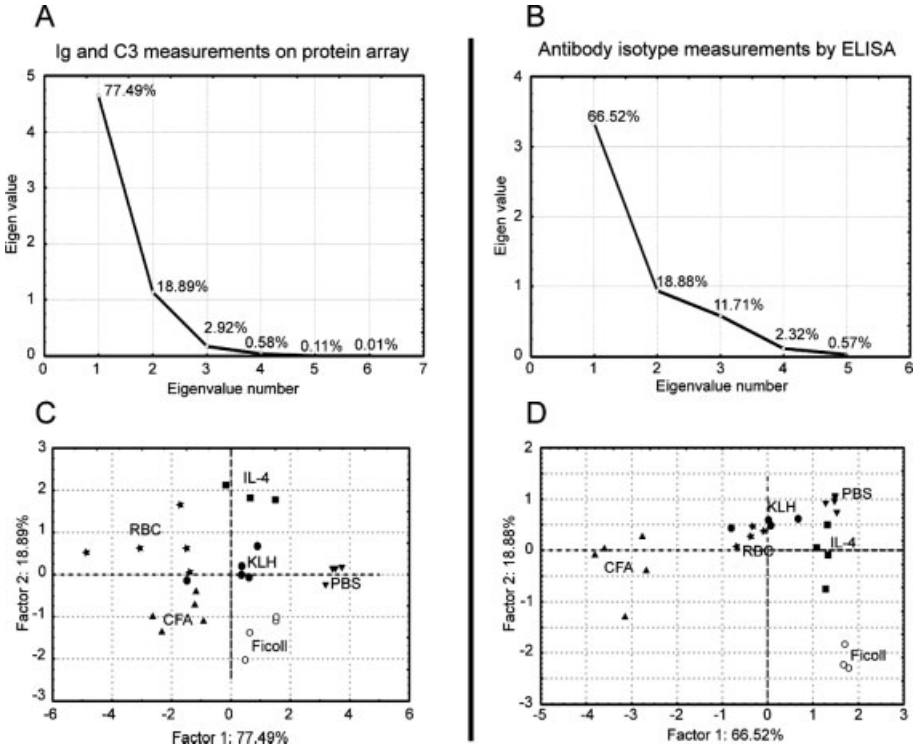

Figure 4. Principal component analysis of isotype measurements by ELISA and of 2-D profiling by antigen array. Scree plots represent Eigenvalues of factors of (A) array-based determinations of Ig binding and C3 deposition at three different dilutions of TNP_{12}–BSA (six variables) and (B) ELISA determinations of levels of five different TNP-specific antibody isotypes. Cumulative percentage of the variance accounted for by the factors is displayed at each inflexion point. (C, D) Projections using the first two calculated factors as coordinates are shown for each set of measurements. Dots represent coordinates of values rendered to individual mice in the respective factor-planes.

functions. To model these differences, we used immunization schemes that are known to result in characteristic, immunologically distinct responses. The variable composition of the immune complexes is reflected by the isotype patterns of our immunization schemes (Fig. 3A). Our approach aims to grasp this complexity by measuring the overall functional effect of antibodies on the complement system instead of determining each component of an immune complex separately. Murine IgG subclasses are quite heterogeneous with respect to effector functions such as complement activating properties [18, 20, 21] and FcγR binding [22]. Accordingly, antibody protectivity against infections can be determined by the dominant circulating isotype [23–26]. Humans possess a similar set of IgG antibodies with distinct effector potentials [6, 27].

For the 2-D characterization of sera we used a reagent which preferentially binds the heavy chains of IgG (γ-chains) but also reacts with Ig light chains of all other isotypes. Therefore IgM is poorly detected on the Ig scale but is efficiently integrated into the detection of C3 products, which is justified by its biological properties. This antibody class is usually produced during the early phase of an immune response by cells that do not go through affinity maturation and do not participate in the generation of memory, similar to innate responses. Additionally IgM can activate complement with the highest efficiency of all antibody classes, this being the primary effector pathway initiated by IgM. In this study, we have not considered antibodies of the IgA class, which are abundant in serum and can initiate complement activation [28]. However, these antibodies are primarily associated with mucosal immune responses and are not expected to influence our results. If required, detection of IgA should preferably be incorporated into the Ig detection channel. The same holds for IgE detection, the class associated with allergies and known to be unable to initiate complement activation.

Although antigens, depending on their biophysical and biochemical properties, may induce complement activation in immunologically naive individuals by both antibody-dependent and -independent ways, immunity profoundly changes this efficiency (Figs. 1A and 3). Differences between animals which were immunized in different ways can be more subtle, underlining the importance of utilizing of antigen features with different antigen densities. Dissimilarity in complement activation for TI and TD Th2 biased immunity groups was most pronounced at lower antigen densities (Fig. 3D).

Our approach allows the direct assessment of functional properties of antibody mixtures against antigens and could therefore be used on arrays containing antigens derived from microbes [29], especially because complement has an important role in antimicrobial protection. Complement can also mediate deleterious effects of autoantibodies [30] pointing to the potential utility of this assay in combination with autoantigen arrays. Here we only followed the changes of 2-D immune profile against a particular model antigen under experimental conditions. When panels of antigens are studied at a time, as in antibody profiling experiments, antigens are expected to show different antibody binding and complement activation even in immunologically naive individuals, both because of the presence or absence of natural antibodies and their distinct intrinsic complement activating properties. In an immunologically experienced or a diseased individual, different antigens are recognized by functionally distinct antibodies of various isotypes [31] and are therefore expected to take up distinct coordinates in this 2-D space. Microarray-based determination of the pattern of positions of relevant antigens and monitoring of their relative movement in this space can indicate fine qualitative changes of the immune response and help observe disease or effectiveness of therapy.

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The authors have declared the following conflict of interest. Eotvos Lorand University (ELTE) and the Hungarian Academy of Sciences (MTA) have applied for a patent on protein microarray-based complement activation detection.

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