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High-throughput, multiplexed IgG subclassing of antigen-specific antibodies from clinical samples

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Abstract

In vivo, the activity of antibodies relies critically on properties of both the variable domain, responsible for antigen recognition, and the constant domain, responsible for innate immune recognition. Here, we describe a flexible, microsphere-based array format for capturing information about both functional ends of disease-specific antibodies from complex, polyclonal clinical serum samples. Using minimal serum, we demonstrate IgG subclass profiling of multiple antibody specificities. We further capture and determine the subclass of epitope-specific antibodies. The data generated in this array provides a profile of the humoral immune response with multi-dimensional metrics regarding properties of both variable and constant IgG domains. Significantly, these properties are assessed simultaneously, and therefore information about the relationship between variable and constant domain characteristics is captured, and can be used to predict functions such as antibody effector activity.

Keywords

antibody; IgG subclass; clinical samples; humoral immunity

1. Introduction

Antibody activity results from the complex interplay of binding interactions between an antibody's Fv (variable) domain and an antigen, and its Fc (constant) domain and a set of Fc receptors (FcR), and other innate immune proteins. Characteristics of both Fv and Fc domains are easily determined for monoclonal or recombinant antibodies. However, the methods used for studying monoclonal samples are often not transferrable for similar characterization of antigen-specific antibodies from complex, polyclonal clinical sera samples, largely due to either large sample requirements, or the need to first perform affinity-purifications to isolate the antibodies of interest.

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Traditional biophysical readouts of the variable domain of polyclonal clinical sera include determination of Ab titer and avidity. Numerous functional assays regarding neutralization, induction of antibody-dependent cellular cytotoxicity, complement fixation, and phagocytosis are also routinely performed (Gomez-Roman et al., 2006; Huber et al., 2006; Polonis et al., 2008; Ackerman et al., 2011). These functional readouts are the cumulative result of multiple molecular interactions; and a means to assess the contributions of both various antibody Fv specificities and specific Fc domain characteristics could provide a comprehensive functional landscape of antibody activity, and better understanding of the characteristics of humoral responses associated with these protective activities.

Here we present the use of coded microsphere arrays to affinity purify antigen specific antibodies “on bead,” prior to IgG subclass assignment using subclass specific fluorescent detection and flow cytometry. Bead array technology allows up to 500 antigen or even epitope-specificities to be tested in parallel, opening a path for ultra-high throughput and multi-dimensional profiling of the humoral immune response, capturing information about both Fv and Fc domain characteristics in parallel. While we have limited the current scope of characterization of Fc domain functions to subclass determination, using alternative reagents, such as Fc receptors, an even greater breadth of Fc functions and characteristics could be determined. Even so, we demonstrate that antibody activity in a cell-based assay can be reliably predicted by array signatures, while this activity was not captured by traditional measurement of antibody titer.

2. Materials and Methods

2.1 Preparation of coded array microspheres

A customized multivariate Luminex assay was developed using a panel of HIV antigens coupled to carboxylated fluorescent beads (polystyrene or magnetic, Luminex Corp.) (Tomaras et al., 2008). A total of 5 million carboxylated beads were covalently coupled to 25 µg HIV antigen using a two-step carbodiimide reaction. Antigens tested included gp140 (Clade B, IT-001-0021p, Immune Technologies), gp120 (YU2, IT-00109927p, Immune Technologies), gp41 (HXBc2, IT-001-005p, Immune Technologies), and p24 (HXBc2, IT-001-017p, Immune Technologies), and resurfaced stabilized cores (Wu et al., 2010) (NIH AIDS Reagent Program). Beads were washed by centrifugation and activated for 20 min by resuspension in 80 µl of 100 mM monobasic sodium phosphate, pH 6.2, followed by the addition of 0.5 mg each of *N*-hydroxysulfosuccinimide (24520, Pierce) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide-HCl (77149, Pierce). Activated microspheres were washed three times in 250 µl of Coupling Buffer (50 mM MES, pH 5.0), resuspended in 100 µl of buffer, and incubated with 25 µg of HIV antigen for 2 h on a rotational mixer. Finally, coupled microspheres were washed three times with 1 ml of PBS-TBN (PBS-1×, 0.1% BSA, 0.02% Tween 20, 0.05% Sodium Azide, pH 7.4) and resuspended in 250 µl of PBS-TBN. After either 30 minute or overnight incubation in PBS-TBN, beads were washed to remove blocking buffer and resuspended in storage buffer (PBS-1×, 0.05% Sodium Azide.) The coupled beads were counted and stored at 4°C for up to 2 months.

2.2 Preparation of clinical plasma antibody samples

Study subjects were recruited from Ragon Institute cohorts and included healthy, acute, and chronically HIV infected subjects, as well as controllers, individuals able to maintain long-term suppression of virus in the absence of anti-retroviral therapy. In order to remove anti-retroviral drugs, antibodies were separated from other serum proteins using Melon Gel according to the manufacturer's instructions (Thermo Scientific), and resuspended at a concentration of 1 mg/ml. Significantly, this purification resin enriches IgG by retaining common plasma components, and thus represents a very gentle means of IgG purification,

not subject to the same subclass preferences or acidic elution conditions that Protein A or G purifications require. The study was approved by the Massachusetts General Hospital Institutional Review Board, and each subject gave written informed consent.

2.3 HIV-specific IgG Isotype detection

The four different HIV antigen-coupled microspheres were mixed together, each diluted to a concentration of 50 microspheres/ μ l in Assay Buffer (PBS-1 $\times$, 0.1% BSA), creating a working mixture of 50 microspheres per bead type, per μ l. Using a pre-wetted 96-well filter plate (for polystyrene beads, HGAM-301, Millipore), or a black, clear bottom 96-well plate (for magnetic beads, Greiner Bio One, 655906) 50 μ l of the working microsphere mixture (2500 beads of each type/well), 40 μ l of Assay Buffer, and 10 μ l of purified clinical IgG sample (10 μ g, approximately equivalent to 1 μ l serum) were added to each well. For monoclonal antibody samples, 10 μ l of antibody at 10 μ g/ml was added per well. In experiments to compare measurements made of serum versus purified IgG, 10 μ l of 1:10 diluted serum was added per well, resulting in roughly equivalent concentrations assuming typical serum IgG concentrations of about 10 mg/ml. For dilution experiments, all samples started at these concentrations and were then further diluted with Assay Buffer. The plate was covered and incubated overnight at 4°C on an XYZ plane plate shaker (IKA). The plate was washed three times with 125 μ l of Assay Wash (PBS-1 $\times$, 0.1% BSA, 0.5% Triton-100 $\times$), and a final wash was conducted with Assay Buffer. HIV-specific antibody isotypes were detected with R-phycoerythrin (PE)-conjugated mouse anti-human IgG1–4 (9052, 9070, 9210, 9200, Southern Biotech), or a murine anti-Hu pan IgG reagent (9040-09 Southern Biotech) at 0.65 μ g/ml, with 100 μ l/well. Under these incubation conditions, and as tested by the manufacturer, cross-reactivity for subclassing reagents was not observed. After 2 h incubation at room temperature on a shaker, the plate was washed three times with 125 μ l of Assay Wash, and microspheres were resuspended in 125 μ l of sheath fluid. Polystyrene beads were washed by vacuum filtration, while magnetic beads were retained using a plate magnet (Life Technologies).

A Bio-plex array reader (Bio-Plex 200 system, Bio-Plex Manager 4.1.1, or FlexMap 3D, Bio-Plex Manager 5.0, Bio-Rad) detected the microspheres and binding of PE detector antibody was measured to calculate a Median Fluorescence Intensity (MFI). Background signal, defined as the average MFI observed for each microsphere set when incubated with the PE detector antibody in the absence of clinical antibody sample, was subtracted from the MFI for each sample. HIV-IG (3957, AIDS Reagent Program) was a positive control and utilized as a means to standardize plate variation, and HuIgG (I2511, Sigma) served as an HIV-1 negative control.

2.4 ADCVI activity and traditional antibody titer

Antibody-dependent cellular viral inhibition (ADCVI) was determined as described previously (Forthal et al., 2006). Briefly, suppression of viral outgrowth was determined as p24 level following co-culture of HIV-infected CD4⁺ cells as targets and autologous NK cells as effectors in the presence of antibody.

The titer of gp120-specific antibody was determined by ELISA according to previously established protocols (Morner et al., 2009).

2.5 Data analysis

Data were organized into an array with a row for each patient and a column for each antigen-IgG subclass pair (antibody feature). In order to facilitate comparisons among the different features, the array was normalized column-wise, i.e., each column was re-centered to a mean of 0 and re-scaled to a standard deviation of 1. The function `heatmap.2` in the R

gplots library was then used to hierarchically cluster and visualize the array, grouping and reordering rows by overall similarity, and likewise (but separately) for the columns.

3. Results

3.1 Array overview

In order to comprehensively map the breadth of characteristics of the humoral immune response to HIV infection, a bead-based array was generated, in which HIV antigens of interest were covalently conjugated to fluorescently coded microspheres using amine-reactive sulfo-NHS chemistry. Both surface antigens (gp41, gp120, and gp160) and structural antigens (p24) were included, each conjugated to a uniquely identifiable bead set. As diagrammed in Figure 1A, these microspheres were then incubated in IgG prepared from clinical serum samples, allowing affinity purification of antigen-specific antibodies “on bead” for each antigen. Beads were subsequently washed, aliquotted, and incubated separately with fluorescent conjugates recognizing total IgG, or individual IgG subclasses (IgG1, IgG2, IgG3, IgG4), prior to analysis by flow cytometry, in which each antigen bead was uniquely identified by fluorescence in red and IR channels, and the amount of plasma antibody bound was determined by the fluorescent intensity observed in the orange channel. Figure 1B presents the total IgG signal observed against each bead set for a group of HIV positive (+) and HIV negative (–), clinical samples, pooled human HIV+ Ig (HIVIG), and 2 control monoclonal antibodies (b12, 2F5). To permit presentation of multiple bead sets on a single y-axis, after subtraction of the background signal observed when beads were incubated with detection reagent alone, signal observed for HIVIG was assigned a value of 1000, and all data was scaled relative to this value, facilitating relative comparisons between subjects for each measured feature.

3.2 Characterizing epitope specific antibodies

Because the assay can capture 500 different specificities, where reagents permit, it is possible to adapt the platform to isolate not only antibodies that recognize specific antigens, but specific epitopes on an antigen of interest. In Figure 1C, resurfaced stabilized cores (RSC)(Wu et al., 2010) have been conjugated to a unique bead set, allowing isolation of antibodies that recognize the CD4 binding site (CD4bs) of gp120. Antibodies against the CD4bs were primarily IgG1 subclass, though a number of subjects additionally demonstrated strong IgG3 subclass responses to this epitope. The absence of IgG3 responses to the CD4bs in treated subjects may support a hypothesis that IgG3 levels may differ in treated subjects experiencing lower levels of immune activation. Additional comparisons, such as the relative proportion of IgG1 antibodies against gp120 directed to the CD4bs may also be determined. Figure 1D presents the MFI of IgG1 subclass antibodies directed against gp120 (green), or the CD4bs on gp120 (red), allowing assessment of the relative contribution of this epitope to IgG1 signal against this antigen. For many subjects, a 1–2 order of magnitude signal difference in total gp120 versus CD4bs binding IgG1 antibodies was observed. However, this fraction varied greatly, and antibodies from one subject gave comparable signal against the gp120 and RSC bead set, indicating that a significant fraction of the gp120-specific IgG1 in this subject may have been directed against this specific epitope.

3.3 Quality Control Characteristics

Figure 2A presents the agreement observed between experimental replicates conducted several weeks apart for all subclass specificities for the gp120 bead set. Similar agreement was observed for other antigens tested, resulting in overall inter-operator, inter-day coefficients of variation in the range of 10–15%, with low intensity measurements tending to have greater variation, and high-intensity measurements tending to have excellent

agreement. Intra-assay CV's typically ranged from 2–5%. The average r^2 value observed across all 16 readouts of paired antigen:subclass combinations was 0.945, with individual r^2 values ranging between 0.89 and 0.99. Similarly, in a regression analysis, the average slope of lines fit to replicate data was 0.99, and demonstrated linearity over almost 5 orders of magnitude. Lastly, good agreement (CV 18–22%) was also observed when the assay was repeated with bead sets prepared in separate conjugation reactions, indicating that good reproducibility of the antigen conjugation procedure is possible. Lastly, when antibody samples are appropriately diluted, array MFI values agree well with traditional ELISA assays (Figure 2B; $r^2 = 0.8$), as has been previously reported for other antigen specificities (Whaley et al., 2010).

3.4 Determining epitope diversity

Intriguingly, as presented in Figure 2C, we observed that at saturation, antibodies from different subjects exhibited different maximal MFI. Likewise, at saturating concentrations, monoclonal antibodies, able to bind only a single site on each conjugated antigen, tended to give lower overall signals than polyclonal mixtures, in which multiple epitope specificities were present. This data is consistent with the notion that at saturation, MFI measurements may capture information about the relative number of antibodies or epitopes that can be bound simultaneously. To explore this possibility, we determined MFI for VRC01 (Zhou et al., 2010) (CD4bs-specific), and F425 (Pantophlet et al., 2007) (V3 loop-specific) individually, as well as in combination at a range of concentrations (Figure 2D). As would be expected, in combination, these non-competitive antibodies exhibited additive signal, with excellent agreement of the experimental data to prediction based on the sum of individual MFI values. In contrast, monoclonal antibodies recognizing the same epitope, competed for binding, and failed to exhibit strictly additive signal (data not shown). Lastly, when IgG from multiple subjects was combined, or if monoclonal antibodies were mixed with polyclonal serum samples, these mixtures likewise exhibited increased MFI signals. Accordingly, when saturating quantities of plasma IgG are utilized, antigen binding sites become limiting, resulting in full occupancy of immunogenic epitopes, with excess HIV-specific antibody remaining in solution. Under such saturation conditions, the total IgG signal appears to reflect a composite of antibody titer, affinity, and epitope diversity.

3.5 Sample format and limit of detection

Because serum components may interfere with other assays of antibody activity, we wished to examine the correlation between array data evaluated in dilute serum versus purified IgG. Figure 2E presents the correlation of total bound IgG for a set of 8 subjects across 6 different antigen bead sets using either serum or purified antibody. Measurements of individual IgG subclasses likewise showed excellent agreement, with average r^2 values exceeding 0.9 (data not shown).

Lastly, in order to assess the limit of detection, a dose response curve for both monoclonal antibodies and polyclonal serum samples was generated (Figure 2F), and exhibited excellent sensitivity. Monoclonal antibodies yielded detectable signal at concentrations of 1–10 pM, while serum samples yielded signal at 10–100 pM (representing a serum dilution of approximately $1:10^6$).

3.6 Multi-dimensional humoral profiling

The microsphere array was used to determine total and IgG subclass-specific responses against 4 HIV antigens for a set of infected and uninfected subjects. Monoclonal IgG1 control antibodies b12 and 2F5 recognized their target antigens (gp120 and gp41, respectively), and were correctly assigned to the IgG1 subclass (data not shown).

Output data was easily analyzed by either antigen-specificity, or by IgG subclass—making different trends in the humoral immune response apparent. While most infected subjects generated antibodies against each HIV antigen, the distribution of IgG subclasses was quite broad, with many subjects restricted to IgG1 dominated responses, while others showed dramatic breadth. As an example, one subject possessed antibodies of all 4 subclasses to multiple antigens. Even in this relatively small data set other trends were apparent: IgG2 responses were most frequently directed to gp41, and gp41 generally drove the most diverse IgG subclass response (data not shown). When coupled to functional, clinical, genetic, or other parameters, this multi-dimensional data may permit mechanistic understanding of the immune response. In particular, inclusion of multiple sequence variants for each antigen, as well as longitudinal study may provide significant insights into the dynamic specificities of humoral immunity.

3.7 Array-based clustering

The true power of this array-based approach to profiling humoral immune responses lies in associating signatures observed on the array with other types of data, whether genetic, functional, or clinical. For example, traditional machine learning methods can be utilized to select features from within the array data that are associated with, or predict other features, such as: clinical outcome, in vitro ADCC activity, FcγR genotype, CD4+ T cell count, or viral load. Figure 3A presents hierarchical clustering of clinical samples from 40 HIV infected subjects, based on array signatures. As would be expected, HIV negative subjects comprise a distinct group using this naïve clustering approach (data not shown). Whereas, among HIV positive subjects, application of hierarchical clustering methods generated novel subject groups defined by the humoral immune response as opposed to commonly utilized clinical parameters such as treatment status. Groups defined by humoral immune profile may differ in viral load, markers of inflammation, or functional assays of antibody activity. While naïve clusters serve as a useful means to visualize data, alternative analyses, such as feature selection can be used to identify complex associations and generate mechanistic hypotheses of antibody activities.

As evidence of the utility of machine learning methods, among HIV positives, subjects in array cluster 1 exhibited significantly improved capacity to inhibit viral outgrowth in an antibody-dependent cellular viral inhibition (ADCVI) assay relative to subjects in cluster 2 (Figure 3B). Because these array clusters were heavily defined by IgG1 responses, we sought to determine whether ADCVI activity was directly predicted by IgG1 responses. When array measurements of gp120-specific IgG1 were plotted against ADCVI activity, a significant correlation was observed (Figure 3C, $p=0.0055$). Indeed, this result from naïve clustering is likewise supported both by feature selection methods, and the known biological potency of IgG1 antibodies in ADCVI activity.

As a comparison, traditional antibody titers against gp120 as assessed by ELISA were used to split infected subjects into 2 groups (high and low titer). As demonstrated in Figure 3D, titer was not indicative of ADCVI activity, and when titer and ADCVI activity were plotted, no correlation was observed (Figure 3E). While this negative result serves to underscore the merits of multiplexed antibody profiling, it also points toward the possible significance of saturation rather than dilution measurements, indicating that the number of epitopes recognized may be an important factor in antibody effector function. Again, this inference based on array data is supported by the biological mechanism of ADCVI, in which avid interactions between multiple antibodies and Fc receptors drive cytotoxic activity.

Moving beyond the clustering of subjects, the clustering of antibody features, as presented on the x-axis in Figure 3A, can also be informative. In this case, clustering of antibody features indicated that the distribution of IgG subclass responses was relatively uniform

across each of the HIV antigens tested. For example, subjects who generated IgG2 antibodies against any one HIV antigen were likely to generate IgG2 responses against multiple HIV antigens. Similar patterns were observed among IgG3 and IgG4 responders. These feature groupings may indicate that in the setting of chronic HIV infection, antigen-specific cues may be dominated by global immune cues in determining subclass selection.

4. Discussion

In vivo, antibody responses to vaccination or natural infection are highly polyclonal, with multiple somatic variants directed to multiple epitopes on multiple antigens. This diversity of variable domain recognition characteristics is further complemented by diversity in constant domain subclass and ability to interact with innate immune receptors such as FcγR. Antibody based protection is thus derived from the sum of specificities and activities of this polyclonal humoral milieu, and techniques capable of parsing this milieu into components that can be associated with other relevant clinical, genetic, or functional characteristics may prove extremely valuable in developing insights into humoral immunity (Yates et al., 2011; Curtis et al., 2012).

We demonstrate measurement of over 20 antibody features associated with both variable and constant domain functions from less than 4 μl of serum. State of the art fluorescent microsphere technology allows 500 Fv features, or antigen specificities, to be profiled in a single well. If a traditional flow cytometer and multi-color rather than single channel readouts of antibody subclass are utilized, subclass assignment can be multiplexed, with some loss in sensitivity. Additional readouts of Fc domain features, such as affinity for Fc receptors may also be determined using this platform. Therefore, it is technologically feasible that some 2,000 measurements of the humoral immune response could be determined in a single well with minimal serum requirements (approximately 1 μl). This number of parameters approaches those achieved in genotyping and gene expression analyses, and is likely to be of significant value in understanding the characteristics and development of both protective and pathological humoral immunity.

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Highlights

We describe the use of luminex bead arrays to characterize antibodies in clinical samples.

Using this technology, up to 500 antigen specificities can be assessed at once.

Simultaneous readouts of antibody subclass can be performed.

This data provides a comprehensive profile of the antibody response.

Array signatures can predict antibody functions.

Figure 1.

Assay schematic and representative data. A) Each antigen of interest is covalently conjugated to a coded microsphere. Microspheres are coded via differential incorporation of 2 internal fluorescent dyes, allowing identification of each bead, and therefore, antigen type. Microspheres are mixed with plasma antibodies to allow capture of antigen-specific antibodies, resulting in “on-bead” affinity purification. Beads are washed, split into replicate wells, and labeled for IgG subclass identification, and subsequently acquired on a Luminex flow cytometer. B) Representative data for quantification of total IgG bound to 4 different antigen bead sets, presented as relative MFI for a series of HIV positive (+) and negative (–) subjects, as well as 2 control monoclonal antibodies. C–D) Analysis of epitope-specific antibodies. C) IgG subclass was determined for antibodies recognizing the CD4bs as defined by recognition of resurfaced stabilized core (RSC). HIV positive subjects included: controllers (ctr), treated (tx), and untreated (untx) progressors. D) Relative signal of RSC-specific (CD4bs) IgG1 antibodies, as compared to total gp120-specific IgG1.

Figure 2.

Assay Quality Control data. A) Subclassing replicate data (MFI) showing low inter-assay variation with different operators on different days with the gp120 bead set. B) Alignment of ELISA titer data (OD) with Luminex titer (MFI) for purified IgG on the gp120 bead set. Error bars represent standard deviation between duplicate measurements. C) Saturation MFI showing how antibody epitope breadth plays an important role in the total signal observed on the gp120 bead set. D) Additive Effects of combining monoclonal anti-gp120 antibodies with different epitope specificities. E) Comparison of subclassing data using either serum or Melon-Gel purified IgG samples from the same patients across multiple bead sets. F) Monoclonal and polyclonal antibody samples were diluted from saturating concentrations until the assay MFI reached baseline (shaded box) on the gp120 bead set. Plotting on a log-log scale indicates a large useful dynamic range for this assay (roughly 10^{-10} to 10^{-6} M for polyclonal and 10^{-12} to 10^{-8} M for monoclonal antibodies).

Figure 3.

Using array signatures to characterize function. A) A cluster plot of array data generates groups of samples with similar humoral immune profiles on the y-axis, and groups antibody features that co-vary on the x-axis. B) Antibody dependent cellular viral inhibition (ADCVI) activity for subjects in array-based clusters. C) Relationship of ADCVI activity with IgG1 responses to gp120. D) ADCVI activity for subjects with high or low titers of gp120-specific antibody. E) Relationship of ADCVI activity with titer.