Published in final edited form as:

Anal Chem. 2018 August 07; 90(15): 9583–9590. doi:10.1021/acs.analchem.8b02479.

Crowd on a Chip: Label-Free Human Monoclonal Antibody Arrays for Serotyping Influenza

Hanyuan Zhang^{1,2}, Carole Henry³, Christopher S. Anderson⁴, Aitor Nogales⁴, Marta L. DeDiego⁴, Joseph Bucukovski¹, Luis Martinez-Sobrido⁴, Patrick C. Wilson³, David J. Topham⁴, and Benjamin L. Miller^{1,2,*}

¹Department of Dermatology, University of Rochester Medical Center, Rochester, New York 14642

²Materials Science Program, University of Rochester, Rochester, New York 14627

³Department of Medicine, University of Chicago, Chicago, Illinois 60637

⁴Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, New York 14642

Abstract

Rapid changes in influenza A virus (IAV) antigenicity create challenges in surveillance, disease diagnosis, and vaccine development. Further, serological methods for studying antigenic properties of influenza viruses often rely on animal models and therefore may not fully reflect the dynamics of human immunity. We hypothesized that arrays of human monoclonal antibodies (hmAbs) to influenza could be employed in a pattern-recognition approach to expedite IAV serology, and to study the antigenic evolution of newly emerging viruses. Using the multiplex, label-free Arrayed Imaging Reflectometry (AIR) platform, we have demonstrated that such arrays readily discriminated among various subtypes of IAVs, including H1, H3 seasonal strains, and avian-sourced human H7 viruses. Array responses also allowed the first determination of antigenic relationships among IAV strains directly from hmAb responses. Finally, correlation analysis of antibody binding to all tested IAV subtypes allowed efficient identification of broadly reactive clones. In addition to specific applications in the context of understanding influenza biology with potential utility in “universal” flu vaccine development, these studies validate AIR as a platform technology for studying antigenic properties of viruses, and also antibody properties in a high-throughput manner. We further anticipate that this approach will facilitate advances in the study of other viral pathogens.

TOC Graphic:

*Corresponding Author: Benjamin L. Miller, Department of Dermatology, University of Rochester Medical Center, 601 Elmwood Avenue Box 697, Rochester, New York 14642; 585-275-9805; Benjamin_miller@urmc.rochester.edu.

Supplementary Material Available: Sources of reagents, validation experiments with hemagglutinin, array layouts, additional hierarchical cluster map including seasonal strains A/WSN/1933 H1N1 and A/Shanghai/1/2013 H7N9, layout information for correlation heat maps, characterization of individual antibodies by ELISA, and experimental protocol and data for HAI assays.

Keywords

influenza virus; antibody microarray; label-free biosensor; influenza-specific human monoclonal antibodies; antigenic cartography; influenza universal vaccine

Introduction

Infection with the influenza A virus (IAV) remains one of the most widespread causes of human disease, with approximately 3 to 5 million cases of severe illness worldwide and more than a quarter million associated fatalities from seasonal influenza each year.^{1,2} IAV pandemics, though rare, remain significant threats to global health.^{3,4} The human toll of IAV is matched by a considerable economic cost, including the direct cost of treatment and the opportunity cost of work lost.^{5,6} IAV infections in livestock are similarly costly,⁷ and are a well-studied reservoir for human infection.⁸ Annual vaccination is recommended to limit the spread of IAV in humans.^{9,10,11} Unfortunately, vaccine efficacy against seasonal IAV is less than ideal (for example, overall vaccine effectiveness was reported to be only 19.8% for the IAV H3N2 subtype in the 2014–2015 season^{12,13}), and pandemic vaccines are typically not available in the early stages of an outbreak. These issues are largely due to the ability of IAV to evolve quickly,¹⁴ such that emerging strains are either poorly antigenically matched to a vaccine,^{15,16} or are able to escape residual immunity from previous exposure or vaccination.^{14,17} To ameliorate this problem, considerable effort is invested in global surveillance,¹⁸ and in monitoring virus evolution.¹⁹ The development of “universal”, or at least more broadly efficacious, vaccines is also recognized as a high priority endeavor.^{20,21} Current strategies for IAV surveillance and vaccine development mainly rely on relatively low-throughput serological tools such as the enzyme-linked immunosorbent assay (ELISA), microneutralization (MN), and hemagglutination inhibition (HAI) assays. While widely used and valuable, these tests most commonly only provide information about one antigen (“1-plex”) at a time. They additionally suffer from significant workflow complexity.^{22,23,24} Full genomic sequencing of virus isolates has emerged as a crucial analytical tool.^{25,26} Genetic analysis provides a useful, but incomplete picture of virus antigenicity: point mutations may disproportionately alter virus recognition by components of the immune system.^{27,28} Posttranslational modification of viral antigens and presentation in the three-dimensional context of the virus are also important, and are not well predicted by sequencing.^{29,30} While antigenic cartography derived from analysis of model organism (ferret) antisera has also proven useful, discrepancies in the immune response elicited by IAV between ferrets and humans have been noted.³¹ Together, these observations suggest that new high-throughput analytical methods providing systematic evaluation of IAV antigenicity at the whole-virus level and focused on human response are needed. Such methods could facilitate

understanding of the relationships among IAV strains, viral evolution, and potentially to accelerate vaccine development.

In previous work, Wrammert et al. demonstrated that immunization produces a clonally diverse repertoire of anti-IAV antibodies, and these antibodies may be rapidly cloned to produce libraries of human monoclonal antibodies (hmAbs) with diverse strain reactivity.³² A small panel of some of these hmAbs was able to discriminate among recombinant hemagglutinins (HA) of IAV H7N9, using fluorophore-tagged secondary antibodies for detection.³³ On the basis of this finding, we anticipated that multiplex arrays employing these anti-IAV hmAbs (a “crowd on a chip”) could prove useful as tools for serology and surveillance, and could provide valuable information for developing broadly effective vaccines. A large number of hmAbs targeting specific antigenic domains would allow for systematic mapping of IAV antigenicity, producing in essence a microarray analog of a “Quick Response” (QR) code, or 2-D barcode, for IAV. Quantitative binding data can be used as a measure of antigenic distance between HA antigens. Visualization of the relative antigenic distances among various strains, a method known as “antigenic cartography”, could then be derived to provide a useful representation of strain relationships.^{29,34,35,36} The microarray could also function as a high-throughput method for characterizing hmAbs, allowing assessment of their specificity against various IAV subtypes. In particular, identification of broadly cross-reactive and conserved stalk-targeting hmAbs would be of substantial interest.³⁷ These preexisting cross-reactive anti-IAV hmAbs due to prior exposure to circulating human influenza viruses or influenza vaccination have been shown to confer immunity to emerging IAV strains.³³ Via competition, one could also use such an array to rapidly compare the specificity of hmAbs.

We have described the use of HA (antigen) arrays as tools for profiling anti-IAV antibodies in human serum,³⁸ and in an avian surveillance context.³⁹ These studies were conducted using Arrayed Imaging Reflectometry (AIR), a label-free, multiplex, and high-throughput biosensing platform developed in our laboratory.^{40,41,42,43} We anticipated that AIR would provide the ideal combination of multiplex capability and quantitative readout needed for hmAb arrays, essentially inverting both the experimental approach and scientific goals of our previous work. In brief, AIR relies on the creation of a near-perfect antireflective condition on the surface of a silicon chip. When target molecules bind probe spots (antibodies, antigens, or other capture molecules) on the surface of the chip, the antireflective condition is degraded, and light reflects from the chip at that spot in proportion to the amount of material bound. Because AIR does not require secondary antibodies or other labeling reagents, multiplex experiments addressing 10's to 100's of targets are as simple, sensitive, and quantitative as single-plex measurements. The work flow is simple, potentially allowing for its use in any laboratory or in the field. Here, human anti-IAV hmAb arrays were tested first for their ability to discriminate among purified recombinant HAs, and then for their utility in providing unique patterns of response with whole IAV.⁴⁴

Experimental Section

Preparation of the hmAb microarrays.

All antibody solutions with the exception of anti-fluorescein isothiocyanate (anti-FITC) control were prepared at a concentration of 250 µg/ml in 10 mM phosphate buffered saline at pH 7.0 and spotted at a droplet volume of 250 pl using a piezoelectric arrayer (Scienion S3). A center-to-center spot distance of 300 µm was used for all arrays. For high-multiplex arrays, duplicate spots were printed for each hmAb. Groups of three anti-FITC concentrations (100, 250, and 500 µg/ml) were printed adjacent to probe antibody spots, and used as a negative control to compensate for intra- and inter-chip thickness variations. Human IgG was also spotted on the array as a control for nonspecific IgG binding. Purified bovine IgG secondary antibodies, which are reactive to bovine sera used for blocking, were printed at both the first and the last spots for quality control of printing. In addition, an hmAb that has been proved to positively react with target IAV antigens was included and printed next to bovine IgG spots to confirm the reactivity of the chips during the assay.

Immunoassay protocols

Experiments with recombinant HA protein and whole IAV particles followed the same general procedure. Two blocking solutions consisting of (1) 10 mg/ml BSA in sodium acetate buffer (50 mM at pH 5.0) and (2) 10% fetal bovine serum (FBS) in modified PBS-EDTA-Tween 20 (10 mM PBS, 5 mM EDTA, and 0.5% Tween 20 at pH 7.4) assay wash buffer (AWB) were prepared and added to separate rows of a 96-well plate. After being incubated in BSA blocking solutions, the chips were washed in AWB solutions thoroughly and then transferred into a BSA pre-blocked row for target exposure. Solutions of HAs were prepared by diluting them in 10% FBS in AWB. Concentrations of recombinant H7 HA proteins derived from A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9) were 0.5, 1, 2, and 4 µg/ml for the titration assays. Concentration of recombinant H3 HA protein derived from A/Wisconsin/67/2005 (H3N2) was 1 µg/ml for the competitive assay. Viral titers of H3N2 human IAVs used for mapping the antigenic evolution were measured and reconstituted to 10⁶ plaque forming units (pfu)/ml for target exposure. Blank 10% FBS solutions were used as negative control groups. In each case, three chips per condition were incubated in target or control solutions overnight at ambient temperature, then washed in AWB several times. Finally, the chips were rinsed in deionized, glass-distilled water and dried under a flow of nitrogen gas before array imaging. Note that the overnight incubation was done primarily for convenience and to maintain a consistent protocol between experiments. Test incubations as short as an hour produced usable data.

Image acquisition and statistical analysis

Once dried, chips were imaged immediately on a prototype AIR reader (Adarza BioSystems, Inc). This system uses a He-Ne laser at a wavelength of 632.8 nm at an incident angle of 70.5° to illuminate the arrays. The beam is linearly s-polarized, collimated, and finally delivered to the chip surface after expansion to allow illumination of the entire array. AIR images were acquired in a 16-bit TIFF format, with exposure times varied from 50 ms to 1 second. The AIR image files were then analyzed using NIH-ImageJ (version 1.46r),⁴⁵ with

pixel intensities of probe spots measured and analyzed in histograms. Final plots of the histograms and distribution charts were generated in OriginPro 2017 (OriginLab Corporation). Considering that each probe spot includes hundreds of pixels, a nonparametric test was further applied to evaluate the significance of differences in the thickness of probes between control and analyte groups. Two data sets of pixel intensities (one from the probe spot on the analyte chip, the other from the same probe spot on the control chip) were compared using a two-sample t-test to determine significant differences. P-values, which give the probability that the reflectivity differences between the control and analyte groups are statistically insignificant, were obtained from this test and a cutoff of 0.05 was used. The median value of the reflection intensities averaged from three chips represents the amount of material bound to each hmAb, and these data were normalized relative to the positive control (human IgG). Error bars were determined by the standard deviation of the difference based on replicate spots. The overall analysis process is shown in Figure S1. All experiments were repeated at least once to confirm observations.

Mapping the genetic and antigenic evolution of H3N2 IAV vaccine strains

Antigenic cartography used to visualize relationships among human H3N2 IAV vaccine strains was obtained by scaling and combining genetic and antigenic maps. All maps were generated by performing a classical multidimensional scaling (principal coordinate analysis) method on the distance matrix calculated from genetic and antigenic data sets. Genetic data were generated from HA sequences of the corresponding strains. These sequences were compiled from the Influenza Resource Database (www.fludb.org) and the World Health Organization's Global Initiative on Sharing Avian Influenza Data (www.gisaid.org). For each HA sequence, the distance was determined by the number of amino acid differences at each residue's position in the HA protein.³⁵ This results in a distance matrix consisting of the number of amino acid differences between all human H3N2 IAV vaccine strains. Antigenic data from the hmAb microarrays were first normalized into the range of highest and lowest values of the responses. Euclidean distances between IAV strains were then calculated to form a distance matrix using the open source library "Pandas" in Python. Antigenic data from HAI assays were analyzed in the same way as microarray data. However, HAI titers were first converted into logarithmic forms based on the observation that antigenic distance is linearly related to the logarithm of the HAI measurement.⁵⁰ Normalized data were then calculated in Python to generate the distance matrix. The genetic, AIR hmAb, and HAI maps were individually scaled and placed on a combined coordinate system.

Clustering methods

Antigenic data used for clustering the strains of interest were first normalized based on the highest and lowest overall response values. Agglomerative hierarchical clustering methods⁴⁶ were then applied to the normalized data sets by using the open source library "Pandas" in Python. Three commonly used linkages (single-linkage, average-linkage, and complete-linkage) were tested and compared for determining the cluster distance. An average-linkage clustering was finally applied because this linkage strikes the balance between chaining and crowding of the clusters and thus, more informative patterns of clustering can be obtained

than other methods.⁴⁶ All the cluster maps and heat maps were generated by the visualization library “Seaborn” in Python.

Results and Discussion

In preliminary experiments, we verified that hmAbs printed on AIR substrates were able to detect recombinant hemagglutinins (Supplementary Information). Efforts to optimize buffer and blocking conditions for preparing chips led to the protocol described above; lower amounts of FBS in the secondary block and diluent were less effective at reducing nonspecific binding than the 10% solutions described here. Next, our efforts turned to detection and discrimination of various subtypes of whole IAV using higher-plex arrays. We prepared an 85-plex microarray consisting of H1 reactive hmAbs, H3 reactive hmAbs (including some known to be cross-reactive to H1), H7 reactive hmAbs cloned from subjects vaccinated against A/Anhui/1/2013 H7N9, and controls (Figure 1a; detailed layout information is provided in Supplemental Figure S2a). Each hmAb selected for the array was previously tested for its reactivity to at least one IAV strain by virus MN, HAI assays and ELISA^{33, 47} (Tables S1 and S2 in Supplementary Information). Exposure of this array to A/WSN/1933 H1N1 and A/mallard/Netherlands/12/2000 H7N3⁴⁴ at 4×10^6 plaque forming units (pfu)/mL produced obvious differences in response patterns. Known H1-reactive hmAbs responded to H1N1 as expected. Many of the H7 hmAbs directed at H7N9 bound to H7N3, as one might expect given the highly-conserved epitopes in these two avian-sourced viruses. In the H1N1 response pattern, three H7 hmAbs derived from the same subject show positive binding. This may be simple cross-reactivity or may indicate a previous exposure of that subject to H1N1 either in a vaccination process or a natural infection. Other cross-reactive readouts from the response patterns of H3 hmAbs are also notable, and consistent with prior ELISA data. Six anti-H3 hmAbs show cross-reactivity to H1N1 (confirmed by ELISA), and five of the H3 hmAbs (SFV005–2G02, 037–10036-5A01, 030–121509-3B01, S6-B01, and 045–051310-2B06) were found to be cross-reactive to H7N3 virus. One of particular interest is the stalk binding H3 hmAb 045–051310-2B06, which had been shown to be cross-reactive with H7N9 HAs as well.^{33,47} This highlights the significant potential of preexisting hmAbs induced by seasonal vaccination or natural exposure for targeting various IAV subtypes, a key goal for universal protection.

Serial dilutions of H7N3 virus were employed to verify quantitative performance of the array, and to select an ideal concentration for subsequent experiments. In essence, this experiment provides 80 separate binding isotherms (one for each of the 80 hmAbs), allowing rapid visualization of the range of antibody affinities for the virus tested (Figure 1b and Figure S2b in Supplementary Information). It is desirable to have the responses of the array spread across the full dynamic range of the sensor so that minor changes in response patterns can be demonstrated and quantified. As a virus concentration of 10^6 pfu/mL yielded the broadest range of antibody binding for both viruses, this concentration was employed in all subsequent experiments. The ability of the method to discriminate between closely related viruses was then tested by exposing a 90-plex array (Table S1 and Figure S3a in Supplementary Information) to IAV A/Anhui/1/2013 H7N9 and A/Shanghai/1/2013 H7N9. H7N9 Anhui and Shanghai strains were examined as a particularly stringent test of the array, since they are nearly identical, differing by only six amino acids in their HA sequences.⁴⁸

Responses were compared with arrays exposed to recombinant A/Shanghai/1/2013 H7N9 HA, recombinant A/Shanghai/1/2013 H7N9 neuraminidase (NA), and carrier solution alone (10% FBS in mPBS-EDTA-Tween 20; see Methods). AIR image results can be found in Supplemental Figure S3b. Antibody responses were scaled relative to the IgG positive control signal. A/Shanghai/1/2013 H7N9 HA generated binding to fewer antibodies on the array than the two viruses, potentially reflecting both differences in HA structure (the recombinant HA is a soluble protein and potentially lacks some epitopes proximal to the deleted membrane domain) and molecular context (isolated protein vs. viral capsid). NA and carrier controls did not yield any signal, demonstrating that the binding responses generated from virus are due strictly to HA binding. The response of each hmAb on the array to A/Shanghai/1/2013 H7N9 relative to its response to A/Anhui/1/2013 H7N9 is plotted in Figure 1c. Linear regression of these data (with confidence and prediction bands) reveals the expected antigenic similarities between the two viruses (i.e. hmAbs yielding similar or identical response on the array), as well as three antibody clusters preferentially binding A/Shanghai/1/2013 H7N9 (boxed). Antibodies known to bind specific epitopes are color-coded, with their corresponding binding locations color coded in the published X-ray crystal structure of A/Shanghai/1/2013 H7N9 HA⁴⁹ in Figure 1d. Notably, antibodies binding the same epitope cluster in the plot in Figure 1c.

Antigenic mapping of seasonal vaccine strains

The antigenic properties of IAV constantly change in order for the viruses to retain their ability to evade the human immune system. To establish the utility of AIR as rapid method for revealing relationships among IAV strains as they evolve, we prepared a 115-plex AIR microarray (Supplemental Figure S4) consisting of 83 hmAbs directed against human H3N2 IAV derived from subjects vaccinated with different strains (A/Perth/16/2009, A/Wisconsin/67/2005, A/Texas/50/2012, and A/Uruguay/716/2007), along with seven H1 hmAbs. Several of the 90 hmAbs were known to be cross-reactive based on previous immunoassays. Array responses to seven H3N2 vaccine strains from the 2010 to 2015 influenza seasons were examined: A/Wisconsin/67/2005 (abbreviated WI05), A/Brisbane/10/2007 (BR07), A/Uruguay/716/2007 (UR07), A/Perth/16/2009 (PE09), A/Victoria/361/2011 (VI11), A/Texas/50/2012 (TE12) and A/Switzerland/9715293/2013 (SW13). Resulting AIR images are shown in Figure 2a. Quantitative array responses to each strain were used to produce a hierarchical cluster analysis (Figure 2b). The dendrogram reveals that IAV strains tested cluster largely as expected, with earlier (WI05, UR07, BR07) and later (PE09, VI11, TE12) strains forming two primary clusters. SW13 behaves as an outlier. The lower portion of the clustering analysis includes several hmAbs with strong, broadly cross-reactive responses. These are potentially useful for their ability to reveal epitopes able to induce cross-reactivity. Several hmAbs towards the center of the chart are H1 reactive and show no binding to H3 strains. The response patterns at the upper part of the clustering, though less strong, are nevertheless useful for differentiating among the strains tested (for example, hmAb 062860p153-E05 shows binding to SW13 and UR07, but essentially no binding to other strains; it is the only hmAb tested with this pattern).

Relationships among IAVs can be visualized in low-dimensional space and studied via antigenic cartography.⁵⁰ This method has previously been used to transform HAI data

(derived from ferret serum) or viral genetic sequence data via metric multidimensional scaling (MDS) to provide relative antigenic distances between IAV strains.²⁹ Traditional antigenic determination assays depend on the ability of antibodies to block HA sialic acid binding and are therefore biased to antibodies specific to the receptor binding domain. Recently, Anderson et al.⁵¹ demonstrated that stalk-specific antigenic changes occur in the stalk region in addition to the sialic acid binding region. Since our AIR assay is not limited to sialic-acid-based inhibition, we tested its suitability for measuring antigenic distances between HA antigens and as input for antigenic cartography. We hypothesized that data from AIR anti-IAV hmAb arrays would provide data based solely on the human immune response to IAV, as well as providing cost and speed advantages over experiments requiring ferret serum. Analysis of array data via metric MDS produced the antigenic cartography shown in Figure 2c. For comparison, we used another high-throughput antigenic distance estimation based on HA genetic sequence data and, for a subset of viruses, traditional ferret serum HAI data. Results of this analysis for AIR hmAb array data are similar to the hierarchical clustering in Figure 2b, demonstrating the robustness of the approach. All three methods gave similar results with regard to the positioning of BR07 and PE09. VI11 and TE12 have been demonstrated to be antigenically similar when isolated from the same source⁵² and indeed clustered together on the map (albeit at different locations depending on technique). Interestingly, SW13 was found to be spaced a considerable distance from other strains, and the results are comparable between the sequence based methods and the hmAb array, but different for the ferret antisera. Differences between human and ferret immunoreactivity are known,³¹ and this observation is consistent with recent studies suggesting that ferrets are more prone than humans to immunodominance bias, reacting to a limited number of epitopes.⁵³ These data also allow for direct comparison between genetic and antigenic distance. In the cartography, the interval between grid lines corresponds to one amino acid change in the HA genetic sequence. This corresponds to five units in the antigenic distance from the hmAb array data. HAI assays measure the antibody titers against each strain, and thus the distances between antiserum-antigen pairs are averaged in determining the resolution of the map. Here, one antigenic distance unit determined by hmAb array corresponds to a four-fold dilution of the ferret antiserum in HAI assays. As a result, the cartography could be used to map the antigenic evolution. For example, although TE12 and VI11 clustered together with both approaches, the AIR approach positioned these strains closer to PE09 and further from SW13. Additionally, the distance between SW13 and TE12 determined by hmAb arrays is larger than distances determined by HAI assays and genetic sequencing (a titer difference equivalent to a more than ten-fold dilution in the cartography compared with about four to six-fold dilutions of HAI and genetic distances). Therefore, CDC criteria⁵⁴ would indicate a replacement of the vaccine for that year based on the observation of antigenic mismatch measured by hmAb arrays. Interestingly, the large antigenic distance between TE12 and SW13 is consistent with the observed low vaccine efficacy seen during the 2014–2015 season (19.8%^{12,13}); viruses emerging during this season were antigenically similar to SW13.⁵⁵

Rapid screening of broadly-reactive antibodies

The observation that several hmAbs were broadly cross-reactive to H3 strains tested (Figure 2b) suggested that additional examination of this array with other IAV subtypes would be

useful. Responses to A/WSN/1933 H1N1 and A/Shanghai/1/2013 H7N9 were quantified, and compared with the previously obtained H3 results using the same agglomerative hierarchical approach (Supplemental Figures S4b and S5). As expected, H1N1 and H7N9 strains are grouped as outliers, with a long antigenic distance from the H3 vaccine strains. Several hmAbs on the array showed moderate cross-reactivity between H1N1, H7N9, and seasonal H3 strains (Supplemental Figures S4b and S5). Clustered antigenic cartographies of these data were generated to examine relationships among hmAb responses (Figure 3a and 3b). When binding to H3 and H1 viruses are considered (Figure 3a), arrayed hmAbs form two clusters. One of these consists primarily of H3-binding hmAbs, while the other includes H1-binders and H1/H3 cross-reactive hmAbs (one cross-reactive hmAb clusters with H3). Incorporation of H7 responses (Figure 3b) reveals that most H3/H7 cross-reactive hmAbs cluster independently. Of particular interest, several hmAbs bind to all subtypes tested, and one (042–100809-2F04) responds to all strains, further highlighting the ability of the platform to rapidly identify broadly cross-reactive hmAbs.

The cluster analysis presented above is useful for examining relationships among hmAbs, but it is not convenient for assessing the behavior of individual antibodies. In order to further explore the H3N2 hmAbs that also bind H1 and H7 strains, a correlation method was developed to cluster responses for each hmAb on the microarray. Here, distances are represented by non-parametric correlation coefficients that measure the degree of proximity between each pair of hmAbs based on the antigenic binding data.³⁴ These non-parametric correlation coefficients were then grouped for visualization by applying a hierarchical clustering method as in the strain clustering maps shown in Figure 2 and Supplementary Information Figure S5. Results of the application of this method are shown in Figure 3c and 3d. In these plots, the color scale indicates the degree of correlation for antibody pairs in their antigenic response to test strains. The antigenic distance between the binding domains can be quantified by these correlation coefficients. In other words, values closer to +1 (red) indicate a high probability that the hmAbs bind antigenic domains shared by the test strains. In contrast, the deep blue zones scoring negatively in the correlations demonstrate low or “non-relatedness” of the hmAbs responding to the same strains. Data generated from H3N2 and H1N1 IAV responses are plotted in Figure 3c. Antibodies with H1 reactivity or H1/H3 cross-reactivity cluster separately from a large cluster of H3-reactive hmAbs. Within this large cluster, two major subdomains are visible. Introduction of binding responses to H7N9 (Figure 3d) causes this block to further separate. In detail, the upper left block of hmAbs that are highly correlated in the binding responses mostly bind H1N1 epitopes, and most likely target the head globular domains. The second cluster of positively correlated (red) hmAbs in Figure 3d are mostly H3/H7 cross-reactive antibodies, consistent with the cluster analysis shown in Figure 3b. These include antibodies previously shown to target residues in the head domains of H3 HA and stalk domains of H1N1 and H7N9, respectively. The third group of the hmAbs at bottom right are mostly H3 reactive antibodies directed at conserved domains across the seasonal vaccine strains. It is also clear that some hmAbs from the second red zone have more interactions with those of the third zone, highlighting a set of hmAbs with binding ability to all tested strains. Homology among these strains is primarily in the stalk domain of the HA, and therefore we can hypothesize that these hmAbs most likely bind in the stalk. These results suggest correlation heat maps may be utilized as visualized

projections of the HA antigenic structure and evolution. Further study of these antibodies in the context of vaccine development will be warranted.

Conclusions

Current strategies for preventing infection by IAV are insufficient to counteract the endless capacity of the virus to escape the human immune response. To rectify this problem, new tools for understanding antigenic relationships among historical, current, and newly emerging strains of IAV are needed to expand our understanding of the virus and accelerate development of broadly (if not universally) effective vaccines.⁵⁶ To that end, we have developed a label-free methodology employing microarrays of anti-influenza hmAbs for assessment of IAV antigenicity. Since AIR chips are compatible with a 96-well microplate format this may be considered “high throughput”; there is also sufficient space on the chip to allow expansion of the array. These “crowd on a chip” arrays are able to discriminate recombinant HAs, and is analytically well behaved, as binding by both recombinant HAs (Figure S1) and viruses (Figure 1b) follows the Langmuir isotherm. Experiments with IAVs confirm that hmAb arrays are able to distinguish between strains, even when closely related. We note that this platform has the potential for broad distribution and use: while we have not explicitly tested the limits of array shelf stability, arrays stored for a period of months at 4 °C provided the same performance as freshly printed arrays.

The patterns of hmAb reactivity provided by individual IAV strains constitute unique antigenic identifiers for the strain. In that sense, they are similar to 2-D barcodes or “Quick Response” (QR) codes widely employed as unique identifiers in commerce. Antigenic cartography using these data is complementary (but not identical) to analysis using sequence data and ferret antisera (HAI), and the AIR method potentially provides advantages over both. In addition to being a high-throughput technique, the hmAb array is able to reveal antigenic differences due to post-translational modification and contextual antigen presentation, both factors that may not be readily detectable at the genetic level. Results obtained via the hmAb array, collected from subjects with diverse immune histories, are also less likely to be affected by immunodominance bias, a known complication of analyses derived from ferret antisera.^{31,55} Previous studies based on HAI assays demonstrated that antigenic evolution was more punctuated than genetic evolution, and small genetic changes may yield a disproportionately large antigenic effect.²⁹ While our work represents a relatively small data set, this report is consistent with our results in which both genetic and antigenic evolution are mapped in the same cartography.

Conversely, array data reveals relationships among hmAbs, allows rapid identification of cross-reactive clones, and potentially highlights epitopes on HA suitable for inducing broad strain reactivity. This helps to address the challenge of preventing IAV infection: designing vaccines that induce rapid and long-lasting immunity in the face of antigenic drift and shift in virus components. Our results show that many hmAbs are grouped together with known neutralizing hmAbs in both clustered response maps and hmAb correlation heat maps. While it is tempting to extrapolate strongly correlated antibody pairs to specific HA epitopes, the complexity of antibody-antigen affinity landscapes suggests this would be unwise. However, we note that hmAbs previously shown to bind the same epitope strongly correlate in this

analysis (an example is H7 mAbs 07-d105-4B03, 07-d105-4E02, 07-d105-4D05). We anticipate that AIR hmAb arrays will also prove useful in the assessment of anti-influenza immunoreactivity in individual human serum samples; efforts along these lines are in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This project has been funded with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under CEIRS Contract No. HHSN272201400005C.

References:

1. Thompson WW ; Shay DK ; Weintraub E ; Brammer L ; Cox N ; Anderson LJ JAMA 2003, 289, 179–186.12517228
2. WHO | Influenza (Seasonal) WHO. Available at: <http://www.who.int/mediacentre/factsheets/fs211/en/> (Accessed August 22, 2017).
3. Kilbourne ED Emerg. Infect. Dis 2006, 12, 9–14.16494710
4. Centers for Disease Control and Prevention (CDC) (2009) Update: infections with a swine-origin influenza A (H1N1) virus--United States and other countries. MMWR Morb. Mortal Wkly. Rep 2009, 58, 431–433.19407737
5. Molinari N ; Ortega-Sanchez IR ; Messonnier MK ; Thompson WW ; Wortley PM ; Weintraub E ; Bridges CB Vaccine 2007, 25, 5086–5096.17544181
6. Bridges CB ; Thompson WW ; Meltzer MI ; Reeve GR ; Talamonti WJ ; Cox NJ ; Lilac HA ; Hall H ; Klimov A ; Fukuda K JAMA 2000, 284, 1655–1663.11015795
7. Narrod C ; Zinsstag J ; Tiongco M Ecohealth. 2012, 9, 150–162.22395956
8. Webster RG ; Bean WJ ; Gorman OT ; Chambers TM ; Kawaoka Y Microbiol. Rev 1992, 56, 152–179.1579108
9. Grohskopf LA ; Sokolow LZ ; Broder KR ; Olsen SJ ; Karron RA ; Jernigan DB ; Bresee JS MMWR Recomm. Rep 2016, 65, 1–54.
10. Ferguson NM ; Cummings DA ; Fraser C ; Cajka JC ; Cooley PC ; Burke DS Nature 2006, 442, 448–452.16642006
11. Prevention and control of influenza: Recommendations of the Advisory Committee on Immunization Practices (ACIP). Available at: <https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5306a1.htm> (Accessed August 22, 2017).
12. Zimmerman RK Nowalk MP ; Chung J ; Jackson ML ; Jackson LA ; Petrie JG ; Monto AS ; McLean HQ ; Belongia EA ; Gaglani M ; Murthy K ; Fry AM ; Flannery B ; US Flu VE Investigators Clin. Infect. Dis 2016, 63, 1564–1573.27702768
13. Seasonal influenza vaccine effectiveness, 2005–2017. Available at: <https://www.cdc.gov/flu/professionals/vaccination/effectiveness-studies.htm> (Accessed August 22, 2017).
14. Taubenberger JK ; Kash JC Cell Host Microbe 2010, 7, 440–451.20542248
15. Belshe RB ; Gruber WC ; Mendelman PM ; Cho I ; Reisinger K ; Block SL ; Wittes J ; Iacuzio D ; Piedra P ; Treanor J ; King J ; Kotloff K ; Bernstein DI ; Hayden FG ; Zangwill K ; Yan L ; Wolff MJ Pediatr. 2000, 136, 168–175.
16. Ohmit SE ; Victor JC ; Rotthoff JR ; Teich ER ; Truscon RK ; Baum LL ; Rangarajan B ; Newton DW ; Boulton ML ; Monto AS N. Engl. J. Med 2006, 355, 2513–2522.17167134
17. Hillman MR Vaccine 2002, 20, 3068–3087.12163258

18. WHO | WHO Global Epidemiological Surveillance Standards for Influenza WHO. Available at: http://www.who.int/influenza/resources/documents/influenza_surveillance_manual/en/ (Accessed August 22, 2017).
19. Ginsberg J ; Mohebbi MH ; Patel RS ; Brammer L ; Smolinski MS ; Brilliant L *Nature* 2009, 457, 1012–104.19020500
20. Gerhard W ; Mozdzanowska K ; Zharikova D *Emerg. Infect. Dis* 2006, 12, 569–574.16704803
21. Lambert LC ; Fauci AS N. *Engl. J. Med* 2010, 363, 2036–2044.21083388
22. Katz JM ; Hancock K ; Xu X *Expert. Rev. Anti. Infect. Ther* 2011, 9, 669–683.21692672
23. Lebarbenchon C ; Brown JD ; Luttrell MP ; Stallknecht DE J. *Vet. Diagn. Invest* 2012, 24, 161–165.21908267
24. Pedersen JC *Methods Mol. Biol* 2014, 1161, 11–25.24899416
25. Nelson MI ; Edelman L ; Spiro DJ ; Boyne AR ; Bera J ; Halpin R ; Ghedin E ; Miller MA ; Simonsen L ; Viboud C ; Holmes EC *PLOS Pathog.* 2008, 4, e1000133.18725925
26. Ghedin E ; Laplante J ; DePasse J ; Wentworth DE ; Santos RP ; Lepow ML ; Porter J ; Stellrecht K ; Lin X ; Operario D ; Griesemer S ; Fitch A ; Halpin RA ; Stockwell TB ; Spiro DJ ; Holmes EC ; St George K J. *Infect. Dis* 2011, 203, 168–174.21288815
27. Glaser L ; Stevens J ; Zamarin D ; Wilson IA ; Garcia-Sastre A ; Tumpey TM ; Basler CF ; Taubenberger JK ; Palese P J. *Virol* 2005, 79, 11533–11536.16103207
28. Xu L ; Bao L ; Lv Q ; Deng W ; Ma Y ; Li F ; Zhan L ; Zhu H ; Ma C ; Qin C *Virol. J* 2010, 7, 325.21083933
29. Smith DJ ; Lapedes AS ; de jong JC ; Bestebroer TM ; Rimmelzwaan GF ; Osterhaus ADME ; Fouchier RAM *Science* 2004, 305, 371–376.15218094
30. Igarashi M ; Ito K ; Yoshida R ; Tomabechi D ; Kida H ; Takada A *PLOS ONE* 2010, 5, e8553.20049332
31. Li Y ; Myers JL ; Bostick DL ; Sullivan CB ; Madara J ; Linderman SL ; Liu Q ; Carter DM ; Wrammert J ; Esposito S ; Principi N ; Plotkin JB ; Ross TM ; Ahmed R ; Wilson PC ; Hensley SE J. *Exp. Med* 2013, 210, 1493–1500.23857983
32. Wrammert J ; Smith K ; Miller J ; Langley T ; Kokko K ; Larsen C ; Zheng NY ; Mays I ; Garman L ; Helms C ; James J ; Air GM ; Capra JD ; Ahmed R ; Wilson PC *Nature* 2008, 453, 667–671.18449194
33. Henry DCJ ; Leon PE ; Kaur K ; Tan GS ; Zheng NY ; Andrews S ; Huang M ; Qu X ; Huang YP ; Salgado-Ferrer M ; Ho IY ; Taylor W ; Hai R ; Wrammert J ; Ahmed R ; Garcia-Sastre A ; Palese P ; Krammer F ; Wilson PC J. *Clin. Invest* 2015, 125, 1255–1268.25689254
34. Zand MS ; Wang J ; Hilchey S *Math. J* 2015, 17, doi:10.3888/tmj.
35. Anderson CS ; DeDiego ML ; Thakar J ; Topham DJ *PLOS ONE* 2016, 11, e0160510.27494186
36. DeDiego ML ; Anderson CS ; Yang H ; Holden-Wiltse J ; Fitzgerald T ; Treanor JJ ; Topham DJ *Immunology* 2016, 148, 160–173.26854888
37. Wrammert J ; Koutsonanos D ; Li GM ; Edupuganti S ; Sui J ; Morrissey M ; McCausland M ; Skountzou I ; Hornig M ; Lipkin WI ; Mehta A ; Razavi B ; Del Rio C ; Zheng NY ; Lee JH ; Huang M ; Ali Z ; Kaur K ; Andrews S ; Amara RR ; Wang Y ; Das SR ; O'Donnell CD ; Yewdell JW ; Subbarao K ; Marasco WA ; Mulligan MJ ; Compans R ; Ahmed R ; Wilson PC J. *Exp. Med* 2011, 208, 181–193.21220454
38. Mace CR ; Topham DJ ; Mosmann TR ; Quataert SA ; Treanor JJ ; Miller BL *Talanta* 2011, 83, 1000–1005.21147350
39. Bucukovski J ; Latorre-Margalef N ; Stallknecht DE ; Miller BL *PLOS ONE* 2015, 10, e0134484.26241048
40. Mace CR ; Striemer CC ; Miller BL *Anal. Chem* 2006, 78, 5578–5583.16878898
41. Sriram R ; Yadav AR ; Mace CR ; Miller BL *Anal. Chem* 2011, 83, 3750–3757.21517019
42. Carter JA ; Mehta SD ; Mungillo MV ; Striemer CC ; Miller BL *Analysis of inflammatory biomarkers by arrayed imaging reflectometry. Biosens. Bioelectron* 2011, 26, 3944–3948.21474297
43. Carter JA ; Triplett E ; Striemer CC ; Miller BL *Biosens. Bioelectron* 2016, 77, 1–6.26385730
44. Nogales A ; Baker SF ; Domm W ; Martínez-Sobrido L *Virus Res.* 2016, 216, 26–40.26220478

45. Schneider CA ; Rasband WS ; Eliceiri KW ; NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 2012, 9, 671–675.22930834
46. Johnson SC *Psychometrika* 1967, 2, 241–254.
47. Henry DCJ ; Leon PE ; Kaur K ; Tan GS ; Zheng NY ; Andrews S ; Huang M ; Qu X ; Huang YP ; Salgado-Ferrer M ; Ho IY ; Taylor W ; Hai R ; Wrammert J ; Ahmed R ; Garcia-Sastre A ; Palese P ; Krammer F ; Wilson PC *J. Clin. Invest* 2015, 125, 1255–1268.25689254
48. Ren X ; Yang F ; Hu Y ; Zhang T ; Liu L ; Dong J ; Sun L ; Zhu Y ; Xiao Y ; Li L ; Yang J ; Wang J ; Jin Q *Emerg. Infect. Dis* 2013, 19, 1881–1884.24206919
49. Yang H ; Carney PJ ; Chang JC ; Villanueva JM ; Stevens JJ *Virology* 2013, 87, 12433–12446.
50. Lapedes A ; Farber RJ *Theor. Biol* 2001, 212, 57–69.
51. Anderson CS ; Ortega S ; Chaves FA ; Clark AM ; Yang H ; Topham DJ ; DeDiego ML *Sci. Rep* 2017, 7, 14614.29097696
52. Barr IG ; Russell C ; Besselaar TG ; Cox NJ ; Deniels RS ; Donis R ; Engelhardt OG ; Grohmann G ; Itamura S ; Kelso A ; McCauley J ; Odagiri T ; Schultz-Cherry S ; Shu Y ; Smith D ; Tashiro M ; Wang D ; Webby R ; Xu X ; Ye Z ; Zhang W *Vaccine* 2014, 32, 4713–4725.24582632
53. Fonville JM Fraaij PLA ; de Mutsert G ; Wilks SH ; van Beek R ; Fouchier RAM ; Rimmelzwaan GF *J. Infect. Dis* 2016, 213, 31–38.26142433
54. Use of antigenic characterization in the selection of viruses for seasonal flu vaccine: <https://www.cdc.gov/flu/professionals/laboratory/antigenic.htm> (accessed December 15, 2017).
55. Xi H ; Wan XF ; Ye Z ; Plant EP ; Zhao Y ; Xu Y ; Li X ; Finch C ; Zhao N ; Kawano T ; Zoueva O ; Chiang MJ ; Jing X ; Lin Z ; Zhang A ; Zhu Y *Sci. Rep* 2015, 5, 15279.26472175
56. Paules CI ; Marston HD ; Eisinger RW ; Baltimore D ; Fauci AS *Immunity* 2017, 47, 599–603.29045889

Figure 1:

(a) Discrimination of A/mallard/Netherlands/12/2000 H7N3 and A/WSN/1933 H1N1 using an 85-plex array of hmAbs including anti-H1 and anti-H3 mAbs directed against seasonal influenza vaccines (white outline), anti-H7 isolated in early stages post-vaccination (light blue), and anti-H7 isolated after 105 days (burnt orange). Note that additional exposures were employed to mitigate spot intensity saturation. Titration experiments with A/mallard/Netherlands/12/2000 H7N3 shown in (b) were used to determine the virus concentration yielding the broadest spread in antibody signals. (c) The 90-plex array is able to discriminate A/Anhui/1/2013 H7N9 and A/Shanghai/1/2013 H7N9. Antibody responses (reflection change relative to the controls) are plotted. Boxed areas indicate antibodies primarily responsive to A/Shanghai/1/2013 H7N9. Colored dots correspond to antibodies with known epitopes, shown in the X-ray crystal structure of A/Shanghai/1/2013 H7N9 HA (PDB: 4LN3).

Figure 2:

(a) Images from exposure of 115-plex AIR microarrays to H3N2 vaccine strains. (b) Hierarchical cluster map of relative hmAb responses and antigenic similarity determined from clustering. (c) Antigenic cartography derived from hmAb array data (red), and compared with sequence data (black) and selected HAI data from ferret sera (blue).

Figure 3:

Antigenic cartographies (a), (b) and correlation cluster analyses (c), (d) reveal cross-reactive hmAbs. (a) Antigenic clusters of hmAb responses to seasonal H3N2 vaccine strains and H1N1. (b) Antigenic clusters of hmAb responses to H3N2 vaccine strains, H1N1 and H7N9 viruses. Red ellipses in (a) and (b) indicate clusters derived via Kmeans testing. (c) Correlation map of hmAb responses for seasonal H3N2 vaccine strains and H1N1. (d) Correlation map of hmAb responses for seasonal H3N2 vaccine strains, H1N1, and H7N9 viruses. Color bars to the right of plots in (c) and (d) indicate hmAb binding as: H1 (dark grey), H1/H3 cross-reactive (light grey), H3 (yellow), H3/H7 cross-reactive (orange), H1/H3/H7 cross-reactive (green), weak response (white). A detailed key to the hmAbs in (c) and (d) is provided in supplementary information Figure S6.