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Proteomic Assessment of Humoral Immune Responses in Smallpox Vaccine Recipients

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Abstract

The availability of effective smallpox vaccines was a critical element of the successful eradication of smallpox in 1980. Antibody responses play a primary role in protective immunity and neutralizing antibody is an established correlate of protection against smallpox. In this study we used a poxvirus proteome array to assess the antibody response to individual viral proteins in a cohort of 1,037 smallpox vaccine recipients. Several statistically significant differences were observed in the antibody response to immunodominant proteins between men and women, including B5R—a major target of neutralizing antibody in vaccinia immune globulin, and the membrane proteins D8L and A27L, both of which have been used as vaccine antigens providing protection in animal models. We also noted differences across racial/ethnic groups. In this cohort, which consisted of both ACAM2000 and Dryvax recipients, we noted minute differences in the antibody responses to a restricted number of viral proteins, providing additional support for the use of ACAM2000 as a replacement smallpox vaccine. Furthermore, our data indicate that poxvirus proteome microarrays can be valuable for screening and monitoring smallpox vaccine-induced humoral immune responses in large-scale serologic surveillance studies and prove useful in the guidance of developing novel smallpox candidate vaccines.

Keywords

Smallpox; Smallpox Vaccine; Immunity; Humoral; Antibodies; Neutralizing; Microarray Analysis

Introduction

Smallpox is the only human disease to have been eradicated. The eradication was made possible through a concerted, worldwide effort in surveillance and immunization campaigns,

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the lack of an animal reservoir for disease, as well as the availability of a temperature-stable, easily administered, effective vaccine containing live vaccinia virus (VACV) that induces cross-protective immunity against a broad range of poxviruses. The vaccine elicits strong humoral and cellular immunity in >97% of recipients; immune responses are detectable decades after immunization, and protection against disease lasts at least 5 years.¹ Humoral immunity is believed to play a major role in protection and elimination of the virus from the host and neutralizing antibody (Ab) titer was identified as a correlate of protection during the eradication campaign.^{2, 3, 4} Data from more recent animal studies and clinical vaccine studies in humans also support the critical role of antibody responses in protection against poxvirus disease.^{5, 6, 7, 8} Poxviruses are dsDNA viruses with large genomes containing >200 open reading frames (ORFs). Infected cells produce two major forms of viral particles: 1) the intracellular mature virion (MV); and 2) the extracellular virion (EV), which contains one additional membrane and several unique proteins and is released from the surface of infected cells, mediating long-distance viral spreading within the infected host.⁹ MVs are the most abundant virion form produced, are environmentally stable, and are believed to be responsible for host-to-host transmission. The MV and EV virions differ antigenically and immune responses targeting both particles are believed to provide superior protection,^{10, 11, 12, 13, 14} although some EV proteins can be removed without compromising protection in animal models.¹⁵

Proteome microarrays containing the complete set of proteins for a given pathogen enable researchers to comprehensively screen the humoral response against that pathogen. An orthopox array was initially described in 2004.¹⁶ This array has been used to do the following: screen the diversity of humoral immune responses in Dryvax recipients;^{17, 18} evaluate vaccinia immune globulin (VIG) preparations;¹⁶ identify major targets of humoral immunity;¹⁶ compare modified vaccinia Ankara (MVA) with Dryvax in animal models and human vaccine recipients;^{19, 20} evaluate Dryvax, ACAM2000, and IMVAMUNE smallpox vaccines and monkeypox virus challenge;²¹ and assess antigenic differences in between MV and EV virions.²²

In the current study we examined a cohort of 1,037 smallpox vaccine recipients in order to further characterize the breadth and depth of the humoral responses against vaccinia virus. We identified a number of viral proteins where the level of antibody (Ab) correlated with neutralizing antibody titer and/or cellular cytokine responses. Furthermore, we investigated race, ethnicity, and sex differences in antibody response following vaccination.

Methods

Subject Recruitment

The study cohort, which is a subset of a previously described cohort, consisted of 1,037 armed forces personnel who were recruited from 2011–2013.²³ The individuals in this report were 18–40 years old and in good general health. 914 of the subjects had received one and only one dose of ACAM2000® 1 month–4 years prior to enrollment, while 123 subjects had received one and only one dose of Dryvax® in the same time period prior to enrollment. Subjects were recruited from Fort Bliss, Fort Campbell, Fort Lewis, and the Naval Health Research Center (NHRC) in San Diego, CA. The Institutional Review Boards

for both the Mayo Clinic and NHRC approved all study procedures and written informed consent was obtained from each participant upon enrollment. Subjects underwent a single blood draw. Whole blood was collected in red-top collection tubes without anticoagulant. After incubation for at least 30 minutes at room temperature, serum was collected by centrifugation, aliquoted and stored at -80°C until use.

Neutralization assay

We used a vaccinia-specific neutralizing antibody assay developed by the Food and Drug Administration (FDA) and optimized for high throughput in our laboratory as previously described.^{24, 25} The vSC56 strain of vaccinia virus expressing β -galactosidase (obtained from Dr. Bernard Moss, National Institutes of Allergy and Infectious Diseases, NIAID) was grown, purified, and titered according to established protocols.^{26, 27} Serum dilutions were incubated with a set quantity of VACV-vSC56 for 1 hour, the mixture was added to HeLa cells overnight. Cells were lysed (Igepal CA630, Sigma-Aldrich, St. Louis, MS) and beta-galactosidase activity measured by the addition of a colorimetric substrate (CPRG, Calbiochem, San Diego, CA). The reaction was quenched with Na_2CO_3 and plates were read with a ThermoMax plate reader (Molecular Devices, Sunnyvale, CA). Sera were tested at least three times (Dryvax cohort) or twice (ACAM2000 cohort), and results were defined as the serum dilution that inhibits 50% of virus activity (ID_{50}).^{24, 25}

Orthopox pathogen array

Serum samples were used to probe Orthopox Full Proteome Microarrays in duplicate (Antigen Discovery; Irvine, CA) using the manufacturer's established protocols.²⁰ Briefly, serum samples were incubated on a protein microarray to allow VACV-specific antibodies to bind to the immobilized poxvirus proteins. Fluorochrome-labeled secondary reagents were used to detect the presence of bound serum antibody. Plates were scanned and fluorescent intensity values were used to determine the quantity of antibody specific for each protein on the array. Values are presented in fluorescence units: "Median Normalized Antibody Measurements". The proteome microarray contained 246 proteins, 10 replicate spots coated with human IgG as internal positive controls, and negative controls - 'noDNA' spots comprising *in vitro* transcription/translation reaction products lacking template DNA.

Cytokine measurements—Cytokine responses to VACV were evaluated as previously described.²³ Briefly, commercial ELISAs were used to measure cytokines present in cell culture supernatants following *in vitro* virus stimulation. Results are reported as pg/mL.

Statistical Analysis

For each sample, the median value of the 12 noDNA spots was calculated and the median of the noDNA spots was subtracted from each protein for the sample. After the control subtraction, a protein was considered a positive target of humoral immune responses if more than 50% of subjects in either vaccine group had detectable antibody response to that protein. The data were normalized using the Variance Stabilization and Calibration for Microarray Data method.²⁸ This method introduces a transformation for the raw intensity values and an estimate of the variance that is constant across the intensity range. This allows one to calibrate the data, measure differences, and quantify measurement error. Penalized

elastic-net logistic regression was used to identify predictors of high vs. low antibody response in the 194 ACAM2000 recipients who had the highest and lowest neutralizing antibody titers. Ten proteins were excluded from the model to help reduce noise, using Harrell's redundancy analysis method.²⁹ The model was chosen by selecting the penalty parameter " λ " utilizing 10-fold cross validation. This method selects a range of λ values, and then fits a model to 90% of the data, applies the model to the remaining 10% of the data and calculates the number that are not classified correctly, this is repeated 10 times, holding out a different 10% of the data. The λ value is selected as the one that minimizes the classification error.³⁰ The predictive accuracy of the model is the area under the ROC for the elastic-net regression model. Specific statistical tests for comparisons are documented in each table or figure. Time since vaccination was used as a controlling variable in the linear models, with vaccine as the predictor, i.e. $\log(\text{neut}) = \text{ACAM2000} + \log(\text{Time.Since.Vacc})$.

Results

Cohort characteristics

We screened sera samples from a cohort of first-time smallpox vaccine recipients in order to characterize proteome-wide antibody responses following smallpox vaccination. Overall, the cohort had a median neutralizing antibody titer of 113.5 ID₅₀ (serum dilution inhibiting 50% of viral activity), with a substantial amount of inter-individual variation as evidenced by the interquartile range of responses (ID₅₀: 62.6 – 189.8). Comparisons between ACAM2000 recipients and Dryvax recipients did not identify any significant differences between cohorts in terms of sex, race, ethnicity, or neutralizing antibody titer (ID₅₀). These comparisons are outlined in Table 1.

Comparison between ACAM2000 and Dryvax.

Our study cohort was comprised of individuals vaccinated with two different smallpox vaccines, providing an opportunity to directly compare Dryvax recipients (n=123) with ACAM2000 recipients (n=914). The demographics for each sub-cohort are presented in Table 1 and demonstrate that neutralizing antibody titers are 8.8% higher in the ACAM2000 recipients, but the difference was not significant. However, linear modeling of neutralizing antibody titer as a function of vaccine received, after correcting for time since vaccination, indicated that this difference was not statistically significant (p=0.23). When we compared these vaccine groups by individual protein array response, we noted that antibody responses to five proteins exhibited significant differences between ACAM2000 and Dryvax vaccine recipients (Supplemental Table 1 and Supplemental Figure 1: Wilcoxon Rank-Sum test). Protein-specific antibody responses were higher in ACAM2000 recipients to the A13L (1.3% higher, p=0.00031), WR148 (0.5% higher, p=0.019), D13L (0.3% higher, 0.024), and B5R (0.3% higher, p=0.035) proteins and with slightly lower responses to D8L (6.4% lower, p=0.0018).

Correlation between neutralizing antibody titers and the humoral response to individual proteins

Within the cohort, only a portion of the viral proteome was targeted by humoral immune responses. We divided the cohort by vaccine received and calculated the percent of subjects

with a positive protein antibody reading (i.e., above background) within each group (Table 2). A protein was considered a positive target of humoral immune responses if more than 50% of subjects in either vaccine group had detectable antibody response to that protein. These 28 positive proteins and an additional 11 proteins (i.e., A10L, A17L, A25L, A33R, A34R, B2R, B5R, E2L, F13L, L1R, L4R) found to be targets of neutralizing antibody in previously published reports were selected for further investigation. The relative magnitude of the immune response to each of these selected proteins is illustrated in Figure 1. A number of proteins were targeted at different rates in the two vaccine groups however, these were among the least immunogenic proteins where responses were barely above background and the differences are likely due to variation in the assay.

Our data indicated that antibody levels to 22 viral proteins were significantly correlated with neutralizing antibody titer to MV (Table 3), suggesting that these proteins may be targets for neutralization.²⁵ Note that four of the proteins in Table 3 (A33R, A34R, A56R, and B5R) are expressed on EV and not on MV. The strongest correlations were found with abundant membrane and structural proteins. From within the cohort, we selected the 194 ACAM2000 recipients with the highest 10% and lowest 10% of neutralizing antibody titers and performed regression modeling (elastic-net models) in order to determine whether or not antibody responses to a set of viral proteins could predict humoral immunity following vaccination. The modeling identified a set of viral proteins prominently on MV virions and expressed late in infection as targets of protein-specific antibody responses that collectively could predict individuals in the high and low neutralizing antibody titer groups a predictive ability of 84%. (Figure 2). Note that the antibody response to these proteins have variable correlations with one another (Supplemental Figure 2). These results largely overlap with the 22 proteins previously found to correlate with neutralizing antibody (Table 3). The exceptions include: A9L, WR218, F7L, WR146, A4L, C5L, and WR169, which are predominantly membrane and structural proteins.

Race and sex

The regression model also included sex, a result consistent with prior studies we and others have published on the known effect of sex on humoral responses to vaccines.^{31, 32, 33, 34, 35, 36, 37} When we compared antibody responses to individual proteins between men and women in the entire cohort, we identified five viral proteins in which antibody titers differed significantly by sex (Figure 3). We also examined potential differences in antibody responses to each protein due to race and/or ethnicity (Table 4). The proteins differentially targeted by race/ethnicity comprise both EV and MV proteins, membrane proteins, structural proteins, and secreted proteins. Compared to responses in Caucasian subjects, the consistent finding was that African-American subjects exhibited a trend to lower responses. Compared to non-Hispanic subjects, Hispanic subjects exhibited higher responses to D13L, WR148, A17L, and WR169.

Correlation with cellular cytokine responses

We compared the humoral response to individual viral proteins with the cytokine response upon *in vitro* stimulation of each subject's PBMCs with VACV.(ref 17) A number of significant associations were identified (Table 5). Each cytokine was associated with an

overlapping but not identical set of protein-specific antibody responses. Antibody responses to A27L, A33R, and D8L were associated with both IL-2 and IFN γ secretion, while the antibody response to the L1R protein (an envelope protein required for cellular entry)³⁸ was negatively associated with TNF α , IL-1 β , and IL-12p40 production. Of note, a large number of protein-specific antibody response associations were observed with IFN γ and these matched the list of protein-specific antibody responses with the strongest associations with neutralizing antibody.

Discussion

Our study examined antibody responses targeting specific vaccinia virus proteins in a large cohort of smallpox vaccine recipients. In terms of viral proteins targeted by humoral immune responses to the vaccine, our data were largely similar to what has been previously reported.^{17, 20, 21} Protein-specific antibody responses in our cohort targeted core vaccinia virus proteins (A4L, H5R, I1L), proteins found in mature virions (A9L, A13L, A27L, D8L, H3L), extracellular enveloped virion proteins (A33R, A34R, A56R, B5R, and F13L), immunomodulatory proteins (WR001, WR218, E3L, N1L) and hypothetical proteins (B20R, C5L, WR169, WR214). These targeted proteins included proteins expressed both early and late in the viral life cycle. Interestingly, our cohort did not respond strongly to several proteins previously described^{17, 20, 21} as commonly targeted by neutralizing antibodies. These included B5R, the major target of the neutralizing activity of vaccinia immune globulin³⁹ that is known to contain multiple neutralization epitopes.⁴⁰ Interestingly, titers of B5R-specific antibody were low and only 11.7% of our cohort had detectable responses to B5R – potentially due to the lack of proper folding after *in vitro* transcription and translation on the microarray. The WR148 (VACV: A26L) protein is expressed on MV and binds to laminin,⁴¹ is another protein reported to be widely recognized,^{17, 20, 21} and was not a major target in our cohort. These differences may reflect the nature of the protein antigens used in the array (expressed using an *in vitro* E. coli-based transcription/translation system) with possible loss of conformational epitopes found in the native context of viral particles.

Neutralizing antibody titer has served as a correlate of protection against poxvirus infection and previous studies have shown that antibody titers to specific poxvirus proteins exhibit strong correlations with plaque reduction neutralization assay titers. Davies *et. al.* reported that the average antibody signal across all proteins correlated with neutralizing antibody titer as measured by plaque reduction neutralization tests ($r^2=0.54$).¹⁷ When we examined the correlation between protein-specific antibody levels and neutralizing antibody titer, we identified a large number of individual viral proteins whose antibody levels were significantly correlated with neutralizing antibody titer (Table 3). These include the following: H3L, the MV heparin binding protein; D8L, the MV membrane protein that binds chondroitin sulfate; A14L, an MV membrane phosphoprotein; A27L the MV attachment and fusion protein;⁴² I1L, a telomere-binding protein required for production of mature virions;⁴⁴ and A10L, a major core protein.⁴⁵ the viral hemagglutinin protein A56R that also functions as a virulence factor;⁴⁶ H6R, a DNA topoisomerase required for both RNA transcription and DNA replication;⁴⁷ A17L, a viral membrane protein found on the intracellular mature virus that participates in viral assembly and morphogenesis;⁵⁰ and D13L, a scaffold protein important for the structural integrity of viral membranes.⁵¹ The

majority of these proteins are structural or membrane proteins and four of the five with the strongest correlation were membrane components of MV (i.e., H3L, A14L, D8L, A27L) with relatively high expression in infected cells and known roles in viral adhesion, binding, and/or entry. H3L is recognized as an important target of neutralizing antibody response, associated with protection in animal models on poxvirus infection.⁵² These results are not surprising, given that both vaccines consist almost entirely of MV, and the majority of the virus produced following immunization is also MV. Interestingly, neutralizing antibody titers also correlated with protein-specific antibody responses to several expressed on EV, despite the fact that the overwhelming majority of the viral particles present in our neutralization assay were MV. These proteins included: A33R is also a major target of antibody responses and can serve as a protective immunogen;⁵³ B5R, a glycosylated membrane protein involved in release of EV from the cell;^{43, 54, 55} and A33R, a membrane glycoprotein found on extracellular virions that forms a complex with other membrane proteins (A34R, A36R, and B5R) enabling viral budding from the plasma membrane and cell to cell spread;^{48, 49} It is possible that the correlations noted in Table 3 simply reflect the fact that these EV proteins are highly immunogenic structural proteins expressed at levels similar to that of highly immunogenic structural proteins on MV virions. Of note, the protein with the fifth-strongest correlation, the EV protein B5R, was recognized by relatively few members of our cohort, which may reflect the fact that neutralizing antibodies to this protein are targeting highly conformational epitopes and are under-represented with the expression system used in the poxvirus antigen array.

The results from our regression modeling identified a collection of viral proteins for which antibody responses were predictive of neutralizing antibody titer. The four proteins with positive regression coefficients (A27L, B5R, A14L, and I1L) are each highly correlated with neutralizing antibody titer (Table 3). It remains to be determined why antibody responses to the other 7 proteins (A9L, WR218, F7L, WR146, A4L, C5L, WR169) have a negative impact on neutralizing antibody titer. One potential application for these results would be the development of a diagnostic test utilizing a subset of the proteins in order to predict protective efficacy in vaccine recipients.

We also assessed correlations between antibody titers to individual proteins and cellular immune responses. Interestingly, the protein-specific antibody responses that correlated with IFN γ production were exactly the same as those that were correlated with neutralizing antibody titers, suggesting that the two arms of immunity (i.e., cellular and humoral) are tightly linked for poxviruses. Some of the same protein-specific Ab responses were also observed with IL-2 (A27L, D8L, and A33R) but the correlations were smaller and the strength of the associations was considerably weaker. Antibody responses to several viral proteins were similar between TNF α , IL-12b, and IL-2 (Table 5) and we have previously reported a strong correlation between pro-inflammatory cytokines in smallpox vaccine recipients.⁵⁶ Regarding the linkage between humoral and cellular immune responses, Sette *et al.* have previously reported on a structural linkage between CD4 $^{+}$ T cell epitopes and antibody epitopes.⁵⁷ In that report, T cell help was only available when the T cell and B cell epitopes came from the same protein. This represents one possible mechanism to explain our results, but confirmation of this would require additional investigations. It should be noted that CD8 $^{+}$ T cells preferentially recognize proteins expressed early in infection with

less frequent targeting of structural proteins, while CD4⁺ T cells preferentially target late antigens as well as structural proteins,^{58, 59, 60, 61} and are therefore more likely to share epitopes with proteins targeted by antibody responses.

With regard to sex, we have previously reported that women in our study cohort had higher neutralizing antibody titers than men (median ID₅₀ 144 vs 110, p=0.024).²³ When we examined the antibody response to individual proteins, we found that men had statistically significant higher responses to five proteins (Figure 3), however the differences were extremely small (L1R: 0.4% increase, L4R: 0.28% increase, A12L: 0.19% increase, C5L: 0.11% increase) and a 0.49% decrease in the WR169-specific antibody response. These findings suggest that the prior reported sex differences in the overall antibody response to smallpox vaccination reflect small differences in response across most proteins rather than large differences in antibody titers to a subset of proteins. Another possible explanation is that neutralizing antibodies (found to be more prevalent in women)³² are targeting mostly conformational epitopes that are not readily detected on the proteome array chip. Interestingly, studies with IMVAMUNE, an MVA-based non-replicating smallpox vaccine, have reported higher antibody titers (ELISA, not neutralizing Ab) in men.⁶²

Our study cohort had sufficient racial and ethnic diversity to examine differential antibody responses to individual viral proteins between racial/ethnic groups. Our findings suggested that the Caucasian subjects had potentially stronger humoral responses than African-American subjects to 18 viral proteins (with a wide array of expression kinetics, and functional category [membrane, structural, secreted, etc.], while individuals of Hispanic ethnicity had lower antibody responses to four viral proteins: A17L and D13L, both MV membrane proteins; WR148, a lamin-binding protein found on the envelope of MV particles; and WR169, an uncharacterized protein with unknown function. We do not know if these differences, which are all relatively minor, are biologically or clinically significant; nor is it clear whether or not they would affect protective efficacy at the population level. In fact, the effectiveness of the smallpox vaccines on a global scale would argue against this. Our results are similar to what we have previously reported on race and sex-based differences in cellular immune responses to smallpox vaccines.³¹ In that study, we found that Caucasian subjects had higher IFN γ ELISPOT responses than African-American and Hispanic subjects. Caucasian subjects also secreted higher levels of IL-2 and IFN α in response to *in vitro* vaccinia stimulation of subject's PBMCs. In contrast, a study examining humoral immunity, side effects, and adverse events following smallpox vaccine response in a civilian smallpox vaccination campaign did not identify any associations between racial groups and adverse events, take rates, or antibody titer.⁶³ These disparate results may reflect differences in pre-existing immunity, differences in race/sex/ethnicity between the two cohorts, differences in baseline immunity (our cohorts were confirmed first time vaccinees), or the assays used to measure the various immune response outcomes after vaccination.

Our study does have several limitations: 1) the cohort is predominantly male; nevertheless, we were able to detect sex-specific differences in individual protein responses; 2) the Dryvax subcohort was much smaller than the ACAM2000 cohort, which limited the power to detect small effects; 3) the protein microarray uses an *in vitro* translation system that may not

fully present or display conformation epitopes targeted by some neutralizing antibodies. We found five statistically significant differences among antibody responses to individual proteins (Supplemental Table 1: A13L, D8L, WR148, D13L, B5R) between ACAM2000 and Dryvax recipients; however, the differences in magnitude were small and unlikely to be of clinical significance. The remaining 39 protein-specific antibody responses did not differ between vaccine groups, and the data provided in Figure 1 demonstrate that protein-specific antibody responses were similar between the two vaccines. Other groups have reported that the antigenic targets following vaccination with MVA and Dryvax are comparable,^{19, 20} and Townsend *et al.* found similarities between the humoral response in animal models of Dryvax, ACAM2000, and Imvamune vaccination.²¹ This matches our findings that (with only a few exceptions) antibody responses to individual viral proteins were similar in magnitude between our ACAM2000 and Dryvax recipients.

In conclusion, pathogen proteome microarrays provide a powerful tool for dissecting humoral immunity, which is increasingly important when dealing with complex pathogens such as poxviruses. Tools such as these also provide insights into humoral immune responses at the individual protein level. This finer resolution information can be valuable for understanding critical targets of humoral immunity and has implications for the development of novel vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest/Disclosures

Dr. Poland is a paid scientific advisor for Johnson & Johnson/Janssen Global Services LLC. Dr. Poland is the chair of a Safety Evaluation Committee for novel investigational vaccine trials being conducted by Merck Research Laboratories. Dr. Poland offers consultative advice on vaccine development to Merck & Co., Medicago, GlaxoSmithKline, Sanofi Pasteur, Emergent Biosolutions, Dynavax, Genentech, Eli Lilly and Company, Kentucky Bioprocessing, Bavarian Nordic, AstraZeneca, Exelixis, Regeneron, Janssen, Vynria, Moderna, and Genevant Sciences, Inc. Drs. Poland and Ovsyannikova hold patents related to vaccinia and measles peptide vaccines. Dr. Kennedy holds a patent related to vaccinia peptide vaccines. Drs. Poland, Kennedy, and Ovsyannikova have received grant funding from ICW Ventures for preclinical studies on a peptide-based COVID-19 vaccine. Dr. Kennedy has received funding from Merck Research Laboratories to study waning immunity to mumps vaccine. These activities have been reviewed by the Mayo Clinic Conflict of Interest Review Board and are conducted in compliance with Mayo Clinic Conflict of Interest policies. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and are conducted in compliance with Mayo Clinic Conflict of Interest policies.

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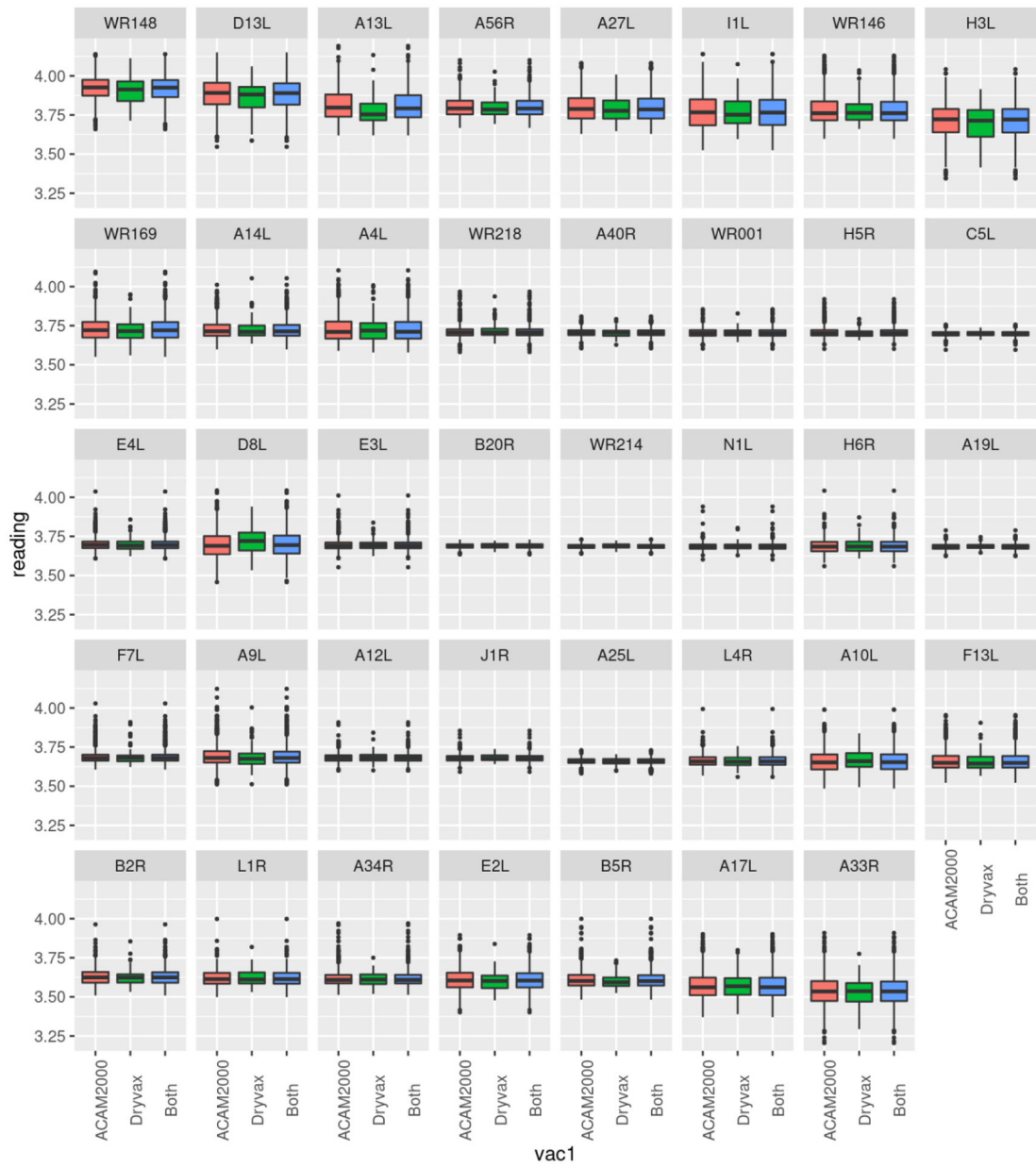


Figure 1. Median Antibody Responses for Selected VACV proteins. Each box and whisker plot provides the median normalized antibody measurements (log₂ scale) for each selected protein in ACAM2000 recipients (red), Dryvax recipients (green), or the entire cohort (blue). The top and bottom of the box represent the 75th and 25th percentile respectively, with outliers above and below the 90th and 10th percentile shown as individual points.

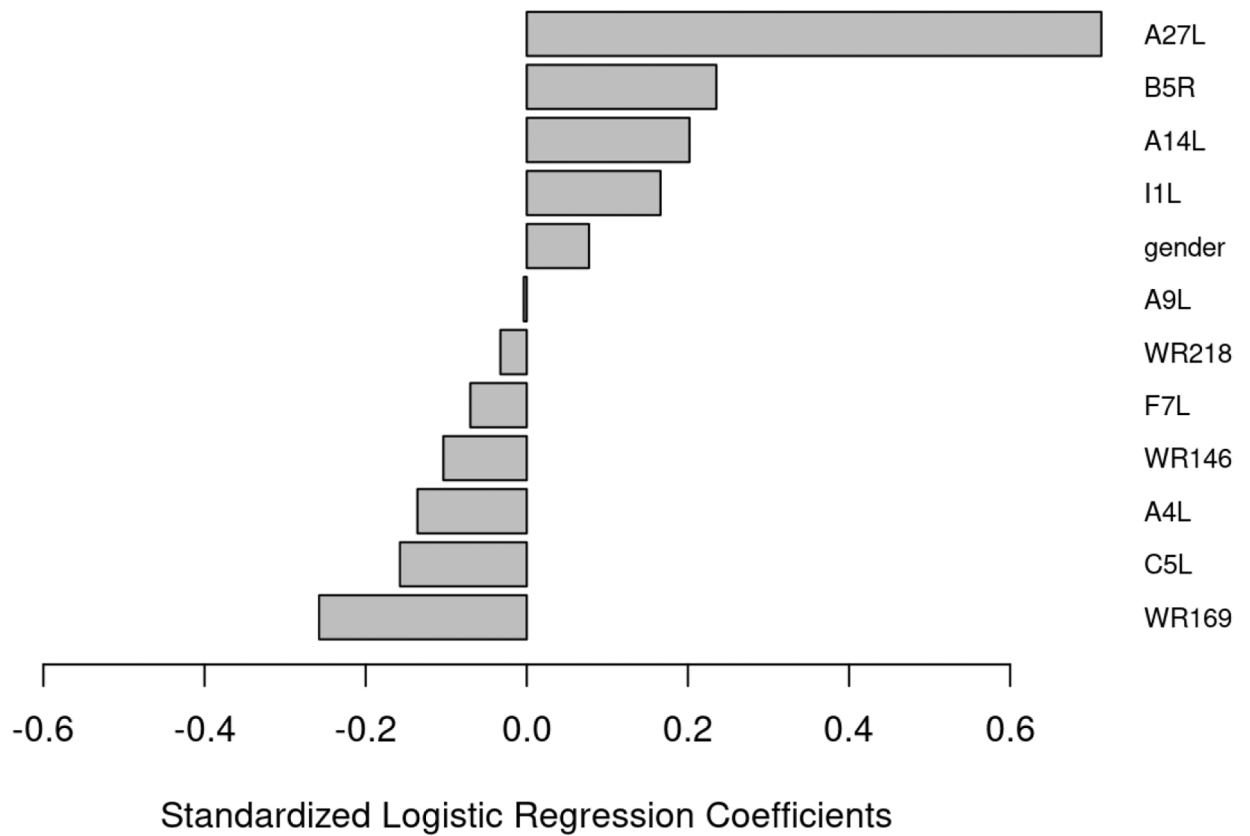


Figure 2. Logistic Regression Predictive Model of Neutralizing Antibody Response .

The bar graph depicts the regression coefficients for the variables present in the model with the lowest classification error. Providing a model to predict a vaccine recipient's neutralizing antibody titer based on the observed antibody response to specific viral proteins.

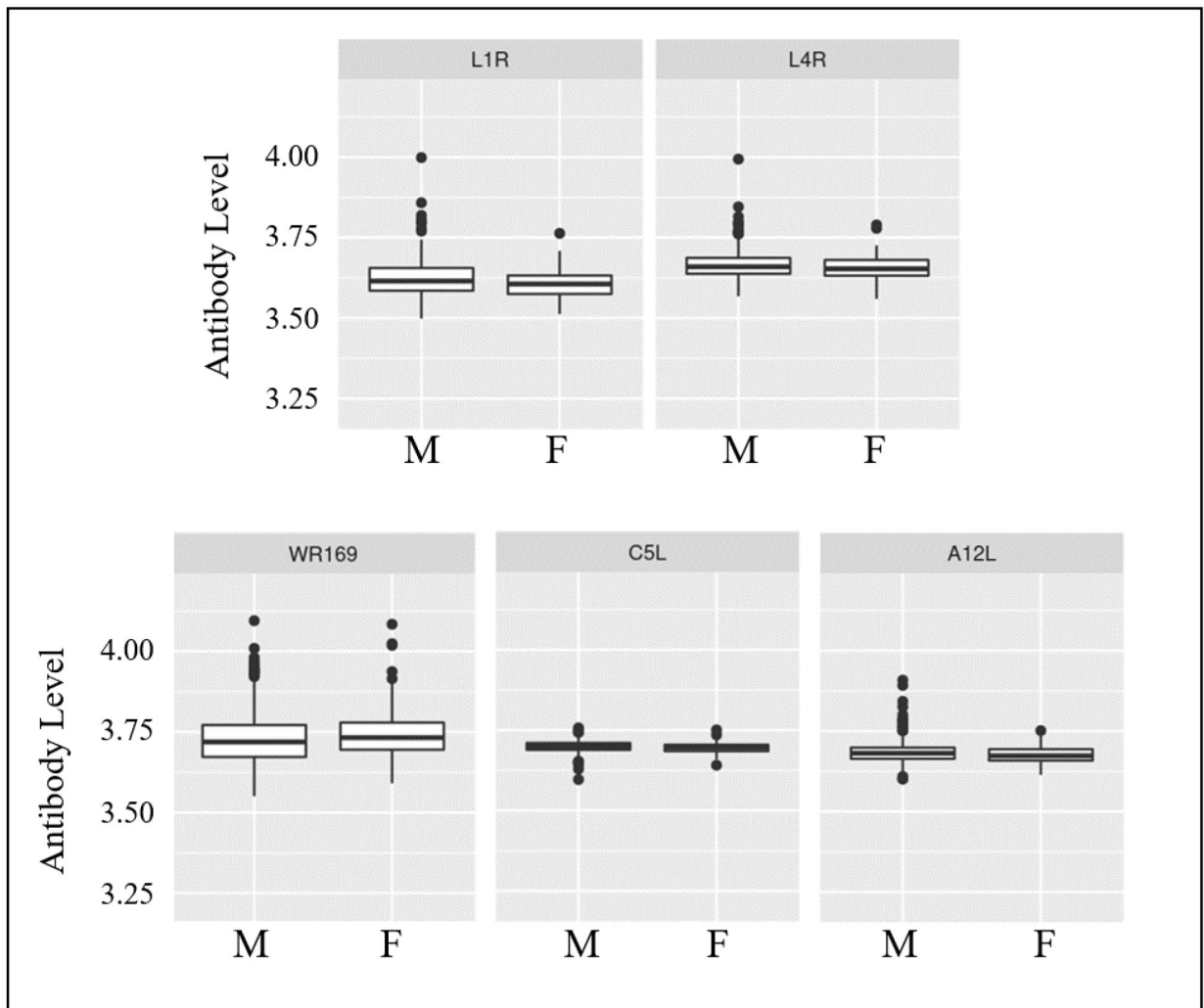


Figure 3. Sex Specific Differences in Antibody Response.

Median normalized antibody measurements for each protein (log₂ scale) are provided in the box and whisker plots. M = male, F = female. p-values calculated using the Welch Two Sample t-test.

Table 1.

Cohort Demographics

Variable	ACAM2000 (n=914)	Dryvax (n=123)	Total (n=1,037)	p-value
Sex				0.302
Female	94 (10.3%)	9 (7.3%)	103 (9.9%)	
Male	820 (89.7%)	114 (92.7%)	934 (90.1%)	
Race				0.380
American Indian/Alaska Native	26 (2.8%)	3 (2.4%)	29 (2.8%)	
Asian	16 (1.8%)	2 (1.6%)	18 (1.7%)	
Black or African American	109 (11.9%)	14 (11.4%)	123 (11.9%)	
More Than One Race	14 (1.5%)	5 (4.1%)	19 (1.8%)	
Native Hawaiian or Other Pacific Islander	10 (1.1%)	3 (2.4%)	13 (1.3%)	
Unknown	19 (2.1%)	1 (0.8%)	20 (1.9%)	
Caucasian	720 (78.8%)	95 (77.2%)	815 (78.6%)	
Ethnicity				0.362
Don't Know	9 (1.0%)	1 (0.8%)	10 (1.0%)	
Hispanic	160 (17.5%)	28 (22.8%)	188 (18.1%)	
Not Hispanic	745 (81.5%)	94 (76.4%)	839 (80.9%)	
Neutralizing Ab titer (ID₅₀)				0.491
Mean (SD)	152.7 (189.8)	140.3 (176.0)	151.2 (188.2)	
Median	117.0	98.6	113.5	
Q1, Q3	63.4, 191.4	52.8, 178.4	62.6, 189.8	
Range	10.7 – 2979.8	15.9 – 1636.1	10.7 – 2979.8	

* Difference between vaccine types were calculated using Pearson's chi-square test and one-way analysis of variance for continuous variables.

Table 2.

Percentage of Vaccinated Subjects with Antibodies Against Vaccinia Proteins

> 75% of subjects	50–75% of subjects	25–50% of subjects	< 25% of subjects			
A13L	A12L	A10L	A14.5L	B1R	F4L	WR002
A14L	A19L	A11R	A15L	B2R	F5L	WR003
A27L	A4L	A12L	A17L	B3R	F6L	WR004
A40R	A9L	A16L	A18R	B4R	F8L	WR005
A56R	B20R	A22R	A1L	B5R	F9L	WR006
C5L	D8L	A36R	A20R	B6R	G1L	WR007
D13L	E3L	A42R	A21L	B7R	G2R	WR008
H5R	E4L	A43R	A23R	B8R	G3L	WR009
I1L	F7L	A49R	A24R	C12L	G5R	WR010
WR001	H3L	A50R	A25L	C14L	G8R	WR011
WR146	H6R	A52R	A28L	C1L	G9R	WR012
WR148	J1R	B15R	A29L	C2L	H1L	WR013
WR218	N1L	B16R	A2L	C4L	H2R	WR014
	WR169	B17L	A30L	C6L	H4L	WR015
	WR214	B19R	A31R	C7L	H7R	WR016
		B9R	A32L	C8L	I2L	WR018
		C17L	A33R	D10R	I3L	WR019
		C3L	A34R	D11L	I4L	WR053.5
		E1L	A35R	D12L	I5L	WR083
		E5R	A37R	D1R	I7L	WR121
		E9L	A38L	D2L	I8R	WR149
		F11L	A3L	D3R	J2R	WR153.5
		F13L	A41L	D4R	J3R	WR161
		F14L	A44L	D5R	J4R	WR163
		F17R	A45R	D6R	J5L	WR164
		G4L	A46R	D7R	J6R	WR181.5
		G6R	A47L	D9R	K1L	WR199
		G7L	A48R	E10R	K3L	WR201
		I6L	A51R	E11L	K4L	WR204
		J1R	A53R	E2L	K5L	WR204.5
		K2L	A55R	E6R	K6L	WR204f
		L4R	A57R	E7R	K7R	WR207
		L5R	A5R	E8R	L1R	WR209
		WR036	A6L	F10L	L2R	WR210
		WR147	A7L	F12L	L3L	WR211
		WR203	A8R	F15L	M1L	WR212
		WR208	B10R	F16L	M2L	WR213
		WR220	B11R	F1L	N2L	WR215
			B12R	F2L	O1L	WR216

> 75% of subjects	50–75% of subjects	25–50% of subjects	< 25% of subjects			
			B14R	F3L	O2L	WR217

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Table 3.

Viral Proteins with Antibody Levels Significantly Correlated with Vaccinia-Specific Neutralizing Antibody Titer

Protein ID	Correlation	p-value (raw)
<i>Neutralizing Antibody (Median: 113.5, IQR: 62.6 – 189.8)</i>		
H3L	0.356	2.31E-32
A14L	0.353	1.06E-31
D8L	0.321	2.44E-26
A27L	0.319	6.52E-26
B5R	0.277	1.10E-19
I1L	0.276	1.30E-19
A10L	0.268	1.69E-18
A56R	0.233	2.68E-14
H6R	0.232	3.88E-14
A33R	0.216	1.88E-12
A17L	0.214	3.02E-12
D13L	0.214	3.73E-12
F13L	0.199	9.64E-11
WR148	0.196	1.84E-10
E2L	0.191	5.16E-10
L4R	0.187	1.23E-09
H5R	0.178	8.14E-09
A13L	0.158	3.43E-07
E3L	0.130	2.75E-05
L1R	0.112	0.00031
B2R	0.104	0.00077
A34R	0.104	0.001
A40R	0.067	0.032
WR001	0.059	0.056
E4L	0.055	0.080
A12L	0.048	0.12
F7L	0.047	0.13
C5L	0.032	0.31
N1L	0.018	0.57
WR218	0.006	0.86
WR169	0.004	0.91
A19L	-0.010	0.75
J1R	-0.014	0.66
WR214	-0.017	0.58
WR146	-0.024	0.44

Protein ID	Correlation	p-value (raw)
<i>Neutralizing Antibody (Median: 113.5, IQR: 62.6 – 189.8)</i>		
A4L	-0.035	0.27
A9L	-0.037	0.24
A25L	-0.038	0.22
B20R	-0.047	0.13

* Spearman's rank correlation

Correlations with p < 0.01 are in bold font.

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Table 4.

Race- and Ethnicity-Specific Differences in Antibody Responses

Race			
Protein	Race comparison	% Difference in Ab response	p-value*
D13L	Caucasian - Black or African American	-1.8%	2.35E-11
WR148	Caucasian - Black or African American	-1.5%	4.20E-11
A33R	Caucasian - Black or African American	-1.6%	5.98E-08
H3L	Caucasian - Black or African American	-1.7%	1.36E-07
A27L	Caucasian - Black or African American	-1.2%	1.65E-06
E2L	Caucasian - Black or African American	-1.0%	3.77E-06
A14L	Caucasian - Black or African American	-0.7%	0.00011
F13L	Caucasian - Black or African American	-0.7%	0.00019
D8L	Caucasian - Black or African American	-1.0%	0.00023
B5R	Caucasian - Black or African American	-0.7%	0.00079
A17L	Caucasian - Black or African American	-0.9%	0.0021
A56R	Caucasian - Black or African American	-0.6%	0.0021
H5R	Caucasian - Black or African American	-0.3%	0.0028
A10L	Caucasian - Black or African American	-0.7%	0.0047
A13L	Caucasian - Black or African American	-0.9%	0.0072
E3L	Caucasian - Black or African American	-0.3%	0.026
C5L	Caucasian - Black or African American	-0.1%	0.028
L4R	Caucasian - Black or African American	-0.3%	0.029
Ethnicity			
Protein	Race comparison	Difference	p-value
D13L	Non-Hispanic - Hispanic	-0.7%	0.0025
WR148	Non-Hispanic - Hispanic	-0.6%	0.0038
WR169	Non-Hispanic - Hispanic	-0.5%	0.019
A27L	Non-Hispanic - Hispanic	1.8%	0.043
A17L	Non-Hispanic - Hispanic	-0.5%	0.044

% Difference = difference in antibody response in African Americans compared to Caucasians or difference in antibody response in Hispanics compared to Non-Hispanic participants.

* p-values calculated using one-way analysis of variance. Differences with p 0.0001 are in bold font.

Table 5.

Correlations Between Antibody Responses to Individual Viral Proteins and Cellular Immune Responses

Protein	Mean reading	R	p-value	protein	Mean reading	R	p-value
<i>IFNα (Median: 184.3pg/ml, IQR: 102.4 – 284.4)</i>							
A40R	3.71	-0.09	0.003	WR214	3.69	-0.09	0.005
<i>IFNγ (Median: 274.1 pg/ml, IQR: 34.9 – 933.0)</i>							
H3L	3.71	0.17	6.7E-08	A10L	3.66	0.12	7.7E-05
D8L	3.70	0.15	2.6E-06	A13L	3.81	0.12	0.00015
A17L	3.57	0.14	4.0E-06	A14L	3.73	0.12	0.00017
B5R	3.61	0.14	5.3E-06	A56R	3.80	0.11	0.0003
A33R	3.54	0.13	2.3E-05	WR148	3.92	0.11	0.0003
D13L	3.88	0.13	5.5E-05	E2L	3.61	0.11	0.0007
A27L	3.80	0.12	7.5E-05	F13L	3.66	0.10	0.001
<i>IL-12p40 (Median: 94.4 pg/ml, IQR: 33.7 – 218.1)</i>							
L1R	3.62	-0.1	2.0E-5	J1R	3.68	0.09	0.003
A25L	3.66	0.1	0.0002				
<i>IL-1β (Median: 64.1 pg/ml, IQR: 25.2 – 160.9)</i>							
L1R	3.62	-0.09	0.002	J1R	3.68	0.08	0.009
L4R	3.66	-0.09	0.004				
<i>IL-2 (Median: 3.8 pg/ml, IQR: -11.2 – 33.8)</i>							
A27L	3.80	0.10	0.002	A33R	3.54	0.09	0.003
D8L	3.70	0.10	0.002				
<i>IL-6 (Median: 1,661.2 pg/ml, IQR: 547.3 – 3,439.7)</i>							
F7L	3.69	-0.12	0.0002				
<i>TNFα (Median: 115.5 pg/ml, IQR: 50.8 – 259.6)</i>							
L1R	3.62	-0.17	7.9E-9	H6R	3.69	-0.10	0.002
B2R	3.63	-0.14	9.0E-6	B20R	3.69	0.09	0.004
L4R	3.66	-0.13	1.7E-5				

R=spearman correlation