

shev's Inequality p-value less than 0.00502. For cluster analysis, data were preprocessed to remove all controls, average the duplicate protein spots and normalize all the arrays with the global median. A cut-off value of 128 was used to avoid negative values and reduce the influence of noise. Agglomerative hierarchical clustering was performed on log 2-transformed data using Euclidean distance as the dissimilarity metric. All computation and heatmap visualizations were accomplished using the statistics package R.

[0257] Vaccinia virus (Copenhagen) genomic DNA was used as a template for PCR amplification in 96-well plates, using a high fidelity polymerase to minimize introduction of spurious mutations. The resulting amplified products were examined for the expected size and sequenced-verified throughout the entire insert length. A total of 251 out of 273 genes (92%) were successfully cloned, and 212 bacmid clones (78%) were successfully converted into baculovirus with correct sequence and used for subsequent protein expression.

[0258] An insect cell-based system was used to express the recombinant proteins to ensure high yield and proper folding of proteins, with post-translational modifications that are similar to mammalian cells. Protein expression and purification was optimized and performed in an automated fashion using 96-well plates, resulting in greater than 80% success rate in obtaining soluble recombinant proteins from insect cell cultures. Each protein expressed from the insect cells was tagged with an N-terminal GST tag to facilitate affinity-based purification. Following purification, samples were analyzed by SDS-PAGE gel electrophoresis, stained for the determination of purity, and correct protein size was confirmed by detection with an anti-GST antibody. Out of 212 sequence-verified viruses, 176 unique proteins were successfully purified and passed Western blot QC.

[0259] Following confirmation of purity and size, the recombinant proteins were dispensed into 384-well plates for microarray printing. Every slide was printed with a dilution series of known quantities of a GST tagged protein for the calculation of a standard curve that was used to convert the signal intensities for each spotted vaccinia proteins probed with anti-GST antibody. A statistical sampling of each lot of microarrays printed was evaluated for quality and consistency before use. The intraslide and intra-lot variability in spot intensity, morphology, and a full inventory of all arrayed proteins were also confirmed.

[0260] The completed vaccinia microarrays were first examined with pooled human vaccinia hyperimmune globulin (VIg) produced for therapeutic treatment of adverse vaccine reactions. The microarrays were incubated with diluted VIg or a pool of sera from nonvaccinated individuals and bound antibody was visualized using fluorescently-labeled antihuman IgG antibody and a confocal laser scanner. Each block of proteins printed on the array had a standard set of positive and negative control protein spots that included anti-GST antibody, an antibiotin antibody and a concentration gradient of human IgG. To aid in the proper orientation and alignment of the scanned array, duplicate spots of ALEXA FLUOR®-647 labeled antimouse antibody were also spotted on the same position of each block.

[0261] Incubation of the microarray with VIg identified nine proteins (C3L (complement regulatory protein), I1L (putative DNA-binding virion core protein), I3L (DNA binding phosphoprotein), H3L (IMV membrane associated protein), H5R (late transcription factor), D13L (rifampicin resistance

protein), A27L (cell fusion protein), A33R (extracellular enveloped virus ("EEV") glycoprotein), and B20R (function unknown, but highly homologous to variola ankyrin-like protein B18R)) that consistently bound IgG, while antibody interactions with all other proteins were insignificant, requiring no further treatment to suppress nonspecific signals. These antigens were diverse in function, consisting of regulatory, surface, core and secreted proteins. Six of these vaccinia proteins were previously reported to interact with immune sera, while C3L and I1L are newly identified antibody-recognized antigens. The nine antigenic proteins did not bind antibody from nonvaccinated sera, confirming the specificity of these antibody-antigen interactions. However, O2L (glutaredoxin) and H7R (hypothetical protein) were reactive with antibodies from both VIg and nonvaccinated control sera, suggesting that these were crossreactive or non-specific interactions.

[0262] Antibody responses to recent vaccination were next examined. Sera were collected from individuals before and 28 days after receiving a primary or secondary administration of DRYVAX® and a control group of volunteers who had never received the vaccine. All vaccinated volunteers recorded a pustule blister and scab formation at the site of inoculation. Dilutions of sera collected from the control and vaccinated subjects were individually incubated with the vaccinia proteome microarray to measure antibody binding to specific antigens. All proteins recognized by VIg were also detected with antibodies from one or more vaccinated individuals. The hypothetical vaccinia protein B20R, identified by VIg binding, only bound antibody from one individual subject receiving a secondary vaccination, suggesting that antibody responses to this protein on the microarray may only occur with hyperimmune sera. Sera from the majority of control subjects contained IgG that bound to O2L and H7R, confirming that these two antigens were not useful for determining specific immunity to vaccinia. Sera from more than half of the vaccines contained IgG that recognized at least 4 vaccinia proteins, while the remaining samples recognized 1-3 proteins. Among the four individuals receiving secondary vaccinations, all but one responded to a greater number of antigenic proteins recognized by IgG after vaccination compared to prevaccination. Antibody binding to O2L (glutaredoxin) and H7R, frequently observed among IgG obtained from both primary and nonvaccinated individuals, was absent in sera from secondary vaccines.

[0263] The antibody recognition of O2L and H7R was restored in serum from only one individual following secondary vaccination. Consistent with the results shown above, cluster analysis demonstrated that the eight vaccinia proteins H5R (VACVgp128), C3L (VACVgp031), I3L (VACVgp093), A27L (VACVgp188), D13L (VACVgp150), I1L (VACVgp091), H3L (VACVgp126), and A33R (VACVgp196) group together. In addition, serum samples from vaccinated individuals clustered together while proteins from controls or prevaccinated individuals form different clusters. Conversely, antibody responses of individuals who received secondary vaccinations were similar to primary vaccinations, either before or after secondary vaccination. Vaccinated individuals appear to form two clusters associated with the eight vaccinia proteins, one more distinct from controls and naïve, another less distinct. The intensity values are highest in the strong cluster, lower in the weak cluster and lowest in controls or prevaccinated individuals. Relative levels of virus-neutralizing antibodies were examined in sera