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Microarray Profiling of Antibody Responses against Simian-Human Immunodeficiency Virus: Postchallenge Convergence of Reactivities Independent of Host Histocompatibility Type and Vaccine Regimen

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We developed antigen microarrays to profile the breadth, strength, and kinetics of epitope-specific antiviral antibody responses in vaccine trials with a simian-human immunodeficiency virus (SHIV) model for human immunodeficiency virus (HIV) infection. These arrays contained 430 distinct proteins and overlapping peptides spanning the SHIV proteome. In macaques vaccinated with three different DNA and/or recombinant modified vaccinia virus Ankara (rMVA) vaccines encoding Gag-Pol or Gag-Pol-Env, these arrays distinguished vaccinated from challenged macaques, identified three novel viral epitopes, and predicted survival. Following viral challenge, anti-SHIV antibody responses ultimately converged to target eight immunodominant B-cell regions in Env regardless of vaccine regimen, host histocompatibility type, and divergent T-cell specificities. After challenge, responses to nonimmunodominant epitopes were transient, while responses to dominant epitopes were gained. These data suggest that the functional diversity of anti-SHIV B-cell responses is highly limited in the presence of persisting antigen.

Human immunodeficiency virus (HIV) infection causes significant morbidity and mortality (43), and a tremendous need exists for an effective vaccine. Simian-human immunodeficiency virus (SHIV) infection in macaques is a model for HIV infection in humans. SHIVs are chimeric viruses that include the *env*, *tat*, *vpu*, and *rev* genes from HIV-1 combined with the *gag*, *pol*, *vif*, *vpr*, and *nef* genes of simian immunodeficiency virus (SIV) to enable infection of macaques (30, 39). SHIV89.6P, a pathogenic variant of SHIV89.6, induces $CD4^+$ T-cell lymphopenia 2 to 3 weeks after infection and death within months due to opportunistic pathogens (26). The SHIV model allows one to study antibody responses against HIV Env, the ultimate target for an HIV vaccine.

A better understanding of the evolution of anti-SHIV immune responses could provide further insights into the mechanisms by which HIV subverts immune clearance and could enhance our ability to develop an effective vaccine. Furthermore, examination of immune responses elicited by successful experimental SHIV vaccines may illuminate protective mechanisms.

In both HIV and SHIV infection, $CD8^+$ T cells play a critical role in suppressing viral replication (22, 39, 52). SHIV DNA vaccines codelivered with interleukin-2 or followed by a recombinant viral boost appear to suppress viral replication in macaques by cytotoxic T-cell-mediated immunity (4, 7, 10). However, vaccines whose effects are mediated by cytotoxic T cells do not prevent the initial infection (4, 7, 10), a phenomenon that likely requires the presence of neutralizing antibodies that bind to virions and block their entry into cells (reviewed in references 12 and 34). Antibody-dependent cellular cytotoxicity may contribute further to the response against HIV-1 (2). Finally, passive transfer experiments demonstrated that purified antibodies alone can protect macaques against SHIV challenge (9, 32, 44).

The current study was undertaken to profile the evolution of antiviral antibody responses elicited by multiprotein modified vaccinia virus Ankara (MVA) and DNA/MVA vaccines (5–8) and to test whether there might be a relationship between the fine specificity of the immune epitopes recognized by T cells and B cells in anti-SHIV immunity. Previous investigators used peptides synthesized on pins (19) to study antibody responses elicited by gp120 protein vaccines and viral infections (21, 31, 36–38). That method suffered from several drawbacks, including the absence of whole proteins, uncontrolled peptide purity, low throughput rates, and loss of binding capacity with required reuse. In this study we avoided many of those problems and conducted a wider survey of reactivities with antigen microarrays to follow the specificity of antiviral B-cell responses. Our arrays contained 430 SHIV-derived peptides and proteins applied to the surface of derivatized microscope slides, where they were analyzed for interactions with serum antibodies (41). Integration of array results with prior data on the specificity of T-cell responses revealed a remarkable convergence of anti-

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TABLE 1. Antibodies used for validation

Specificity[a]	Figure	Hybridoma	Catalog no.[b]	Type	Manufacturer or contributor(s)	Reference(s)
HIV-1 Env gp120 aa 81–110	1A	Chessie 6	810	Mouse IgG1	G. Lewis	1
HIV-1 Env gp120 aa 308–320	1B	5F7	2533	Mouse IgG1	A. von Brunn	40, 47, 48
HIV-1 Env gp41 aa 735–752	1C, 2A	1577	1172	Mouse IgG3	M. Ferguson	16, 18
SIV Gag p17 aa 11–30	1D	KK59	2320	Mouse IgG1	K. Kent & C. Arnold	28
SIV Gag p27 aa 286–315	1E	KK64	2321	Mouse IgG1	K. Kent & C. Arnold	28
HIV-1 Tat aa 1–16	1F	15.1	1974	Mouse IgG1	DAIDS, NIAID[c]	13
HIV-1 Pol p15	1G	N/A[d]	4105	Rabbit serum	DAIDS, NIAID	
HIV-1 Pol p31 aa 1–16	1H	N/A	756	Rabbit serum	D. Grandgenett	11, 23
HIV-1 Pol p31 aa 142–153	1I	N/A	3514	Rabbit serum	D. Grandgenett	23

[a] aa, amino acid.
[b] Catalog number from the AIDS Research and Reference Reagent Program (http://www.aidsreagent.org).
[c] DAIDS NIAID, Division of AIDS, National Institute of Allergy and Infectious Diseases.
[d] N/A, not applicable.

SHIV B-cell responses in the presence of strongly divergent T-cell responses.

MATERIALS AND METHODS

Peptides, proteins, antibodies, and sera. HIV and SHIV proteins and peptides were obtained from the National Institutes of Health's AIDS Research and Reference Reagent Program (McKessonHBOC BioServices, Rockville, Md.) or synthesized at the Emory University branch of the U.S. Centers for Disease Control. SHIV proteins spotted included eight preparations of Env, four of Gag, four of Pol, one of Tat, two of Nef, and one of Rev. Spotted SHIV peptides included 186 overlapping peptides derived from Env, 100 from Gag, 101 from Pol, and 23 from Tat. Seven irrelevant peptides and proteins were included as negative controls. Positive controls included antibodies to rhesus immunoglobulins for normalization and Cy3-labeled protein as positional reference markers. Full descriptions for these reagents, including acknowledgments for original sources, are given on our web site (http://www.stanford.edu/group/antigenarrays). The monoclonal and polyclonal antibodies used for the studies shown in Fig. 2 were also from the National Institutes of Health's AIDS Research and Reference Reagent Program and are listed in Table 1. Macaque sera were from the vaccine trials described previously (5–8).

Array production. Viral peptides and proteins were diluted to 0.2 mg/ml in phosphate-buffered saline or water. A robotic microarrayer (see http://cmgm.stanford.edu/pbrown/mguide/index.html) with ChipMaker 2 Micro Spotting Pins (TeleChem International, Sunnyvale, Calif.) was used to attach viral antigens to addressable locations on poly-L-lysine-coated microscope slides as previously described (41). The 2,304-feature SHIV proteome array was used for all studies described herein.

Probing and scanning of viral antigen arrays. Arrays were circumscribed with a hydrophobic PAP pen and blocked overnight at 4°C in phosphate-buffered saline with 3% fetal bovine serum and 0.5% Tween 20 (blocking buffer). Arrays were incubated with 1:125 dilutions of macaque serum for 1.75 h at 4°C, followed by three washes in blocking buffer. The arrays were then incubated for 1 h with goat anti-monkey immunoglobulin G (IgG) (Nordic Immunology, Tilburg, The Netherlands) covalently conjugated to indocarbocyanine with an *N*-hydroxysuccinimidyl (NHS)-ester-activated dye pack according to the manufacturer's instructions (Amersham Pharmacia, Piscataway, N.J.). Arrays were washed three times with blocking buffer, twice with phosphate-buffered saline, and twice with water. Arrays were spun dry and scanned with a GenePix 4000 Scanner (Axon Instruments, Union City, Calif.). In preliminary titration experiments, the 1:125 dilutions of serum yielded the greatest signal without significant background. Incubations with more concentrated serum resulted in nonspecific binding. The images presented are false-colored derivatives of the digital scans. Detailed protocols were published previously (41) and are available on the website http://www.stanford.edu/group/antigenarrays.

Analysis of array data. The median feature and background pixel intensities for each antigen feature were determined with GenePix Pro 3.0 software (Axon Instruments, Union City, Calif.), from which the net fluorescence intensity (expressed as digital fluorescence units [DFUs]) was calculated for individual features. For each antigen, a raw value was generated from the median of net fluorescence values for all features representing that antigen. In order to perform a log transformation of array values for subsequent statistical analysis, an adjustment of raw values was performed to eliminate negative values. If the raw value for an antigen was less than 1.0 on any array in the data set, the values for that antigen were adjusted for all arrays in the data set as follows: 1.0 plus the absolute value of the lowest raw value was added to the raw value for that antigen for all arrays in the data set to generate adjusted raw values.

Normalization between arrays was then performed. For each array, the median of raw values for 16 identical anti-rhesus IgG features were used to normalize data sets between arrays: adjusted raw values for each array were multiplied by a normalization factor so that the normalized median for the anti-rhesus IgG reactivity was equal to 20,000 (see Fig. 3D). These normalized, adjusted median values were used for all further analysis.

Software for significance analysis of microarrays (SAM) (45, 46) was obtained from G. Chu and R. Tibshirani (http://www-stat.stanford.edu/~tibs/SAM/index.html) and used to analyze log base 2-normalized values. Threshold parameters (delta values and fold change) were selected so that negative control antigens were excluded and false discovery rates (q values) were at most 5%. Differences in input data sets and response type (paired, multiclass, or survival data) required that the threshold parameters be set differently for each type of analysis. For Fig. 4B and C and Table 2, pairwise SAM identified peptides with significant increases in reactivities at the indicated times relative to preimmune values for the same animals. Thresholds for positive values for SAM included a delta of 0.65 and a change of greater than 1.5-fold, resulting in a false discovery rate (q) of less than 0.04 (4%). The list of reported hits was restricted further to reactivities that increased by at least 3.5-fold over the preimmune values.

For Fig. 5, multiclass SAM identified antigens with significant differences in reactivity in at least one treatment group, with a delta threshold of 0.2 that resulted in a q of <0.05. For Fig. 9, SAM was performed on survival data with postchallenge week 22 reactivities from the Gag-Pol DNA-vaccinated, rMVA-boosted group with reported hits having a q of <0.04. Antigens with average reactivities of $>1,500$ DFUs in at least one subgroup (survivors or nonsurvivors) were selected for cluster analysis. Positive SAM hits were subject to hierarchical clustering of both antigens and macaques with Cluster software, and the results were displayed with TreeView software (17). Clusters were determined by the complete linkage algorithm, with GWEIGHTs (http://rana.lbl.gov/manuals/clustertreeview.pdf) assigned to each antigen in proportion to its SAM score and with centered and uncentered correlation measurements for the arrays and antigens, respectively. For Table 3, pairwise SAM was performed between week 0 and 75 data with a fourfold change threshold to yield hits with a q of <0.004. Reactivities of $>1,500$ DFUs were considered positive. Identification numbers for monkeys correspond to those in previous reports (5–8).

Elispot assays. Peptide pools were used at a concentration of 10 μg per ml of each peptide. Stimulations were for approximately 36 h. Only Elispot counts at least twice background were considered significant. For details, see Amara et al. (7).

RESULTS

SHIV proteome array construction and validation. We used a robotic microarrayer to print 430 distinct peptides and proteins derived from SIV Gag, Pol, and Nef and HIV-1 Env, Tat,

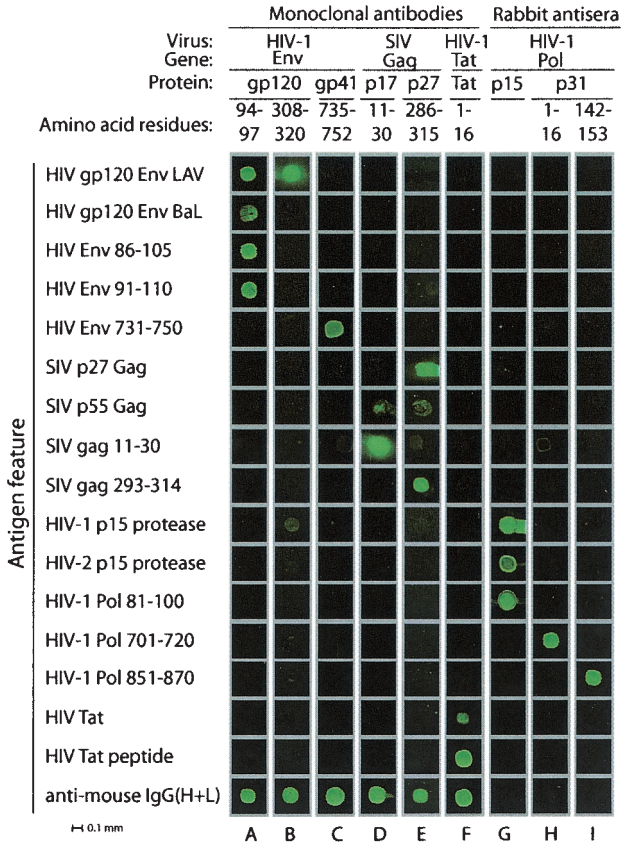


FIG. 1. Validation of SHIV proteome arrays with specific sera and monoclonal antibodies. SHIV proteome arrays similar to those in Fig. 3 were incubated with monoclonal antibodies specific for HIV Env gp120 amino acids 94 to 97 and 308 to 320, HIV Env gp41 735 to 752, SIV Gag p17 11 to 30 and p27 286 to 315, and HIV Tat 1 to 16 or with rabbit antisera specific for HIV Pol p15 or p31 1 to 16 or 142 to 153 (as described in Table 1). Antigen features are indicated on the left, and each column of features is derived from a single array.

and Rev in ordered arrays on poly-L-lysine-coated microscope slides. Additional negative, positive, and reference reagents were spotted on the arrays. To validate SHIV arrays, individual arrays were incubated with monoclonal and polyclonal antibodies specific for nine distinct HIV-derived proteins and peptides (Table 1), demonstrating specific reactivity only at their corresponding antigen features (Fig. 1). Integration of array data with known concentrations or with enzyme-linked immunosorbent assay (ELISA) results for detection of anti-Env antibodies demonstrated linear relationships (Fig. 2), a property previously described for this system (24, 41).

Use of arrays to profile antibody responses in vaccine trials. SHIV proteome arrays were used to characterize sera from three macaque vaccine trials. In two trials, macaques were primed at 0 and 8 weeks with 2.5 mg of plasmid DNA expressing SHIV89.6 Gag-Pol-Env or SHIV89.6 Gag-Pol and boosted at 24 weeks with 2×10^8 PFU of recombinant modified vaccinia virus Ankara (rMVA) expressing the same SHIV proteins as the prime, e.g., SHIV Gag-Pol-Env or SHIV Gag-Pol (5–8). For the third trial macaques were inoculated at 0, 8, and 24 weeks with rMVA expressing SHIV89.6 Gag-Pol-Env (8). A control group received empty vectors without vaccine inserts. All groups were challenged at 7 to 8 months after the final immunization with a pathogenic derivative of SHIV89.6, SHIV89.6P (26).

All animals in the Gag-Pol-Env DNA/rMVA and rMVA-only groups controlled the viral challenge. In the Gag-Pol DNA/MVA group, two of six animals failed to control their challenge infection. In the control group, the levels of virus remained high, and five of six macaques succumbed to AIDS by 28 weeks. In each trial, 50% of the macaques were selected to have at least one *A*01* allele, and 50% were selected to have at least one *B*01* allele. These alleles were present in a background of otherwise unselected histocompatibility types.

Images of SHIV proteome arrays probed with serum derived from an individual macaque before vaccination (Fig. 3A), after three immunizations with a Gag-Pol-Env-expressing MVA (Fig. 3B), and after SHIV89.6P challenge (Fig. 3C) are representative results. In this example, vaccination raised antibodies to three gp120 Env proteins (one from clade B and two from recombinant clade AE), six gp120 Env peptides, three gp41 Env peptides, the p55 precursor protein of SIV Gag, one Gag peptide, and one integrase peptide. Four of the reactive gp120 peptides included overlapping sequences from the V3 region. All but one of these 15 reactivities (that against the gp120 peptide 101-120) were enhanced by SHIV challenge (Fig. 3D). The challenge also raised responses against new peptides; one in gp120 and two in gp41 are shown.

A color reactivity map for the SHIV array data for the three vaccine trials revealed distinct patterns of responses to the vaccines and in the anamnestic responses immediately post-

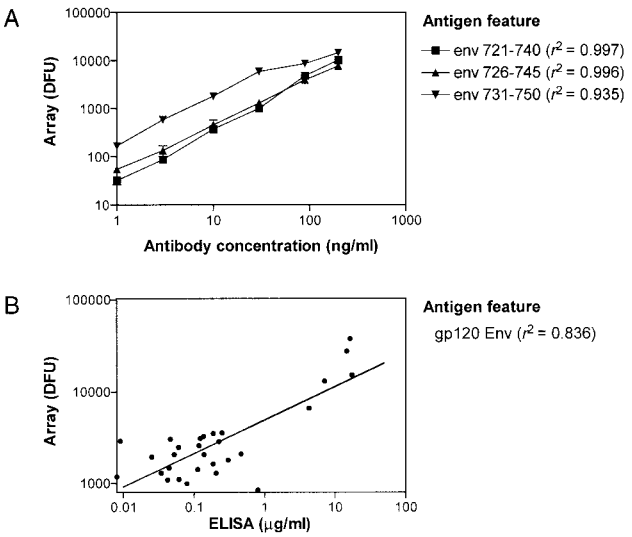
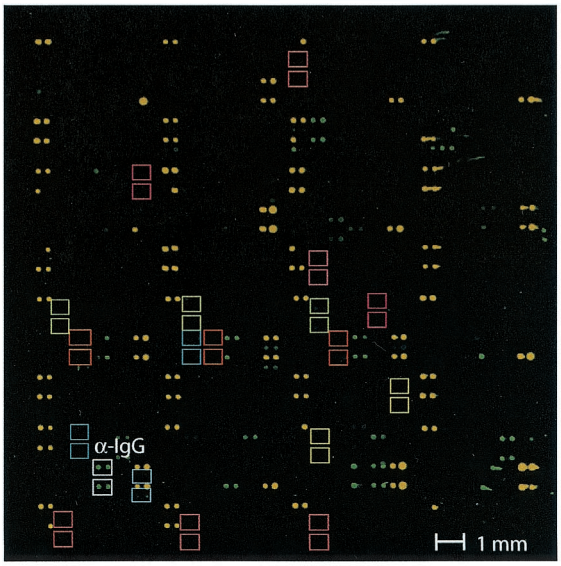
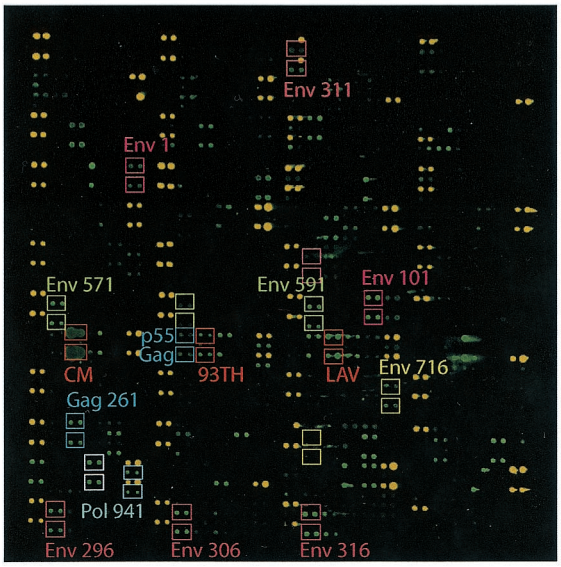


FIG. 2. Comparison of antiviral antibody detection with microarrays and ELISA demonstrates concordant results. SHIV arrays were incubated with various concentrations of Env epitope-specific monoclonal antibodies (A). Array results are presented as normalized mean net digital fluorescence units (DFUs). Macaque samples were assayed by SHIV array and ELISA for anti-Env antibodies (B). Array results are presented as normalized median net DFUs, and ELISA results (7) as optical densities (O.D.).

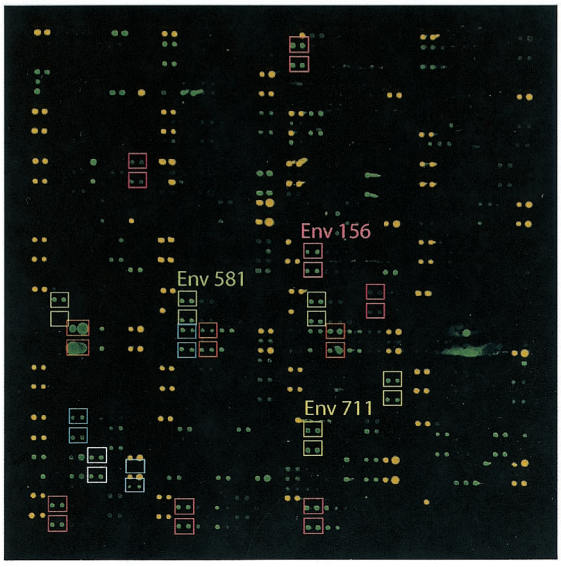
A



B



C



D

Array feature	Protein domain(s)	Fluorescence Pre-immune	Post-MVA	Post-challenge
HIV-1 CM (clade AE) gp120 Env	gp120 all	47	2037	5802
HIV-1 93TH975.15 (clade AE) gp120 Env	gp120 all	473	2997	5868
HIV-1 LAI/IIIB (clade B) gp120 Env	gp120 all	871	19860	27730
HIV-1 89.6P (clade B) Env 001-020	gp120 Leader	29	2244	2911
HIV-1 89.6P (clade B) Env 101-120	gp120 C1	73	7550	1856
HIV-1 89.6 (clade B) Env 156-175	gp120 V2	54	535	8286
HIV-1 89.6 (clade B) Env 296-315	gp120 V3	606	4054	6777
HIV-1 89.6 (clade B) Env 306-325	gp120 V3	478	5642	18416
HIV-1 89.6P (clade B) Env 311-330	gp120 V3	134	2091	12537
HIV-1 89.6 (clade B) Env 316-335	gp120 V3	206	7069	14119
HIV-1 89.6P (clade B) Env 571-590	gp41 Wang/Gnann	1	4206	21469
HIV-1 89.6P (clade B) Env 581-600	gp41 Wang/Gnann	62	1168	9538
HIV-1 89.6P (clade B) Env 591-610	gp41 Wang/Gnann	1379	4352	31413
HIV-1 89.6P (clade B) Env 711-730	gp41 MPI & Kennedy	159	331	5261
HIV-1 89.6 (clade B) Env 716-735	gp41 MPI & Kennedy	174	2163	28162
SIV mac251 p55 Gag	p55 precursor	689	1498	9329
SIV mac239 Gag 261-280	p24 core	214	3528	4131
HIV-1 HXB2R (clade B) Pol 941-960	p31 integrase	800	3366	2509
anti-Rhesus IgG	goat IgG	20000	20000	20000

challenge, followed by a strong convergence of the response to common epitopes (Fig. 4A). Consistent with the origins of SHIV chimeras, antisera reacted to gp120 Env proteins from HIV-1 but not SIV and to Gag proteins from SIV but not HIV-1. Analysis of peptide reactivities revealed that most of the reactivity was directed against Env, with the strongest responses against the V3 region of gp120 (amino acids 299 to 332), the Wang/Gnann region of gp41 (residues 590 to 619, also known as cluster I) (51), and the Kennedy region of gp41 (amino acids 724 to 742). During the immunizations, reactivities were strongest in the rMVA-only group (Fig. 4A).

After challenge, Gag-Pol-Env-vaccinated macaques exhibited marked acceleration in the kinetics of anti-Env antibody responses relative to Gag-Pol and control vaccinated macaques (Fig. 4A). However, by 20 to 22 weeks postchallenge, antiviral antibody profiles in all groups had converged (Fig. 4A). At this time, reactivities directed against the V1, V3, and C5 regions of gp120 and the Wang/Gnann peptide, C-helix, and C terminus of gp41 were similar in all groups. Responses against Gag were primarily detected against protein, not peptides. Reactivities for proteins and peptides representing Pol, Nef, Rev, and Tat occurred at only low intensities and frequencies and were not included in Fig. 4A.

Breadth of antiviral B-cell responses. Significance analysis of microarrays (46) was performed to identify antigen features with statistically significant increases in reactivity in samples obtained at specific time points in the vaccine trials relative to paired preimmune samples. The rMVA-only inoculations induced the highest average number of Env-reactive features postvaccination and in the postchallenge anamnestic response (Fig. 4B). Consistent with the visual inspection of data (Fig. 4A), the Gag-Pol-Env DNA/rMVA group had a lower tally after vaccination but a similar tally in the anamnestic postchallenge response. By 20 to 22 weeks after challenge, all groups, including the two surviving animals in the unvaccinated control group, had mounted anti-Env antibody responses with similar numbers of reactive Env peptides (Fig. 4B). Although neither the Gag-Pol nor the control empty vector group were primed for Env, anti-Env responses appeared earlier and more uniformly in the Gag-Pol group. In the Gag-Pol group, T-cell responses to Gag and Pol likely protected CD4 cells sufficiently to allow generation of antibody responses.

Consistent with the visual inspection (Fig. 4A), anti-Gag responses were detected against fewer peptides, with the number of reactivities being fairly similar between groups (Fig. 4C). In contrast to the kinetics for the appearance of reactivities to Env, the reactivities against Gag appeared at similar times (albeit at different heights) in the vaccine groups. In the control group, anti-Gag reactivities, like anti-Env reactivities, showed the slowest appearance, not rising until 22 weeks after challenge.

Identification of novel epitopes targeted by anti-SHIV B-cell responses. The statistical analysis revealed reactivities to 18 peptides that did not overlap any previously described linear epitopes (Table 2) (29). Antibodies to three of these were detected in at least three of the test groups and at more than one test time and thus appeared to represent consistent targets for antiviral B-cell responses. One of these was directed against the C terminus of gp41 Env and two were directed against p6 Gag.

Reactivities indicative of challenge. A problem encountered during clinical trials for vaccines is the need to distinguish responses elicited by the vaccine from those against the pathogen itself. Compared to postvaccination responses, reactivities after challenge were stronger and broader (Fig. 4A). Furthermore, after challenge, epitopes of the virus that were absent from the vaccine became reactive. For example, the carboxy terminus of gp41 was deleted from the rMVA vaccine. Most peptides from this region are not reactive after immunization three times with this vaccine. However, after challenge, they become reactive in this group by 9 weeks and in the empty-vector control monkeys by 22 weeks (Fig. 4A, Table 2).

Statistical analysis demonstrates ultimate convergence of anti-SHIV antibody responses. Interestingly, statistically significant differences in antiviral antibody profiles were observed only immediately postvaccination and immediately postchallenge (Fig. 5). In contrast to pairwise SAM analysis for individual macaques, multiclass SAM analysis across groups at each time point identified no statistically significant difference in reactivities in the preimmune samples, the memory vaccine response samples at week 49, or the samples obtained 20 to 22 weeks postchallenge (Fig. 5A). However, 19 statistically significant differences in reactivities were identified between the groups at the peak vaccine response at week 27 (Fig. 5A and

FIG. 3. The 2,304-feature SHIV proteome array. Ordered antigen arrays were generated by spotting 430 distinct SHIV- and HIV-derived peptides and proteins in four or eight replicate sets with a robotic microarrayer. Antibodies specific for macaque IgG ($\alpha-$IgG) and antibodies labeled with Cy3 and Cy5 (yellow features), to serve as reference features to orient the arrays, were also spotted. Individual arrays (A to C) were incubated with prevaccination serum (week 0) (A), postvaccination prechallenge serum (week 27, 3 weeks after final boost) (B), or postchallenge serum (week 64, 9 weeks after challenge) (C), all derived from an individual macaque from the rMVA-only vaccine trial (group receiving three rMVA immunizations) (8). Bound antibodies were detected with indocarbocyanine-labeled goat anti-macaque IgG. Colored squares identify targets of anti-SHIV antibody responses induced by vaccination and challenge. Orange and yellow boxes demarcate reactivities against gp120 Env proteins from various strains of HIV and SIV, respectively, induced by vaccination. Dark red, pink, and light red boxes locate peptides from the amino-terminal, V2, and immunodominant V3 domains, respectively, of gp120 Env. Dark and light green boxes indicate reactivities to gp41 Env peptides from the immunodominant Wang/Gnann and Kennedy domains, respectively, detected initially after immunization and then more intensely after challenge. Blue boxes demarcate reactivities against HIV Gag p55 precursor protein and a p24 Gag peptide detected following immunization and challenge. Light blue boxes locate reactivities against a HIV p31 Pol (integrase) peptide. Antigen features measure approximately 200 μm in diameter. These 2,304-feature SHIV proteome arrays were used for all array experiments reported in this article. Quantitative analysis (D) of highlighted features from panels A to C is displayed. Median digital fluorescence units (DFUs), net of local background, were normalized to the net median DFU values for the anti-rhesus IgG features on each array. Antigen features with positive reactivity, defined as >1,400 DFUs, are highlighted in a color-matched fashion to the boxes outlining their corresponding antigen features in panels A to C.

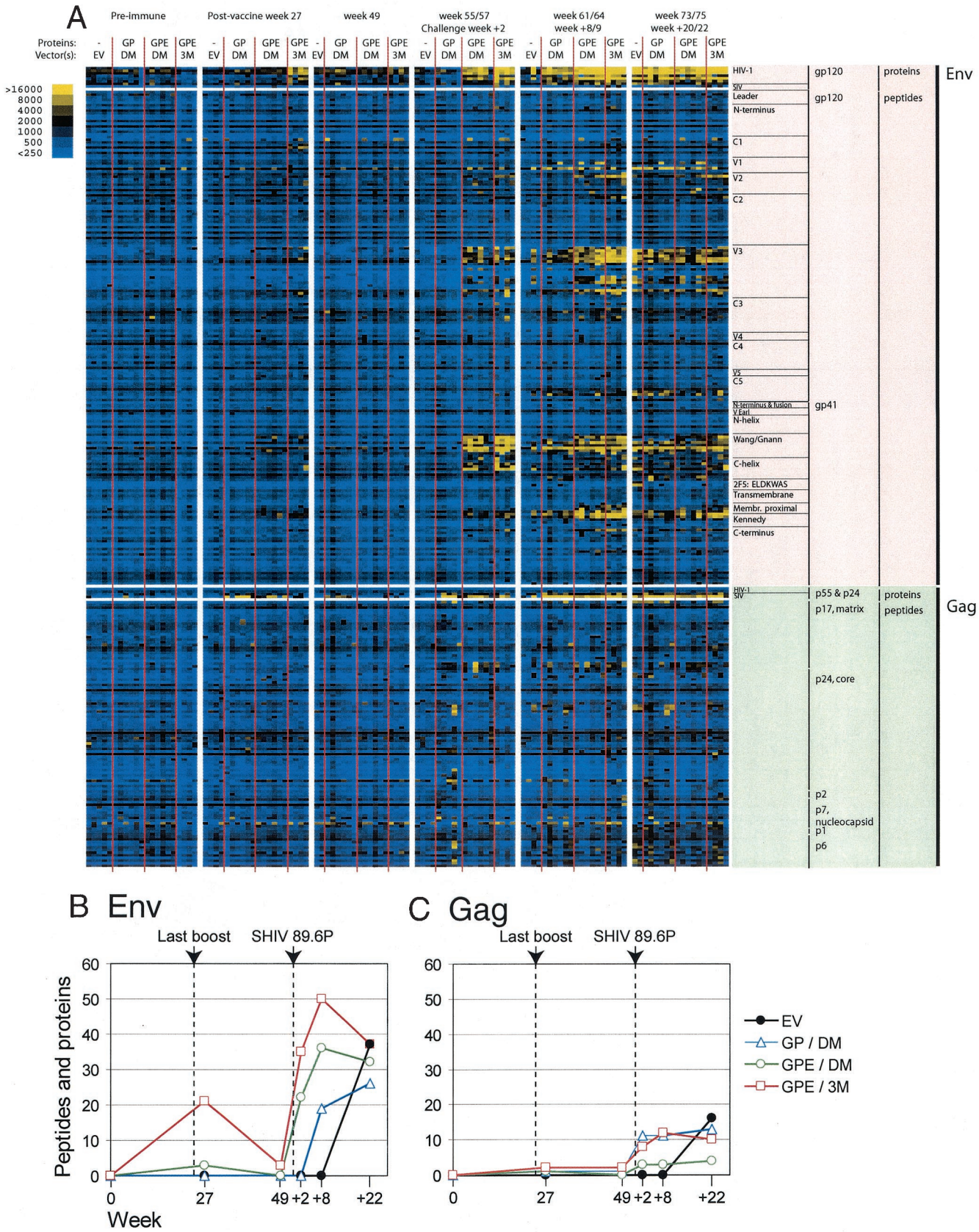


FIG. 4. Reactivity of macaque sera on SHIV proteome arrays: accelerated postchallenge antiviral antibody responses in vaccinated macaques. A false-color map (A) presents antibody reactivities detected against Env and Gag proteins and sequential overlapping peptides contained on SHIV proteome arrays. Time points and groups of animals are indicated along the top. Results from individual animals are represented in

TABLE 2. Identification of novel SHIV epitopes[a]

Peptide	Sequence[b]	Reactive time point(s)[c] (wk)			
		EV	GP/DM	GPE/DM	GPE/3M
Env 336–355 gp120 C3	RAKWNNTLQQIVIKLREKFR				27
Env 621–640 gp41 C-helix	WNNMTWMEWEREIDNYTDYI			+20	+2
Env 701–720 gp41 MPI	SIVNRVRQGySPLSFQTLLP			+8	
Env 746–765 gp41 C terminus*	VNGFLALFWVDLRNLCLFLY	+22		+8	+2, +9, +22
Env 756–775 gp41 C terminus	DLRNLCLFLYHLLRNLLLIV				+9
Gag 452–473 p6	VHQGLMPTAPPEDPAVDLLKNY	+22			+22
Gag 461–480 p6	PPEDPAVDLLKNYMQLGKQQ	+22			+22
Gag 472–493 p6*	NYMQLGKQQREKQRESREKPYK	+22	+9, +20		49, +9, +20
Gag 482–503 p6	EKQRESREKPYKEVTEDLLHLN	+22			+9, +20
Gag 491–510 p6	PYKEVTEDLLHLNSLFGGDQ	+22	+2, +20		
Gag 492–510 p6*	YKEVTEDLLHLNSLFGGDQ	+22	+2, +9, +20		+9, +20
Pol 031–050 N terminus	LQVWGRDNNSPSEAGADRQG				+9
Pol 271–290 RT	FSVPLDEDFRKYTAFTIPSI		+9		+2
Pol 281–300 RT	KYTAFTIPSINNETPGIRYQ				27
Pol 541–560 RT	TPKFKLPIQKETWETWWTEY				+2
Pol 621–640 RNase	VVTLTDTTNQKTELQAIYLA				+2
Tat 025–039	CYCKKCCFHCQACFI				+2, +9
Tat 089–100	KVERETETDPVH			27	27

[a] Peptides from regions of SHIV proteins that have statically significant antibody reactivity but do not overlap epitopes previously described are shown with the experimental groups and time points at which their reactivities were detected. Peptides marked with asterisks were detected in at least three of the test groups and at more than one time point and thus appear to represent consistent targets for antiviral B-cell responses.

[b] Lowercase y in Env 701 indicates a tyrosine residue that was mutated to cysteine in the DNA vaccine. Underlined residues were deleted from Env in the rMVA vaccine.

[c] Time points 27 and 49 refer to weeks after initial vaccination; time points +2, +8, +9, +20, and +22 refer to weeks after SHIV challenge. See the legend to Fig. 4 for definitions of groups.

B), 27 statistically significant differences 2 weeks postchallenge (Fig. 5A and C), and 33 statistically significant differences 8 weeks postchallenge (Fig. 5A and D).

Hierarchical cluster analyses of epitopes with statistically significant differences revealed that macaques clustered based on their vaccination group and antigens clustered into Gag, Env, and/or Pol (Fig. 5B to D, see dendrograms at the top and side of reactivity maps). Within the Env epitopes, further clustering of overlapping peptides was observed. Combined with observations described earlier (Fig. 4), these clusters revealed that the Gag-Pol-Env rMVA-only animals followed by the Gag-Pol-Env DNA/rMVA animals had the broadest and strongest postvaccination and postchallenge antibody responses. These cluster analyses also revealed anti-Env responses appearing in the Gag-Pol group prior to their appearance in the control group (Fig. 5D).

The remarkable convergence by 20 to 22 weeks postchallenge of antiviral antibody responses in all vaccine groups as well as the two surviving control animals was confirmed by measuring Pearson correlation coefficients (Fig. 6). At 2 weeks postchallenge, anti-SHIV antibody responses in each of the vaccine groups were divergent from those in the control group, with Pearson correlation coefficients of approximately 0.1 to 0.2 (Fig. 6A). At this time, comparisons within each group yielded Pearson coefficients above 0.5 (data not shown). By 20 to 22 weeks postchallenge, the responses had converged, with Pearson correlation coefficients between the vaccinated and unvaccinated groups rising to approximately 0.7 for all intergroup comparisons. Further comparisons revealed moderate convergence of antiviral B-cell responses between the Gag-Pol-Env DNA/rMVA and rMVA-only groups as early as 2 weeks postchallenge, with a Pearson correlation coefficient of 0.5 (Fig. 6B).

This convergence resulted in the targeting of a specific set of 25 peptides and seven proteins contained on SHIV arrays (Table 3). Seventeen of the 25 convergent peptides include regions that have been described previously as being immunodominant, the V3, Kennedy, Wang, and Gnann epitopes (14, 20, 27, 50, 53). Particularly notable was the range of reactivity to the V3 loop, a principal neutralizing and tropism-determining domain (Fig. 7). Cross-reactivity to V3 peptides from certain other clade B strains was apparent. Eight of nine convergent peptides from the V3 region contained a common sequence (GPGRAFY), with the remaining V3 peptide having a conserved basic (K) substitution at the fourth residue of this sequence. Less-conserved substitutions in the last four residues rendered other clade B V3 peptides nonreactive regardless of vaccine regimen. The only V3 peptides from non-clade B strains were two peptides from clade E, neither of which showed reactivity postvaccination or postchallenge. To detect

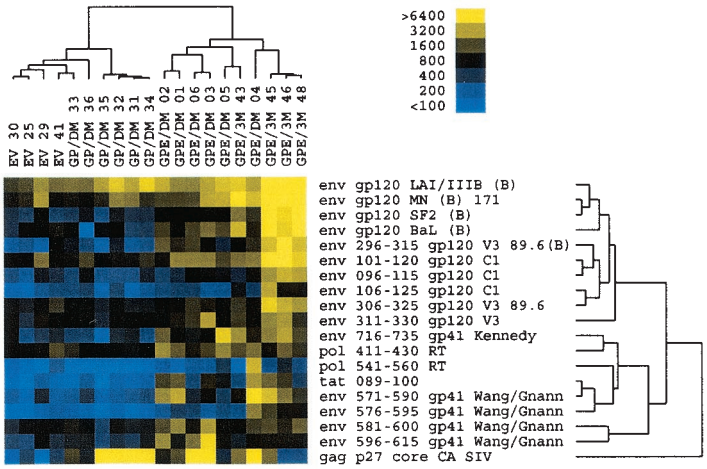
individual columns. Groups include vector-vaccinated (controls, EV), Gag-Pol-DNA-vaccinated and rMVA-boosted (GP DM), Gag-Pol-Env DNA-vaccinated and rMVA-boosted (GPE DM), and Gag-Pol-Env rMVA-primed and -boosted (GPE 3M) animals. Antigen features derived from Env and Gag are indicated along the right border, with proteins indicated first followed by overlapping peptides spanning each polypeptide. As indicated by the color key, blue represents lack of reactivity, black represents low reactivity, and yellow represents high reactivity. The average number of reactive Env (B) and Gag (C) features for macaques in each group was determined by pairwise SAM comparisons of macaques at the individual time points relative to their preimmune samples.

A

week	SAM hits	
Pre-immune:		
0	0	
Post-vaccination:		
27	19	see B
49	0	
Post-challenge:		
55/57	27	see C
61/64	33	see D
73/75	0	

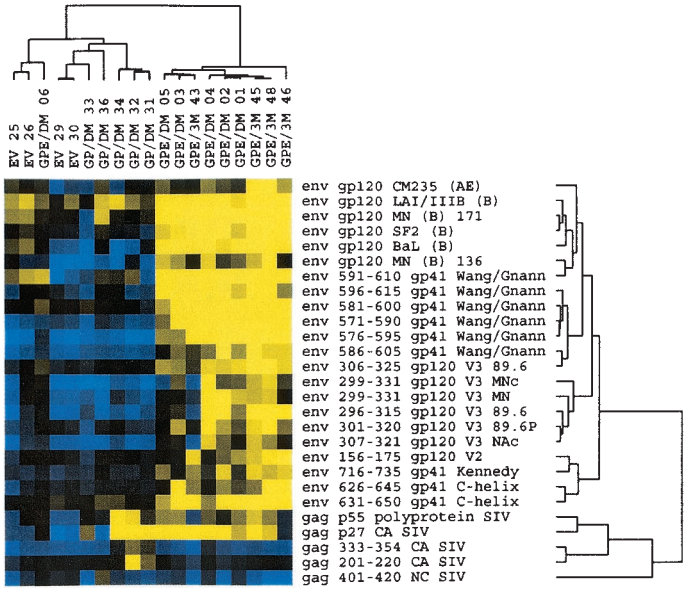
B

Post-vaccine week 27



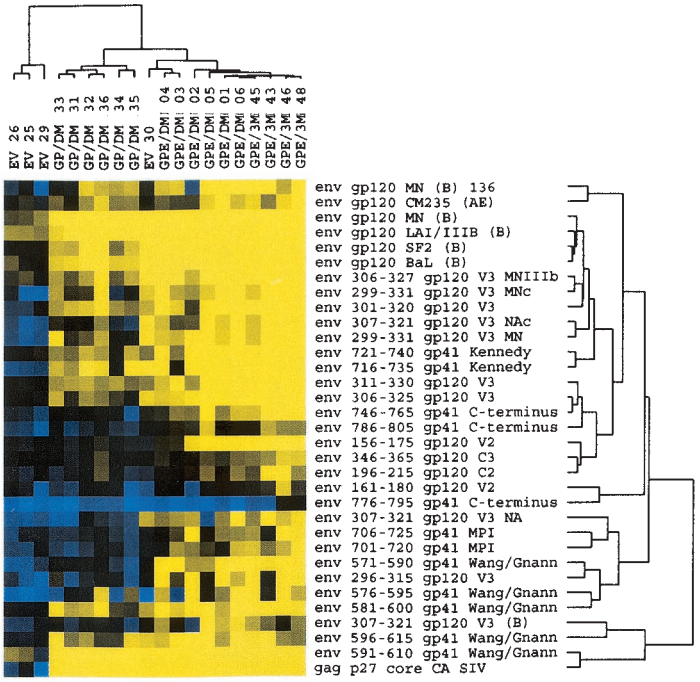
C

Post-vaccine week 55/57 (Post-challenge week +2)



D

Post-vaccine week 61/64 (Post-challenge week +8/9)



responses to diverse strains of HIV, Env peptides and proteins from non-clade B strains could be included in later versions of the "SHIV proteome" array.

The convergence of antiviral B-cell specificity resulted from preferential expansion of antiviral antibody reactivities against convergent epitopes (Fig. 8; Table 2) and preferential drop-out of reactivities against nonconvergent epitopes (Fig. 8; Table 3). Following challenge, the empty-vector-treated animals sequentially gained reactivities against epitopes within the convergent class without significant loss at any time point. In contrast, the vaccinated and challenged animals exhibited a dynamic focusing of antiviral antibody responses, with both significant expansion of reactivities against epitopes within the convergent class and drop-out of reactivities against epitopes in the nonconvergent class (Fig. 8).

Anti-SHIV antibodies converge despite divergent T-cell specificities. To test the specificity of raised $CD4^+$ and $CD8^+$ T cells, interferon gamma Elispot assays were performed with pools of Env and Gag peptides. In contrast to the B-cell responses for the same animals, Elispot analyses demonstrated no consistent patterns of T-cell responses to specific pools of Env peptides, with individual monkeys showing different distributions of reactivities throughout Env (data not shown). Comparisons of T-cell Elispot analyses revealed no convergence, with Pearson correlation coefficients of 0.1 to 0.2 (Fig. 6C and D). These studies were performed on outbred macaques that had been selected only for the presence of at least one *A*01* or *B*01* allele. The lack of convergence of their divergent T-cell responses was likely determined by the diversity of expressed major histocompatibility complex alleles (7). Comparison of the B-cell and T-cell specificities for individual animals also revealed no correlations in reactivity to either Env or Gag epitopes, suggesting that the epitope specificity of T-cell responses did not have a strong influence on the epitope specificity of B-cell responses against Env and Gag (data not shown).

Failure of anti-SHIV antibody responses in macaques that succumbed to challenge. Failure to develop and/or loss of antiviral antibodies was associated with the development of AIDS and death. Three of four unvaccinated control animals (macaques 25, 26, and 29) failed to develop significant anti-SHIV antibodies (Fig. 5D) and succumbed to AIDS by 32 weeks postchallenge. Two of six Gag-Pol DNA/rMVA-vaccinated animals (macaques 31 and 34) succumbed to AIDS by 52 weeks postchallenge. These macaques initially mounted antiviral antibody responses indistinguishable from those of other macaques in their group (Fig. 5B to D) but lost anti-SHIV antibody responses against a spectrum of antigens by 22 weeks postchallenge (Fig. 9). Thus, the breadth and persistence of antiviral antibody responses have prognostic utility in SHIV-infected macaques.

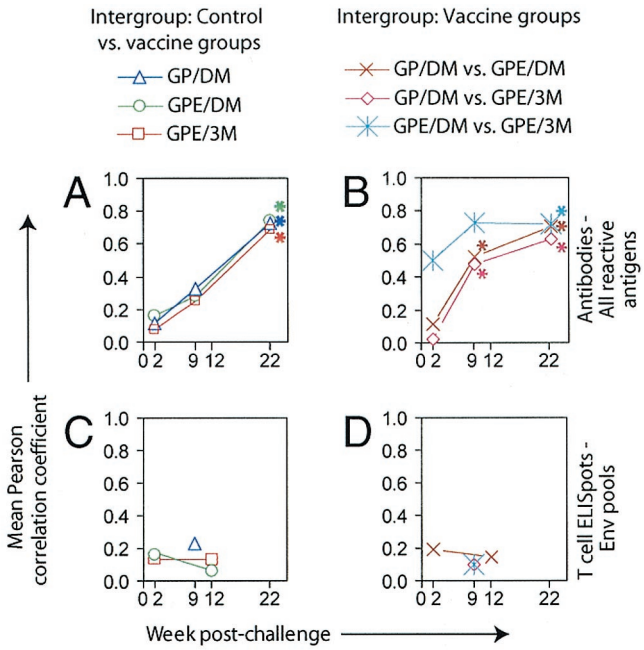


FIG. 6. Postchallenge convergence of anti-SHIV antibody specificities but not T-cell specificities independent of vaccination regimen and macaque genotype. Pearson correlation coefficients were determined for antibody reactivities to all array reactive antigens (A and B) and for T-cell gamma interferon Elispot responses to pooled Env peptides (C and D). Mean Pearson correlation coefficients were plotted from pairwise comparisons between samples from the control group and each vaccine group (A and C) and between samples from different vaccine groups (B and D). For panels A and B, an antigen was classified as reactive if antibodies in at least one sample bound to it to generate a signal of >3,500 DFUs. Asterisks indicate time points with values that had statistically significant differences ($P < 0.05$) from postchallenge week 2 results, as determined by the Mann-Whitney test.

DISCUSSION

We developed and applied SHIV antigen microarrays to profile the evolution of antiviral antibody specificities in vaccinated and challenged macaques. These proteomic studies represent one of the most detailed analyses to date of B-cell responses against linear regions of viral antigens and provide insights into mechanisms regulating the evolution and specificity of B-cell responses. The most intriguing observation was the strong convergence of antiviral B-cell specificities to a restricted set of linear epitopes within Env (Fig. 4A, Fig. 6A and B, Fig. 8, and Table 3). This convergence occurred in an outbred population receiving vaccines encoding different SHIV proteins and included infected controls that survived (Fig. 5A and Fig. 6A and B). The converging specificities of B-cell

FIG. 5. Evolution of anti-SHIV antibody responses postvaccination and postchallenge. Multiclass SAM analysis (A) was performed on SHIV proteome array results to identify the number of antigen features with statistically significant differences in reactivities at the indicated time points. (B to D) Hierarchical cluster analyses of SAM-identified antigen features at weeks 27, 55, and 64. Antigen features are indicated to the right of each panel, and individual macaques and their respective groups are indicated along the top. Dendrograms represent the hierarchical relationship between the individual macaques (dendrograms at top) and individual antigen features (dendrograms to right). Treatment groups are designated as in Fig. 4.

TABLE 3. Convergent SHIV peptides and proteins[a]

Protein	Region(s)	Antigen feature[b]	Peptide sequence[c]	% Positive
gp120	All	Env gp120 BaL	Protein	100
		Env gp120 CM235	Protein	94
		Env gp120 LAI/IIIB	Protein	100
		Env gp120 MN136	Protein	83
		Env gp120 MN171	Protein	100
		Env gp120 SF2	Protein	94
	V1	Env 141–160 89.6P	TNLTSSSWGMMEEGEIKNCS	78
	V2	Env 156–175	KNCSFYITTSIRNKVKKEYA	72
	V3	Env 299–331 MN	TRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAH	72
		Env 299–331 MN-c	CTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHC	89
		Env 301–320 89.6P	RPNNNTRERLSIGPGRAFYA	100
		Env 306–325 89.6	RRRLSIGPGRAFYARRNIIG	89
		Env 306–327 MNIIIB	YNKRKRIHIQRGPGRAFYTTKNIIC	94
		Env 307–321 not specified	KSIPMGPGKAFYATG	56
		Env 307–321 MN	KRIHIGPGRAFYTTK	56
		Env 307–321 NA	KSIHIGPGRAFYTTG	56
		Env 307–321 NA-c	CKSIHIGPGRAFYTTGC	72
		Env 311–330	SIGPGRAFYARRNIIGDIRQ	83
	C5	Env 486–505	VRIEPIGVAPTRAKRRTVQR	61
		Env 491–510	PIGVAPTRAKRRTVQREKRA	61
gp41	Wang/Gnann	Env 571–590	IKQLQARVLALERYLRDQQL	56
		Env 581–600	LERYLRDQQLMGIWGCSGKL	94
		Env 586–605	DQQLMGIWGCSGKLICTTSV	56
		Env 591–610	MGIWGCSGKLICTTSVPWNV	94
		Env 596–615	SGKLICTTSVPWNVSWSNKS	89
	C-helix	Env 631–650	REIDNYTDYIYDLLEKSQTQ	56
	MPI[d] & Kennedy	Env 711–730	SPLSFQTLLPASRGPDRPEG	61
		Env 716–735	TLLPASRGPDRPEGTEEEGG	83
		Env 721–740	ASRGPDRPEGTEEEGGERDR	72
	C-terminus	Env 746–765 89.6	VNGFLALFWVDLRNLCLFLY	61
p24	All	Gag CA SIV	Protein	94
p17	C-terminus	Gag 121–142 MA SIV	TSRPTAPSSGRGGNYPVQQIGG	56

[a] Convergent antigens were determined by pairwise SAM comparisons of reactivities at weeks 0 and 75. Antigens that were reactive in the majority of the 18 monkeys alive 20 to 22 weeks after challenge are displayed.

[b] Approximate amino acid residue numbers are shown for peptides. Strains are also indicated for Env antigens that are not derived from sequences that are identical in strains 89.6 and 89.6P. All Env antigens are from HIV clade B isolates except for the gp120 from CM235, a clade AE recombinant. Abbreviations: −c, circular; NA, North American consensus; 136 and 171, separate preparations.

[c] Underlining designates a conserved sequence in reactive V3 peptides.

[d] MPI, membrane-proximal interior.

responses superseded concomitant divergent T-cell responses driven by the expression of different major histocompatibility complex alleles by macaques in this outbred population (Fig. 6) (8). Convergence resulted from preferential expansion of anti-SHIV B-cell responses to target immunodominant linear epitopes and temporal loss of reactivity against nonimmunodominant epitopes (Fig. 8 and Tables 3 and 4). These data suggest that B-cell responses are functionally limited and undergo selection to recognize a highly restricted set of immunodominant determinants within Env (Table 3).

The B-cell repertoire has a theoretical complexity exceeding 10^{10}, while observed complexities range from 10^5 to 10^7 (3, 15, 33). The striking convergence of anti-SHIV B-cell reactivities challenges a paradigm in which B-cell responses develop against diverse and heterogeneous epitopes in viral antigens. Although our methodology largely limited us to analysis of linear epitopes, our data demonstrate that antiviral B-cell responses in all immunocompetent animals ultimately target and are almost entirely restricted to a set of immunodominant determinants in SHIV (Fig. 4A, Fig. 6, and Table 3). The ability of HIV immunogens to induce antibody directed against the same set of dominant epitopes in genetically outbred populations and even in different species has been observed previously (25, 31, 36–38). The convergence of antiviral antibody responses has also been described for hepatitis B virus (49) and herpes delta virus (35) infections. Together, our data and the data from others suggest that the B-cell repertoire is fundamentally restricted in its ability to recognize viral antigens and is only capable of maintaining sustained B-cell responses against a limited set of immunodominant viral epitopes.

The strong convergence of linear B-cell epitopes in SHIV infection is in sharp contrast to our observations in autoimmune disease. In experimental autoimmune encephalomyelitis in mice, autoreactive B-cell responses directed against self-proteins maintain divergent profiles, with the inducing autoantigens persisting as dominant targets of autoantibody responses (42). This likely reflects fundamental differences in the regulation of immune responses against foreign microbial proteins compared to autoantigens, for which mechanisms promoting tolerance may inhibit diversification of autoreactive B-cell responses.

Our SHIV antigen arrays revealed that Env raised stronger responses than other viral proteins following both vaccination and challenge, consistent with better exposure of Env relative to internal viral proteins. The strongest responses against Gag

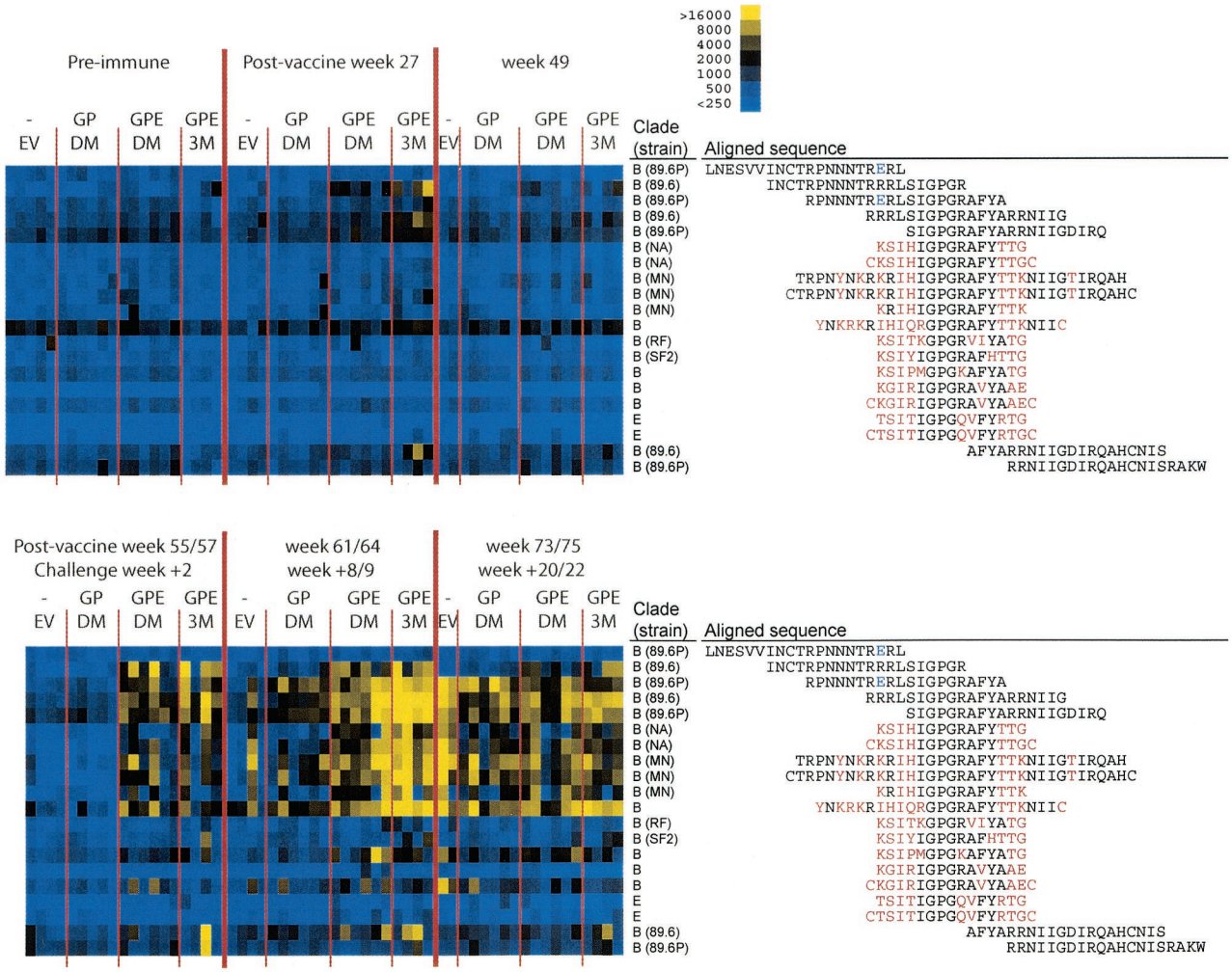


FIG. 7. Reactivity of macaque sera against peptides from the V3 region of gp120: cross-reactivity to peptides containing a core GPGRAFY sequence. Treatment groups are designated as in Fig. 4. Amino acid residues shown in black are from the vaccine strain SHIV89.6; the distinct glutamic acid residue (E) of the challenge virus SHIV89.6P is indicated in blue; and the remaining divergent residues from other strains are shown in red. NA refers to a North American consensus sequence. Peptides that were not reactive to any serum (e.g., the first one listed) may not have bound to the slide or may not have been presented in an appropriate conformation.

and Env were against proteins, not peptides (Fig. 4A). This is consistent with the proteins containing more than one epitope and with the proteins presenting conformational as well as linear epitopes. For the DNA/rMVA vaccines, the arrays confirmed induction of very low levels of prechallenge antibodies (7, 10). The rMVA-only vaccine was the only vaccine to raise substantial levels of anti-Env titers (Fig. 4 and 5). Nevertheless, postchallenge, the Gag-Pol-Env DNA/rMVA- and rMVA-only-vaccinated animals demonstrated similar anamnestic antiviral antibody responses (Fig. 4 and 6B). Thus, the two vaccines may differ in their ability to stimulate naïve B cells to develop into antibody-secreting cells but seem overall similar in their ability to generate memory B cells.

Our antigen arrays primarily monitor linear epitopes and thus do not necessarily score neutralizing antibodies, which can be directed at conformational and discontinuous as well as linear epitopes. In general, the intensity of responses detected in the arrays correlated with the heights of antibody responses determined in ELISAs (Fig. 2B). Although arrays could be used in studies addressing the avidity of antibody binding to different epitopes, such studies were not undertaken in this analysis.

Our results demonstrate the power of antigen arrays for monitoring immune responses in vaccine trials. The development of analogous arrays may be particularly useful for analyzing vaccine trials for viruses, such as hepatitis B virus, Ebola virus, and respiratory syncytial virus, where neutralizing antibodies play a critical role in protection (recently reviewed in reference 12). Arrays may also be employed to analyze vaccine trials for or to detect infection with bioterrorism agents such as anthrax and smallpox.

SHIV antigen arrays distinguished vaccinated from challenged individuals (Fig. 4A and 5), identified novel (Table 2) and convergent (Table 3) viral epitopes, monitored the kinetics of epitope-specific responses (Fig. 4), surveyed the breadth and strength of antiviral antibody responses elicited by vaccination and challenge (Fig. 4 and 5), and served as a predictor of mortality (Fig. 5D and 9). Array profiles distinguished merely

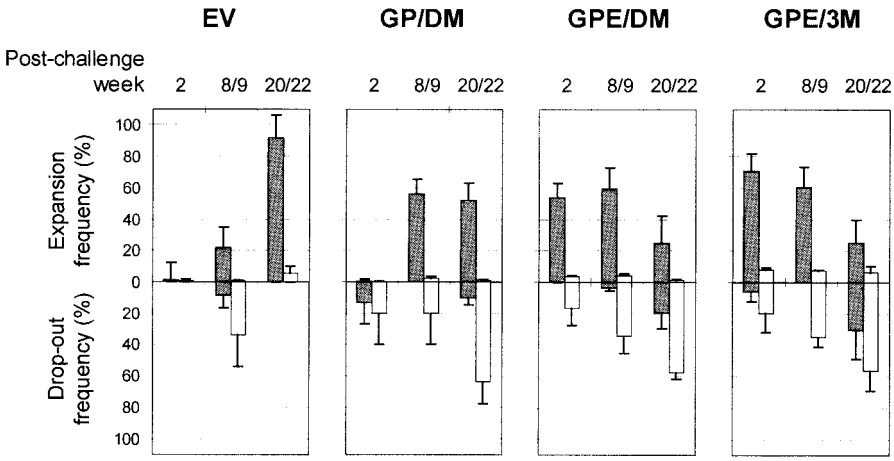


FIG. 8. Expansion and drop-out frequencies of reactivities to convergent and nonconvergent Env epitopes postchallenge. SAM analysis was performed on separate classes of epitopes: 24 convergent Env peptides listed in Table 3 (gray bars) and the remaining 162 nonconvergent Env peptides listed in Table 4 (open bars). Expansion frequency was defined as the number of newly positive epitopes within the specified class at the indicated time point (with new reactivity above 1,500 DFUs) divided by the number of negative epitopes within that class at the prior time point (with reactivity less than 1,500 DFUs), multiplied by 100%. Conversely, drop-out frequency was defined as the number of newly negative epitopes within the specified class (with reactivity falling below 1,500 DFUs) at the indicated time point divided by the number of positive nonconvergent epitopes within that class at the previous time points, multiplied by 100. Data are means ± standard error of the mean for individual monkeys within each treatment group. For designations of groups, see the legend to Fig. 4.

vaccinated from challenged animals based on the number and intensity of recognized epitopes as well as on the reactivity against several peptides derived from the C terminus of gp41which were absent from the rMVA vaccine (Fig. 4A, Fig. 5, and Table 2). Immediately following challenge, array profiles grouped animals according to responses associated with and predictive of specific vaccine regimens (Fig. 5). Antiviral antibody profiles also provided prognostic value, with failure to

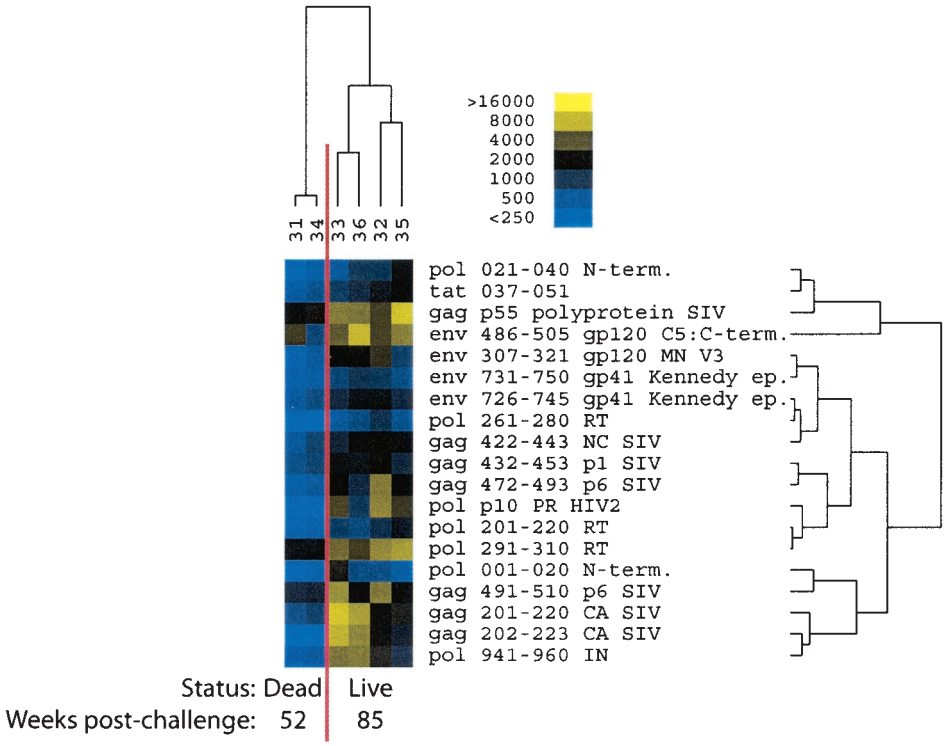


FIG. 9. Survival is associated with increased breadth and intensity of anti-SHIV antibody responses. SAM analysis based on survival data was used to identify differences in anti-SHIV array reactivities in serum obtained at postchallenge week 22 between macaques in the Gag-Pol DNA/rMVA group that died of AIDS versus those that controlled their infections. Array data for antigens with significant differences in reactivity were subjected to hierarchical clustering.

TABLE 4. Nonconvergent reactive SHIV Env peptides

Env protein	Region(s)	Total no. of nonconvergent peptides in region	Significantly reactive nonconvergent peptides[a]	
			No. (% of total) nonconvergent peptides	% that overlapped convergent peptides[b]
gp120	Leader & N terminus	17	2 (12)	0
	C1	8	2 (25)	0
	V1	5	1 (20)	100
	V2	7	3 (43)	100
	C2	19	1 (5)	0
	V3	10	3 (30)	100
	C3	13	2 (15)	0
	V4, C4, V5	16	0 (0)	n/c[c]
	C5	8	3 (38)	67
gp41	Fusion peptide, V, N-helix	12	0 (0)	n/c
	Wang/Gnann	4	2 (50)	100
	C-helix	7	4 (57)	75
	2F5	4	3 (75)	0
	Transmembrane	5	0 (0)	n/c
	MPI & Kennedy	6	3 (50)	100
	C terminus	21	3 (14)	33
		162	32	
	Avg for all regions		(20)	56

[a] Nonconvergent peptides that also demonstrated significant reactivity by pairwise SAM between samples at week 0 and any postchallenge time within each treatment group (Fig. 4B and C).

[b] Percentage significantly reactive nonconvergent peptides that overlapped convergent peptides (Table 3) by at least five amino acid residues.

[c] n/c, not calculable.

mount or maintain broad antiviral antibody responses correlating with the development of AIDS (Fig. 5D and 9).

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