Full-Length HIV-1 Immunogens Induce Greater Magnitude and Comparable Breadth of T Lymphocyte Responses to Conserved HIV-1 Regions Compared with Conserved-Region-Only HIV-1 Immunogens in Rhesus Monkeys

Kathryn E. Stephenson,^a Adam SanMiguel,^a Nathaniel L. Simmons,^a Kaitlin Smith,^a Mark G. Lewis,^b James J. Szinger,^c Bette Korber,^c and Dan H. Barouch^{a,d}

Division of Vaccine Research, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA^a; Bioqual, Inc., Rockville, Maryland, USA^b; Los Alamos National Laboratory, Los Alamos, New Mexico, USA^c; and Ragon Institute of MGH, MIT, and Harvard, Boston, Massachusetts, USA^d

A global HIV-1 vaccine will likely need to induce immune responses against conserved HIV-1 regions to contend with the profound genetic diversity of HIV-1. Here we evaluated the capacity of immunogens consisting of only highly conserved HIV-1 sequences that are aimed at focusing cellular immune responses on these potentially critical regions. We assessed in rhesus monkeys the breadth and magnitude of T lymphocyte responses elicited by adenovirus vectors expressing either full-length HIV-1 Gag/Pol/Env immunogens or concatenated immunogens consisting of only highly conserved HIV-1 sequences. Surprisingly, we found that the full-length immunogens induced comparable breadth ($P = 1.0$) and greater magnitude ($P = 0.01$) of CD8⁺ T lymphocyte responses against conserved HIV-1 regions compared with the conserved-region-only immunogens. Moreover, the full-length immunogens induced a 5-fold increased total breadth of HIV-1-specific T lymphocyte responses compared with the conserved-region-only immunogens ($P = 0.007$). These results suggest that full-length HIV-1 immunogens elicit a substantially increased magnitude and breadth of cellular immune responses compared with conserved-region-only HIV-1 immunogens, including greater magnitude and comparable breadth of responses against conserved sequences.

An effective global vaccine for human immunodeficiency virus type 1 (HIV-1) will need to afford protection against a tremendous diversity of HIV-1 variants worldwide (10, 16). Though the induction of HIV-1-specific antibodies will likely be necessary to block HIV-1 acquisition (2, 8), an effective HIV-1 vaccine will also likely need to induce HIV-1-specific cellular immune responses to control breakthrough infections and to provide T helper function (15). It would therefore be desirable for a vaccine to elicit HIV-1-specific T lymphocyte responses that target epitopes that are highly conserved across diverse HIV-1 subtypes and that impose a steep fitness cost on viral escape mutants (10, 11, 12, 21). It is currently unclear, however, how best to elicit responses against conserved regions by vaccination.

DNA- and viral vector-based vaccines expressing strings of linked HIV-1 epitopes that included highly conserved sequences showed early promise in preclinical studies (7, 27, 28), but these vaccines proved poorly immunogenic in phase 1 clinical trials (5, 6, 9). An alternative strategy to improve the induction of responses against highly conserved regions is based on concatenating longer conserved stretches of proteins that would capture the most conserved epitopes but that would also provide some local protein context, thus improving the chances of biologically appropriate epitope processing (7, 17, 21, 29). This strategy is under active investigation and has been shown to induce T lymphocyte responses to conserved regions in mice and rhesus monkeys (11, 22, 23). However, direct comparisons of responses to conserved regions when such sequences are embedded in full-length immunogens versus conserved-region-only immunogens have not previously been reported.

An alternative strategy to contend with global HIV-1 diversity is to use polyvalent full-length mosaic immunogens (4, 10) that

are designed to maximize immunologic coverage of HIV-1 sequence diversity while maintaining conserved sequences in their natural context (4, 18). These full-length mosaic immunogens have been shown to induce an increased breadth and depth of HIV-1-specific cellular immune responses compared to those of natural and consensus HIV-1 antigens in rhesus monkeys (3, 24). There is concern, however, that full-length immunogens may redirect immune responses away from conserved regions to more variable regions (12).

We therefore investigated whether conserved-region-only HIV-1 immunogens derived from mosaic sequences would more robustly focus immune responses on conserved HIV-1 epitopes than full-length mosaic HIV-1 immunogens in rhesus monkeys. As a secondary objective, we also compared the breadth of cellular immune responses elicited by 2-valent and 3-valent full-length HIV-1 immunogens. Our data demonstrate that the full-length immunogens elicited greater magnitude and breadth of cellular immune responses than the conserved-region-only immunogens, including greater magnitude and comparable breadth of CD8⁺ T lymphocyte responses against the conserved regions.

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Address correspondence to Dan H. Barouch, dbarouch@bidmc.harvard.edu.

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FIG 1 Immunogen design strategy. The 2-valent full-length mosaic HIV-1 Gag/Pol/Env immunogens were designed to provide optimal immunologic coverage of HIV-1 M group sequences in the Los Alamos National Laboratory HIV-1 Sequence Database (3). Regions rich in conserved epitopes were selected from the 2-valent full-length immunogens and concatenated into the 2-valent conserved-region-only immunogens. The sequences are provided in Fig. S1 in the supplemental material.

MATERIALS AND METHODS

Animals, vectors, and immunizations. Adult Indian-origin rhesus monkeys ($n = 18$) were housed at Bioqual, Inc., Rockville, MD, and were selected to be Mamu-A*01, B*17, and B*08 negative. Animals were primed at week 0 by the intramuscular route in the quadriceps muscles with 2×10^{10} viral particles of replication-incompetent adenovirus serotype 35 (Ad35) vectors and were boosted at weeks 12 and 48 with adenovirus serotype 26 (Ad26) vectors expressing 2-valent full-length ($n = 6$), 3-valent full-length ($n = 6$), or 2-valent conserved-region-only ($n = 6$) mosaic HIV-1 Gag/Pol/Env antigens (Fig. 1). Ad26 and Ad35 vectors were constructed using an adenovirus cosmid and adapter plasmid system with E1/E3 deletions as described previously (1). The HIV-1 antigens were expressed under the control of a human cytomegalovirus promoter. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Harvard Medical School.

ELISPOT assays and epitope mapping. Comprehensive T lymphocyte epitope mapping was performed utilizing 1,285 Gag, Pol, and Env potential T cell epitope (PTE) peptides that represented global HIV-1 potential epitopes found in >15% of HIV-1 sequences in the Los Alamos National Laboratory (LANL) HIV Sequence Database (NIH AIDS Research and Reference Reagent Program [13]), as well as 1,017 Gag, Pol, and Env overlapping peptides that matched the sequences of the 2-valent full-length vaccine (Mos1 and Mos2 peptides; JPT Peptide Technologies). These peptides allowed an analysis of both breadth (recognition of a particular epitope) and depth (cross-recognition of variants of that epitope) and were organized into subpools of 10 peptides (subpools of up to 16 peptides were allowed in regions of variable length or terminal regions of the proteins).

Gamma interferon enzyme-linked immunosorbent spot (ELISPOT) assays were conducted at week 4 following the boost immunizations utilizing PTE, Mos1, and Mos2 peptide pools and subpools with 2×10^5 peripheral blood mononuclear cells (PBMCs) per assay. All subpools with positive responses were then deconvoluted to individual 15-amino-acid PTE, Mos1, or Mos2 peptides and confirmed with CD8- and CD4-depleted PBMCs (MACS nonhuman primate CD8 and CD4 MicroBead kits; Miltenyi Biotec) to determine if reactive peptides represented CD8⁺ or CD4⁺ lymphocyte epitopes. For deconvolution and cell depletion assays, we utilized PBMCs from weeks 4, 8, and 12 postimmunization. The numbers of spot-forming cells (SFCs) per 1×10^6 PBMCs were calculated. Medium backgrounds were typically <15 SFCs per 1×10^6 PBMCs. Positive responses were defined as >55 SFCs per 1×10^6 PBMCs and >4-fold background.

Humoral immune assays. We evaluated Env-specific humoral immune responses by direct enzyme-linked immunosorbent assay (ELISAs) with seven variants of HIV-1 Env gp140: UG37 (clade A), UG92 (clade A), CN54 (clade C), ZA97 (clade C), UG21 (clade D), BR29 (clade F), and Mos1.

Statistical analysis. To calculate the breadth of the cellular immune responses, we evaluated the number of PTE, Mos1, and Mos2 peptide responses for each animal, and we aligned the reactive peptide sequences to eliminate overlap. If any reactive peptide sequences shared 9 or more amino acids, we assumed that the peptides were recognized by the same T lymphocyte; these overlapping sequences were conservatively defined as a single positive-response region. If the first and last overlapping peptide in a string of overlapping peptides shared 8 or less amino acids, we assumed that the peptides were recognized by a minimum of two T lymphocytes and represented two positive-response regions. The magnitudes of responses were calculated as the maximum number of SFCs per 1×10^6 PBMCs detected for any given positive-response region, where response regions were defined as the set of several overlapping and variable peptides that was positive in the ELISPOT assays. We assumed that the maximum response represented the optimal peptide among the set of positive overlapping peptides in terms of susceptibility to a given T lymphocyte response. The distributions of the breadth (number of positive-response regions per monkey) and magnitude of the responses were compared between vaccine groups using a nonparametric Wilcoxon rank-sum test with a continuity correction. Two-tailed P values of <0.05 were considered significant. All statistical analysis was performed using the statistical package R (<http://www.r-project.org/> [20]).

RESULTS

Immunogen construction. The 2-valent full-length mosaic HIV-1 Gag/Pol/Env immunogens were designed to provide optimal immunologic coverage of HIV-1 M group sequences in the LANL HIV-1 Sequence Database and have been described previously (3). The 3-valent full-length mosaic immunogens were designed to include one extra set of immunogens, in addition to the 2-valent mosaic immunogens, and aimed to augment immunologic coverage of potential T cell epitopes (10). Full-length mosaic proteins do not contain unnatural HIV-1 junctional regions by deliberate exclusion of such junctions during the design phase, and Gag, Pol, and Env antigens are not fused together. Pol antigens contained reverse transcriptase and integrase without pro-

FIG 2 Breadth and magnitude of HIV-1-specific CD8⁺ T lymphocyte responses following immunization with 2-valent mosaic full-length, 2-valent mosaic conserved-region-only, and 3-valent mosaic full-length immunogens. All monkeys received Ad35 vectors at week 0 and Ad26 vectors at week 12 (time point 1) and week 48 (time point 2). The breadth of cellular immune responses was assessed with Gag, Pol, and Env PTE peptides, as well as overlapping peptides that matched the sequences in the 2-valent full-length vaccine (Mos1, Mos2). The magnitude of individual peptide responses is depicted on the y axis. Individual monkeys are depicted on the x axis. Reactive peptide sequences that included 9 or more shared amino acids are assumed to be recognized by the same T lymphocyte and are clustered together as positive-response regions labeled in numerical order by monkey.

tease and included point mutations to eliminate catalytic activity, as previously described (19). In addition, Env gp140 antigens contained point mutations to eliminate cleavage and fusion activity.

The conserved-region-only vaccine was constructed by excising and concatenating six highly conserved regions (3 in Gag, 1 in Pol, and 2 in Env) from the 2-valent full-length mosaic antigens described above. These sequences included 12% of the total protein length and were identical in the 2-valent full-length mosaic and conserved-region-only vaccines. These sequences were selected by meeting three criteria: (i) the contiguous sequences were found in >80% of all HIV-1 M group viruses in the LANL database, (ii) the sequences were rich in known human cytotoxic T lymphocyte epitopes to allow further clinical vaccine development if desirable, and (iii) the sequences contained known rhesus epitopes to optimize our ability to detect responses in the nonhuman primate model. We concatenated these six peptides (ranging in length from 30 to 70 amino acids) into two conserved-region-only polypeptide immunogens of 247 amino acids each. The immunogen design strategy is depicted in Fig. 1, and the immunogen sequences are provided in Fig. S1 in the supplemental material.

The full-length immunogens induced comparable breadth of T lymphocyte responses to the conserved HIV-1 sequences as compared with the conserved-region-only immunogens. The primary goal of our study was to determine whether the conserved-

region-only immunogens would focus and enhance immune responses against highly conserved HIV-1 sequences compared with those of the full-length immunogens. We therefore immunized 18 rhesus monkeys by the intramuscular route with Ad35/Ad26 vectors expressing (i) full-length 2-valent mosaic Gag/Pol/Env immunogens, (ii) conserved-region-only immunogens derived from the 2-valent mosaic sequences as described above, or (iii) full-length 3-valent mosaic Gag/Pol/Env immunogens ($n = 6$ per group).

We first determined which vaccine elicited the greatest breadth of responses to the conserved HIV-1 regions contained in the conserved-region-only vaccine. We assessed the number of positive epitope-specific responses elicited by each vaccine at week 4 following the boost immunizations and detected by epitope mapping utilizing the combined sets of PTE, Mos1 and Mos2 peptides (see Materials and Methods). In this analysis, positive responses detected by peptides that overlapped by 9 or more amino acids were defined as a single response, to provide a minimum estimate of the number of responses needed to explain the response data. We observed that the 2-valent full-length immunogens induced CD8⁺ T lymphocyte responses to conserved HIV-1 regions comparable in number to those induced by the conserved-region-only vaccine after the first boost immunization (Fig. 2; see Fig. S2 in the supplemental material). Specifically, there were 6 total conserved-region CD8⁺ T lymphocyte responses in each group, and at the second time point, there were 7 responses detected in each group

FIG 3 Breadth and magnitude of HIV-1-specific CD4⁺ T lymphocyte responses following immunization with 2-valent mosaic full-length, 2-valent mosaic conserved-region-only, and 3-valent mosaic full-length immunogens. The magnitude of individual peptide responses is depicted on the y axis. Individual monkeys are depicted on the x axis.

($P = 1.0$, Wilcoxon rank-sum test). As expected (3), CD4⁺ T lymphocyte responses were sporadic in both groups (Fig. 3). These results indicate that the 2-valent full-length and the conserved-region-only immunogens induced comparable breadths of responses to the conserved regions.

The full-length immunogens induced higher-magnitude cellular immune responses to the conserved HIV-1 epitopes compared with those induced by the conserved-region-only immunogens. We next assessed the magnitude of the positive responses against the conserved HIV-1 epitopes induced by the two vaccines. We sought to determine whether the conserved-region-only vaccine might focus and thereby amplify cellular immune responses to conserved HIV-1 regions compared to the full-length immunogens, which could have theoretically redirected immune responses toward more variable epitopes. Surprisingly, we observed that the full-length immunogens generated significantly greater magnitude CD8⁺ T lymphocyte responses to the conserved HIV-1 regions (median, 638 SFCs per 1×10^6 PBMCs; interquartile range [IR], 566 to 660 SFCs per 1×10^6 PBMCs) than the conserved-region-only vaccine (median, 288 SFCs per 1×10^6 PBMCs; IR, 156 to 438 SFCs per 1×10^6 PBMCs) after the early boost ($P = 0.01$, Wilcoxon rank-sum test) (Fig. 4) and still showed a trend toward higher-magnitude responses to the conserved regions after the final boost (medians, 480 versus 160 SFCs per 1×10^6 PBMCs; $P = 0.1$) (Fig. 4). Thus, the full-length immunogens elicited higher-magnitude cellular immune responses against the conserved regions than the conserved-region-only immunogens, perhaps reflecting a benefit of presenting and processing conserved epitopes in a more natural context.

The full-length immunogens induced greater total breadth of HIV-1-specific T lymphocyte responses than the conserved-region-only immunogens. We next evaluated whether the full-length immunogens induced greater total breadth of HIV-1 epitope-specific cellular immune responses than the conserved-region-only immunogens. Total breadth was measured as the number of positive HIV-1 response regions detected by PTE, Mos1, and Mos2 peptides via epitope mapping (see Materials and Methods). As expected, the full-length immunogens induced a significantly 4-fold greater total breadth of HIV-1-specific CD8⁺

T lymphocyte responses than the conserved-region-only immunogens (4 versus 1 median epitopes, $P = 0.007$) (Fig. 2). After the final boost immunization, the full-length immunogens induced a significantly 5-fold greater breadth of HIV-1-specific CD8⁺ T lymphocyte responses than the conserved-region-only immunogens (5 versus 1 median epitopes, $P = 0.007$). These data demonstrate that the full-length immunogens induced a markedly increased breadth of total HIV-1-specific cellular immune responses compared to the conserved-region-only immunogens, which is not surprising, given their much greater length, but they also induced greater magnitude and comparable breadth of responses to the actual conserved sequences encoded in the conserved-region-only vaccine.

FIG 4 Magnitude of positive CD8⁺ T lymphocyte responses against conserved HIV-1 epitopes induced by 2-valent full-length and conserved-region-only mosaic HIV-1 immunogens. Box-and-whisker plots depict the magnitude of responses in each group for each time point. P values were determined by Wilcoxon rank-sum tests.

HIV-1-specific CD4⁺ T lymphocyte responses were generally lower than CD8⁺ T lymphocyte responses in both vaccine groups, although the breadth of CD4⁺ T lymphocyte responses was still greater with the 2-valent full-length vaccine than the conserved-region-only vaccine (Fig. 3). We also assessed responses to peptides spanning the junctions of the conserved regions in the conserved-region-only vaccine, and we did not observe any CD4⁺ or CD8⁺ T lymphocyte responses specific for junctional epitopes (data not shown). We also did not observe any unusual spatial clustering of epitopes indicative of changes in immunodominance patterns induced by the 2-valent conserved-region-only immunogen compared to the 2-valent full-length immunogen, apart from the fact that the immune responses induced by the 2-valent conserved-region-only immunogen were focused solely on conserved regions (see Fig. S2 in the supplemental material).

The 2-valent full-length mosaic immunogens induced an equivalent breadth of HIV-1-specific T lymphocyte responses as the 3-valent full-length mosaic immunogens. A secondary objective of our study was to assess whether the 3-valent full-length mosaic immunogens would induce greater breadth of cellular immune responses than the 2-valent full-length mosaic immunogens. Theoretically, increasing the valency of the mosaic vaccine should improve immunologic coverage (4, 10), but this may be counterbalanced by reduced doses of each vaccine component as well as diminishing theoretical coverage benefits with increasing valency. We therefore compared the total breadth of HIV-1-specific responses elicited by the 2-valent full-length mosaic HIV-1 vaccine with the 3-valent full-length mosaic HIV-1 vaccine. Interestingly, we found that the 2-valent full-length vaccine induced an essentially comparable breadth of HIV-1-specific CD8⁺ T lymphocyte responses as the 3-valent full-length vaccine after the initial boost ($P = 0.75$) and after the final boost ($P = 0.94$) (Fig. 2). There was also no difference in HIV-1-specific CD4⁺ T lymphocyte responses between the 2-valent and 3-valent full-length mosaic vaccines ($P = 0.26$). These data suggest at most a minimal benefit of increasing the valency of the mosaic vaccine.

It should be noted that our analysis may not have been powered to detect small increases in breadth attributable to the 3-valent vaccine. The theoretical benefit of including additional mosaics beyond the first two in a set is relatively small (10), and with only 6 animals per group, we would not expect to discern subtle benefits. In addition, since we did not include a dedicated matched Mos3 peptide set for epitope mapping and the rarer epitopes included in the 3-valent vaccine were not fully represented in the PTE peptide set, it is possible that additional vaccine responses against the third mosaic may have been missed. As the first two mosaic immunogens generally included the most common epitopes, the third mosaic immunogen included sequences that are rarer at the population level. The PTE set is based on coverage of the most common variants in the population, and so 33% of the new variants introduced in the third mosaic immunogen were not included in the PTE set; thus, responses to these variants would not have been detected in our assays. Nevertheless, these data suggest that the benefits of increasing the mosaic valency from 2 to 3 are likely to be small.

Taken together, our results demonstrate that the 2-valent full-length mosaic HIV-1 immunogens are immunologically advantageous in terms of T cell responses to the 2-valent conserved-region-only immunogens and, consistent with prior findings (25),

FIG 5 HIV-1 Env-specific binding antibody titers induced by 2-valent mosaic full-length, 3-valent mosaic full-length, and 2-valent mosaic conserved-region-only immunogens. Titers of antibody binding to 7 strains of Env determined by ELISA at multiple time points (weeks) are depicted.

are noninferior to the 3-valent full-length mosaic HIV-1 immunogens.

Env-specific antibodies were induced by the full-length mosaic immunogens. Finally, we evaluated Env-specific humoral immune responses induced by full-length mosaic HIV-1 immunogens. Despite being designed to optimize contiguous linear peptide sequences, both full-length mosaic vaccines induced Env-specific binding antibody titers by ELISA that peaked 4 weeks after the first boost immunization (Fig. 5). The conserved-region-only vaccine did not contain intact Env immunogens and thus did not induce any detectable Env-specific binding antibodies. Though not surprising, these results underscore additional potential benefits of the full-length immunogens compared to the conserved-region-only immunogens, since it is believed that the induction of Env-specific antibodies will likely be necessary to block HIV-1 acquisition (2, 8).

DISCUSSION

One of the key obstacles to the development of an effective HIV-1 vaccine is the tremendous genetic diversity of globally circulating strains of HIV-1 (10, 16, 26). This genetic diversity makes it difficult to design a single vaccine immunogen that will provide immunologic coverage against so many different strains. One vaccine strategy proposed to overcome this challenge is to design HIV-1 immunogens that consist solely of highly conserved HIV-1 regions. The hypothesis underlying this strategy is that immune responses specific for conserved HIV-1 regions will recognize a multitude of different circulating HIV-1 strains and that these responses will be particularly beneficial because they impose a high fitness cost on HIV-1 escape mutants (11, 12, 21, 29). We therefore directly investigated the possibility that responses to conserved epitopes would be enhanced if they were presented in isolation.

We observed that full-length HIV-1 Gag/Pol/Env immunogens induced a substantially greater total breadth of HIV-1-specific T lymphocyte responses, greater magnitude of T lymphocyte responses against conserved HIV-1 epitopes, and comparable breadth of T lymphocyte responses against conserved HIV-1 epitopes compared to conserved-region-only HIV-1 Gag/Pol/Env immunogens. These data suggest that the conserved-region-only immunogens provided no clear benefit compared with full-length immunogens. We also showed that

the 2-valent full-length mosaic immunogens induced an essentially comparable breadth of HIV-1-specific cellular immune responses as the more complex 3-valent full-length mosaic immunogens.

Several HIV-1 vaccine immunogens have been designed to induce cellular immune responses against conserved and/or immunodominant epitopes, including the polyepitope immunogens HIVA (7) and EP HIV-1090 (28), although these immunogens proved poorly immunogenic in clinical trials (5, 6, 9). These data suggest that polyepitope immunogens may not have been optimally processed for or presented to the immune system (10). To place conserved HIV-1 sequences within a more natural context, another strategy involves immunogens such as HIV_{consv}, which was designed to include longer fragments of conserved HIV-1 regions (11). Preclinical studies of this strategy in mice and rhesus monkeys have shown that HIV_{consv} is immunogenic when expressed by DNA and viral vector vaccines, particularly when boosted by injections of adjuvanted synthetic long peptides (22, 23). An ongoing clinical trial of HIV_{consv} (clinical trial NCT01151319) will provide important information regarding the safety and immunogenicity of this immunogen. Nevertheless, a direct comparison of conserved-region-only immunogens and full-length immunogens has not previously been reported.

The peptide fragments in our conserved-region-only immunogens were roughly comparable to those in HIV_{consv} (11) in terms of peptide length. Contrary to our hypothesis, we observed that epitopes embedded in the conserved regions elicited greater magnitude and comparable breadth of responses when embedded in full-length mosaic HIV-1 immunogens compared to concatenated in the conserved-region-only vaccine. A likely reason for the enhanced magnitude of the responses is that the conserved HIV-1 sequences embedded in the full-length proteins were processed and presented optimally by antigen-presenting cells. Moreover, we demonstrate that full-length immunogens can induce an increased total breadth of HIV-1-specific cellular immune responses without diminishing responses to conserved HIV-1 regions. Given that the number of vaccine-induced responses has been shown to correlate with the control of viremia and survival following simian immunodeficiency virus challenge of rhesus monkeys (14), eliciting an increased breadth of responses may be beneficial. These data suggest potential immunologic advantages to utilizing full-length immunogens over conserved-region-only immunogens. We also showed that full-length mosaic HIV-1 immunogens induced Env-specific antibodies, despite being designed to optimize coverage of contiguous epitopes (3, 25).

In conclusion, our data demonstrate that an HIV-1 mosaic vaccine expressing full-length immunogens elicited greater cellular immune responses to conserved epitopes than a concatenated conserved-region-only vaccine. These data indicate that responses to epitopes in variable regions did not compromise responses to the conserved regions. Moreover, our results suggest that presentation and processing of conserved epitopes in the more natural context of full-length immunogens may be critically important for the induction of immune responses targeted to particular HIV-1 regions. In addition, the 2-valent and 3-valent full-length mosaic vaccines induced essentially comparable breadths of HIV-1-specific T lymphocyte responses. These results support the clinical evaluation of the 2-valent full-length HIV-1 mosaic immunogens.

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