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Mosaic Vaccines Elicit CD8+ T Cell Responses in Monkeys that Confer Immune Coverage of Diverse HIV Strains

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Abstract

Creation of a successful HIV vaccine will require the development of a strategy to generate cellular immunity with sufficient cross-clade breadth to deal with the extreme genetic diversity of the virus. Polyvalent mosaic immunogens derived from *in silico* recombination of natural strains of HIV are designed to induce cellular immune responses that maximally cover the sequence diversity of circulating virus isolates. Immunization of rhesus monkeys with plasmid DNA and recombinant vaccinia virus vaccine constructs expressing either consensus immunogens or polyvalent mosaic immunogens elicited a CD4+ T lymphocyte-biased response with comparably broad epitope-specific total T lymphocyte specificities. However, immunization with the mosaic immunogens induced HIV-specific CD8+ T lymphocyte responses with markedly greater depth and breadth. Therefore, the use of polyvalent mosaic immunogens is a promising strategy for a global vaccine for HIV.

An HIV vaccine must elicit immune responses that recognize genetically diverse viruses (*1, 2*). Accruing data suggest that both cellular and humoral immune responses will be needed to contain HIV spread. Therefore, an HIV vaccine must generate CD8+ T lymphocytes that control HIV replication as well as CD4+ T lymphocytes that provide help for the generation and maintenance of both cellular and humoral immune responses against the virus (*3-5*). Creating immunogens that can elicit cellular immune responses against the genetically varied circulating isolates of HIV presents an important challenge for creating an AIDS vaccine.

Recent studies have suggested that vaccines based on centralized HIV gene sequences might provide a solution to the problem posed by the genetic heterogeneity of the circulating strains of HIV (*6-9*). Proteins created from consensus envelope sequences, generated by selecting the most commonly used amino acid of all catalogued HIV sequences at each residue of the protein, were shown to have functional properties of naturally occurring HIV envelopes and conserved neutralizing antibody epitopes (*7, 10, 11*). It was also shown in a rhesus monkey model that the cross-reactive potential of T lymphocyte responses generated by immunization with a consensus envelope immunogen was significantly enhanced compared to T lymphocyte responses generated by immunization with a single natural envelope protein immunogen (*12*).

Building on the successes of a centralized gene approach to immunogen design, we have begun to explore the use of a polyvalent mosaic vaccine strategy that optimizes the population coverage of potential T cell epitopes for selected antigens (*13*). These vaccines are designed to elicit immune responses with the greatest potential to recognize

genetically diverse HIV variants. The mosaic immunogen sequences are derived from *in silico* recombination of natural strains of HIV that are constrained to have recombination breakpoints that are found in nature, and are complete protein sequences. The computational design uses a genetic algorithm that mimics evolution by recombination that naturally occurs in HIV. However, contrast to natural evolution, where, viral fitness is the selection criteria, the selection criteria for mosaics are the population coverage of potential T cell epitopes (*13*). Potential epitopes are defined as all epitope-length fragments (we use 9-mers, the most common optimal length of MHC class I presented epitope, and the common span between anchor residues in MHC class II epitopes (*14*) The present study was initiated to explore the breadth of CD4+ and CD8+ T lymphocyte responses generated through vaccination with polyvalent mosaic as compared to vaccination with consensus immunogens.

We selected two HIV genes for use in the immunogens in this study: HIV-1 *gag* and *nef*. This choice was originally motivated by the high frequency of both CD4+ and CD8+ T cell responses to these proteins in natural HIV-1 infection ((*13, 15, 16*). These antigens essentially correspond to the design proposed in the original mosaic concept paper (*13*). Although we originally proposed to use only the conserved core of Nef, we chose to use intact Nef proteins in this study because the immunogenicity of the Nef fragment was poor (*17*).

Thirty *Mamu-A*01*-negative rhesus monkeys were distributed into three groups, two experimental groups each consisting of 12 monkeys and a control group consisting of

6 monkeys. The experimental groups of monkeys received priming immunizations by the intramuscular route at weeks 0, 4 and 8 with a total of 5 mg of *gag* and a total of 5 mg of *nef* plasmid DNA for each immunization. At week 33, monkeys were boosted by intramuscular and intradermal inoculation with a total of 10^9 PFU of a recombinant vaccinia virus expressing *gag* and a total of 10^9 PFU of a recombinant vaccinia virus expressing *nef*. One of these experimental groups received immunogens containing single consensus gene inserts and the other received immunogens containing a cocktail of 4 complementary mosaic gene inserts. The control monkeys were immunized with empty vectors.

The breadth of the vaccine-elicited cellular immune responses was determined by assessing peripheral blood T lymphocyte recognition of 10 different Gag and 10 different Nef sequences using a peptide/IFN-γ ELISpot assay. Each of the 10 sets of indicator proteins included two clade A, two clade B, four clade C, and two clade G sequences, and were selected according to the same strategy we used in our previous evaluation of the consensus Env immunogen (*12*). These sequences were recent and were sampled from diverse geographic locales to represent the contemporary diversity of the sequences catalogued in the Los Alamos database (*12*). By assessing T lymphocyte immune responses to each of these selected proteins, we simulated the exposure of the vaccine-induced cell populations to representative, diverse real virus proteins (*12*). This strategy allowed us to evaluate the precise number of responses per strain elicited by the vaccine in each animal and characterize the cross-reactive potential of each epitope-specific response (Fig.1).

We first determined the minimum number of epitope-specific T cell responses to each of the 10 strains in the monkeys induced by vaccination with either the consensus or the mosaic immunogens (Fig. 1A). These data are displayed as the minimum number of responses to each of the 10 series of indicator peptides, where responses to overlapping peptides from the same strain are treated as a single epitope-specific response. The median minimum number of epitope-specific T cell responses generated by each monkey against a single strain of Gag and Nef following vaccination with consensus immunogens was 5.5 [interquartile range (IR) 3.0-11.75], and the median minimum number of epitope-specific T cell responses generated by each monkey following vaccination with mosaic immunogens was 7.75 (IR 6.0-11.4). There was no statistically significant difference between these values in the two experimental groups of monkeys (Wilcoxon rank sum test). In comparing the minimum number of epitope-specific T cell responses of these monkeys to individual proteins, either Gag alone or Nef alone, we observed a trend (Wilcoxon rank sum, $p = 0.07$) toward a significant advantage in using mosaic immunogens for inducing Nef epitope-specific T cell responses, but not for inducing Gag epitope-specific T cell responses. Statistical modeling using Poisson regression was not appropriate as the data were highly overdispersed, and the there was significant vaccine/protein (Gag and Nef) interaction. We therefore used a more appropriate quasipoisson regression model, and considered the Gag- and Nef-specific responses individually. This approach also showed no advantage for using either the mosaic or consensus immunogens for total CD4+ and CD8+ T cell responses. There were significantly fewer responses to Nef than to Gag by a factor of 0.6 ($P < <2e\text{-}16$). However, Nef is ~200 while Gag is ~500 amino acids in length. If one normalizes by

antigen size, there is a greater density of Nef- than Gag-specific responses (the peptides recognized by PBLs of each animal are displayed in Fig. S1).

We then assessed the total number of these minimum epitope-specific T lymphocyte responses generated by each vaccinated monkey. We defined the minimum number of epitope-specific T cell responses each animal, counting all responses to variant forms of the same epitope only once. For example, if T cells from a vaccinated monkey recognized peptide number 25 in 4 different Gag peptide series, that recognition would be scored as the recognition of a single epitope. Overlapping reactive peptides were also counted as one potential epitope, As shown in Fig. 2A, no significant difference was observed in the minimum number of epitope-specific T cell responses comparing those elicited by vaccination with consensus immunogens [15.0 (IR 6.5-22.5)] and those elicited by mosaic immunogens [15.5, (IR 11.75-22.25)]. There were also no significant differences between the numbers of epitope-specific T cell responses elicited by the consensus and mosaic immunogens when considering responses to the individual virus proteins (data not shown).

We then determined whether one or the other of these vaccine strategies induced cellular immune responses that recognized a greater diversity of total epitope containing peptide variants per animal (Fig. 2B). To carry out this analysis, we quantified the responses to variant forms of the same epitope (SOM methods). There was no difference in these values between the groups of vaccinated monkeys as determined by the Wilcoxon rank sum test, assessing the total number of recognized variants ($p = 0.24$), the

Gag variants alone ($p = 0.45$), or the Nef variants alone ($p = 0.17$). These data were massively over dispersed, and therefore we concluded that neither a Poisson or quasipoisson regression analysis was an appropriate statistical model for the data. The overdispersion suggests that unexplained factors are impacting the level of these T cell responses – this is consistent with the extremely highly numbers of responses observed in s small number of the vaccinated animals. Both groups included animals that recognized more than 100 distinct variant peptides; the two highest responding animals were in the mosaic vaccinated group and responded to 250 and 300 variant peptides (Fig. 2B) associated with a minimum of ~40 distinct T cell responses (Fig. 2A). Because the extent of animal-to-animal variation in response could mask a benefit of the mosaic vaccine approach, we evaluated the recognition of epitope variants by defining the typical diversity of variants recognized by PBLs of each experimental animal. We determined the average number of variants recognized per response by dividing the total number of variants responses by the minimum number of responses for each vaccinated monkey. In comparing these ratios between the two groups of vaccinated monkeys, we found that the mosaic-vaccinated monkeys had higher values than the consensus-vaccinated monkeys ($p = 0.045$, Wilcoxon rank sum test).

While theoretical reasoning suggested that the mosaic immunogens should generate cellular immune responses with a greater breadth of immune recognition than consensus immunogens ((*13*), these data showed that any advantages regarding total CD4+ and CD8+ T cell responses associated with using mosaic immunogens were modest. We reasoned, however, that both vectors employed in the present immunizations

generate CD4+ T lymphocyte-biased cellular immune responses (*18*), and any benefit derived from using mosaic immunogens might be obscured in known promiscuous CD4+ T lymphocyte responses (*19*). That is, since CD4+ T lymphocytes recognize peptide antigen in association with MHC class II molecules, and there is a greater promiscuity of peptide/MHC class II binding than peptide/MHC class I binding, a benefit associated with using mosaic immunogens may be more apparent in CD8+ T lymphocyte than in CD4+ T lymphocyte responses (*20, 21*).

To determine whether the mosaic immunogens elicited a broader CD8+ T lymphocyte response than the consensus immunogens, we re-evaluated the breadth of T cell responses to HIV Gag and Nef that were generated by mosaic and consensus gene-based immunizations using unfractionated and CD8+ T lymphocyte-depleted PBL populations. Responses that persisted in CD8+ T lymphocyte-depleted PBLs were likely CD4+ T lymphocyte responses, and the unfractionated lymphocyte responses minus the CD8+ T lymphocyte-depleted responses represented the CD8+ T lymphocyte responses. Sufficient lymphocytes were available from 7 monkeys in each of the groups of vaccinated animals to carry out this evaluation; the animals that had responded to more than 100 variant peptides (above the orange band in Fig. 2B) could not be evaluated in these cell fractionation studies as the initial mapping of the response to the peptide level had required the use of the available PBLs. In the 7 consensus immunized and 7 mosaic immunized monkeys that were evaluated, there were no sustained differences in the magnitude of the vaccine-induced anti-Gag and anti-Nef antibody responses (Fig. S2); therefore, any detected differences in the cross-reactivity of the cellular responses

between these groups of vaccinated monkeys were not simply a manifestation of a more robust general immune response. In the evaluation of the cellular immune response, if greater than one half of a SFC response by unfractionated PBLs was eliminated by CD8+ T lymphocyte depletion, we designated that response as CD8+ T lymphocyte-mediated.

We then repeated the analyses we had previously performed on the CD8+ and CD4+ T cell subsets for the seven animals. As illustrated in Fig.1B and 1C, the vaccine-elicited cellular immune responses in these monkeys were indeed biased toward CD4+ T lymphocyte responses by a factor of 2.2 (Poisson regression, $p = 2 \times 10^{-16}$) in the Gag-specific responses and by a factor of 11.8 (Poisson regression, $p = 2 \times 10^{-16}$) in the Nef-specific responses. The data were not overdispersed, but there was a significant interaction between the vaccine and the Gag and Nef proteins. We therefore modeled the T cell responses separately for the two proteins, using vaccine, clade of the peptide test strain, and T cell type as explanatory variables. The number of responses per strain was enhanced in the mosaic group relative to consensus in Gag by a factor of 1.9 (P= 0.05), and the CD8+ T cell responses were increased relative to the CD4+ T cell response among the Nef mosaic responses by a factor of 3.8 (P = 0.00009). (See supplement figure S2 for a full breakdown of responses by protein and by CD4/CD8 T lymphocytes). The median minimum number of epitope-specific CD4+ T lymphocyte responses per strain in the monkeys induced by vaccination with the mosaic immunogens was 4.5 (IR 2.0-7.0), while those induced by vaccination with the consensus immunogens was 2.0 (IR 1.3-4.0). The median minimum number of total epitope-specific CD8+ T lymphocyte responses per strain in the monkeys induced by vaccination with the mosaic immunogens was 2.0

(IR 1.0-3.0), while those induced by vaccination with consensus immunogens was 1.0 (IR 0.0-1.0).

We then compared total minimum number of epitope-specific responses across all strains per animal in each group using the Wilcoxon rank sum test (Fig. 2C and 2D). For CD4+ T lymphocyte responses, there was no evidence for differences in the number of total ($p = 0.28$), Gag-specific ($p=0.18$), or Nef-specific responses ($p = 0.45$). However, for CD8+ T lymphocyte responses, there were significantly more total ($p = 0.001$) and Gag-specific ($p = 0.006$) but not Nef-specific ($p = 0.158$) responses. This finding was supported by the Poisson regression modeled separately for each protein. For Gag-specific there were more CD4+ than CD8+ T cell responses by a factor of 2.1 ($P = 1 \times 10^{-14}$), and the mosaic immunogen elicited more responses per monkey specific for Gag by a factor of 2 ($P = 0.02$). For Nef-specific responses, there were more CD4+ than CD8+ T cell responses per monkey by a factor of 11.3 ($P = 2 \times 10^{-13}$), and the CD8+ T cell responses were increased relative to CD4+ T cell response in the Nef mosaic vaccinees by a factor of 3.9 ($P = 0.005$).

We also assessed whether the mosaic vaccinated monkeys developed cellular immune responses that recognized a greater diversity of epitope peptide variants than the consensus vaccinated monkeys by quantifying the responses to variant forms of the same epitope. For CD4+ T lymphocyte responses, there were significantly more Gag-specific ($OR = 2.8$, $p = 0.058$) but not Nef-specific ($OR = 0.9$, $p = 0.833$) responses to variant peptides in the mosaic immunized group. Strikingly, for CD8+ T lymphocyte responses,

there were more total responses by a factor of 2.5 ($p = 0.0001$), Gag-specific responses by a factor of 2.4 ($p = 0.003$), and Nef-specific responses by a factor of 2.5 ($p = 0.139$) in the mosaic immunized than in the consensus immunized monkeys. These findings are consistent with the hypothesis that the benefit of the mosaic immunogens was more apparent for CD8+ T lymphocyte than CD4+ T lymphocyte responses.

Finally we assessed the breadth and depth of anti-HIV CD8+ T lymphocyte coverage generated by each of the vaccine strategies. Examples of several types of typical responses and our strategy for illustrating them are shown in Fig. 3; the complete alignment of all peptides where at least a single response was made is provided in Fig. S1. As illustrated in Fig. 3 A and C and shown comprehensively in Fig. 4, it was very common for a vaccine-induced CD8+ T cell response to be highly strain specific and capable of recognizing peptide variants from only 1 or 2 of the 10 test strains. Recognition of a single variant among the 10 test strains occurred in 11 of the consensus group responses, and 20 of the mosaic group responses. In the 11 strain-specific CD8+ T lymphocyte responses in the consensus group of monkeys, most recognized peptides closely resembled the consensus sequence of the vaccine immunogen (Fig. 3A, example; Fig S1, complete details). In contrast, in 17 of the 20 strain-specific CD8+ T lymphocyte responses in the mosaic vaccinees, only 1 of the 4 mosaic immunogen proteins closely matched the strain-specific peptide recognized in the ELISpot assay. This suggests that the mosaic immunogens extended the breadth of the CD8+ T cell responses by generating additional variant-specific responses. Broadly cross-reactive responses to highly conserved epitopes were very rare, with all 10 peptide variants recognized only 6 times in

all vaccinated monkeys (Fig. 3B, 4). As shown in Fig. 3B, a response occurs for two overlapping peptides, and therefore likely represents the recognition of a single epitope; the same two overlapping peptides accounted for the breadth of responses in consensus vaccinated animals 93 and 228 (Fig. 4, C18/19 and C15/16). Finally, extending the breadth of response to more than 2 variant peptides occurred only 6 times among the consensus vaccine-induced CD8+ T cell responses and 17 times among those responses in mosaic vaccine recipients (Fig. 3D, 4). Modeling the depth of the peptide-specific responses as a function of vaccine, protein and T cell type, we found that mosaic vaccine-elicited CD8+ T cell responses were more likely than consensus vaccine-elicited CD8+ T cell responses to recognize variant peptides by a factor of 1.4 ($P = 0.021$). Variants of Nef were less likely to be recognized by CD8+ T cells than variants of Gag by a factor of 1.4 ($P = 0.024$). Finally, CD4+ T cell responses had greater depth than CD8+ T cell responses by a factor of 1.5 ($P=0.008$).

Because we assessed the reactivity of the PBL of each vaccinated monkey to peptides spanning the entire Gag and Nef proteins of 10 genetically disparate HIV-1 isolates, we were able to determine the vaccine coverage of these distinct virus strains in the vaccinated monkeys. As shown in the white, blue, purple, red and aqua shaded bars in the boxes adjacent to each monkey number in Fig. 4, a substantial number of peptides from an entire strain of virus were not recognized by PBL of the consensus vaccine immunized monkeys, while almost all of the indicator strains of peptides were recognized by at least one epitope-specific CD8+ T lymphocyte response in the PBL of the mosaic vaccine recipients ($P = 0.057$, wilcoxon rank sum). These observations underscore the

breadth and depth of CD8+ T lymphocyte responses elicited by the mosaic vaccine constructs.

These observations highlight the substantial difference in the breadth of vaccine-elicited CD4+ T lymphocyte and CD8+ T lymphocyte responses in monkeys. Vaccine-induced epitope-specific CD4+ T lymphocytes can recognize quite divergent sequences, while vaccine-induced epitope-specific CD8+ T lymphocytes are, for the most part, highly sequence restricted. We previously demonstrated the increased breadth of recognition specificity that can be generated through vaccinating with a construct expressing a centralized gene (*12*). The present study demonstrates the extraordinary cross-reactivity of CD8+ T lymphocyte recognition that can be generated using mosaic immunogens.

The breadth of the mosaic vaccine-induced CD8+ T lymphocyte responses in this study was manifested in a number of ways. The mosaic immunogens elicited CD8+ T cell responses to more epitopes of a viral protein. Perhaps more importantly, however, these immunogens generated responses to more variant sequences of the CD8+ T lymphocyte epitopes. This latter measure of breadth, an increased depth of epitope recognition, may contribute to the control of variant viruses that emerge as the virus mutates away from recognition by cytotoxic T lymphocytes. These findings provide a compelling rationale for assessing this vaccine strategy in humans for use to protect against HIV and other viruses that readily mutate.

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Figure Legends

Fig. 1. Epitopes of indicator Gag and Nef proteins recognized by T lymphocytes of vaccinated monkeys. (A) Total lymphocytes from each vaccinated monkey were assessed for their recognition of specific epitopes of each of the 10 indicator Gag and Nef proteins. Each animal's responses to each peptide series are shown in different colored bars: clade A (aqua), clade B (red) clade C (purple) and clade G (blue). Epitope recognition was determined by matrix mapping and confirmed using individual peptides in IFN-γ ELISpot assays. A single animal in each of the experimental groups did not generate measurable ELISpot responses following vaccination; therefore, data from 11 monkeys per group are shown. Minimum numbers of T lymphocyte responses to Gag and Nef combined are shown. (B) Minimum numbers of CD4+ T lymphocyte and (C) minimum numbers of CD8+ T lymphocyte responses to Gag and Nef combined in 7 monkeys from each of the vaccine groups. Unfractionated and CD8+ lymphocyte-depleted PBL were assessed for their recognition of specific epitope peptides from each of the 10 indicator Gag and Nef proteins in IFN-γ ELISpot assays. A response to a particular peptide was considered to be CD8+ T lymphocyte-mediated if following stimulation with that particular peptide the spot forming cell (SFC) responses by CD8+ T lymphocyte-depleted lymphocytes PBL at least 50% less than the response by unfractionated PBLs. If SFC responses to a peptide were not reduced by CD8+ lymphocyte depletion, that response was considered to be mediated by CD4+ T lymphocytes.

Fig. 2. Breadth and depth of Gag- and Nef-specific T lymphocyte responses in individual monkeys in the two vaccine groups. (A) The breadth of T lymphocyte responses by individual monkeys. The minimum number of T lymphocyte responses by individual animals was determined as follows: if a 15-mer peptide from one or more of the 10 sets of indicator proteins scored positive, the response was counted as one positive response. For the Mosaic-vaccinated monkeys, epitopes in the Gag protein that were recognized are shown in blue bars and epitopes in the Nef protein that were recognized are shown in aqua bars. For the Consensus-vaccinated monkeys, recognized Gag and Nef epitopes are shown in green and light green bars, respectively. (B) The depth of T lymphocyte responses by individual monkeys. The number of peptides that scored positive in (A) was expanded to include all of the variant peptides from each reactive region of the protein to estimate the depth of the responses. (C) The breadth of CD4+ and (D) CD8+ T lymphocyte responses in individual monkeys.

Fig. 3. Examples of the depth of vaccine-elicited CD8+ T lymphocyte responses. Consensus and mosaic vaccine-induced CD8+ T lymphocyte responses were assessed for recognition of variant forms of the same region of the viral protein. For each example shown, the variant HIV-1 sequences are displayed aligned to the M group consensus of that sequence. Amino acid identity to the consensus sequence is shown by a dash. For some sequences, a blank space is inserted to maintain the alignment. The peptides recognized by PBLs of a vaccinated monkey are shown in black at the top and are preceded by the number of the responding monkey. The variant peptides in the same region that are not recognized are shown in red. Every unique peptide sequence

recognized by PBLs is shown in a different shade of green, and white boxes represent peptides that are not recognized. A large box represents an exact match of a number of sequences. (A) A highly restricted CD8+ T lymphocyte response that is commonly elicited by a consensus immunogen. CD8+ T lymphocytes from monkey 58 recognize only the peptide sequence that matches the vaccine sequence. (B) A cross-reactive CD8+ T lymphocyte response that is rarely elicited by a consensus immunogen. Three different variants of peptide 15 and 16 sequences exist in 10 indicator gag proteins, and PBLs from monkey 228 recognize all three variants. (C) A highly restricted CD8+ T lymphocyte response that is commonly elicited by a mosaic immunogen. PBLs of monkey 65 recognize only the variant peptide that matches one of the four mosaic sequences used in the mosaic immunogen cocktail. (D) A cross-reactive CD8+ T lymphocyte response that is commonly elicited by a mosaic immunogen. PBLs of monkey 65 recognize four different variant forms of the peptide, all of which differ in sequence from the vaccine immunogens.

Fig. 4. Breadth and depth of Gag- and Nef-specific CD8+ T lymphocyte responses in 7 individual monkeys from each vaccine group. All of the Gag and Nef peptides recognized by PBLs of individual monkeys were aligned to the HIV-1 M group consensus sequences as described in the legend of Figure 3. PBL recognition of any peptide sequence is shown in rectangles of a different shade of green, and non-recognition of a peptide is shown in unshaded rectangles. The stacks of 10 rectangles on the left represent the 10 sets of indicator peptides. If at least one peptide from an indicator peptide sets is recognized by PBLs of the designated monkey, a rectangle is

shaded, with A1 and A2 shown in turquoise; B1 and B2 in red; C1, C2, C3 and C4 in purple; and G1 and G2 in dark blue. If no PBL responses to a particular set of indicator peptides are detected, the rectangle is shown unshaded.