HHS Public Access

Author manuscript

Nat Med. Author manuscript; available in PMC 2010 September 01.

Published in final edited form as:

Nat Med. 2010 March ; 16(3): 324–328. doi:10.1038/nm.2108.

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

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Abstract

An effective HIV vaccine must elicit immune responses that recognize genetically diverse viruses1, 2. It must generate CD8+ T lymphocytes that control HIV replication and CD4+ T lymphocytes that provide help for the generation and maintenance of both cellular and humoral immune responses against the virus3–5. Creating immunogens that can elicit cellular immune responses against the genetically varied circulating isolates of HIV presents an important challenge for creating an AIDS vaccine6, 7. Polyvalent mosaic immunogens derived by *in silico* recombination of natural strains of HIV are designed to induce cellular immune responses that recognize genetically diverse circulating virus isolates8. In the present study we immunized rhesus monkeys by plasmid DNA prime/ recombinant vaccinia virus boost using vaccine constructs expressing either consensus or polyvalent mosaic proteins. The mosaic immunogens elicited CD8+ T lymphocyte responses to more epitopes of each viral protein than the consensus

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AUTHOR CONTRIBUTIONS

B.K., W. F., G.N.P. and B.K.F. designed the vaccine gene inserts. H.L., R.Z. and B.F. H. generated the recombinant vaccinia virus constructs. B.K., B.F.H., S.S. and N.L.L. designed the study. A.C. and K.G.M. performed all the immunizations. H.B., A.B., D.Q. and S.S. designed and conducted the cellular immunologic assays. R.J.P., C-Y.T. and B.F.H. designed and conducted the antibody assays. M.M., S. W., W.F., J. T., J.S. and B.K. performed the data analysis.

immunogens, and to more variant sequences of CD8+ T lymphocyte epitopes. This increased breadth and depth of epitope recognition may contribute both to protection against infection by genetically diverse viruses and to the control of variant viruses that emerge as they mutate away from recognition by cytotoxic T lymphocytes.

Mosaic immunogen sequences are designed with an algorithm that simulates evolution by recombination, using coverage of potential T lymphocyte epitopes as the selection criterion8. Nine-amino-acid fragments are considered potential epitopes since 9 amino acids is the most common length of MHC class I-presented peptides and the most common span between anchor residues of MHC class II-presented peptides9. These sequences form complete proteins and are created using recombination breakpoints that are found in nature. The present study was initiated to explore the breadth of CD4+ and CD8+ T lymphocyte responses generated through vaccination with polyvalent mosaic and consensus immunogens.

We selected two HIV genes for use in the immunogens employed in this study8: HIV-1 *gag* and *nef*. Thirty rhesus monkeys were distributed into three cohorts, two experimental groups consisting of 12 monkeys each and a control group consisting of 6 monkeys. One experimental group received mosaic immunogens, the other experimental group received consensus immunogens, and the control group received empty vectors. We chose a DNA-prime/recombinant vaccinia virus boost regimen for this study since these immunogens elicit both CD4+ and CD8+ T lymphocyte responses10–12.

The breadth of the vaccine-elicited cellular immune responses was determined by assessing peripheral blood T lymphocyte recognition of 10 different natural Gag and Nef sequences using a peptide/IFN-γ ELISpot assay and matrix epitope mapping. The Gag and Nef sets of 10 indicator proteins included two clade A, two clade B, four clade C, and two clade G sequences. We used the same set of sequences we had previously used to evaluate vaccine-elicited responses to a consensus Env immunogen13. These isolate sequences were selected to represent recently sampled viruses, diverse geographic origins, and genetically distinct subregions of each clade. This strategy allowed us to assess vaccine-induced responses to sequences representative of the diverse HIV-1 strains to which human vaccinees will be exposed. Many 9-mers in an HIV-1 strain are rare in the overall population of viruses (44% of Gag 9-mers and 67% of Nef 9-mers are present in <15% of wild-type sequences, based on the Los Alamos database collection of M group sequences). As rare epitope variants are such a large component of the potential response, it is useful to know how often a vaccine can induce responses to rare 9-mers in representative strains. This peptide design strategy allowed us to determine the precise number of vaccine-elicited responses per virus strain that were induced in each animal, and to characterize the cross-reactive potential of each epitope-specific response.

The magnitudes of the vaccine-induced anti-Gag and anti-Nef antibody and ELISpot responses between these groups of monkeys were comparable (Supplemental Fig. 1a–c); 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. The median number of epitope-specific total T lymphocyte

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]; the median number per strain following vaccination with mosaic immunogens was 7.75 (IR 6.0–11.25). There was no statistically significant difference between these numbers of responses in the two experimental groups of monkeys (Wilcoxon rank sum test).

To assess whether either vaccine strategy induced cellular immune responses that recognized a greater diversity of epitope variants, we determined the average number of variants recognized per epitope-specific response by dividing the total number of variant peptides recognized by the number of epitope-specific responses in each vaccinated monkey. The mosaic-vaccinated monkeys had higher ratios than the consensus-vaccinated monkeys ($p = 0.045$, Wilcoxon rank sum test) (Supplemental Table 1).

The vectors employed in the present immunizations generated higher levels of CD4+ than CD8+ T lymphocyte immune responses12, and any benefit for CD8+ T lymphocyte responses derived from using mosaic immunogens might be obscured in the overall response by the CD4+ T lymphocyte responses14. Since CD4+ T lymphocytes recognize peptide antigens associated with MHC class II molecules, and peptide/MHC class II binding is more promiscuous than peptide/MHC class I binding, benefits associated with mosaic immunogens might be more apparent in CD8+ than CD4+ T lymphocyte responses15, 16.

To determine separately the relative impact of mosaic and consensus immunogens on CD8+ and CD4+ T lymphocyte responses, we evaluated the breadth of 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. Sufficient lymphocytes were available from 7 monkeys in each of the groups of vaccinated animals to carry out this evaluation.

We determined the total number of epitope-specific responses to all tested strains of Gag and Nef by PBL of each animal and compared the groups using the Wilcoxon rank sum test (Fig. 1a). The median 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), and was 2.0 (IR 1.25–4.0) in animals vaccinated with the consensus immunogens. The median number of 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), and with the consensus immunogens was 1.0 (IR 0.0–1.0). (See Supplemental Fig. 2 for a full breakdown of responses by protein and by CD4/CD8 T lymphocytes). There were more total T lymphocyte responses (CD4+ plus CD8+) per strain induced by Gag mosaic immunogens than by consensus immunogens by a factor of 2, $p = 0.023$ (Poisson regression), but vaccine induced Nef responses did not differ significantly between the two groups.

We then compared the number of epitope-specific CD4+ and CD8+ T lymphocyte responses per animal, counting overlapping peptides or sequence variants as one event (Fig. 1b). There was no significant difference in the number of CD4+ T lymphocyte responses elicited by consensus or mosaic immunogens, total (Wilcoxon rank-sum statistics; $p = 0.28$), Gag-specific ($p=0.18$), or Nef-specific ($p = 0.45$). However, mosaic immunogens induced

significantly more CD8+ T lymphocyte total (p = 0.001) and Gag-specific (p = 0.006) responses, and a trend towards more Nef-specific (p = 0.158) responses.

We also assessed whether mosaic-immunized monkeys developed CD4+ and CD8+ T lymphocyte responses with greater depth than consensus-immunized monkeys by quantifying the number of responses to all the variant forms of a given epitope (Fig 1c). There were more Gag-specific (p = 0.05) but not Nef-specific (p = 0.40) CD4+ T lymphocyte responses to variant peptides per response in the mosaic immunized group: a median of 1 (range 1–3) Gag epitope-specific CD4+ T lymphocyte response in the consensus immunized and a median of 3 (range 2–8) such responses in the mosaic immunized monkeys. For CD8+ T lymphocyte responses, there were more total (Gag-plus Nef-specific) responses (p = 0.005), Gag-specific responses (p = 0.001), and a trend toward more Nef-specific responses (p = 0.16) in the mosaic immunized than in the consensus immunized monkeys. These findings are comparable to those reported by Barouch et al. in the accompanying manuscript [a median of 1 (range 0–2) in the consensus immunized and a median of 3 (range 2–4) in the mosaic immunized monkeys]. Together these data suggested that the benefit of the mosaic immunogens was more apparent for CD8+ T lymphocyte than for CD4+ T lymphocyte responses.

Because we evaluated the reactivity of the PBLs of each vaccinated monkey to peptides spanning the entire Gag and Nef proteins of 10 genetically disparate HIV-1 isolates, we were able to define the immune recognition of actual proteins from distinct circulating virus strains in the immunized monkeys. Examples of typical responses and our approach to illustrating them are shown in Fig 2; the complete alignment of all peptides to which at least a single CD8+ T lymphocyte response was made is shown in Supplemental Fig. 3.

As illustrated in Figs. 2a and c and as shown comprehensively in Fig. 3, some vaccine-induced CD8+ T lymphocyte responses were highly strain specific, capable of recognizing peptide variants from only 1 or 2 of the 10 test strains. Broadly cross-reactive responses to individual epitopes were observed, but were very rare, with all 10 peptide variants recognized only 6 times in all vaccinated monkeys (Fig. 2b, Fig. 3). A response can occur to two overlapping peptides, likely representing the recognition of a single epitope (Fig. 2b). CD8+ T lymphocyte responses were usually highly specific. Extending the breadth of responses to more than 2 variant peptides was rare in both groups of vaccinated monkeys. This type of potentially valuable response occurred only 6 times among the consensus vaccine-induced CD8+ T lymphocyte responses and 17 times among those responses in mosaic vaccine recipients (Fig. 2d, 3) [binomial test p = 0.03].

Modeling the depth of the peptide-specific responses as a function of vaccine and T lymphocyte type, we found that mosaic vaccine-elicited CD8+ T lymphocyte responses were more likely than consensus vaccine-elicited CD8+ T lymphocyte responses to recognize variant peptides by a factor of 1.6 (p= 0.0006, binomial regression) [Fig. 3, Supplemental Fig. 4]. Extending the breadth of a response to more than 2 variant peptides occurred 42 times among CD4+ T lymphocyte responses in the mosaic immunized animals, and only 23 times in the consensus vaccinated animals (binomial test p = 0.05) [Fig. 3]. CD4+ T lymphocyte responses usually had greater depth (encompass a larger fraction of

variants) than CD8+ T lymphocyte responses by a factor of 1.48 ($p = 0.0004$); this was true for both vaccine prototypes and may explain why the benefit of mosaic immunogens was more pronounced for CD8+ T lymphocytes.

As shown in the shaded bars in the boxes adjacent to each monkey number, almost all of the indicator strains of Gag and Nef proteins were recognized by one or two epitope-specific CD4+ T lymphocyte responses in the PBLs of both groups of vaccinated monkeys (Fig. 4a). However, more CD4+ T lymphocyte responses that recognized three epitopes were detected in mosaic-vaccinated monkeys than in consensus-vaccinated monkeys. Almost all of the indicator strains of peptides were recognized by at least one epitope-specific CD8+ T lymphocyte response in the PBLs of the mosaic vaccine recipients, while a substantial number of viral strains were not recognized by epitope-specific CD8+ T lymphocyte peptide responses in the consensus-immunized monkeys ($p = 0.057$, Wilcoxon rank sum test) [Fig. 4b]. The mosaic immunogens also elicited a significantly larger number of CD8+ T lymphocyte responses that recognized a minimum of two ($p = 0.0022$) and three epitopes ($p = 0.0018$) of these natural virus strains as compared to the consensus immunogen.

In the recent STEP human vaccine trial, an adenovirus-based HIV-1 Gag/Pol/Nef vaccine induced a median of only 2 epitope-specific responses to the vaccine in each subject (Nicole Frahm, personal communication), only 40% of the subjects mounted a response to Gag, and only 32% of vaccinees generated both a CD4+ and CD8+ T lymphocyte response17. It is possible that this vaccine failed to confer protection because most of the vaccine-elicited responses were not able to recognize the circulating strains of HIV-1.

The present study demonstrates that immunization of nonhuman primates with mosaic immunogens induced CD8+ T lymphocytes with greater cross-reactivity than the consensus immunogen. The mosaic immunogens elicited CD8+ T lymphocyte responses to more epitopes of each viral protein than the consensus immunogens and to more variant sequences of the CD8+ T lymphocyte epitopes. This increased breadth and depth of epitope recognition could contribute to protection against infection by genetically diverse viruses and, in some instances, may block the emergence of common variant viruses.

METHODS

Experimental groups and vaccination schedule

Monkeys were housed at New England Regional Primate Research Center (NERPRC), Southborough, MA. The animals were maintained in accordance with standards set forth by American Association for Accreditation of Laboratory Animal Care. Thirty *Mamu-A*01, Mamu-B*01, and Mamu-B*17*-negative rhesus monkeys were distributed into three groups, two experimental groups each consisting of 12 monkeys and a control group consisting of 6 monkeys. One of the experimental groups received immunogens containing single consensus gene inserts and the other received immunogens containing a cocktail of 4 complementary mosaic gene inserts. On weeks 0, 4 and 8 the experimental groups of monkeys received priming immunizations by the intramuscular route with either a total of 5 mg of M Consensus *gag* or Mosaic *gag* and a total of either 5 mg of M Consensus *nef* or Mosaic *nef* plasmid DNA for each immunization. At week 33, monkeys were boosted by

simultaneous intramuscular and intradermal inoculations with a total of 10^9 PFU of a recombinant vaccinia virus expressing the same antigens. The control monkeys were immunized with empty vectors.

Pooled peptide and Peptide Matrix IFN–γ ELISpot assays

We coated multiscreen ninety-six well plates overnight with 100 μl per well of 5 μg^{-ml} anti-human interferon-γ (IFN-γ) (B27; BD Pharmingen) in endotoxin-free Dulbecco's-phosphate buffered saline (D-PBS). The plates were then washed three times with D-PBS containing 0.1% Tween-20, blocked for 2 h with RPMI-1640 containing 10% FBS. Then we added peptide pools and 2×10^5 PBLs to each well in 100 μl reaction volumes in triplicate for pooled peptides assays and in duplicate for peptide matrix assays. Each peptide pool was comprised of 15 amino acid peptides overlapping by 11 amino acids. The pools covered the entire HIV-1 Gag and Nef proteins. Each peptide in a pool was present at a 1 μg^{-ml} concentration. Following an 18 h incubation at 37°C, the plates were washed nine times with D-PBS containing 0.1% Tween-20 and once with distilled water. We then incubated the plates with 2 μg^{-ml} biotinylated rabbit anti-human IFN-γ (U-Cytech) for 2 h at room temperature, washed six times with D-PBS containing 0.1% Tween-20, and incubated for 2.5 h with a 1:500 dilution of streptavidin-AP (Southern Biotechnology Associates). After five washes with D-PBS containing 0.1% Tween-20 and three washes with D-PBS, we developed the plates with NBT/BCIP chromogen (Pierce), stopped by washing with tap water, air dried, and read with an ELISpot reader (Cellular Technology Ltd.). The number of spot forming cells per 10^6 PBLs was calculated. The responses to the indicator peptides in the control group of 6 animals were in the range of 5–108 $SFC^{-million}$ PBLs. These values were always less than 4× below the medium alone values.

CD8+ T lymphocyte depletion

We incubated PBL from the monkeys with phycoerythrin-labeled anti-CD8 antibody and then with antiphycoerythrin-labeled magnetic beads (Miltenyi Biotech) following manufacturer's protocol. We then sorted the labeled PBL using a Miltenyi AutoMACS cell sorter to deplete CD8+ T lymphocytes. The efficiency of CD8+ T cell depletion in this study using the AutoMACS cell sorter was 94.3–99.7%. We measured IFN-γ ELISPOT responses in unfractionated and CD8+ T-lymphocyte-depleted PBL from these monkeys. 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. The responses of CD8+ T lymphocyte-depleted PBL were designated as CD4+ T lymphocyte responses. These highly stringent criteria may underestimate the magnitude of the vaccine-elicited CD4+ T lymphocyte responses. Methods for Construction of plasmid DNA vaccines, recombinant vaccinia virus production and Statistical Analyses and associated references are available as Supplemental on-line material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank Georgia Tomaras for generous advice and assistance with reagents and antibody data. This work was supported by NIH grants AI-0678501 (Center for HIV AIDS Vaccine Immunology), HIVRAD P01 AI-61734, NIAID contract N01 AI-50010 and Los Alamos National Laboratory-directed research grant to B.T.K., W.F., S.W.

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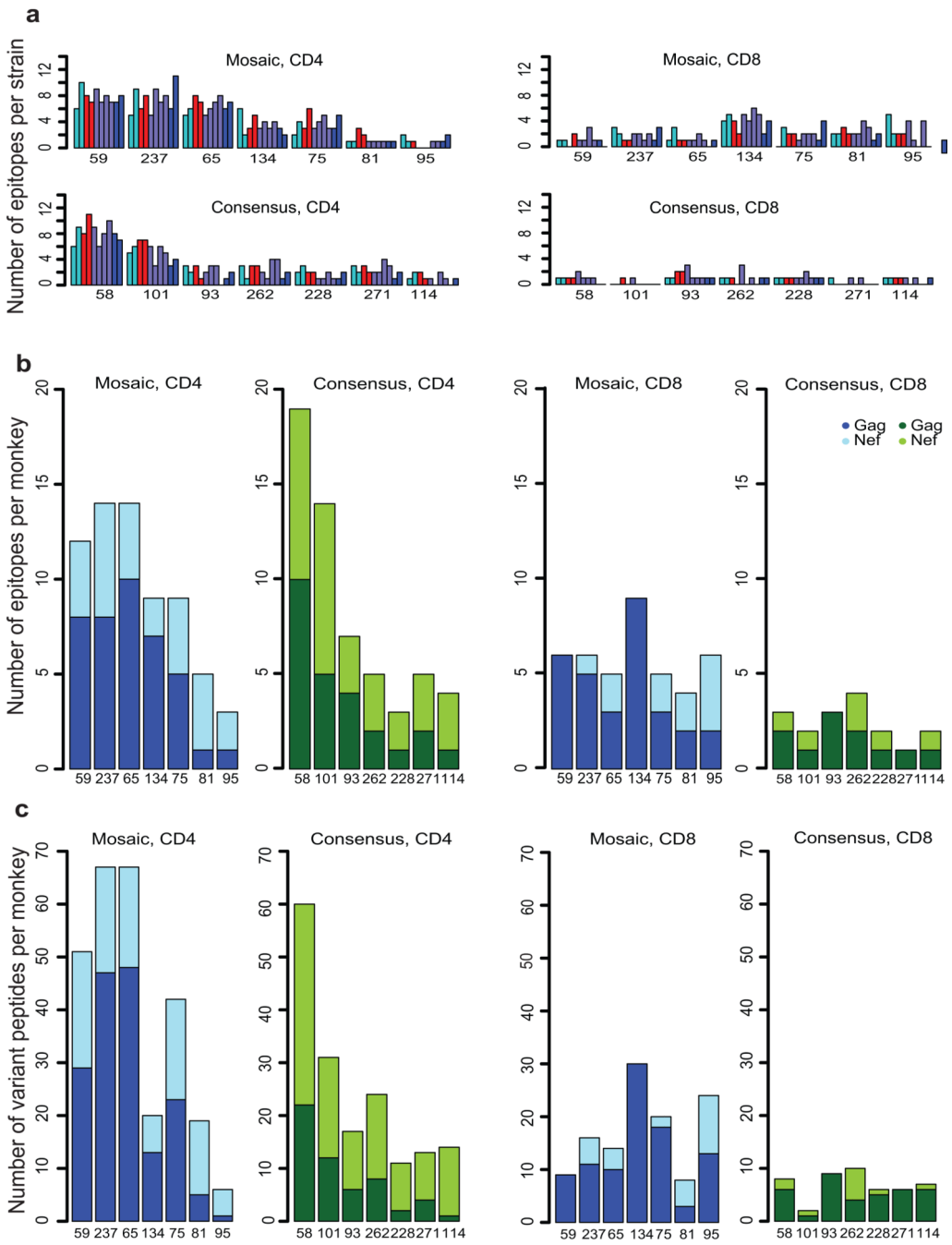

Fig. 1.

Number of epitope-specific responses by CD4+ and CD8+ T lymphocytes of vaccinated monkeys. (a) CD4+ and CD8+ T lymphocyte responses from 7 monkeys per group were assessed for their recognition of specific epitopes of each of the 10 indicator Gag and Nef proteins in IFN-γ ELISpot assays. Unfractionated and CD8+ lymphocyte-depleted PBL were assessed for their recognition of specific epitope peptides. 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). (b) The breadth of CD4+ and CD8+T lymphocyte responses

by individual monkeys was determined as follows: if a 15-mer peptide from one of the 10 sets of indicator proteins was recognized by a T lymphocyte population, it was scored as one positive; if multiple variant forms of the same peptide from different indicator proteins were recognized by a T lymphocyte population, they were also scored 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. (c) The depth of CD4+ and CD8+T lymphocyte responses by individual monkeys was determined by counting the responses made to all variant peptides from each epitopic region of either the Gag or the Nef protein.

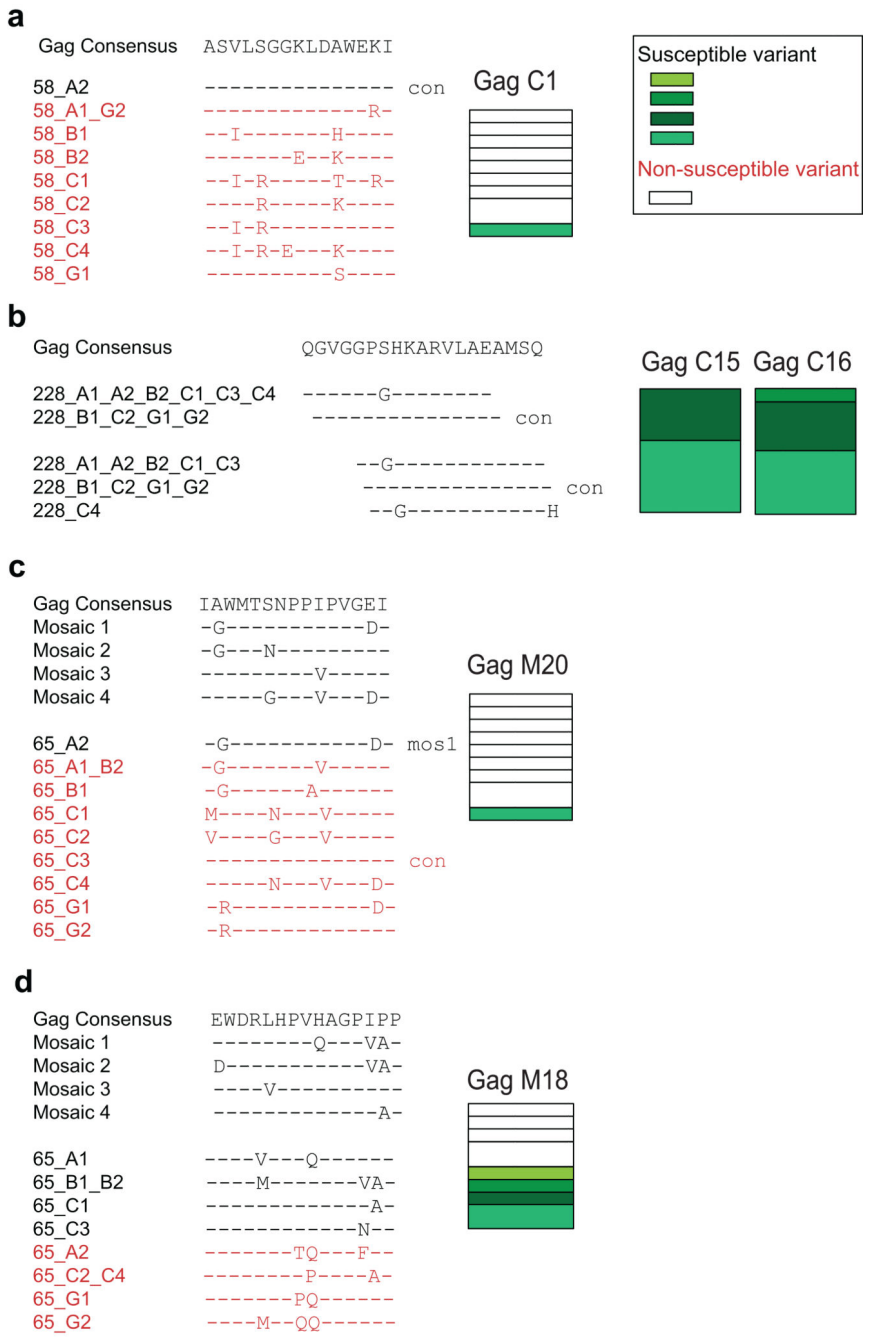

Fig. 2.

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 a 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: CD8+ T lymphocytes from monkey 58 recognize only the peptide sequence that matches the vaccine sequence. (b) A cross-reactive CD8+ T lymphocyte response: three different variants of peptide 15 and 16 sequences exist in 10 indicator gag proteins, and CD8+ T lymphocytes from monkey 228 recognize all three variants. (c) A highly restricted CD8+ T lymphocyte response: CD8+ T lymphocytes 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: CD8+ T lymphocytes from monkey 65 recognize four different variant forms of the peptide, all of which differ in sequence from the vaccine immunogens.

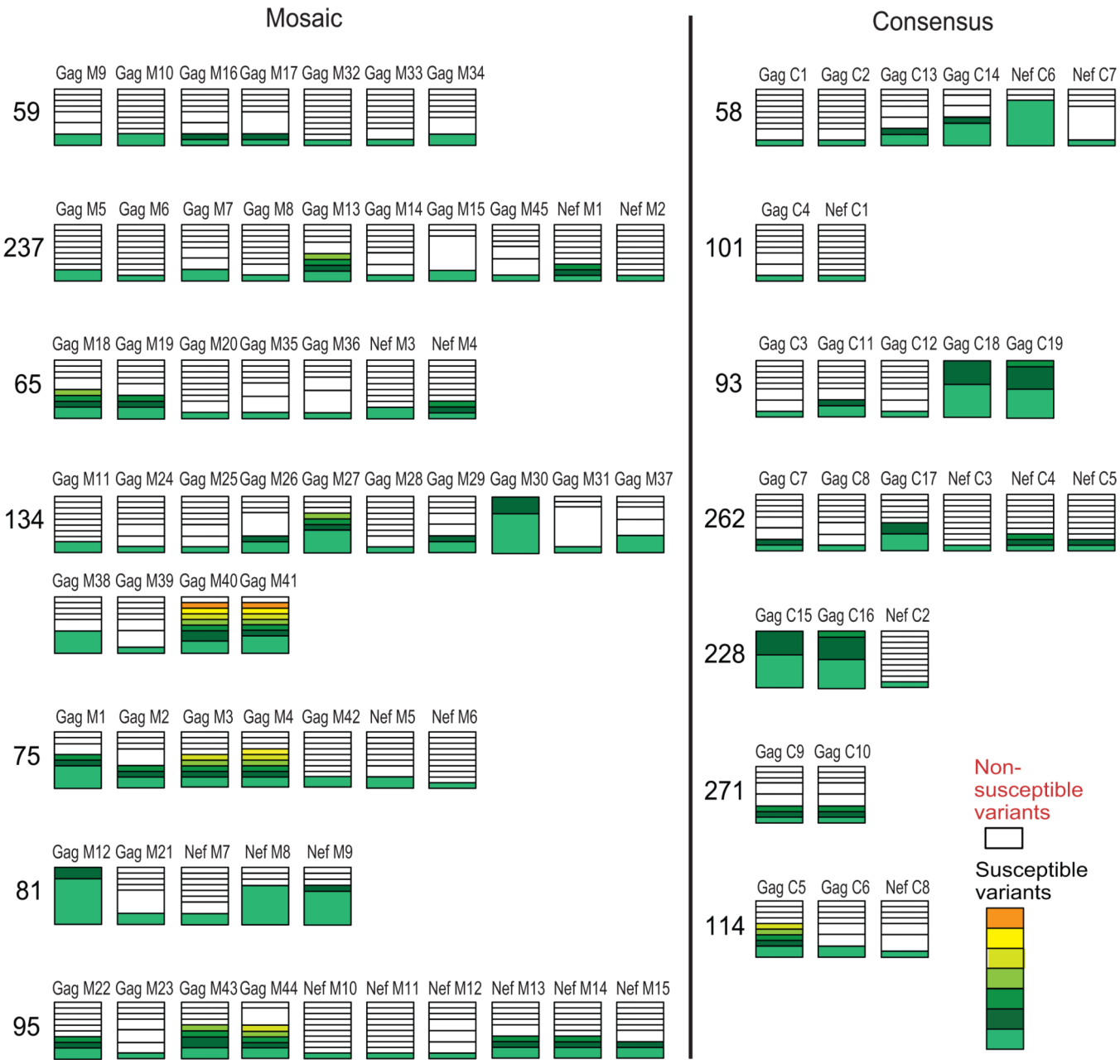

Fig. 3.

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 2. Lymphocyte recognition of any peptide sequence is shown in rectangles of a different shade of green, orange, or yellow and non-recognition of a peptide is shown in unshaded rectangles.

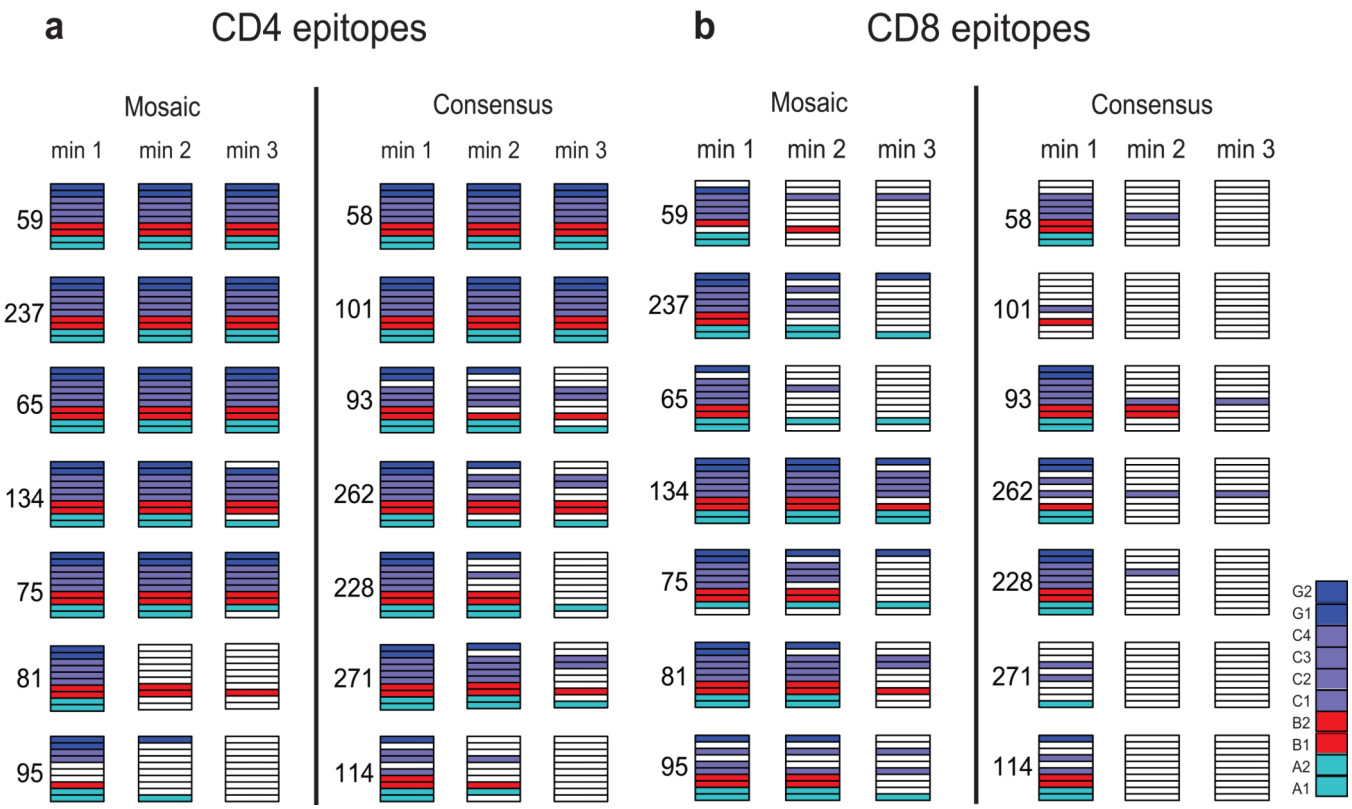

Fig. 4.

Cross-clade epitope recognition by CD4+ and CD8+ T lymphocytes of 7 individual monkeys from each vaccine group. The stacks of 10 rectangles represent the 10 sets of indicator peptides 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. For each monkey, in the left hand column (min1) if at least one peptide from an indicator peptide set was recognized by either (a) CD4+ or (b) CD8+ T lymphocytes, the rectangle is shaded with its representative color. If no PBL responses to a particular set of indicator peptides are detected, the rectangle is shown unshaded. Recognition of one, two or three epitopes per strain of indicator proteins by CD4+ and CD8+ T lymphocytes of each of the 7 monkeys in both vaccine groups are shown as min1, min 2 and min 3, respectively.