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## Focus and Breadth of Cellular Immune Responses Elicited by a Heterologous Insert Prime-Boost Vaccine Regimen in Rhesus Monkeys

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### Abstract

The global sequence diversity of HIV-1 presents a daunting challenge for vaccine development. We investigated whether a heterologous insert prime-boost regimen could expand global coverage by selectively boosting cellular immune responses to conserved epitopes. Rhesus monkeys were primed and boosted with recombinant adenovirus vectors expressing homologous or heterologous HIV-1 Gag sequences that were optimized to focus responses on highly conserved epitopes. We observed comparable responses directed to specific regions of the Gag protein in all experimental groups without evidence of improved coverage or expanded breadth in the heterologous insert group. These data suggest that antigen-independent factors contribute to the immunodominance patterns of vaccine-elicited cellular immune responses.

### 1. Introduction

HIV-1 infects 40 million individuals worldwide, with 2 million new infections reported annually. Therefore, a prophylactic vaccine is urgently needed. HIV-1 vaccine strategies must contend with the enormous global sequence diversity of HIV-1. Both population level and experimental data suggest that cellular immune responses play a critical role in containing viral replication and determining the rate of HIV-1 disease progression [1–7]. However, HIV-1 vaccine candidates capable of eliciting broadly reactive cellular immunity remain a challenge. Multiple cellular and immunologic constraints may shape vaccine-elicited immunodominance hierarchies and limit the breadth and global epitope coverage of vaccine-elicited cellular immunity. These include restrictions on proteolytic processing, transport and MHC binding of antigen-derived peptides, and constraints on T-cell receptor-MHC interactions that shape thymic selection and antigen-specific T-cell responses [8–10]. To date, HIV-1 vaccine candidates have elicited primarily narrowly focused T-cell responses in clinical trials [11, 12]. However, recent nonhuman primate studies have suggested that both vector and antigen selection may impact the breadth of vaccine-elicited cellular immunity [13–15]. In this study, we assessed a novel “heterologous insert” prime-boost regimen that utilizes different antigen sequences in the prime and boost immunizations

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as a strategy to focus cellular immune responses on conserved regions in nonhuman primates.

## 2. Materials and methods

### 2.1 Animals, vectors and immunizations

Adult Indian-origin rhesus monkeys (n=18) were housed in the biosafety level 3 containment facility at BIOQUAL, Inc., and all studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC). Animals were vaccinated by the intramuscular route with  $10^{11}$  viral particles of replication-incompetent, E1-/E3-deleted vectors in the quadriceps muscles. rAd26 and rAd35 vectors expressing Gag<sub>ConM</sub> and Gag<sub>CZA</sub> were constructed using an E1/E3-deleted adenovirus cosmid and adapter plasmid system as described previously [16, 17]. The HIV-1 antigens were expressed in the adenovirus E1 region under control of a human cytomegalovirus promoter.

### 2.2 ELISPOT assays and epitope mapping

Comprehensive T lymphocyte epitope mapping was performed utilizing Gag PTE peptides (NIH AIDS Research and Reference Reagent Program). IFN- $\gamma$  ELISPOT assays were conducted at week 4 following both the prime and the boost immunization initially with complete peptide pools as well as with subpools containing 10 PTE peptides that were pooled sequentially by peptide number. All peptide subpools with positive responses were then deconvoluted, and epitopes were confirmed with individual 15 amino acid PTE peptides. Cell-depleted IFN- $\gamma$  ELISPOT assays were performed to determine if reactive peptides represented CD8+ or CD4+ T lymphocyte epitopes. Cell depletions were performed by negative bead selection and were >98% efficient.

## Results

### 2.3 Heterologous insert sequence selection

We employed the Los Alamos National Laboratory HIV-1 sequence database to select complementary HIV-1 Gag sequences that shared multiple highly conserved potential T-cell epitopes (PTEs) [18, 19] but were divergent at nonconserved sequences. Screening of clade C HIV-1 Gag sequences against the M consensus HIV-1 Gag sequence (Gag<sub>ConM</sub>) identified primary isolates that shared multiple high frequency PTEs (Fig. 1A). To optimize the sensitivity for detecting selective boosting of conserved epitopes, we selected the primary clade C isolate C.ZA.03.03ZASK016MB2.DQ351233 (Gag<sub>CZA</sub>) that shared 247 high frequency PTEs representing 51% of all possible PTEs with Gag<sub>ConM</sub> but was divergent at other low frequency PTEs (Fig. 1A). The mean circulating frequency of shared PTEs in M group and clade C, as represented in the Los Alamos National Laboratory sequence database, was 0.72 and 0.86, respectively, while the mean circulating frequency of divergent PTEs was 0.16 and 0.09 (Fig. 1B). We therefore selected the combination of Gag<sub>ConM</sub> and Gag<sub>CZA</sub> as heterologous antigen inserts that theoretically could optimize responses to conserved PTEs while also expanding PTE breadth. We hypothesized that a heterologous insert prime boost regimen that utilized Gag<sub>ConM</sub> and Gag<sub>CZA</sub> would selectively focus cellular immune responses on conserved PTEs, leading to an increase in the frequency of circulating strain recognition relative to homologous insert prime boost regimens that utilized Gag<sub>ConM</sub> or Gag<sub>CZA</sub> alone.

### 3.2 Comparative immunogenicity of heterologous and homologous insert prime boost regimens in Rhesus monkeys

We utilized replication-incompetent recombinant adenovirus (rAd) vectors expressing Gag<sub>ConM</sub> and Gag<sub>CZA</sub> to assess the breadth of cellular immune responses elicited by

homologous and heterologous insert prime-boost regimens. We generated rAd serotype 26 (rAd26) and rAd serotype 35 (rAd35) vectors expressing Gag<sub>ConM</sub> and Gag<sub>CZA</sub> as previously described [16, 17]. Rhesus monkeys (n=6/group) were primed IM at week 0 with 10<sup>11</sup> viral particles (vp) rAd35 vectors expressing either Gag<sub>ConM</sub> or Gag<sub>CZA</sub> and boosted at week 12 with 10<sup>11</sup> vp rAd26 vectors expressing either homologous or heterologous Gag antigens. The magnitude and epitope specificity of vaccine-elicited cellular immune responses were determined at week 4 following both the prime and the boost immunizations by IFN- $\gamma$  ELISPOT assays as previously described [14]. Individual epitope responses were mapped using HIV-1 Gag PTE peptides, which consist of 325 15-mer peptides optimized for global PTE coverage [19]. Positive responses were confirmed using CD4- and CD8-depleted IFN- $\gamma$  ELISPOT assays [14].

The overall magnitude of Gag-specific cellular immune responses was comparable at week 4 following priming with rAd35-Gag<sub>ConM</sub> and rAd35-Gag<sub>CZA</sub> (Fig. 2A). The magnitude of Gag-specific cellular immune responses was also comparable following the boost immunization, with similar magnitude responses elicited by the homologous insert Gag<sub>ConM</sub>/Gag<sub>ConM</sub> and Gag<sub>CZA</sub>/Gag<sub>CZA</sub> regimens as well as the heterologous insert Gag<sub>ConM</sub>/Gag<sub>CZA</sub> regimen (Fig. 2B). Additionally, the median number of individual epitope-specific responses proved comparable following both the prime (Fig. 2C) and the boost (Fig. 2D) immunizations in all groups. These data suggest that the heterologous insert prime-boost strategy did not detectably augment the magnitude or breadth of vaccine-elicited cellular immune responses as compared to traditional homologous insert prime-boost immunization strategies in this model. We cannot exclude, however, that minor differences could have been missed given the limited number of animals utilized in this study.

The epitope distribution of responses following the boost immunization was also comparable among all three experimental groups (Fig. 3A). Overall, 66% of epitopes detected following the prime immunization were also observed following the boost immunization, although additional epitope-specific responses were detected following the boost immunization (Fig. 3A). Overall, responses were not evenly distributed across all HIV-1 Gag PTE peptides but rather exhibited a stereotypical pattern of clustering that was independent of the specific Gag insert sequence utilized for the prime and boost immunizations (Fig. 3A). Moreover, the heterologous insert prime-boost regimen did not prove superior to the homologous insert prime-boost regimens for improving the global epitope coverage of vaccine-elicited cellular immune responses (Fig. 3B). The mean circulating frequency of M group epitopes elicited by the various vaccine regimens was 0.18 for the Gag<sub>ConM</sub>/Gag<sub>CZA</sub> group, 0.31 for the Gag<sub>ConM</sub>/Gag<sub>ConM</sub> group and 0.25 for the Gag<sub>CZA</sub>/Gag<sub>CZA</sub> group (P = nonsignificant, 2-sided Mann-Whitney tests). These data demonstrate that the heterologous insert prime-boost strategy did not detectably alter the focus of vaccine-elicited cellular immune responses or improve immunologic coverage of HIV-1 global sequence diversity in this study.

### 3. Discussion

The protective efficacy of T-cell based HIV-1 vaccines may be critically dependent on the breadth of vaccine-elicited cellular immunity. Indeed, the breadth of vaccine-elicited cellular immune responses has been positively correlated with control of viral replication following SIV challenge of rhesus monkeys [13]. Previous studies have shown that both the choice of the vector and the choice of the insert may influence the breadth and global epitope coverage of vaccine-elicited cellular immune responses. For example, heterologous vector prime-boost regimens (i.e. rAd26/rAd5) have proven superior to homologous vector prime-boost regimens (i.e. rAd5/rAd5) for enhancing cellular immune breadth [13]. Moreover, a

combination of mosaic antigens has been shown to augment the breadth of cellular immune responses as compared with individual consensus or natural sequences [14, 15]. In contrast, heterologous insert prime-boost immunization strategies have not previously been evaluated and represent a potential strategy to increase the breadth and global epitope coverage of vaccine-elicited cellular immunity. This is of particular interest, since heterologous inserts are currently being explored in phase 1 HIV-1 vaccine clinical trials.

We investigated whether different HIV-1 Gag sequences that shared highly conserved epitopes could improve the global epitope coverage of vaccine-elicited cellular immune responses when delivered as a heterologous insert prime-boost regimen. We observed that the heterologous insert regimen did not prove superior to the homologous insert regimens with respect to global epitope coverage or cellular immune breadth. Vaccine-elicited cellular immune responses in all groups were focused on stereotypical regions of HIV-1 Gag regardless of the insert used for the prime and boost immunization. These data indicate that this heterologous insert prime-boost regimen was unable to overcome immunodominance constraints, suggesting that antigen-independent constraints on antigen processing, presentation and T-cell development and/or vaccine-specific constraints established during the priming immunization may limit the utility of heterologous inserts to expand cellular immune breadth or coverage. It is certainly possible that heterologous insert prime-boost regimens may have more subtle effects on breadth, coverage, and qualitative features of the T-cell repertoire that we were not powered to detect in this study. Future studies should therefore be directed to address these questions in both nonhuman primates and humans.

#### Highlights

- A heterologous HIV-1 Gag prime-boost regimen was evaluated in rhesus monkeys
- No improvement in cellular immune breadth or coverage was observed
- Similar patterns of responses were induced by homologous and heterologous inserts

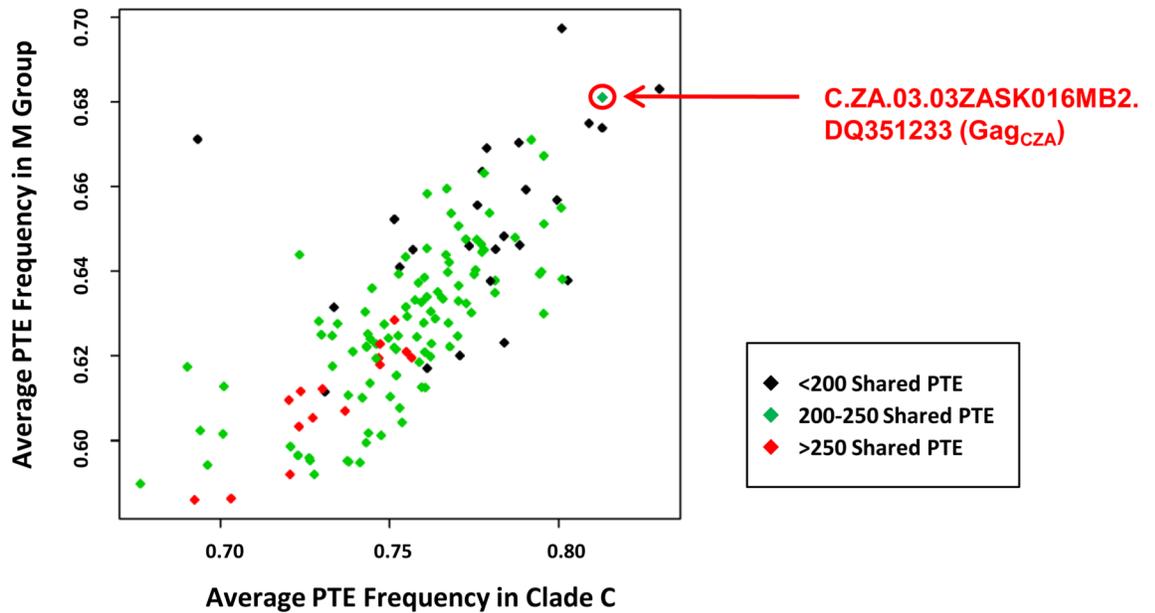
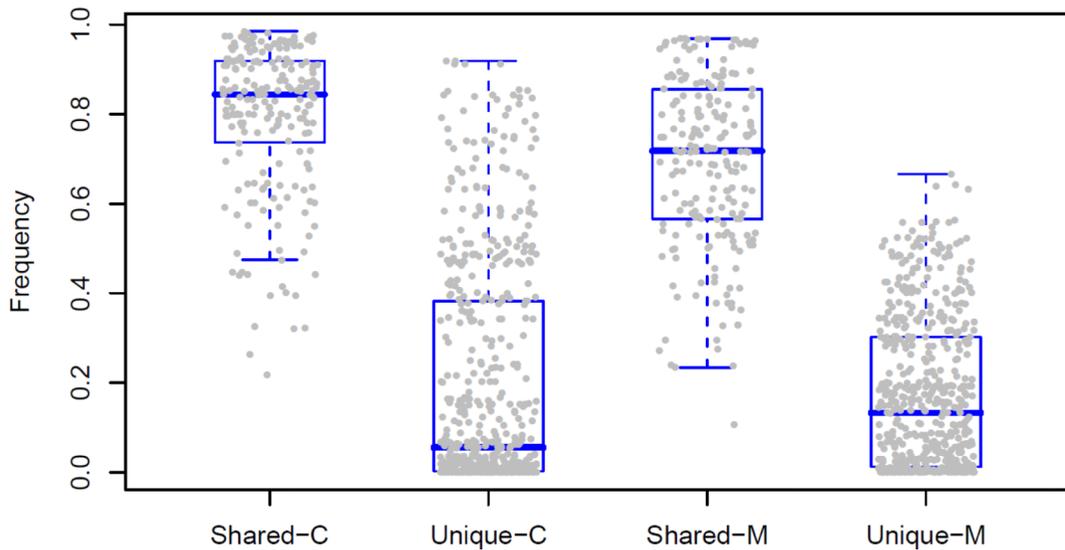
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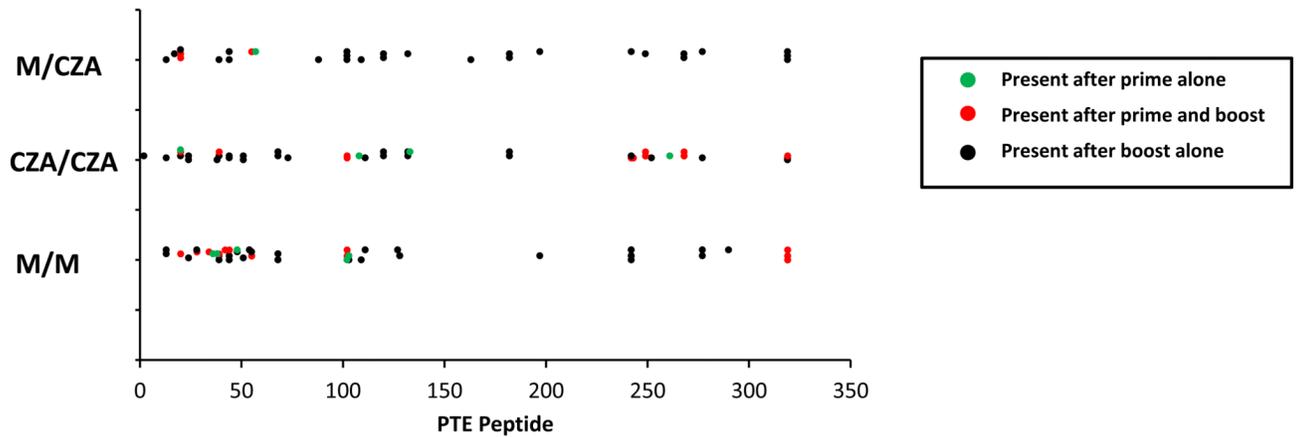
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**A****B**

**Fig. 1.** Selection of HIV-1 Gag antigens for heterologous insert prime-boost immunization. (A) Individual clade C sequences were compared against the M consensus sequence to determine the circulating frequency of shared PTEs among clade C isolates or in the M group as a whole. Isolates with a high circulating frequency by both criteria were evaluated for the total number of shared PTEs with Gag<sub>ConM</sub> in order to maximize the sensitivity for detecting selective boosting of conserved epitopes. (B) The mean circulating frequency of PTEs shared between Gag<sub>ConM</sub> and Gag<sub>CZA</sub> was compared with the mean circulating frequency of divergent PTE among clade C isolates or in the M group as a whole.



A



B

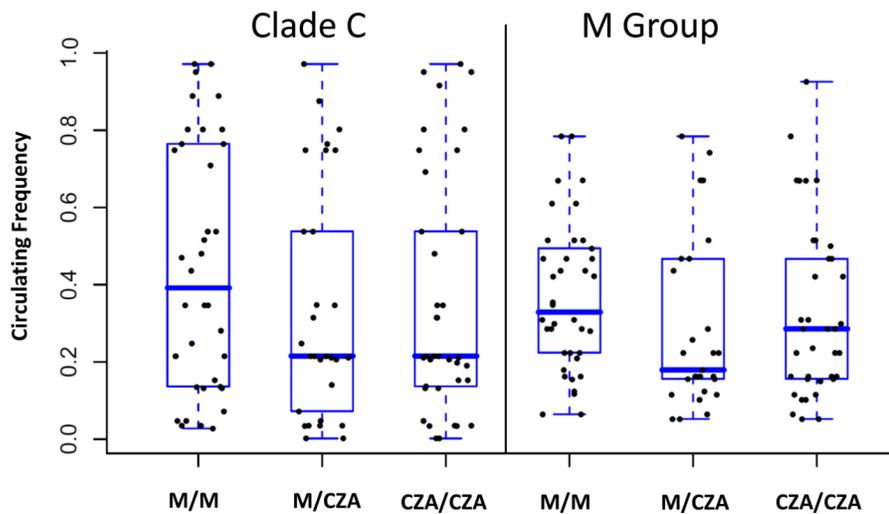


Fig. 3.

Epitope distribution of vaccine-elicited cellular immune responses following heterologous and homologous insert prime-boost immunization. (A) Comparative distribution of vaccine-elicited, epitope-specific cellular immune responses following the prime and boost immunizations. PTE numbers on the X axis represent relative position within the HIV-1 Gag protein. (B) Mean circulating frequency of clade C and M group PTE-specific responses elicited by the homologous and heterologous insert prime-boost regimens.