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Characterization of T cell responses to cryptic epitopes in recipients of a noncodon-optimized HIV-1 vaccine

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Abstract

Introduction—Cryptic epitopes (CE) can be encoded by any of the 5 alternate reading frames (ARF, 2 sense and 3 antisense) of a known gene. While CE responses are commonly detected during HIV-1 infection, it is not known whether these responses are induced following vaccination.

Methods—Using a bioinformatic approach, we determined that vaccines with codon-optimized HIV inserts significantly skewed CE sequences and are not likely to induce cross-reactive responses to natural HIV CE. We then evaluated the CE- and protein-specific T-cell responses using Gag, Pol and ARF peptide pools among participants immunized with a noncodon-optimized vaccine regimen of two pGA2/JS7 DNA primes followed by two MVA/HIV62 Gag-Pol-Env vector boosts or four saline injections.

Results—Vaccinees had significantly more IFN- γ ELISpot responses toward Gag ($p=0.003$) but not Pol protein than placebo recipients. However, CE-specific T-cell responses were low in magnitude and their frequencies did not differ significantly between vaccine and placebo recipients. Additionally, most positive CE responses could not be mapped to individual peptides. After expanding responses in a cultured assay, however, the frequency and median magnitude of responses to ARF peptides was significantly greater in vaccinees ($p<0.0001$), indicating CE-specific T cell responses are present but below an *ex vivo* assay's limit of detection.

Conclusions—Our data demonstrates that HIV-1 vaccines currently in clinical trials are poorly immunogenic with regards to CE-specific T cell responses. Therefore, the context of HIV-1 immunogens may need to be modified as a comprehensive strategy to broaden vaccine-induced T cell responses.

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Keywords

HIV-1; cryptic epitopes; alternative reading frames; CD8+ T cells; HVTN 205 vaccine trial; codon-optimized

INTRODUCTION

The extensive sequence diversity of human immunodeficiency virus type 1 (HIV-1) poses a formidable challenge in vaccine development [1]. The search for a universal HIV-1 vaccine has focused on induction of adaptive immune responses through multiclade, polyvalent viral vectors like the recombinant canarypox vector used in RV-144, which offered some efficacy in protection against infection [2,3]. While modest protection against HIV-1 acquisition during the RV-144 trial in Thailand mainly correlated with binding antibodies, preclinical studies in non-human primates and observational studies in HIV-infected adults have established that epitope-specific T cell responses play a critical role in controlling viral replication following infection [4–10]. Furthermore, simian immunodeficiency virus (SIV) vaccines that induce a greater breadth of virus-specific T cell responses at high frequencies correlated with better viral control [11–13]. A broader CD4 and CD8 T cell response has also correlated with better markers of HIV disease progression [14–17]. Thus, mitigating cell-mediated immunity through T cell responses may be imperative for protection against disease progression in those where antibodies failed to protect against infection. Such optimization will likely require identification of new targets to induce a more broadly reactive effector population.

Cryptic epitopes (CE) are a unique set of major histocompatibility class I (MHC-I)-restricted T cell epitopes recognized during infection with SIV, HIV, murine leukemia virus (LP-BM5), and influenza virus and provide a novel source of HIV-1 antigens for vaccine development [18–22]. In contrast with traditional, protein-derived epitopes, CE are encoded by forward (F2, F3) and reverse (R1, R2, R3) alternative reading frames (ARFs) of existing genes, corresponding to translation of sense and antisense transcripts of messenger RNA [23–26]. Thus, a double-stranded DNA vector can express traditional epitopes from the primary reading frame (F1) of the sense transcript while expressing CE in the remaining two frames (F2–3) of the same transcript or the three reverse frames (R1–3) of the antisense transcript. Therefore, the prevalence of epitopes could be 5 times greater in ARFs, making CE ideal for expanding the breadth of vaccine-induced responses without expanding the current size of the vector inserts. Despite this advantage, all vector inserts to date have been designed to preserve targets encoded by the primary reading frame (F1) without considering the effects on the ARFs. While insert sequences still contain ARFs (F2–3, R1–3), neither the sequence conservation of CE from these frames or the potential of current vaccine vectors to induce CE-specific T cells responses has been directly evaluated.

Because of the importance of broadening T cell response for HIV vaccine design and the potential implications of broadening these responses through CE targeting, we evaluated whether select HIV vaccines in clinical development are able to induce these types of responses. We found that recombinant vaccines with codon-optimized inserts skewed CE to such an extent that they poorly represented naturally occurring HIV strains. Analysis of samples from recipients of a vaccine regimen encoding noncodon-optimized HIV inserts revealed vaccination could induce CE-specific T cell populations but at frequencies that were low in comparison to protein-derived epitopes. These findings suggest that current HIV vaccines have the capacity to express CE and, if the ARFs that encode them are prioritized, vaccination could further increase the breadth of vector-induced T cell responses.

MATERIALS AND METHODS

Bioinformatic Analyses

The *gag* and *RT* region nucleotide sequences for each full length HIV-1 clade B transmitted founder virus (TFV) genome that was available from 12 acutely-infected patients in our cohort [53] were aligned before determining a single consensus sequence for each reading frame (LANL's Consensus Maker tool, <http://www.hiv.lanl.gov/content/sequence/CONSENSUS/SimpCon.html>). This reference set was then translated (ExPASy's Translate tool, <http://web.expasy.org/translate/>), along with 1 noncodon-optimized vaccine insert (MVA/HIV62, Acc. AY528646), 2 codon-optimized vaccine inserts (MRKAd5, obtained through personal communication with M. Betts; VRCrAd5, Acc.VRC-4306), and the 2 *wild type* parent sequences that were optimized (*wt*MRKAd5 *gag*, CAM-1 Acc. D10112, *pol* LAV-1 Acc. K02013 and *wt*VRCrAd5, NL4.3 Acc. M19921). Pairwise alignment scores were computed by Geneious (BLOSUM62 cost matrix with a gap open penalty of 12.0 and gap extend penalty of 3.0) for each frame of the TFV consensus sequences and all 5 test sequences [27]. Homology was plotted (GraphPad Prism6 v6.0b) as the proportion of TFV consensus residues that were conserved in the sequence. Significance was determined using two-tailed Fisher's exact tests calculated by GraphPad QuickCalcs (<http://www.graphpad.com/quickcalcs/ConfInterval1.cfm>).

In a separate analysis, vaccine inserts and their *wild type* parent amino acid sequences were compared to individual TFV consensus sequences. Alignment scores for all amino acids translated from all six reading frame of *gag* and *RT* were computed by Geneious using the matrix described above and the Jukes-Cantor correction model. Unrooted, neighbor-joining phylogenetic trees were plotted to infer amino acid sequence divergence among the sequences without assuming any ancestral relationships.

Study Cohort

Previously cryopreserved peripheral blood mononuclear cells (PBMCs) were obtained from 126 participants based on their chronological enrollment in a Phase 2a trial (HVTN 205). Five individuals were excluded after their response rate for negative controls repeatedly exceeded the statistical threshold for positivity (described below) and one was excluded because the individual seroconverted three months post final vaccination. Statistical analyses were performed for 120 participants who received intramuscular injections of either a 0.9% sodium chloride placebo (40) or vaccine treatment (80) in a testing schema with two 3 mg DNA primes followed by two 1 mL of $1 \times 10^{7.5}$ TCID₅₀ MVA boosts at 0, 2, 4, and 6 months, respectively. Vaccine doses of the pGA/JS7 DNA prime contained JS7DNA plasmid encoding clade B HIV-1 HXB2 *gag*, HXB2/ADA *env-tat-rev-vpu*, and BH10 *PR-RT* with an inactivating point mutation in *PR*; the MVA boost, HIV/MVA62, is a modified vaccinia Ankara vector encoding the same *gag*, *PR-RT*, and *env* sequences [40]. PBMC samples were obtained 2 weeks post last vaccination. Stratification of participants into responders and non-responders was finalized prior to reporting results to the Statistical Center for HIV/AIDS Research & Prevention (SCHARP) for unblinding.

Peptide Design

Consensus clade B 15mer peptides overlapping by 11 amino acids (aa) for HIV-1 Gag and Pol proteins were obtained through the NIH AIDS Reagent Program (catalog #8117, #6208, respectively). Full length Gag, Protease (PR), and Reverse Transcriptase (RT) proviral DNA regions of the MVA/HIV62 vector were used to design overlapping ARF peptides (OLPs; 8 to 18mers overlapping by 10 residues) for potential CE using PeptGen (<http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html>) and synthesized in a 96 well array format by New England Biolabs [28–30]. Individual OLPs were combined by reading

frame into subpools (approximately 10 peptides/pool) and large pools of no more than 248 peptides for traditional proteins and 98 peptides for potential CE (range of 33 – 98 with means of 42 and 86 for *gag* and *PR-RT* regions, respectively; Supplemental Table 1). Individual peptide, subpool, and large pool stocks were reconstituted at 40mM in DMSO, diluted to 100uM in deionized water, and stored at –80C until use.

Ex vivo IFN γ ELISpot Assay

Previously cryopreserved PBMCs were thawed and rested overnight at 37°, 5% CO₂ to improve recovery rates, which retained approximately 85% of the cells per vial with average viabilities of 90% by trypan blue staining. ELISpot assays were performed as previously described using protein (NIH AIDS Reagents Program) and ARF peptide pools (New England Biolabs) at final concentrations of 5 μ M per peptide per subpool and 2 μ M per peptide per large pool. Antigens were tested in duplicate with 100,000 PBMCs/well in Millipore nitrocellulose 96-well plates while unstimulated cells were plated in quadruplicate as a negative control and phytohemagglutinin (PHA) in duplicate as a positive control. Spots that developed on the nitrocellulose membrane were counted by an ELISpot reader (ImmunoSpot; CTL) as a measure of interferon gamma (IFN γ) cytokine production. T cell responses to HIV-1 Gag, Pol, or their respective ARF peptide pools were reported as positive in the IFN γ ELISpot if 1) the negative control response averaged less than 55 SFU/10⁶ PBMCs, 2) the average positive control response was greater than 500 SFU/10⁶ PBMCs, and 3) the duplicate average of the wells was greater than the mean negative control response of all samples plus 3 standard deviations and 4) greater than or equal to 3 times the average response of the sample's negative control. Responses between both groups were analyzed for differences in magnitude using two-tailed non-parametric Mann-Whitney U tests calculated by GraphPad Prism v6.0b and frequency using two-tailed Fisher's exact tests calculated by GraphPad QuickCalcs (www.graphpad.com/quickcalcs/ConfInterval1.cf).

Cultured IFN γ ELISpot Assay

In a subset of PBMC samples, a 12 day *in vitro* cultured assay was performed concurrently with *ex vivo* ELISpot assays. Cryopreserved PBMCs were prepared for overnight resting and stimulated with the same antigens as described above. In brief, cells were suspended after resting in 10% HyClone FBS culture medium with 25 ng/mL IL-7 (R&D Systems, #207-IL) before being plated in 24 or 48-well culture plates at 1 mil PBMCs/mL. Cells were stimulated with subpools (5 μ M final concentration per peptide) and/or large pools (2 μ M final concentration per peptide) corresponding to the previous positive large pool responses in the participant. As a control, each sample was stimulated with a previously negative ARF large pool in addition to using unstimulated cells and PHA as negative and positive controls, respectively. On days 2 and 7, IL-2 (BD, #354043) was added to each well at a final concentration of 40–45 BRMP units/mL. Cultures were incubated an additional 2 days before washing cells three times with PBS and resting in IL-2-free R10 media for 30–36 h. After resting, cultured cells were tested in an IFN γ ELISpot assay as described above. Based on previous studies using cultured cells, samples with media background values greater than the media mean for all samples plus 2 standard deviations (210 SFU/1 mil PBMCs) were excluded [32–36]. Positive responders were identified when the magnitude of a subpool response exceeded the 3x the sample's background and media mean for all samples plus two standard deviations. Groups were compared with the Mann-Whitney statistical U test using background-subtracted responses.

RESULTS

Codon-optimized vector inserts significantly skew all ARF sequences

Because CE responses are dominantly due to CD8 T cells, our strategy was to analyze HIV recombinant adenoviral serotype 5 (Ad5) vector vaccines as these can be potent inducers of CD8 T cells. In order to enhance protein expression without changing the natural amino acid sequence, some HIV-1 vaccines (including all recombinant Ad5 vectors) are codon-optimized [37,38]. However, CE are encoded in ARFs, and their conservation is not prioritized during codon optimization of traditional genes.

To quantify the impact on ARF sequences following codon-optimization, we compared the amino acid identities of synthetic constructs to ARFs of naturally-occurring transmitted founder virus sequences (TFV). Since TFV sequences are derived from sequencing at the earliest disease stage, each TFV sequence corresponds to the transmitted virus's genome after it established infection and prior to little or any experience of immune pressure in the new host [18]. Thus, our use of TFV sequences ensured epitopes encoded by vector inserts would be scored against a reference sequences that accurately represents vaccine targets [39]. Using 12 full length HIV-1 clade B TFV sequences that were available from our acutely infected cohort, we compiled a reference set containing 1 amino acid consensus sequence per reading frame and gene region. In a preliminary analysis of a previously recognized CE encoded by *gag* (Gag-CE), we found that both the Gag and CE residues encoded by a noncodon-optimized vector insert were homologous to the TFV sequence (Supplementary Figure 1). In contrast, a codon-optimized vector encoded the same Gag sequence but the CE incurred an 11% loss in homology (1 of 9 residues) when compared to the TFV sequence and the *wild type*, noncodon-optimized sequence from which its insert was designed. Given these results at the epitope level, pairwise alignments for the full length of the inserts were then performed to measure amino acid conservation with respect to the TFV reference set for gene regions that were common (*gag* and *reverse transcriptase*, *RT*) to 1 noncodon-optimized vector insert (MVA/HIV62), 2 codon-optimized vector inserts (MRKAd5, VRCAd5), and both *wild type* sequences from which the codon-optimized inserts were derived. We found that codon optimizing the protein-coding frame (F1) amplified the effect seen at the epitope level, leading to a significant decrease (Fisher's exact, $p < 0.0001$) in amino acid identity for all ARFs of *gag* (mean, 46.3%, range of 34.0% – 79.2%; Fig. 1A) and *RT* (mean, 40.4%; range of 27.7% – 78.9%, Fig. 1B) that could encode CE (ARFs F2–3, R1–3; [27]).

To further evaluate the cumulative effect of codon optimization on vector inserts, we used phylogenetic analyses to compare individual TFV and insert sequences from two vaccine vectors (MVA/HIV62 and VRCrAd5) for which we had access to clinical trial samples. Genetic distances between the TFV reference set, all vector inserts, and *wild type* sequences were calculated for both proteins (F1) from the *gag* and *reverse transcriptase* (*RT*) regions and all ARF amino acid sequences (F2, F3; R1–3), which would include any potential CE (Fig. 2). Tight clustering of tree branches indicates amino acid sequences for Gag and RT proteins (F1) are genetically similar (Figs. 2A and 2B, left panels). Noncodon-optimized sequences demonstrated tight clustering for both proteins (F1) and potential CE (F2–3, R1–3; Fig. 2, all panels). Similarly, codon-optimized Gag and RT (F1) did not significantly diverge from the TFV reference set. However, codon-optimized ARFs had distances 2 to 5 times greater than the TFV reference set and the *wild type* noncodon-optimized sequences (Figs. 2A and 2B, right panels). The genetic disparity shown for F2 of *gag* and *pol* was representative of the codon-optimized CE encoded by the remaining ARFs (F3, R1–3; data not shown). These results confirmed that regardless of the method used to encode the insert, there was little difference between residues of proteins encoded by the noncodon-optimized, codon-optimized, *wild type*, or TFV consensus sequences. Conversely, analysis of the

sequences from each of the 5 ARFs in the *gag* and *RT* regions of the genome demonstrated that codon optimization of inserts can lead to a significant decrease in amino acid conservation for ARFs and a greater than 2-fold decrease in homology between targets encoded by TFVs and vaccine-derived CE.

Lack of CE-specific T cell responses in recipients of a noncodon-optimized vaccine

Based on the results above, we exclusively evaluated CE-specific T cell responses to a noncodon-optimized vaccine vector, MVA/HIV62. Responses to proteins and CE encoded by the *gag* and *PR-RT* inserts were measured with an interferon-gamma (IFN- γ) ELISpot assay using peripheral blood mononuclear cells (PBMC) collected from participants enrolled in HVTN 205 who received two pGA2/JS7 DNA injections at 0 and 2 months followed by two vector boosts at 4 and 6 months or placebo. As expected from prior studies with this product, Gag but not Pol-specific T cell responses were present only in vaccine recipients (Fig. 3, [40]). CE-specific responses were occasionally detected in vaccinees, especially in PR-RT ARF F2. However, these were not significantly greater than the responses seen in the placebo controls for any of the *gag* or *PR-RT* ARFs. Furthermore, subsequent mapping of the PR-RT ARF F2 responses failed to identify any peptide, indicating that the detected peptide pool responses were either weak or not present at all.

In vitro cultured assay detected a higher frequency of responders in vaccinees

A cultured assay was performed on a select set of positive responders to determine if low frequency CE specific responses were present and could be expanded after a 12 day *in vitro* expansion. This assay's extended period of antigenic stimulation allows for proliferation of low frequency HIV-1-specific T cells and therefore achieves greater sensitivity than an *ex vivo* ELISpot assay [32–34]. Eighteen participant samples from both treatment groups were cultured with peptides from *gag* and *PRT-RT* ARFs but high background seen in samples from 7 participants excluded them from further analysis. In the 11 remaining samples with acceptable background, the magnitude of CE-specific T cells was significantly greater in vaccinees ($p < 0.0001$; Fig. 4A). Furthermore, CE-specific responses were only detected in the vaccinees and none of the placebo controls (Fisher's exact test, $p = 0.06$; Fig. 4B). In addition, none of the irrelevant ARF pools (those that were negative at the initial testing) were positive upon culturing. Taken together, these data suggests that CE responses may be induced by this DNA/MVA vaccine regimen, albeit at low frequencies.

DISCUSSION

Translation of cryptic epitopes from alternative reading frames highlights the immense coding capacity of HIV-1's genome and increases the demand for immunologic coverage through vaccination [7,9,10,12]. These immunogens present a new source of HIV-1 antigen diversity that further challenges the development of a broadly protective HIV-1 vaccine [1,4]. In retrospect, vaccine candidates in ongoing clinical trials were designed prior to research that established the immunological relevance of CE in HIV-1-infected patients [18–20]. Consequently, current vaccines prioritize preservation of primary reading frames rather than ARFs, which may affect the generation of CE [37,38,41]. We therefore assessed the CE coding capacity of current vector designs, which supported investigation of CE-specific T cell responses in recipients of a noncodon-optimized HIV-1 vaccine regimen.

Our comparison of vector inserts demonstrated that codon optimization of HIV-1 genes preserves amino acid sequences encoded by the primary reading frame (F1) but decreases amino acid conservation of potential CE from ARFs (F2–3, R1–3). Because of the high magnitude of responses to PR-RT-CE for ARF F2, we tested the same large pool of peptides in participants vaccinated with the VRC DNA prime rAd5 vaccine as part of HVTN 084

[59]. However, no T cell responses specific to ARF F2 were detected when evaluated *ex vivo* (unpublished results). Although not investigated by our group, it is also possible that codon optimization skews the normal CE production by skewing the regulatory sequences governing the synthesis of transcriptional or translational products [54,55]. Similarly, recognition of optimized CE may be skewed because previous immunogenicity studies have shown that at least two-thirds of a peptide's residues must be conserved for cross-recognition by epitope-specific T cells [42]. The average percent (46.3% and 40.4%, *gag* and *PR-RT* regions, respectively) of amino acid conservation for codon-optimized CE fell well below this threshold with exception of frame R2. The higher homology in R2 results from shared positioning of optimized codons in the primary reading frame (F1). Since codon optimization of a gene (F1) frequently changes the nucleotide in the third or wobble position of codons, its complementary base in the second reverse reading frame (R2) is also changed at the wobble position, which minimizes the decrease in homology for this reading frame [43]. In comparison, all frames of noncodon-optimized inserts were nearly identical to TFV sequences from acutely-infected patients, indicating a higher probability of inducing cross-reactive, CE-specific T cells during a transmission event. Moreover, these observations suggest that the native context of HIV-1's genomic architecture is more rigid than was previously suspected, where manipulation of gene sequences may result in decreased antigen production, presentation, and/or recognition [44,45].

In participants of HVTN 205 receiving a vector encoding a noncodon-optimized insert, we were unable to detect significant CE responses *ex vivo*. Such a finding could be due to a number of factors including the use of cryopreserved specimens and the method of resting cells overnight both of which may have contributed to enhanced cell death. We find these possibilities unlikely since both systems are used extensively by our group and the HVTN and do not significantly impact responses seen to epitopes in the protein reading frame [56–58]. It seems more likely that the poor responses observed to CE lies in the fact that even with HIV infection responses to CE are low in magnitude and relatively infrequent [18,19]. Furthermore, the average OLP length (15mer, with a range of 8 – 18mer) exceeded the length of peptides commonly recognized by MHC-I (8 – 11mer), which potentially reduced sensitivity to CD8+ T cell responses in our assays. In addition, the higher frequency of stop codons in ARFs further restricted the size and number of OLPs that could be artificially synthesized. Accordingly, CE generated through non-conventional translation methods, including stop codon readthrough and doublet decoding, may have been undetectable with the described set of OLPs [30,46]. Furthermore, the low immunogenicity of Gag- and PR-RT-CE suggests MVA/HIV62 may not have been processed efficiently or effectively presented to the immune system [47,48]. During infection, translation of *PR-RT* from the *gag-pol* transcript relies on a native –1 ribosomal frameshift, which reduces PR-RT expression to one-tenth the level of Gag protein. Since the vector insert maintained the same out-of-frame nucleotide code for the *gag* and *PR-RT* regions, vaccine-derived PR-RT levels may have been insufficient for priming central memory T cell populations and limited the number of effector responses directed against Pol protein in this study. Alternatively, 90% of *gag-pol* reads do not result in frameshifting, such that translation could occur downstream of *gag* and generate CE from ARF F2 of *PR-RT*. Expression from this region is consistent with the trend seen for PR-RT-CE F2 responses (Fig. 3B) and our previous data, which suggested that epitopes in the *pol* region are predominantly cryptic [18]. Nevertheless, a cultured assay was performed with the same OLPs and detected CE-specific T cell responses exclusively in vaccinees.

Our inability to detect vaccine-induced CE responses *ex vivo* contrast with what has been reported in rhesus macaques immunized with SIV vaccines [26,49]. In fact, CE responses have been reported in up to one-quarter of these vaccinated macaques [50]. However, these macaques were vaccinated with SIV rAd5 vectors that potently induce CD8 T cell responses

unlike MVA recombinant vectors, used in our study, that induce relatively weak CD8 responses and are more skewed towards CD4. Additionally, the breadth of vaccine-induced responses is significantly greater in macaques compared to humans likely due to the fact that the former primates are able to encode many more MHC type I alleles than the six available to humans [51]. As such it seems reasonable to expect that that this greater vaccine-induced breadth would extend to CE as well.

Together, our *in silico* and *in vitro* analyses suggest that codon optimization negatively impacts the breadth of CE-specific T cell responses when compared with the repertoire generated through infection or vaccination with a noncodon-optimized vector. Vaccination with the noncodon-optimized MVA/HIV62 vector induced cellular responses that were low in magnitude and frequency, indicating HIV-1 CE are presented but at levels that may not be adequate for recognition by peripheral T cells. Still, the immunity afforded to animals vaccinated with naturally encoded constructs supports delivery of noncodon-optimized vectors to expand the pool of vaccine-induced epitopes [52]. As future studies determine the importance of CE responses in viral control, our research may provide direction for developing vaccine designs that increase the breadth of T cell responses by either preserving as many natural HIV-1 targets as possible or specifically engineering vaccines to express CE as immune targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CE	cryptic epitopes
ARF	alternative reading frames
TFV	transmitted founder virus
OLP	overlapping peptides
HLA-I	human leukocyte antigen class I
MHC-I/II	major histocompatibility class I or II

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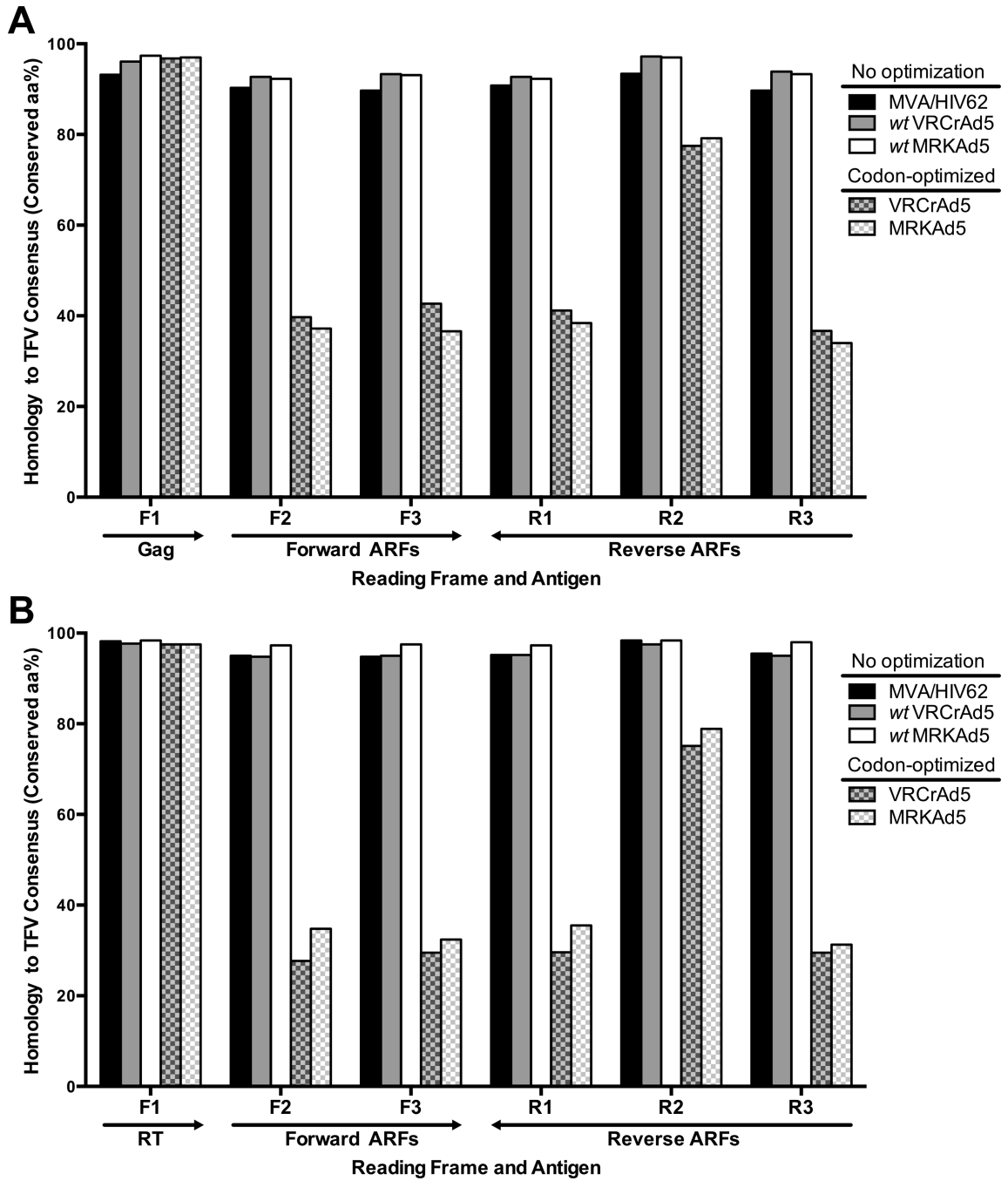


Figure 1. Codon optimization significantly alters natural HIV-1 ARFs

The homology of amino acid sequences is plotted as the percent of residues that were conserved with respect to a reference, the translated TFV consensus sequence, for 1 noncodon-optimized insert (MVA/HIV62; black), 2 codon-optimized inserts (VRCrAd5, MRKAd5; grey and white pattern, respectively), and the 2 *wild type* sequences from which the codon-optimized insert were designed (*wt*VRCrAd5, *wt*MRKAd5; grey and white). Amino acid identities were compared for all reading frames of the *gag* (A) and *RT* (B) regions.

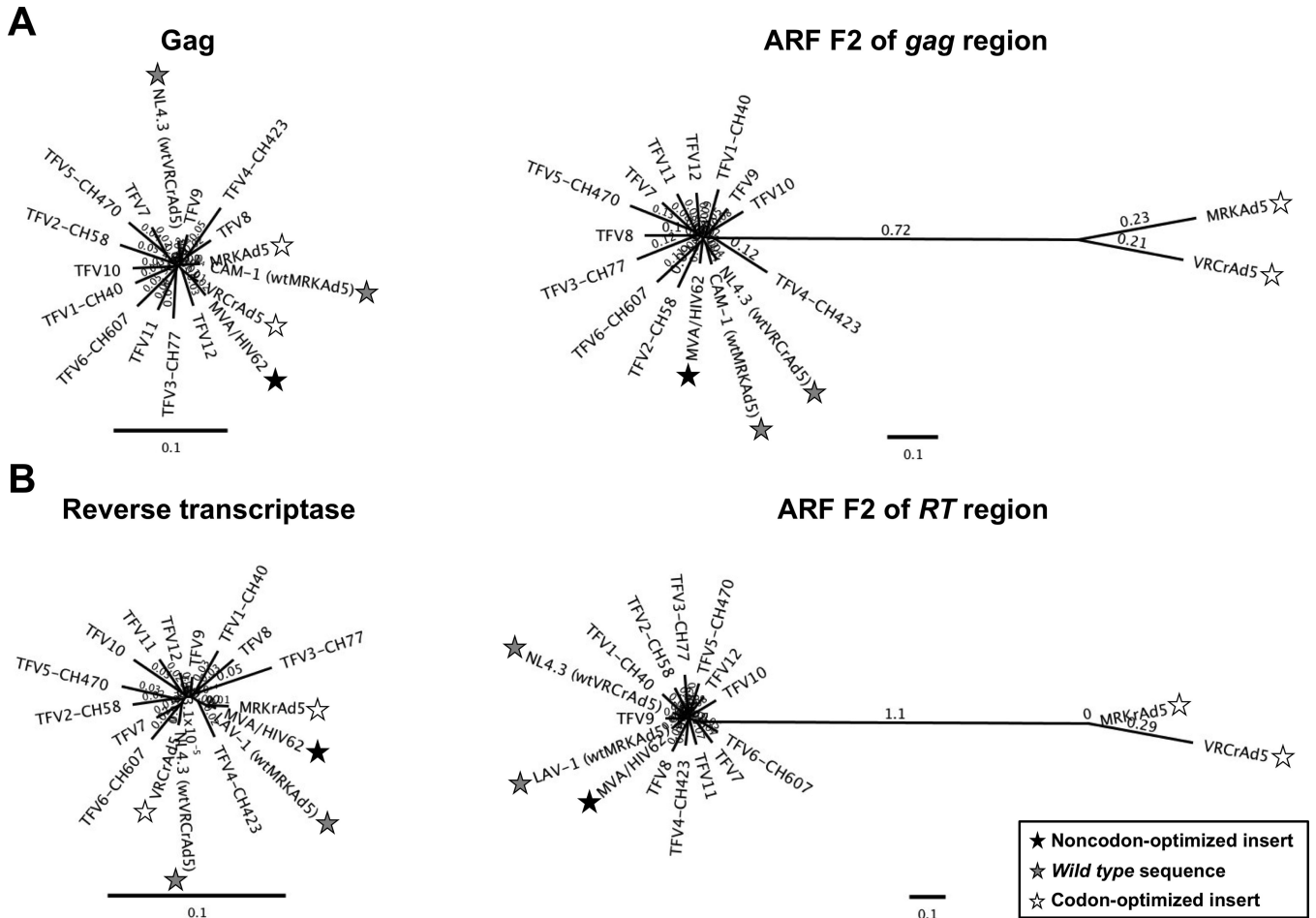


Figure 2. Genetic distances of ARF sequences are greatly increased for codon-optimized vector inserts

Genetic distances were determined for amino acid sequences translated from 12 individual clade B TFV sequences, 1 noncodon-optimized vector insert (MVA/HIV62; black stars), 2 codon-optimized vector inserts (VRCrAd5, MRKAd5; white stars), and 2 *wild type*, noncodon-optimized sequences (*wt*VRCrAd5, *wt*MRKAd5; grey stars). Distances are plotted for Gag and RT proteins (A and B, respectively; left panels) and forward frame 2 (F2), a representative example of distances for an alternative reading frame (ARF; A and B, right panels). Branch lengths reflect sequence divergence. Scale bars indicate 10% genetic distance.

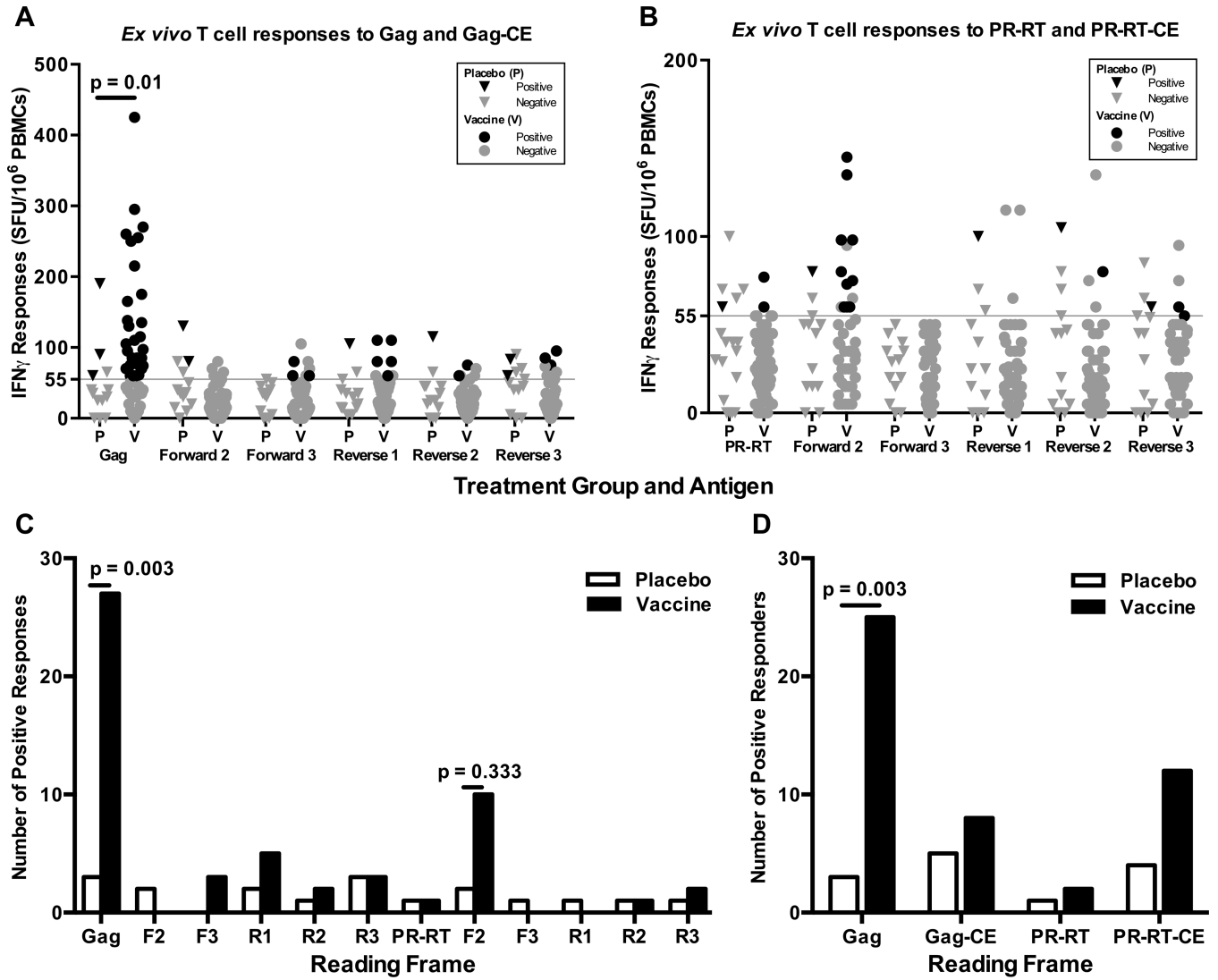


Figure 3. Lack of detectable CE-specific cytotoxic T cell responses to ARF peptide pools
 The magnitude of responses to large, single frame overlapping peptide pools from the *gag* (A) and *PR-RT* (B) regions was measured by IFN γ ELISpot. All responses for are shown for positive responders who received vaccine (N= 32; V, circles) or placebo (N=8; P, triangles) treatment during HVTN 205. Positive values (black) exceeded both the positivity threshold (grey line) and 3 times the sample background. (C) The total number of positive responses to 12 large, single frame peptide pools are shown for positive responders according to treatment group. (D) The proportion of participants with a positive response to either protein or any CE encoded by the *gag* or *PR-RT* regions is shown as a percentage according to group.

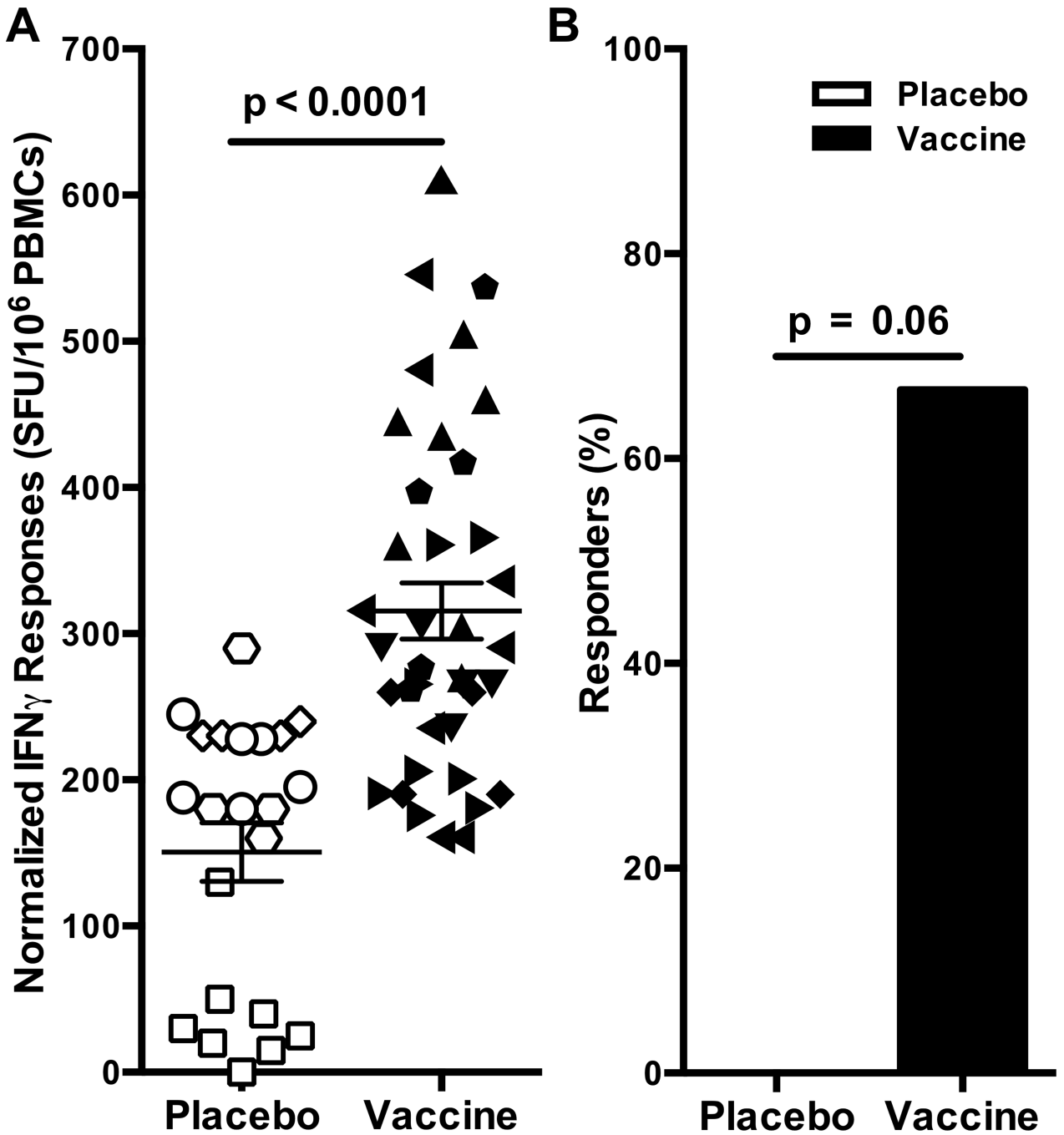


Figure 4. Increased detection of proliferating HIV-1 CE-specific T cells in a cultured IFN γ ELISpot assay

T cells were evaluated following 10 days in culture with subpools of peptides encoded by ARFs. (A) Data are presented as the number of responses normalized to the lowest magnitude subpool response after subtracting background. Each symbol represents all positive and negative responses for an individual vaccinee. Bars represent the standard error of the mean. (B) The proportion of participants with a positive response to any ARF subpool is shown as a percentage according to group.

Table 1

Vaccine dosage and schema for participants of HVTN205.

Participants		Dose		Vaccination Schedule (months)				
Group	Number	DNA (mg)	MVA (TCID ₅₀)	0	2	4	6	6.5
Vaccine	80	3	1×10 ⁸	DNA	DNA	MVA	MVA	Endpoint
Placebo	40	0	0	Placebo	Placebo	Placebo	Placebo	Endpoint

Analyses were performed for HIV-1 uninfected, vaccinia-naïve adults who were vaccinated per protocol. Participants received either a prime-boost regimen with HIV-1 pGA2/JS7 Gag-PR-RT-Env-Tat-Rev-Ypu DNA and a clade B Gag-PR-RT-Env MVA/HIV62 vector or 0.9% sodium chloride via intramuscular injection. Immunogenicity was tested at 6.5 months (2 weeks post last boost).