

# CD8 T cell response and evolutionary pressure to HIV-1 cryptic epitopes derived from antisense transcription

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Retroviruses pack multiple genes into relatively small genomes by encoding several genes in the same genomic region with overlapping reading frames. Both sense and antisense HIV-1 transcripts contain open reading frames for known functional proteins as well as numerous alternative reading frames (ARFs). At least some ARFs have the potential to encode proteins of unknown function, and their antigenic properties can be considered as cryptic epitopes (CEs). To examine the extent of active immune response to virally encoded CEs, we analyzed human leukocyte antigen class I-associated polymorphisms in HIV-1 *gag*, *pol*, and *nef* genes from a large cohort of South Africans with chronic infection. In all, 391 CEs and 168 conventional epitopes were predicted, with the majority (307; 79%) of CEs derived from antisense transcripts. In further evaluation of CD8 T cell responses to a subset of the predicted CEs in patients with primary or chronic infection, both sense- and antisense-encoded CEs were immunogenic at both stages of infection. In addition, CEs often mutated during the first year of infection, which was consistent with immune selection for escape variants. These findings indicate that the HIV-1 genome might encode and deploy a large potential repertoire of unconventional epitopes to enhance vaccine-induced antiviral immunity.

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Abbreviations used: ARF, alternative reading frame; CE, cryptic epitope; HLA-I, HLA class I; ICS, intracellular cytokine staining; ID, immunodominant; LTP, linked transmission pair; MOI, multiplicity of infection; PP, posterior probability; SN, seronegative.

Clinical human vaccine studies have so far failed to demonstrate that induction of either antibody or T cells in isolation confer protection against HIV-1 infection or delay disease progression (Flynn et al., 2005; Buchbinder et al., 2008). A clinical trial recently completed in Thailand (RV-144) demonstrated partial efficacy (Rerks-Ngarm et al., 2009). In this study, the use of an HIV-1 recombinant canarypox vector (ALVAC-HIV) boosted with a bivalent glycoprotein subunit vaccine (AIDSVAX B/E) was able to induce envelope-specific antibody and T cell responses. Because the correlates of protection using this latter vaccine regimen are not understood, it would be prudent to develop strategies to in-

crease both the humoral and cell-mediated immune responses induced by HIV-1 vaccines.

Recent nonhuman primate studies suggest a correlation of viral load with the breadth and magnitude of vaccine-induced CD8 T cell responses (Liu et al., 2009; Wilson et al., 2009). Perhaps importantly, the breadth of the CD8 T cell responses induced by these SIV-based vaccines far exceeded those elicited by any HIV-1 vaccine tested to date (McElrath et al., 2008).

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Foremost among these, the recent Merck trivalent vaccine (Step Trial) induced a median of only three CD8 T cell epitope (a median of one Gag, one Pol, and one Nef response) responses per vaccinee, and only 31% of vaccinees demonstrated both CD8 and CD4 T cell responses (McElrath et al., 2008). These studies would suggest that to be effective, at minimum, vaccines will need to induce a considerably greater number of T cell responses.

The three letter codon alphabet recognized by unique tRNA during protein synthesis allows for three potential overlapping reading frames in each direction of DNA transcription. A reading frame that encodes a potential peptide sequence that is not part of a functional protein is called an alternative reading frame (ARF), and within HIV-1 there are many such ARFs in both the sense and the antisense directions of transcription (Fig. S1). Although all functional HIV-1 proteins are thought to be transcribed from the positive sense DNA strand, several studies have shown that antisense RNA is transcribed during HIV-1 infection and that translation of several ARFs occurs, with the potential to generate immunogenic and antigenic peptides called cryptic epitopes (CEs), a potential source of HLA class I (HLA-I)-presented peptides (He et al., 2008; Seila et al., 2008). After transcription, there are multiple mechanisms through which the RNA strand can be manipulated or otherwise used to generate CEs, including alternative splicing patterns, ribosomal frameshifting (Weiss et al., 1987), internal ribosomal entry sites (McBratney et al., 1993), initiation codon scanthrough (Bullock et al., 1997), doublet decoding (Bruce et al., 1986), and initiation from non-AUG codons (Malarkannan et al., 1999).

CD8 T cell responses targeting CEs derived from the positive-strand RNA have been described previously for both SIV and HIV-1 (Cardinaud et al., 2004; Maness et al., 2007); however, the frequency and biological significance of these responses are unknown. Furthermore, CD8 T cell targeting of CEs derived from antisense transcription has not been described despite evidence that HIV-1 proteins are produced from this process (Michael et al., 1994; Ludwig et al., 2006; Landry et al., 2007). This latter production of peptides could be an important source of antigenic epitopes considering the relatively large amount of CEs that can be generated from antisense open reading frames (Fig. S1).

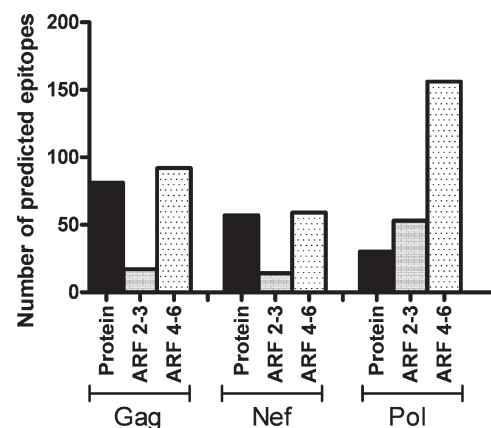
In this report, we predict frequent targeting of CEs derived from ARFs in chronic infection by identifying HIV-1 polymorphisms associated with specific HLA-I alleles. The majority of these CE responses are predicted to occur in the *pol* region and most CEs are derived from antisense transcription, possibly because of the large number of potential peptides synthesized from this region. Using PBMCs from early and chronically HIV-1-infected patients, we show that CD8 T cell targeting of CEs is a frequent occurrence. Finally, we show evidence of evolution at predicted CEs during the first year of HIV-1 infection. Immune targeting of protein products derived from antisense transcription is a previously unrecognized process that, in addition to providing insight

into the natural immune response to HIV-1, could potentially be used to significantly increase the breadth of a vaccine response.

## RESULTS AND DISCUSSION

### CE prediction in ARFs of HIV-1

Analysis of the six possible translational reading frames of HIV-1 *gag*, *pol*, and *nef* reveals a vast number of open reading frames that have the potential to encode proteins and epitopes (Fig. 1 and Fig. S1). To gain a more comprehensive understanding of CD8 T cell recognition of CEs, we determined HLA-I-associated HIV-1 polymorphisms in the main reading frame encoding the functional protein reading frame (RF 1) and ARFs 2–6 of the *gag*, *pol*, and *nef* regions in a large cohort of clade C-infected individuals (Matthews et al., 2008). The resultant polymorphisms predict amino acid changes that have escaped CTL pressure or reverted once that pressure has been lifted. This analysis made corrections for viral phylogeny in addition to HLA linkage disequilibrium and HIV codon variation, and the resultant HIV-1 polymorphisms indicated potential CD8 T cell escape mutations (Carlson et al., 2008). We then used the amino-acid regions flanking the calculated polymorphisms (27mer input peptide, i.e., 13 amino acids on either side of the polymorphism) to better predict a probable CD8 T cell epitope with an epitope prediction program (Epipred), as indicated by a posterior probability (PP) of >0.1 (Heckerman et al., 2007). This latter strategy recognizes that a polymorphism may be HLA linked but represent a processing or compensatory mutation occurring outside of the actual epitope. For a given HLA-peptide pair, PP is a Bayesian measure that quantifies the amount of evidence (based on the HLA and amino acids at various positions in the peptide) that the peptide is indeed an epitope presented by a given HLA. The PP is derived from a predictive model trained using known



**Figure 1. Predicting CE-specific CD8 T cells using HLA-I-associated HIV-1 polymorphisms.** HLA-I-associated HIV-1 polymorphisms in *gag*, *nef*, and *pol* were used to predict potential CD8 T cell epitopes in all reading frames. The number of predicted epitopes is compared between protein (sense RF 1) and the ARFs (the other five reading frames). The ARFs include sense ARFs 2 and 3 as well as antisense ARFs 4–6.

HLA-restricted epitopes. In addition, the predictive model uses a prior probability of 0.1. Consequently, our use of a PP threshold of 0.1 amounts to selecting putative epitopes that have net positive evidence for being actual epitopes. This analysis predicted 391 CEs in the five combined ARFs for *gag*, *pol*, and *nef* genes compared with 168 conventional epitopes encoded in RF 1 (Fig. 1). For each gene, the number of predicted CEs outnumbered the number of conventional epitopes, but this was most prominent for the *pol* region (209 vs. 30, respectively). Despite the fact that antisense transcription has been demonstrated to occur for many cellular and viral genes including HIV-1 (Michael et al., 1994; He et al., 2008), previous studies have only analyzed responses to CEs derived in the forward reading frames (Cardinaud et al., 2004). Therefore, we determined the proportion of CEs predicted to be targeted by CD8 T cells in the forward (ARFs 2 and 3) and reverse (ARFs 4–6) reading frames. Interestingly, for *gag*, *pol*, and *nef*, a greater percentage of the peptides encoded for ARFs 4–6

(79% compared with ARFs 2 and 3) were predicted to be targeted by CD8 T cells (Fig. 1).

### Recognition of predicted CEs by HIV-1-infected patients

These data are the first to suggest that epitopes derived from antisense transcripts are common CD8 T cell targets in HIV-1 infection. Although predicting CD8 T cell epitopes based on HLA-I-associated HIV-1 polymorphisms is a validated approach (Carlson et al., 2008; Goepfert et al., 2008), it has not been used to predict CEs. For functional analysis, we selected CEs that were calculated to have the highest chance of representing a true CD8 T cell epitope (Table I), i.e., predicted epitopes with a PP of >0.2 (i.e., those with a 20% chance of representing a true CD8 T cell epitope). We also excluded from consideration peptides in the ARF that could be associated with a mutation in RF 1. Using these criteria, the dominance of peptides from *pol* still persisted among the 24 peptides that we selected to test ( $P = 0.02$ ; Table I). Interestingly, the majority of the peptides

**Table I.** CEs predicted based on HLA-I-associated HIV-1 polymorphisms

ARF	Protein	HLA	Best epitope <sup>a</sup>	Peptide	PP <sup>b</sup>	PHI <sup>c</sup>	CHI <sup>c</sup>
					%		
2	<i>pol</i>	B*3910	---ELKTFGRF+---	EF8	30	1 (0)	0 (0)
3	<i>gag</i>	A*01	---SSHANvKRY---	SY9*	54	7 (0)	7 (0)
3	<i>pol</i>	B*15	---YRYSISRtY---	YY9	40	1 (0)	3 (0)
3	<i>pol</i>	C*07	--- <u>YRfSISRAY</u> ---	YY9*	20	13 (1)	17 (1)
3	<i>pol</i>	C*07	--- <u>NLWKKGYRf</u> ---	NF9	30	13 (0)	17 (2)
4	<i>pol</i>	A*3001	---FcFPPWYYL---	FL9	51	2 (0)	9 (0)
4	<i>pol</i>	A*34	---NIPCFSYf----	NF8*	26	2 (0)	4 (0)
4	<i>pol</i>	B*15	--- <u>LCFYVATgY</u> ---	LY9	31	1 (0)	3 (2)
4	<i>pol</i>	B*35	---+SPAILFWQL---	SL9	30	4 (0)	7 (0)
4	<i>pol</i>	B*42	---LPKSDLREv---	LV9	51	1 (0)	2 (0)
5	<i>pol</i>	A*0205	---SVnCFSTSLV---	SV9*	35	14 (0)	20 (0)
5	<i>gag</i>	A*3001	-+- <u>CLQPSDVSK</u> ---	CK9*	29	2 (1)	9 (0)
5	<i>pol</i>	A*3002	-+- <u>AYFPVFRFL</u> ---	AL9*	28	2 (0)	9 (1)
5	<i>pol</i>	A*33	---TGHLPANF+---	TF8	26	1 (0)	4 (0)
5	<i>nef</i>	A*6801	---SLTaGHPTM---	SM9	30	1 (0)	8 (0)
5	<i>gag</i>	B*08	--- <u>FPHFQQPf</u> ----	FF8*	36	5 (1)	9 (0)
5	<i>pol</i>	B*35	--- <u>IPNAYcESV</u> ---	IV9	42	4 (0)	7 (1)
5	<i>pol</i>	B*5802	--- <u>ASFIWPPTF</u> -+-	AF9	40	4 (1)	5 (1)
5	<i>gag</i>	C*0801	---NVAPGPNAL--+	NL9*	58	1 (0)	2 (0)
5	<i>pol</i>	C*0804	---+FPTNFCISL---	FL9	27	1 (0)	2 (0)
5	<i>pol</i>	C*18	---DPTYkSSI---	DI8*	26	0 (0)	1 (0)
6	<i>pol</i>	A*0205	---+ <u>SLLVHVWLPL</u> --	SL10	29	14 (1)	20 (1)
6	<i>pol</i>	A*29	--- <u>NMHPPHPVL</u> ---+	NL9	69	4 (0)	1 (1)
6	<i>pol</i>	B*5802	--- <u>LPSFLHKL</u> ---	LL9	28	4 (1)	5 (2)

CD8 T cell epitopes were predicted by EpiPred. Bolded and underlined amino acids were recognized in primary (PHI) and chronic (CHI) HIV-1 infections, respectively. Lowercase letters and plus signs represent HLA-associated HIV-1 polymorphisms within and outside the predicted epitope, respectively. The asterisks indicate evidence for reversion in the predicted epitope.

<sup>a</sup>Predicted epitope from an input 27mer sequence.

<sup>b</sup>PP indicates the percent probability that the predicted epitope represents a real epitope.

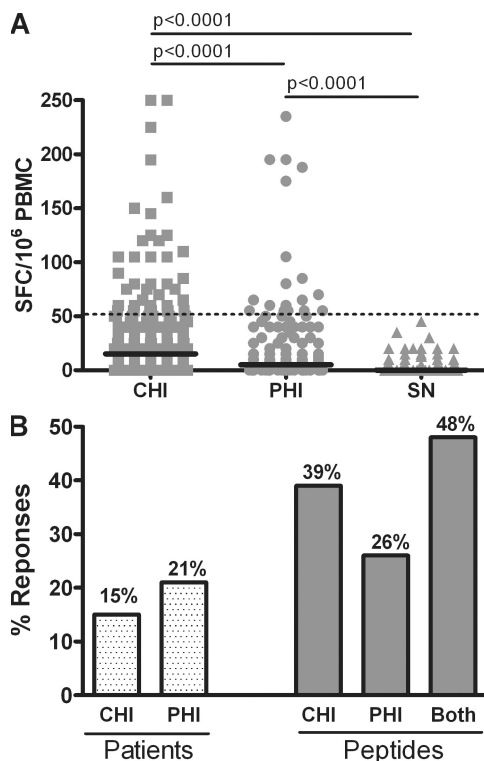
<sup>c</sup>Number of patients with the allele that were tested (number of responses).

were derived from ARFs 4–6 (products of antisense transcription and translation).

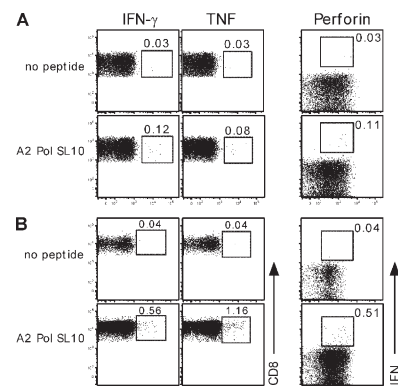
The predicted CE (Table I) were tested using PBMCs from 41 patients with chronic and 24 patients with primary HIV-1 subtype B infection in an overnight IFN- $\gamma$  ELISPOT assay (Bansal et al., 2007). We tested the reactivity of CE in a subtype B cohort, reasoning that HLA-I-restricted CE would be similar across subtypes (including subtype C), as has been demonstrated with conventional epitopes (Kawashima et al., 2009). CE-specific responses were detected in HIV-1-infected individuals during both primary and chronic infection (Fig. 2 A). In contrast, we saw below threshold responses to these CE among the 15 seronegative (SN) controls tested. Similar to those observed for conventional epitopes (Altfeld et al., 2001), the magnitude and breadth of CE responses, as measured by IFN- $\gamma$ , was significantly higher in patients with chronic compared with primary infection ( $P < 0.0001$ ), suggesting that CE responses are progressively targeted with chronic infection. Overall, 48% of the tested CE demonstrated a positive response in at least one

patient (Fig. 2 B), and CD8 T cell responses to at least one epitope were detected in 21% of patients with primary and 15% with chronic infection (Fig. 2 B). The actual number of CE responses is likely to be higher because we evaluated only a small subset of predicted CE, and we tested clade C-predicted CE in the context of clade B infection. Further evidence attesting to the specificity of these CE responses is that none of the predicted CE appeared similar to known HIV-1 epitopes restricted by the indicated HLAs. Additionally, we scanned each of these predicted CE in the BLAST search engine (<http://www.ncbi.nlm.nih.gov/blast>) and did not find any sequence match to known human pathogens in the database.

The CE tested in this paper were predominantly 9–10mers derived from antisense ARFs (Table I) and were predicted to stimulate CD8 T cells. To confirm that CE responses were derived from antisense ARFs and are recognized by CD8 T cells, we stimulated PBMCs from 14 HIV-1-infected individuals (5 chronic and 9 primary) directly ex vivo with selected CE in a 6-h intracellular cytokine staining (ICS) assay and stained the cells with antibodies specific for CD3, CD8, IFN- $\gamma$ , TNF, IL-2, perforin, and CD107. A representative figure demonstrates that the A02-SL10 CE induces production of IFN- $\gamma$ , TNF, and perforin by CD8 T cells (Fig. 3 A). In a subset of individuals, we used a peptide-based cultured assay to quantify the responses elicited to CE and found that low frequency antigen-specific cells expanded 5–10-fold over a 10-d culture of PBMCs stimulated with the cognate peptide (Fig. 3 B). This modest expansion in precursor frequency was also observed in other HIV-1-infected patients (unpublished data) and is consistent with the generally poor proliferative responses seen in patients with chronic progressive HIV-1 infection (Migueles et al., 2002). We next compared the ex vivo functional profile of the CE-specific CD8 T cells to those



**Figure 2. CE-specific responses are recognized by CD8 T cells in HIV-1 infection.** (A) An IFN- $\gamma$  ELISPOT assay using antigen-specific stimulation in duplicate was used to determine the magnitude of T cell responses in primary (PHI;  $n = 24$ ) and chronic (CHI;  $n = 41$ ) HIV-1 subtype B-infected patients. These responses were compared with SN controls ( $n = 15$ ). Each symbol represents the response magnitude to an individual CE. The dotted line indicates the cutoff for a positive response (>55 SFCs/10<sup>6</sup> PBMCs), and horizontal bars represent medians. (B) The percentage of patients or peptides that elicited a CE-specific response are shown. The percent positive is shown on top of the bar.



**Figure 3. CE-specific CD8 T cells produce cytokines and effector molecules.** (A and B) PBMCs were stained for phenotype and functionally characterizing CE-specific T cell responses. The production of cytokines (IFN- $\gamma$ , IL-2, and TNF) and up-regulation of CD107 and perforin by antigen-specific CD8 T cells were measured (percentages are shown). Representative data from a chronically infected patient showing CE-specific responses as measured ex vivo (A) and after a 10-d culture (B) are shown.

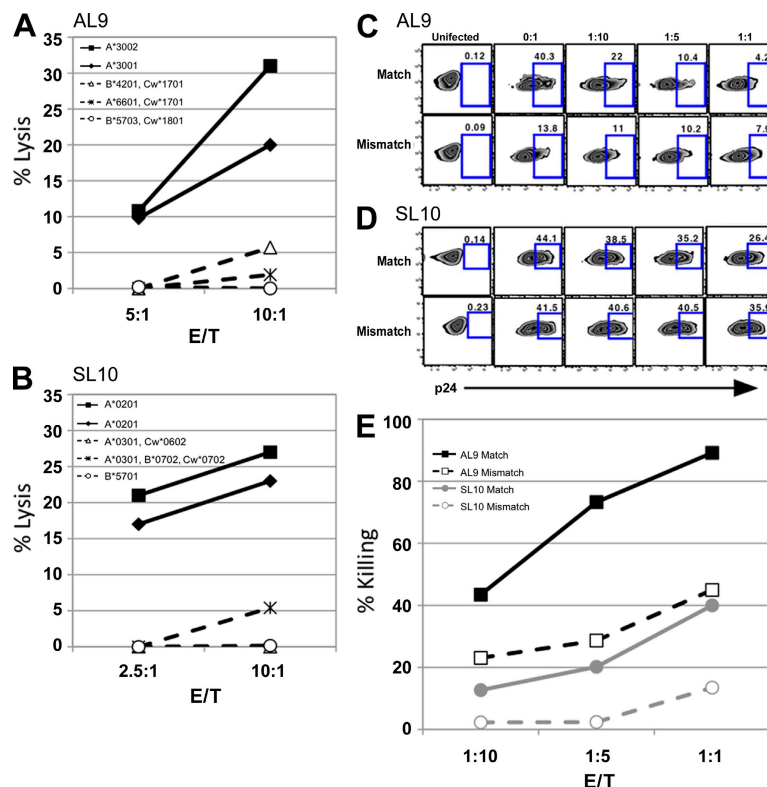
elicited by conventional immunodominant (ID) HIV epitopes in the same patient samples. The CE-specific CD8 T cells were able to produce all of the functional molecules analyzed and did not differ significantly from the ID responses (Fig. S2).

#### Antisense reading frame–derived CEs are restricted by the predicted HLA-I alleles and presented by HIV-1–infected cells

To verify that CE responses were in fact restricted by the predicted HLA-I alleles, we derived two CE-specific CD8 T cell lines (A\*3002-AL9 and A\*0205-SL10; Table I) from two chronic patients that recognized epitopes derived from the pol antisense reading frames (ARFs 5 and 6, respectively). In a  $^{51}\text{Cr}$  release assay, each of these T cell lines lysed peptide-pulsed HLA-I–matched but not –mismatched targets (Fig. 4, A and B). Next, we infected HLA-I–matched and –mismatched activated CD4 T cells from healthy SN donors with HIV-1 NL4.3 at a multiplicity of infection (MOI) of 0.1. 2 d later, infected cells were used as targets and co-cultured with either the A\*3002-AL9–specific or the A\*0205-SL10–specific cell line at

varying E/T ratios for 24 h (Fig. 4, C and D). The relative amount of target cell lysis was calculated by the diminution of intracellular p24 by the effectors when compared with infected targets alone. The HLA-I–matched HIV-1–infected targets demonstrated significant killing of targets cells by the CE-specific cell line when compared with the HLA-I–mismatched control (Fig. 4 E). This finding demonstrates that a peptide encoded by an HIV-1 antisense reading frame is generated in infected CD4 T cells, transported to the cell surface, and presented to CE-specific CD8 T cells in an HLA-I–dependant manner.

To be certain that CE responses do not represent cross-reactive responses induced by conventional HIV-1–specific epitopes, we determined whether an A\*0205-SL10 CE-specific cell line could recognize other known HLA-A\*02–restricted epitopes. As expected, the CE-specific cell lines lysed targets pulsed with the A\*0205–restricted CE but not other conventional epitopes nor another CE restricted by the same allele (Fig. S3 A). We also stained the CE-specific T cells from a 10-d culture assay with an A\*0205 pentamer to further demonstrate the specificity of CE responses (Fig. S3 B).



**Figure 4. CE-specific T cells recognize peptide-pulsed and HIV-1–infected targets.** (A and B) T cell lines were derived by peptide-specific stimulation followed by limiting dilution cloning. MHC restriction of the T cell lines was determined using matched (continuous lines) and mismatched (dashed lines) HLA-I–expressing B cell lymphoblastic cell lines as targets in a  $^{51}\text{Cr}$  release assay for an A\*3002–restricted AL9–specific T cell line (A) and an A\*0205–restricted SL10–specific T cell line (B). The HLA-I–s of the patient from whom the T cell line was derived are A\*3001/A\*6601, B\*4201/B\*5703, and Cw\*1701/Cw\*1801 (A), and A\*0201/A\*0301, B\*0702/B\*5701, and Cw\*0602/Cw\*0702 (B). The HLA-I–s of B cell lymphoblastic cell lines that are shared with the patient are shown inside the panel. Results are representative of three independent experiments. (C–E) CD8 T cell–depleted PBMCs from HLA-I–matched or –mismatched donors were activated with PHA for 2 d and infected with HIV-1 NL4.3 at an MOI of 0.1 for 2 d. The infected targets were co-cultured with effectors, i.e., a CE-specific line (A\*3002-AL9 [C] or A\*0205-SL10 [D]), at 1:10, 1:5, and 1:1 E/T ratios for 24 h. The killing of infected targets was measured by p24 reduction in an ICS assay. (C and D) Density plots showing the percentage of p24 staining of target cells after co-culture with the effector line. (E) The percentage killing of HLA-I–matched versus –mismatched targets by the A\*3002-AL9 and A\*0205-SL10 cell lines are shown graphically.



### CEs evolve after primary HIV-1 infection

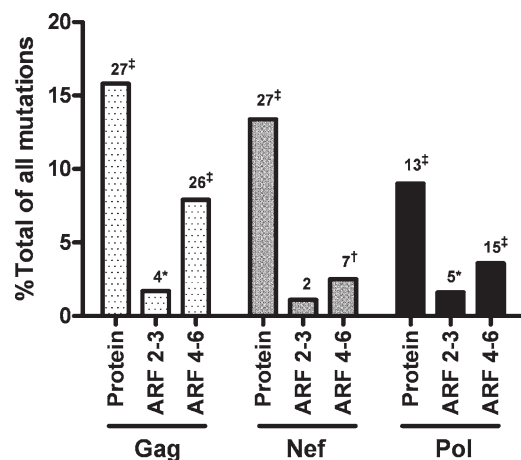
Perhaps the best indicator of immune potency is viral escape in response to immune pressure and reversion when the pressure no longer exists. Although we predicted the presence of CD8 T cell targeting based on evidence of escape and reversion, these data were obtained cross-sectionally in a chronically infected cohort and, hence, provide indirect evidence of selection. Therefore, we sought direct evidence of selection by determining if CEs (predicted in the chronic cohort) mutate during the first year of HIV-1 infection, similar to what has been observed for conventional epitopes (Brumme et al., 2008). In 37 epidemiologically linked transmission pairs (LTPs) from Zambia, we sequenced HIV-1 *gag*, *nef*, and *pol* at baseline and every 3 mo for 1 yr. We hypothesized that mutations in the main reading frame, especially the synonymous mutations, may actually reflect CD8 T cell escape mutations in CEs derived from overlapping ARFs. Therefore, we analyzed the number of nonsynonymous mutations (those that result in amino acid changes in the protein) as well as the number of synonymous mutations in the main reading frame of Gag, Pol, and Nef proteins that resulted in amino acid changes in one or more of the five ARFs during the first year of infection. This analysis enabled us to determine if any of the HLA-I-associated HIV-1 polymorphisms predicted in the chronic cohort (in all six reading frames) were observed to mutate in these linked recipients. As expected from previous studies (Brumme et al., 2008), several nonsynonymous mutations (RF 1) occurring in the Gag, Nef, and Pol proteins during the first year of infection matched the predicted conventional epitope escape mutations in the chronic South African cohort (Fig. 5). Additionally, for all of the antisense reading frames (ARFs 4–6, occurring within *gag*, *nef*, and *pol*), several synonymous mutations in the main reading frame corresponded to amino acid changes in predicted CEs that are likely caused by immune pressure (escape or reversion) and not to chance alone ( $P < 0.01$ ). Predicted CE mutations were also significantly observed in the sense reading frames (ARFs 2 and 3) of *gag* and *pol* ( $P < 0.04$ ) but not *nef*. Similar to what has been observed previously for conventional epitopes (Li et al., 2007), the majority of CE changes occurring during the first year of infection were predicted to be reversions, although some CD8 T cell escapes were also noted.

Collectively, our results support the notion that epitopes derived from antisense RNA transcripts frequently serve as CD8 T cell targets in HIV-1 infection. Indeed, 307 out of 559 (55%) of all of the predicted CD8 T cell responses (conventional and cryptic) targeted CEs that were derived from antisense reading frames. The highest number of predicted CEs is derived from antisense transcription in the *pol* region, exceeding the epitopes derived from the Pol protein and epitopes derived from sense RNA transcripts of either *gag*, *pol*, or *nef* (Fig. 1). The dominance of CEs from *pol* was confirmed with the IFN- $\gamma$  ELISPOT results whereby 9 out of 11 (82%) of the positive epitopes were encoded in this region (Table I). It is interesting that a ribosomal frameshift is necessary for pol translation (Gaudin et al., 2005) and in that sense would have a similar mechanism of synthesis compared with epi-

topes derived from ARFs. This frameshift requirement results in an  $\sim 10$ -fold decrease in Pol protein production compared with Gag (Gaudin et al., 2005), with a concomitant decrease in epitope production (Tsomides et al., 1994). Therefore, we speculate that CE production, which is also dependant in part on defective ribosomal translation, might be better able to compete with epitopes derived from the Pol protein.

Importantly, the CE predictions were biologically confirmed to be targeted by CD8 T cells in the context of primary and chronic HIV-1 infection. The functionality of these cells did not differ substantially when compared with those targeting conventional epitopes in their ability to secrete cytokines and synthesize perforin. CD8 T cells were shown to kill CE-specific targets, including those that presented epitopes derived from antisense transcription after HIV-1 infection. Additionally, the specificity of these cells was demonstrated by the fact that they could not recognize target cells pulsed with HIV-1-specific conventional epitopes that were restricted by similar HLA-I alleles as the CE analyzed (Fig. S3).

Our findings demonstrate that targeting of CEs appears to be placing immune pressure on the virus. The fact that CEs were predicted based on evidence of CD8 T cell escape indicates at least some level of significant antiviral immune pressure. Additionally, several CEs (Table I, asterisks) were also predicted to revert back to consensus when transmitted to a host that lacks the HLA-I restricting allele. We also demonstrate that both reversions and escape mutations of predicted epitopes and one biologically confirmed epitope (C\*07YY9; reversion) were noted during the first year after infection



**Figure 5. Evolution of predicted CEs after primary HIV-1 infection.** Sequencing of *gag*, *nef*, and *pol* was performed in 37 epidemiologically linked recipients from Zambia at the time of identified infection and every 3 mo for the first year. The number of observed mutations during this time that matched the predicted HLA-I-associated HIV-1 polymorphisms in the chronic cohort (Fig. 1) as a percentage of the total observed mutations (those not predicted in the chronic cohort) is shown. The total number of predicted mutations is shown above each bar. Symbols next to the number of predicted mutations designate the reading frames where mutations, predicted from the chronic cohort, were not caused by chance alone (\*,  $P < 0.04$ ; †,  $P < 0.01$ ; ‡,  $P < 0.0001$ ).

(Fig. 5). The evidence for reversion, in particular, suggests that several CEs are evolutionarily conserved by the virus because of fitness constraints, a phenomenon similar to what is seen for some conventional HIV-1 epitopes (Goepfert et al., 2008). A recent study also suggested that targeting CEs may be important by demonstrating more frequent CE responses in patients with well-controlled HIV-1 infection (Garrison et al., 2009).

Because optimal translation necessitates a variety of factors including an ATG methionine start codon, previous studies have evaluated ARF sequences in the forward reading frame containing ATG (Cardinaud et al., 2004; Maness et al., 2007). In contrast, we evaluated epitopes derived from antisense RNA transcripts with or without an optimized start codon because the evidence obtained from our HLA-I-associated HIV-1 polymorphism analysis indicated that these CEs indeed represent CD8 T cell targets. Notably, a methionine start codon is not always necessary for translation initiation, as several nonmethionine start codons have also been described (Malarkannan et al., 1999). It is also possible that CEs can be generated even when no known start codon is present because of ribosomal slippage (Maness et al., 2010). Such alternative mechanisms of peptide synthesis may well result in a relatively lower epitope production; however, many of these alternatively synthesized peptides may nonetheless be immunogenic (Schwab et al., 2003).

Although the magnitude of IFN- $\gamma$  CE responses was relatively low when compared with CD8 T cells targeting ID epitopes, it is clear from previous studies that the magnitude of CD8 T cell response is a poor correlate of immune control in HIV-1 infection (Betts et al., 2001; Masemola et al., 2004). Escape from CD8 T cells, however, can be one indication of immune potency, and a recent study demonstrated escape from CD8 T cells of low magnitude early after primary HIV-1 infection (Goonetilleke et al., 2009). Perhaps the best correlate of immune control in chronic HIV infection is the breadth of CD8 T cells responding against Gag and possibly Pol epitopes (Kiepiela et al., 2007; Goepfert et al., 2008; Matthews et al., 2008). Clearly, CEs have the potential to contribute to an enhanced breadth of a CD8 T cell immune response for therapy or preventive modalities.

These results may have important implications for vaccine design and suggest that a significant number of new epitopes could be included as vaccine targets. Importantly, codon optimization, which was used to increase expression of HIV-1 proteins by the Merck trivalent vaccine, results in a marked skewing of the translated products obtained from the ARFs, which may differ by as much as 80% compared with non-codon-optimized genes (unpublished data). There are a large number of open reading frames (products of sense and antisense transcription) that can potentially encode for additional CD8 T cell epitopes. In fact, for the *gag*, *pol*, and *nef* regions, an additional 5,376 CD8 T cell epitopes are potentially encoded by the ARF (compared with 1,785 encoded by the main reading frame). Therefore, targeting CEs could greatly increase the epitope breadth of several vaccines including

those for HIV-1 infection. This increased breadth could be achieved using similar-sized vaccine inserts potentially modified to increase CE expression. Although the partial efficacy seen in the RV-144 study seems most likely caused by the induction of HIV-1-specific antibodies (Rerks-Ngarm et al., 2009), it is still possible that T cells played a supportive role. With this in mind, it is interesting to note that the ALVAC-HIV vaccine used in RV-144 is not codon optimized (unlike the Merck trivalent vaccine) and could potentially induce CE responses. Therefore, it may be important to take into account the contribution of CE-induced responses in the overall CD8 T cell repertoire of future vaccine constructs.

## MATERIALS AND METHODS

**Calculation of HLA-I-associated HIV polymorphisms.** HLA-I-associated HIV polymorphisms were identified as previously described (Carlson et al., 2008). In brief, for *gag*, *nef*, and *pol*, a maximum likelihood phylogenetic tree was constructed using chronic clade C sequences (Matthews et al., 2008). For every HLA allele, amino acid position, and amino acid at that position, two generative or directed graphical models of the observed presence or absence of the amino acid in each sequence were created: (1) the null hypothesis, stating that observations are generated by the phylogenetic tree alone, and (2) the alternative hypothesis, stating that additional escape or reversion takes place because of HLA pressure. The likelihood of the observations was then maximized over the parameters of both models using an expectation-maximization algorithm, and a p-value was computed using a likelihood ratio test. To account for HLA linkage disequilibrium and HIV codon covariation, for each HIV amino acid, HLA alleles and other amino acids are iteratively added to the model of selection pressure using forward selection. In each step, the likelihood ratio test compares the model with and without the most recently added predictor. To account for multiple hypothesis testing, the p-values were converted to q-values as previously described (Storey and Tibshirani, 2003). We analyzed all associations with  $q < 0.2$ , corresponding to a 20% false discovery rate.

**Identification of predicted epitopes.** For each HLA-I-associated polymorphism, we constructed a consensus sequence that was consistent with the predicted association. For example, if the association predicted escape from A at position 100 in the presence of an HLA-I allele, then we computed the consensus of all sequences with A at position 100. If the predicted association was reversion to A in the absence of an HLA-I allele, then we constructed the consensus sequence of all sequences without an A at position 100. We then took the consensus sequence  $\pm 13$  amino acids from the association and used EpiPred (Microsoft Corporation) to identify the most likely epitope in the candidate sequence.

**Identification of mutations after HIV transmission in LTPs.** Early and late sequences from 37 LTPs were aligned to the consensus sequence from the Durban cohort, which was used to identify HLA-I-associated polymorphisms. Each sequence was then translated into each reading frame, and mutations were identified and classified as HLA-I-associated or not, as previously described (Brumme et al., 2008). In brief, an observed mutation was classified as HLA-I-associated if it was exactly consistent with the predicted association from the Durban dataset. For example, if the association was escape from A100 in the presence of an HLA-I allele, then the patient must have the HLA-I variant and we must see a mutation away from A at position 100. If the association was reversion to A100 in the absence of the HLA-I allele, then the patient must not have the HLA-I allele, and we must see a mutation toward A. For ARFs, we removed all mutations that were nonsynonymous relative to the primary reading frame.

**Patient cohort for CE testing.** PBMC samples from clade B HIV-1 early ( $n = 24$ ) and chronically infected ( $n = 41$ ) patients were used in this study. These patients were enrolled in the 1917 Clinic at the University of Birmingham

at Alabama. In addition, PBMCs from healthy SN donors ( $n = 15$ ) were used as controls. The Zambian patients ( $n = 37$ ) used in this study were HIV-1 clade C-infected and antiretroviral therapy-naïve individuals from the Zambia-Emory HIV Research Project. The study was approved by the Institutional Review Board of the University of Alabama at Birmingham and the ethics committee in Lusaka, Zambia, and all participants consented for the study.

**IFN- $\gamma$  ELISPOT assay.** The predicted HLA-I-restricted CEs (usually 9–11mers) based on clade C were synthesized in a 96-well peptide array format from New England Peptides. Clade B peptides were synthesized and also tested where the peptide sequence differed from clade C. This ELISPOT assay was performed as previously described using 10- $\mu$ M peptide concentrations (Bansal et al., 2007). In brief, nitrocellulose 96-well plates were coated with anti-IFN- $\gamma$  monoclonal antibody, and PBMCs (100,000 cells/well) in duplicate were incubated with the appropriate peptides for 20–24 h at 37°C in 5% CO<sub>2</sub>. After washing, biotinylated anti-IFN- $\gamma$  was added to the plates for 2 h at room temperature. After another round of washing, streptavidin-conjugated alkaline phosphatase was added for 1 h and then NBT/BCIP (SouthernBiotech) was added for color development. Individual cytokine-producing cells were counted by an ELISPOT reader (ImmunoSpot; CTL). The criteria for a positive response was >55 SFCs/10<sup>6</sup> PBMCs and four times the unstimulated control.

**ICS assay.** Cryopreserved PBMCs were stained in an ICS assay as described previously (Bansal et al., 2008). Co-stimulatory monoclonal antibodies (anti-CD28 and anti-CD49d) and Benzonase (EMD) was added to each tube containing 10<sup>6</sup> PBMCs. For co-culture, CD107a-FITC was added. Cells were pulsed with the appropriate peptide, monensin, and Brefeldin A for 6 h at 37°C. The cells were stained with the LIVE/DEAD cell dye (Invitrogen), washed, and surface labeled for 20 min before Cytofix/Cytoperm reagent (BD) was added. After 20 min, the cells were labeled with intracellular antibodies for 20 min at room temperature. The samples were then labeled with anti-CD3 (Pacific blue), anti-CD8 (Q-dot 605), and anti-CD4 (Alexa Fluor 750) conjugated antibodies. ICS was performed using anti-IL-2 (allophycocyanin), anti-TNF (PE-Cy7), and anti-IFN- $\gamma$  (Alexa Fluor 700) and antiperforin conjugated antibodies. CD14 and CD19 labeled with Percpcy5.5 were used as a dump channel. The cells were fixed in 2% paraformaldehyde and analyzed on a flow cytometer (LSR II; BD). At least 100,000 CD3<sup>+</sup> events were acquired and the data were analyzed using FlowJo software (version 8.1.1; Tree Star, Inc.).

**T cell lines.** T cell lines were generated by culturing PBMCs from two chronically HIV-1-infected patients with the cognate cryptic peptide for 10 d in the presence of 25 ng/ml IL-7 and 50 U/ml IL-2. T cells were then cloned in a limiting dilution cloning assay using irradiated allogeneic feeders, IL-2, and anti-CD3 as described previously (Sabbaj et al., 2003). The lytic capacity of these clones, their MHC restriction, and cross recognition of epitopes by CE-specific lines was determined using a <sup>51</sup>Cr release assay (Sabbaj et al., 2003). For the 10-d cultured assay, the PBMCs were stimulated with cognate peptides as described.

**In vitro killing assay.** CD8-depleted PBMCs from HIV SN individuals were activated with PHA for 2 d before infection with HIV-1 NL4.3 at an MOI of 0.1. 2 d after infection, the CE-specific lines were added to HLA-matched (A\*0205 for SL10- and A\*3002 for AL9-specific cell lines) and mismatched targets (10<sup>5</sup>) at various E/T ratios (1:10, 1:5, and 1:1), and the cells were co-cultured in duplicate for 24 h at 37°C and 5% CO<sub>2</sub>. Cells were then stained with surface markers (CD4-Percpcy5.5 and CD3-Pacific blue; BD). After washing with PBS, cells were permeabilized with Perm A reagent followed by intracellular staining with p24-PE (Beckman Coulter) in Perm B buffer (Invitrogen) according to the manufacturer's instructions. The killing of target cells was measured by p24 reduction. The percentage of killing was determined by taking the ratio of the percentage of p24 staining without any effectors to the percentage of p24 at the different E/T ratios and subtracting this number from 100. Gates were set using the uninfected targets.

**Statistics.** Analyses of variables between each group were performed using the nonparametric Mann-Whitney test. All analyses were performed with Prism software (GraphPad Software, Inc.).

**Online supplemental material.** Fig. S1 depicts open reading frame maps for *gag*, *nef*, and *pol*. Fig. S2 shows that CE-specific responses elicit multiple effector functions. Fig. S3 depicts the specificity of an SL10 CE-specific T cell line. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20092060/DC1>.

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