AIDS virus–specific CD8+ T lymphocytes against an immunodominant cryptic epitope select for viral escape

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Cryptic major histocompatibility complex class I epitopes have been detected in several pathogens, but their importance in the immune response to AIDS viruses remains unknown. Here, we show that *Mamu-B*17*+ simian immunodeficiency virus (SIV)mac239-infected rhesus macaques that spontaneously controlled viral replication consistently made strong CD8+ T lymphocyte (CD8-TL) responses against a cryptic epitope, RHLAFKCLW (cRW9). Importantly, cRW9-specific CD8-TL selected for viral variation in vivo and effectively suppressed SIV replication in vitro, suggesting that they might play a key role in the SIV-specific response. The discovery of an immunodominant CD8-TL response in elite controller macaques against a cryptic epitope suggests that the AIDS virus–specific cellular immune response is likely far more complex than is generally assumed.

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CD8+ T lymphocytes (CD8-TLs) play a critical role in control of HIV and simian immunodeficiency virus (SIV) infection. There is a strong temporal association between the onset of the initial HIV/SIV-specific CD8-TL response and the decline of peak viremia to the set point, which is maintained into the chronic phase (1). In addition, experimental in vivo depletion of CD8+ cells in SIV-infected rhesus macaques results in a dramatic rise in viremia (2, 3), including in animals that spontaneously control SIV replication (elite controllers) (4). Finally, there is a close correlation between certain MHC class I (MHC-I) alleles and control of viral replication, both in HIV-infected humans and SIV-infected macaques (5–8). This correlation implies that certain CD8-TL responses are better than others at controlling virus replication. It is, therefore, important to understand the entire AIDS virus–specific CD8-TL response, particularly in elite controllers, which might represent the rare instances of effective CD8-TL responses.

CD8-TLs recognize virus-infected cells by engaging complexes of virally derived peptides and MHC-I molecules. The obvious and best-studied source of these peptides is previously described viral proteins. The SIV genome contains nine described open reading frames (ORFs), *gag*, *pol*, *vif*, *vpx*, *vpr*, *tat*, *rev*, *nef*, and *env*, encoding 15 mature proteins. However, an understudied and potentially important source of such peptides is defective ribosomal products (DRiPs) (9). Transcription and translation are highly error-prone processes. Evidence suggests that such defective products are rapidly degraded, and the resulting peptide fragments presented at the cell surface bound to MHC-I molecules (10, 11).

Peptides derived from translation of alternate reading frames (ARFs; henceforth called cryptic epitopes) are a potentially important class of DRiPs. Immune responses directed against cryptic epitopes have been detected in several infections, including influenza virus (12), murine AIDS (13), and, importantly, HIV (14). Cardinaud et al. (14) used mice that express human MHC-I molecules to demonstrate the immunogenicity of predicted cryptic peptides. They also detected CD8-TL responses directed against these peptides in HIV-infected patients. Although these experiments elegantly demonstrated the existence of

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HIV-derived cryptic epitope-specific CD8-TLs, the importance of these responses in HIV-infected humans or SIV-infected macaques has not yet been addressed.

Previously, we demonstrated that the MHC-I allele *Mamu-B*17* was associated with control of SIVmac239 replication and was overrepresented in a cohort of elite controllers (8). Here, we show that *Mamu-B*17*$^+$ macaques mounted strong, chronic phase responses against a cryptic epitope, RHLAFKCLW (cRW9). This epitope lies in the +2 reading frame relative to the *env* ORF, within the intron between the 5′ and 3′ *rev* exons. CD8-TLs recognizing this epitope were consistently detected in *Mamu-B*17*$^+$ macaques and represented the immunodominant chronic phase response in one elite controller. cRW9-specific CD8-TLs also selected for viral variation in vivo. This mutation was synonymous in the overlapping *Env* ORF but changed the C-terminal tryptophan anchor residue, which is essential for binding of the peptide to the Mamu-B*17 molecule (15). In addition, cRW9-specific CD8-TLs effectively suppressed virus replication in vitro. Collectively, these data suggest both that translation of the ARF that encodes cRW9 is a regular event in SIV infection and that immune responses against cryptic epitopes may be important components of the total SIV-specific repertoire in some individuals.

RESULTS AND DISCUSSION

SIVmac239 encodes a cryptic epitope predicted to bind to Mamu-B*17

The majority (9 of 13 in our cohort) of Indian rhesus macaques that control replication of the highly pathogenic simian AIDS virus SIVmac239 are *Mamu-B*17*$^+$ (8). Reasoning that this association was due to effective CD8-TLs directed at Mamu-B*17–restricted epitopes, we sought to characterize the entire repertoire of SIV-specific Mamu-B*17–restricted responses. Mamu-B*17–restricted CD8-TL responses against epitopes derived from the 15 SIV proteins have been described (15). Therefore, in this study we searched for Mamu-B*17–restricted epitopes encoded by ARFs in the SIVmac239 genome. We scanned all reading frames of the SIVmac239 genome not known to encode proteins. Using epitope prediction software (16), we identified a single peptide, cRW9, predicted to tightly bind (IC_{50} < 50 nM) the Mamu-B*17 molecule. cRW9 is located in the +2 reading frame relative to the ORF encoding the envelope protein (Fig. 1). It is located in the same ORF that encodes exon 1 of the Rev protein but is downstream of the only known splice donor site and so is not predicted to be translated under "normal" biological circumstances.

CD8-TLs from SIV-infected macaques targeted cRW9

cRW9 was predicted to bind the Mamu-B*17 molecule with high enough affinity to be immunogenic, so we next used IFN-γ ELISPOT to determine if any of our MHC-I–defined SIV-infected macaques made detectable cRW9-specific immune responses. To our surprise, we found responses in several (13 of 20) *Mamu-B*17*$^+$ macaques in the chronic phase of SIV infection, including two elite controllers, animals r95071 and r98016 (Fig. 2 a). Responses just below the threshold of positivity were detected in two additional macaques. Response to the cRW9 peptide was not detected in SIV-naive *Mamu-B*17*$^+$ macaques but was detected in one of five SIV-infected *Mamu-B*17*$^-$ animals (r95003) (Fig. 2 a). This animal also made a response to another Mamu-B*17–restricted epitope, $Vif_{65\text{-}73}$HW8 (not depicted). Furthermore, virus from this animal harbored mutations in $Vif_{65\text{-}73}$HW8 consistent with escape from this response (17). These data indicate that this animal likely expresses an unknown MHC-I allele that presents epitopes also bound by Mamu-B*17. We next measured responses in four animals, including the two elite controllers, against other known SIV-derived CD8-TL epitopes to assess the relative contribution of the cRW9-specific cells to the SIV-specific immune response (Fig. 2 b). The cRW9 response comprised a substantial portion of the total SIV-specific CD8-TL response, as measured against responses

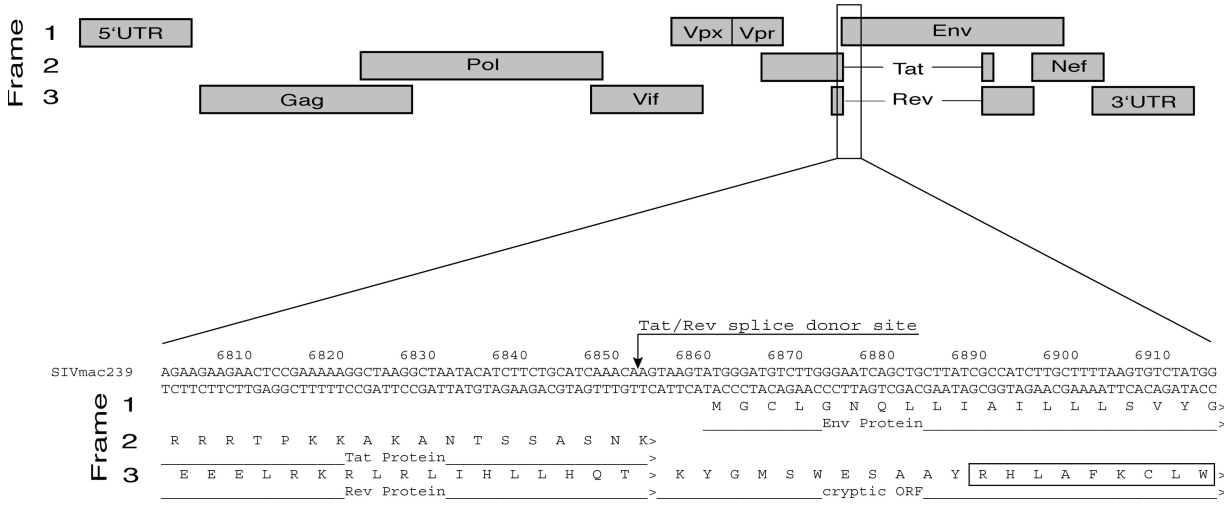

Figure 1. Location of cRW9 in the SIVmac239 genome. The cRW9 epitope (enclosed in box) is located in the same reading frame (frame 3 in this image) as exon 1 of the *rev* ORF but downstream of the only known splice donor site (indicated by an arrow).

restricted by the other MHC-I alleles expressed by these animals and known to present SIV epitopes. In animal r95071, the dominant SIV-specific response was against cRW9. Interestingly, this animal has maintained virus loads of ~1,000 viral RNA (vRNA) copies/ml plasma or less for most of its SIV infection history.

To determine the overall frequency of the cRW9-specific response, we made a tetramer of the Mamu-B*17 molecule folded with the cRW9 peptide and again tested animals r98016 and r95071 (Fig. 2 c). The response was detected in both animals but was very strong in r95071. Because animal r95071 expresses two MHC-I molecules known to bind SIVmac239 epitopes, Mamu-A*02 and Mamu-B*17, we used tetramers to measure the frequency of the cRW9 response relative to that of other responses restricted by these molecules. In agreement with the ELISPOT data, the cRW9 response was the highest frequency chronic phase CD8-TL response in this animal, comprising 41% of the total detectable SIV-directed CD8-TL response (Fig. 2 d).

Viral sequence evolution in cRW9

Because the cRW9-specific response was a consistent and often strong response, we wished to determine whether this response could select for viral variants in vivo. Viral escape from specific CD8-TL responses is a hallmark of AIDS virus infection (17, 18). If cryptic epitope-specific CD8-TLs exert appreciable selective pressure on the virus, escape variants should be selected for in vivo. Thus far, to our knowledge, viral escape from cryptic epitope-specific immune responses has not been documented. To examine this, we isolated

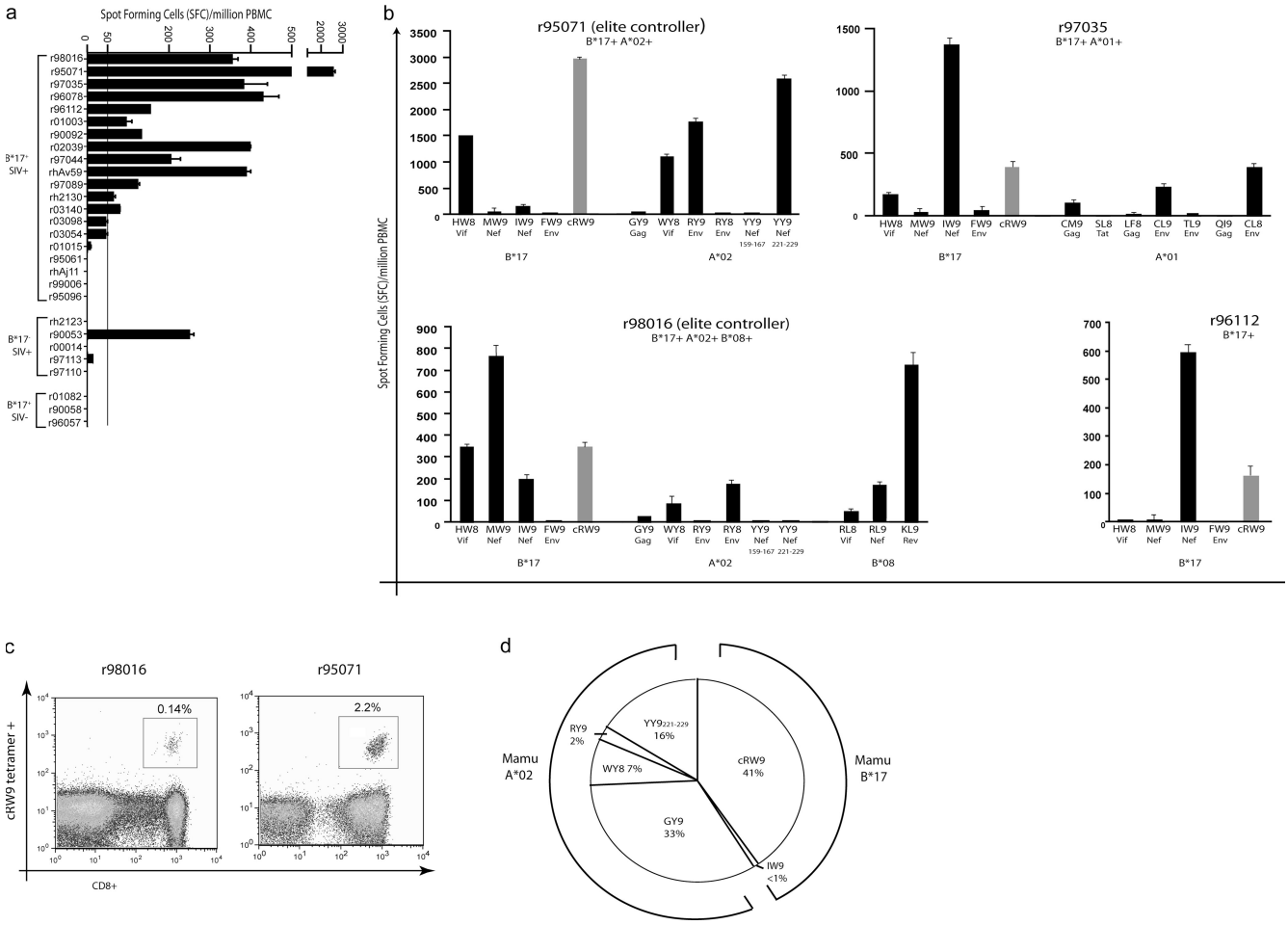

Figure 2. Magnitude of the cRW9-specific response in four animals. (a) IFN-γ ELISPOT to determine if SIV-infected macaques (Mamu-B*17$^+$ or Mamu-B*17$^-$) or SIV-naive animals make cRW9-specific responses. Responses are measured as spot-forming cells (SFC) per million PBMCs. The mean number of spots of the negative control wells was subtracted from each well. Responses were considered positive if the number of spots (per million PBMCs) was >50 (represented by a vertical bar). Error bars represent the mean ± the SE for each measurement. (b) IFN-γ ELISPOT to test four animals in the chronic phase of SIV infection for responses to known SIV epitopes restricted by MHC-I alleles expressed by each animal. For each animal, a gray bar indicates the cRW9 response, while all others are black. On the x axis are the epitopes tested and their restricting alleles. (c) Elite controllers r95071 and r98016 were also tested for response frequency using tetramers of the Mamu-B*17 molecule loaded with the cRW9 peptide. Tetramer$^+$ populations are expressed as percentages of CD3$^+$ lymphocytes. (d) Animal r95071 was also tested for responses to epitopes restricted by the MHC-I molecules Mamu A*02 and Mamu-B*17. Percentages in the pie chart represent the frequency of each response (by tetramer) relative to other measured responses.

vRNA from the plasma of SIV-infected animals and sequenced the region encoding cRW9. SIV derived from 5 of 20 (19 *Mamu-B*17*$^+$ and 1 *Mamu-B*17*$^-$ [r95003]) animals sequenced at time of death contained an identical mutation, T6913C. This mutation is synonymous in the *env* ORF but is predicted to change the C terminus residue in the cRW9 epitope from tryptophan to arginine (Fig. 3 a, wild-type RHLAFKCLW, variant RHLAFKCLR). Because a C-terminal tryptophan is an anchor residue for epitopes presented by Mamu-B*17 (15), this mutation is predicted to drastically reduce binding to the Mamu-B*17 molecule. To test this, we performed competition assays using purified Mamu-B*17 molecules. As expected, the wild-type peptide was found to be a strong Mamu-B*17 binder, with an affinity of 32 nM. Interestingly, the variant peptide bound Mamu-B*17 with an affinity of 948 nM, representing a 97% decrease in binding capacity and demonstrating that the mutation drastically impairs Mamu-B*17 binding (Fig. 3 b).

To determine if the T6913C mutation was maintained due to positive selection, we compared rates of synonymous and nonsynonymous substitutions in the ARF that encodes cRW9.

a

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          CGCCATCTTGCTTTTAAGTGTCTATGG
            A  I  L  L  L  S  V  Y  G
          ______Env Protein_____|___
          R  H  L  A  F  K  C  L  |W
          ______Cryptic ORF_____|___

Rh1937    ........................C..
Rh2129    ........................C..
R97044    ........................C..
R98015    ........................C..
R95003*   ........................C..
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b

Epitope	Sequence	IC50 (nM)	% reduction
cRW9	RHLAFKCLW	32	
mutant	RHLAFKCLR	948	97

c

Region	$d_S \pm$ S.E	$d_N \pm$ S.E	P[1]
Tat exon 1	0.6 ± 0.6	0.2 ± 0.2	NS
Rev exon 1	2.1 ± 1.4	1.5 ± 0.9	NS
Cryptic ORF (excluding cRW9)	2.5 ± 1.0	0.1 ± 0.1	NS
cRW9 epitope	0.0 ± 0.0	4.6 ± 0.5	0.001
Env (first 100 amino acids)	0.0 ± 0.0	2.4 ± 0.0	< 0.001

[1] Paired t-test (2-tailed).

Figure 3. Sequence variation in cRW9 associated with loss of binding and positive selection. (a) Nucleotide alignments of five animals sequenced at time of death reveals possible escape in the cRW9 epitope. Animal r95003 is marked with an asterisk because this animal is *Mamu-B*17*$^-$ yet made characteristic Mamu-B*17 responses (Fig. 2 a and explained in Results and Discussion). (b) Relative binding of the cRW9 and the mutant peptide to the Mamu-B*17 molecule. The tryptophan, W, to arginine, R, mutation at the C terminus of the cRW9 peptide reduces binding to the Mamu-B*17 molecule by 97%. Binding is measured as the concentration of peptide at which binding of a radioactively labeled reference peptide is reduced by 50% (IC_{50}). (c) Means (± SE) for five monkeys of the number of synonymous substitutions per 100 sites (d_S) and of the number of nonsynonymous substitutions per 100 nonsynonymous sites (d_N) in comparisons between time of death and inoculum (SIVmac239) sequences.

We analyzed ~600 bp surrounding the ARF-encoding cRW9 in 5 animals (shown in Fig. 3 a).The analyzed region encompasses most of *tat* exon 1, *rev* exon 1, and the region encoding the first 100 amino acids of the envelope protein. We found evidence for selection in the regions encoding the envelope protein ($P < 0.001$) and the cRW9 epitope ($P = 0.001$), but not in the regions encoding Tat, Rev, or in the rest of the ARF that encodes cRW9 (Fig. 3 c).

Positive selection in the cRW9 epitope, resulting in abrogation of peptide binding, indicates that SIV evolves to evade the cRW9-specific response. However, viral escape from any CD8-TL response should result in a reduction in the ability of CD8-TLs to recognize cells presenting the variant peptide. To test this possibility, we used IFN-γ ELISPOT with PBMCs from animal r95071 to determine whether cRW9-specific CD8-TLs could recognize variant peptides using a series of peptide dilutions. The variant peptide did not stimulate IFN-γ secretion, even at the highest concentration (Fig. 4 a). Complete loss of recognition even at high peptide concentrations suggests that escape is complete in this novel epitope.

Next, we wished to determine whether the T6913C mutation was associated with decay in the frequency of the cRW9-specific response in vivo. To test this, we used tetramers to stain PBMCs from animals r98015 and r97044 at various time points after SIV infection. The frequency of the cRW9-specific CD8-TL response dropped considerably after fixation of the mutation in the cRW9 epitope (Fig. 4 b). These data provide further evidence that the T6913C mutation is an effective escape mutation.

Finally, we tested whether CD8-TLs could recognize cells infected with a virus harboring the escape variant of the cRW9 epitope. We used site-directed mutagenesis to generate a virus (SIV-T6913C) with the T6913C mutation. We infected target cells derived from SIV-naive macaques that were either *Mamu-B*17*$^+$ or *Mamu-B*17*$^-$ using magnetofection (19). 20–30% of the target cells were infected after 24 h with either the wild-type SIVmac239 or the SIV-T6913C escape mutant virus (not depicted). Using intracellular cytokine staining (ICS), we found that a cRW9-specific CD8-TL line recognized the wild-type–infected cells but did not recognize the SIV-T6913C–infected cells (Fig. 4 c). Collectively, these results indicate that cRW9-specific CD8-TLs selected for escape in vivo, drastically reducing epitope recognition and without changing the primary ORF.

Collectively, our escape data provide evidence that cRW9 is encoded by a DRiP. Escape from the cRW9 response always involved changing the tryptophan anchor residue to an arginine, leading to a near complete loss of recognition without diminishing the replicative capacity of the virus (unpublished data). Interestingly, escape from all other known Mamu-B*17–restricted responses involves changes in residues other than the tryptophan anchor, and these "escape variant" peptides are often recognized in cytokine secretion assays (unpublished data). It is possible that epitopes contained in bona fide proteins cannot escape by mutating the tryptophan anchor because, in functional proteins, tryptophan often represents a residue critical for

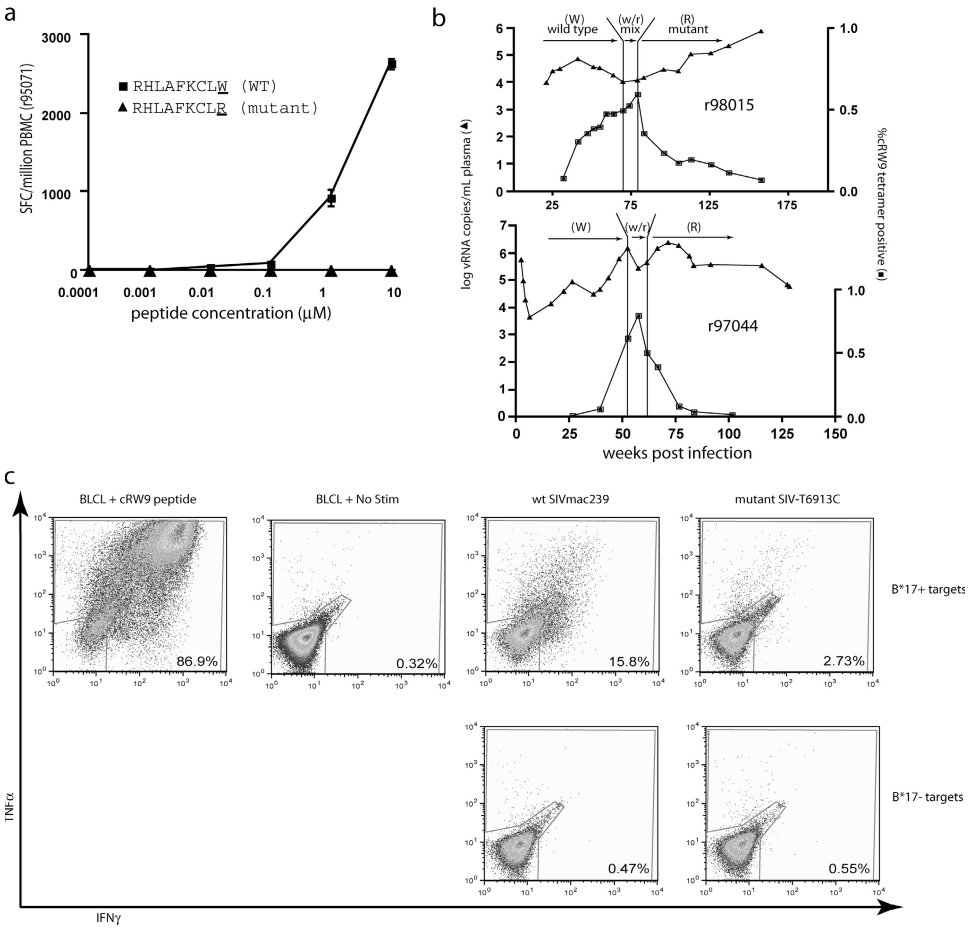

Figure 4. Viral escape in cRW9 leads to loss of recognition. (a) PBMCs from animal r95071 were tested for their ability to recognize (measured by spot-forming cells [SFC] per 10^6 total cells; y axis) the wild-type cRW9 and the mutant peptides at a titration of peptide concentrations (x axis). Error bars represent the mean ± the SE for each peptide concentration. (b) Frequency of the cRW9 response, measured as a percentage of $CD3^+$ $CD8^+$ lymphocytes (right y axis, filled box symbols), from animals r98015 and r97044 was measured by staining PBMCs from various time points with Mamu-B*17 tetramers loaded with cRW9 peptide (x axis) to determine the frequency of the cRW9-specific response before, during, and after apparent escape from the cRW9 response. The virus load of the animals is included for reference (left y axis, filled triangles). (c) cRW9-specific CD8-TLs were measured for their ability to recognize *Mamu-B*17*$^+$ target cells infected with wild-type versus mutant SIV-T6913C. Cells were infected using the magnetofection technique (reference 19). After 24 h, recognition was assayed by ICS using IFN-γ and TNF-α production. The far left panel shows the response of the cRW9-specific cell line to MHC-matched B cells pulsed with the cRW9 peptide, and the adjacent panel shows the response to the same B cells with no peptide pulse.

protein function. In contrast, the ARF-encoded tryptophan in cRW9 is under no such constraints.

cRW9-specific CD8-TLs effectively suppress SIV replication in vitro

Because high-frequency cRW9-specific responses select for viral variants in vivo, we wanted to know whether cRW9-specific CD8-TLs effectively inhibited viral replication in a functional assay (20, 21). We cultured a cell line from animal r95071 that was >99% specific for cRW9 by tetramer after 4 wk. Co-culture of this line with infected primary target cells for 7 d resulted in a >100-fold decrease in vRNA in culture supernatants, as compared with cultures with no CD8-TLs added (Fig. 5 a). This suppression of viral replication was Mamu-B*17 restricted (Fig. 5 b). Additionally, the cRW9-specific line suppressed replication of target cells infected with the escape variant SIV-T6913C virus (Fig. 5 b) but to a much smaller degree than wild type, resulting in a <20-fold decrease in vRNA (Fig. 5 a). This further supports that the T6913C mutation is an escape mutation and drastically reduces epitope recognition without changing the primary ORF.

The mechanism by which the ARF-encoding cRW9 is translated was not determined in this study. This ARF might encode a novel functional protein or a variant form of a known protein. Several splice variants of the SIV Rev protein have been described (22, 23). However, all of these variants use the same splice donor site, and none is predicted to encode the cRW9 epitope. The most parsimonious explanation for the existence of cRW9 is that it is contained within an incompletely spliced transcript that is exported from the nucleus and translated. The location of the cRW9 epitope (in the *rev* ORF, just downstream of the only known splice donor site)

indicates that it might be derived from a transcript that is exported before the removal of the intron between the 5′ and 3′ *rev* exons. The accumulation of Rev protein might lead to the export of such incompletely spliced transcripts, without regard for the ability of these mRNAs to encode functional proteins. Indeed, the Rev responsive element is located within this intron and might explain the translation of the ARF that encodes cRW9.

Our data do not address the role that cRW9-specific CD8-TLs might play in control of SIV replication in vivo. They do, however, demonstrate that cRW9-specific responses are a major component of the Mamu-B*17–restricted repertoire of SIV-specific responses. The remarkable ability of SIV to evade the cRW9-specific response without changing any functional proteins suggests that the role cRW9-specific CD8-TLs play in control of AIDS virus replication might be minimal. However, it is difficult to assess the importance of responses against a single epitope to overall control of a pathogen such as HIV or SIV. CD8-TL responses to HIV/SIV are generally directed at a variety of viral gene products, and in this case, a viral ARF. In addition, viral escape from any one CD8-TL response, even an immunodominant one, can be followed by an altering of the CD8-TL immunodominance hierarchy with little or no change in virus load.

Further experiments will be required to determine the mechanism and timing of cRW9 presentation. However, our data are important for several reasons. This is the first study to indicate that CD8-TL responses directed at cryptic epitopes might play a role in controlling SIV or HIV replication that is equal in all respects to responses against bona fide proteins. We show that cRW9-specific responses are capable of driving viral evolution in vivo and controlling virus replication in vitro. These results are also of interest for vaccine design. Importantly, vaccines designed to elicit virus-specific CD8-TLs can induce such responses (24). There is no consensus on whether or not cryptic epitopes should be included in vaccine regimens, and our data do not directly address this issue. However, if escape-without-consequence is a general feature of cryptic epitopes, they are likely to be poor vaccine immunogens. Most importantly, these data suggest that a complete knowledge of the total AIDS virus–specific cellular immune response will not be possible without accounting for such responses.

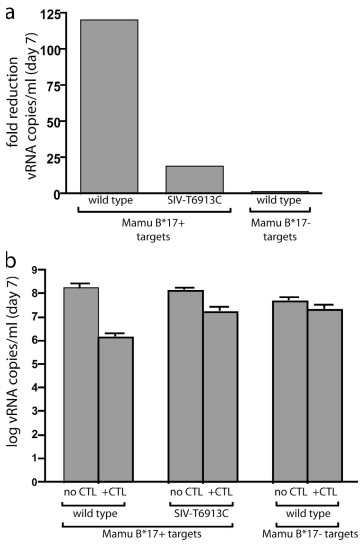

Figure 5. cRW9-specific CD8-TLs effectively suppress SIV replication in Mamu-B*17$^+$ target cells. Quantitative PCR of vRNA on the final day (day 7) of the assay. The ability of a cRW9-specific cell line derived from animal r95071 to suppress wild-type or SIV-T6913C replication in target cells that were either Mamu-B*17$^+$ or Mamu-B*17$^-$ was measured. (a) The data is represented as fold-reduction between the wells with no CD8-TLs added (no CTL) and those with an E/T ratio of 1:20 (+CTL). (b) The same data as in panel a but represented as the total vRNA copies/ml supernatant on day 7 of the assay. Error bars represent the mean ± the SE for each measurement.

MATERIALS AND METHODS

Animals. All animals used in this study were infected with clonal SIVmac239 as part of previous studies, except for animal r97035 and r98015, which were infected with clonal SIVmac239 with escape mutations in the Mamu-A*01 epitopes $Gag_{181-189}$CM9 and Tat_{28-35}SL8 and the Mamu-B*17 epitope $Nef_{165-173}$IW9 as part of a previous study (25). All animals were housed at the Wisconsin National Primate Research Center (WNPRC) and cared for under the guidelines of the National Research Council and the University of Wisconsin Institutional Animal Care and Use Committee (WIACUC). Procedures involving animals were approved by the WIACUC.

Cryptic epitope prediction and MHC binding studies. The entire SIVmac239 genome was conceptually translated into all three potential reading frames using MacVector 8 trial version. All reading frames that encode known viral proteins were excluded from analysis. Remaining sequences were entered into Web-based software (www.mamu.liai.org; reference 16) to predict cryptic epitopes predicted to bind the Mamu-B*17 molecule with $IC_{50} < 50$ nM. Quantitative assays to measure the binding affinity of peptides to purified Mamu-B*17 molecules are based on the inhibition of binding of a radiolabeled standard peptide and were performed as described previously (15).

ELISPOT. IFN-γ ELISPOT assays were conducted as described previously (26). Using precoated ELISpot^{plus} kits (Mabtech), 10^5 fresh or frozen PBMCs were added per well along with 10 μM peptide (or in serial 10-fold dilutions of 10 μM to 10 pM). Minimal optimal peptides for cRW9 and other known epitopes restricted by Mamu-B*17 (15), Mamu-B*08 (unpublished data), Mamu-A*01 (27), or Mamu-A*02 (28) were used. All samples were run in duplicate and positive (concanavalin A) and negative (no peptide) controls were included in each plate.

MHC-I tetramer production and response testing. Mamu-B*17 tetramers were produced as described previously (29), with minor modifications. Tetramer stains were performed as described previously (26) using 5×10^5 fresh PBMCs and 0.5 μg of tetrameric complexes loaded with synthetic peptides representing epitopes described in Results and Discussion.

vRNA sequencing. vRNA sequencing was performed as described previously (17) but using primers designed to amplify the region surrounding the cRW9 epitope (6511-F, TGATCCTCGCTTGCTAACTG; 7348-R, CTCTTGTTCCAAGCCTGTGC).

Mutant virus production. Escape variant SIV (SIV-T6913C) was created using site-directed mutagenesis with the following primers: forward: T6913C-F GCTTTTAAGTGTCTACGGGATCTATTGTAC; reverse: T6913C-R GTACAATAGATCCCGTAGACACTTAAAAGC. Mutagenesis was performed using the Quik Change kit (Stratagene) according to the manufacturer's instructions. Viral plasmid ligation and transfection and viral growth and characterization were performed as described previously (25).

Virus recognition. Target cells ($CD8^-$) were isolated from SIV-naive animals that were either Mamu-$B*17^+$ or Mamu-$B*17^-$ by PCR sequence-specific priming. Cells were infected with SIV (either wild-type SIVmac239 or mutant SIV-T6913C) using the magnetofection technique, and recognition was measured by ICS after 24 h as described previously (19).

Virus suppression. Viral suppression assays were performed similarly as described previously (21) but with modifications. In brief, target cells were generated from PBMC SIV-naive macaques using anti-CD8 nonhuman primate microbeads on MACS LS columns (Miltenyi Biotec) according to the manufacturer's protocol. The $CD8^-$ fraction was stimulated with 5 μg/ml PHA (Sigma-Aldrich) for 24 h, washed, and incubated in R15-50 (RPMI media/15% fetal calf serum/1% L-glutamine plus 50 U/ml interleukin 2; National Institutes of Health [NIH] AIDS Reference and Reagent Program) for an additional day. For use in the assay, target cells were resuspended at 10 million/ml and incubated for 4 h with 300,000 copies of vRNA per million target cells of the clonal SIV stocks. Cells were then washed twice, and 250,000 were added per well in a 48-well plate. cRW9-specific CD8-TLs were added at a ratio of 1:20, and the cells were cultured in 1 ml R15-50 for 7 d. 0.5 ml of culture media was removed on day 7 for quantitation of vRNA. vRNA was isolated from supernatants using an Magattract vRNA kit on a Biorobot M48 workstation (QIAGEN) and quantitated as described previously (26).

Statistical analysis of selection. Numbers of synonymous substitutions per synonymous site (d_S) and numbers of nonsynonymous substitutions per nonsynonymous site (d_N) were estimated using the method of Nei and Gojobori (30), and the results were analyzed as described previously (17, 31).

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