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The Antiviral Efficacy of Simian Immunodeficiency Virus-Specific CD8$^+$ T Cells Is Unrelated to Epitope Specificity and Is Abrogated by Viral Escape$^\nabla$

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CD8$^+$ T lymphocytes appear to play a role in controlling human immunodeficiency virus (HIV) replication, yet routine immunological assays do not measure the antiviral efficacy of these cells. Furthermore, it has been suggested that CD8$^+$ T cells that recognize epitopes derived from proteins expressed early in the viral replication cycle can be highly efficient. We used a functional in vitro assay to assess the abilities of different epitope-specific CD8$^+$ T-cell lines to control simian immunodeficiency virus (SIV) replication. We compared the antiviral efficacies of 26 epitope-specific CD8$^+$ T-cell lines directed against seven SIV epitopes in Tat, Nef, Gag, Env, and Vif that were restricted by either Mamu-A*01 or Mamu-A*02. Suppression of SIV replication varied depending on the epitope specificities of the CD8$^+$ T cells and was unrelated to whether the targeted epitope was derived from an early or late viral protein. Tat$_{28–35}$SL8- and Gag$_{181–189}$CM9-specific CD8$^+$ T-cell lines were consistently superior at suppressing viral replication compared to the other five SIV-specific CD8$^+$ T-cell lines. We also investigated the impact of viral escape on antiviral efficacy by determining if Tat$_{28–35}$SL8- and Gag$_{181–189}$CM9-specific CD8$^+$ T-cell lines could suppress the replication of an escaped virus. Viral escape abrogated the abilities of Tat$_{28–35}$SL8- and Gag$_{181–189}$CM9-specific CD8$^+$ T cells to control viral replication. However, gamma interferon (IFN-γ) enzyme-linked immunospot and IFN-γ/tumor necrosis factor alpha intracellular-cytokine-staining assays detected cross-reactive immune responses against the Gag escape variant. Understanding antiviral efficacy and epitope variability, therefore, will be important in selecting candidate epitopes for an HIV vaccine.

CD8$^+$ T lymphocytes are an important component of the cellular immune response and play a role in controlling human immunodeficiency virus/simian immunodeficiency virus (HIV/SIV) replication. The depletion of circulating CD8$^+$ lymphocytes in SIV-infected macaques results in an increase in plasma viral concentrations (25, 42, 59). CD8$^+$ T lymphocytes have been shown to exert selective pressure on viral sequences in vivo; immune escape variants are observed in both the acute (3, 7, 50) and chronic (6, 15, 23, 54) phases of HIV/SIV infection. Furthermore, recent studies suggest that escape from CD8$^+$ T-cell responses exacts a cost in viral fitness, since transmitted escape variants are not maintained in the absence of the selecting major histocompatibility complex (MHC) class I allele (2, 17, 33).

Several studies have shown associations between certain MHC class I alleles and slow or rapid HIV/SIV disease progression, implying that these alleles restrict CD8$^+$ T-cell responses of varying effectiveness (8, 23, 26, 47, 48, 52, 61, 73, 74). However, it is still unknown which of the many HIV-specific CD8$^+$ T lymphocytes actually contribute to the control of viral replication. Despite technological advances and new methodologies to detect and enumerate CD8$^+$ T-lymphocyte responses against HIV/SIV, most of the current cellular assays do not actually measure antiviral efficacy, the ability to suppress viral replication (70).

Initial reports using such functional assays demonstrated that CD8$^+$ cells (63) and virus-specific cytotoxic T lymphocytes (71) inhibited immunodeficiency virus replication in vitro. Dendritic cells pulsed with inactivated autologous virus initiated the expansion of virus-specific CD8$^+$ T cells that controlled HIV replication (39). Based on these viral-replication inhibition assays, it has been suggested that CD8$^+$ T lymphocytes directed against epitopes derived from early-expressed proteins, particularly Nef and Rev, are more efficacious than CD8$^+$ T lymphocytes directed against epitopes in late-expressed viral proteins (1, 65, 66, 72). However, a recent investigation demonstrated that Pol-specific CD8$^+$ T cells were also effective at suppressing HIV replication (62). While most data suggest that differences exist in the antiviral efficacies of CD8$^+$ T-cell populations, current HIV studies are limited to a small number of well-defined clones.

Initially, we studied two immunodominant epitopes, Tat$_{28–35}$ SL8 and Gag$_{181–189}$CM9, which are bound by the commonly studied Indian rhesus macaque MHC class I molecule Mamu-

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A*01 (3–6, 9, 17, 18, 32, 36, 45, 50–53, 60, 69). $CD8^+$ T-cell lines directed against the $Tat_{28–35}$SL8 epitope were consistently more effective at suppressing $SIV_{mac}239$ replication than $CD8^+$ T-cell lines directed against the $Gag_{181–189}$CM9 epitope in our functional in vitro assay (36). This finding supported the notion that $CD8^+$ T cells directed against early proteins are more efficacious than their counterparts directed against late proteins (1, 65, 66, 72).

In this study, we have conducted a broadened investigation to identify additional SIV-specific $CD8^+$ T-cell responses with strong antiviral activity. Furthermore, we have directly compared the virus-suppressive abilities of $CD8^+$ T cells that recognize early proteins to those of cells that recognize late-expressed proteins. Using the previously developed viral-suppression assay (VSA), we compared the abilities of 26 SIV-specific $CD8^+$ T-cell populations directed against seven epitopes in five SIV proteins, Tat, Nef, Gag, Env, and Vif, to suppress viral replication. We found that suppression of SIV replication varies depending on the epitope specificities of the $CD8^+$ T-cell lines and cannot be generalized by protein location or time of protein expression. The $Tat_{28–35}$SL8- and $Gag_{181–189}$CM9-specific $CD8^+$ T-cell lines were the most effective at suppressing viral replication. Interestingly, low plasma viremia in macaques used to generate the SIV-specific $CD8^+$ T-cell lines was not predictive of effective viral suppression.

We also investigated the effect viral escape had on the abilities of $Tat_{28–35}$SL8- and $Gag_{181–189}$CM9-specific $CD8^+$ T cells to suppress SIV replication. Recent studies of HIV have observed the ability of antigen-specific $CD8^+$ T lymphocytes to recognize variant peptides by using gamma interferon (IFN-γ) production as the readout, providing encouragement that the immune system may be able to cope with viral escape (22, 24, 46, 64). Similarly, we detected cytokine responses to a variant Gag peptide in both IFN-γ enzyme-linked immunospot (ELISPOT) and IFN-γ/tumor necrosis factor alpha (TNF-α) intracellular-cytokine-staining (ICS) assays. However, the cross-reactive cytokine response to the variant Gag peptide did not predict the abilities of $Gag_{181–189}$CM9-specific $CD8^+$ T-cell lines to suppress viral replication of an SIV escape variant. This illustrates the profound effect of viral escape on abrogating the ability of $CD8^+$ T lymphocytes to suppress viral replication. Researchers have also claimed the detection of cross-clade HIV-specific $CD8^+$ T-cell responses on the basis of cytokine production assays alone (13, 21, 68). Our results suggest that such findings may be misleading unless confirmed by functional assays.

MATERIALS AND METHODS

Animals and viruses. Indian rhesus macaques (*Macaca mulatta*) were identified as *Mamu-A*01*$^+$ and/or *Mamu-A*02*$^+$ by sequence-specific primer DNA amplification (PCR-SSP), as previously described (32, 37). SIV-specific $CD8^+$ T-cell lines were derived from *Mamu-A*01*$^+$ and/or *Mamu-A*02*$^+$ macaques infected with the molecularly cloned virus $SIV_{mac}239$, GenBank accession no. M33262 (28). Naïve macaques were used as a source of peripheral blood mononuclear cells (PBMC) for in vitro SIV infections.

For in vitro SIV infections, CD8-depleted PBMC were infected with $SIV_{mac}239$ (28) or an engineered escape variant virus based on $SIV_{mac}239$ (17). This variant virus contained point mutations in two known Mamu-A*01 epitopes, $Tat_{28–35}$SL8 and $Gag_{181–189}$CM9, and contained the previously identified compensatory mutations required for replication of the Gag mutant (18, 53).

SIV-infected animals were maintained at the National Primate Research Center (University of Wisconsin—Madison) and cared for according to the regulations and guidelines of the University of Wisconsin Institutional Animal Care and Use Committee.

Quantification of vRNA in plasma. Viral RNA (vRNA) was isolated from EDTA-anticoagulated plasma and detected in quantitative reverse transcription-PCR (QRT-PCR) using a modification of a published protocol with a one-step QRT-PCR kit (Invitrogen, Carlsbad, CA) (12). The final reaction mixtures (20-μl total volume) contained 0.2 mM of each deoxynucleoside triphosphate, 5 mM $MgSO_4$, 0.015% bovine serum albumin (Sigma, St. Louis, MO), 150 nanograms random hexamer primers (Promega, Madison, WI), 0.8 μl SuperScript III reverse transcriptase and Platinum *Taq* DNA polymerase in a single enzyme mix, 600 nM of each amplification primer—forward (SIV1552), 5′-GTCTGCGTCATC TGGTGCATTC-3′; reverse (SIV1635), 5′-CACTAGCTGTCTCTGCACTATGT GTTTTG-3′—and 100 nM probe 5′-6-carboxyfluorescein-CTTCCTCAGTGTGT TTCACTTTCTCTTCTGCG-6-carboxytetramethylrhodamine-3′. Temperature cycling was performed on the LightCycler 1.2 (Roche, Indianapolis, IN) with slightly altered parameters. The reverse transcriptase reaction was performed at 37°C for 15 min and then at 50°C for 30 min. An activation temperature of 95°C for 2 min was followed by 50 amplification cycles of 95°C for 15 seconds and 62°C for 1 min with ramp times set to 3° per second. Data were acquired and analyzed using LightCycler 4.0 software.

Each QRT-PCR assay was run with a set of internal standards made up of a dilution series of synthetic transcript containing a fragment of the SIV *gag* gene. The theoretical concentrations of these standards range from 15 million copy eq to 3 copy eq per sample. Copy numbers for samples were determined by interpolation onto the standard curve using the LightCycler software version 4.0.

The interassay variation in observed crossing points for each point on the standard curve is less than 2%. Each assay is also run with an internal plasma standard to control for both vRNA isolation and detection in QRT-PCR. The interassay variability in the crossing point for this standard is 2.8%, and its observed concentration (average, 179,000 copy eq/ml) varies by 48%. Under normal assay conditions, the quantification threshold for this assay is 30 vRNA copy eq/ml.

Generation and maintenance of $CD8^+$ T-cell lines. Peptide-specific $CD8^+$ T-cell lines were generated using previously described methods (67). Briefly, PBMC were separated from whole heparin- or EDTA-treated blood by Ficoll-Paque PLUS (GE Health Sciences, Piscataway, NJ) density centrifugation. $CD8^+$ cells were separated from freshly isolated PBMC using the CD8 nonhuman primate microbead kit (Miltenyi Biotec, Auburn, CA) and the Miltenyi Biotec AutoMACS magnetic cell separation unit according to the manufacturer's protocol. Fresh, unseparated PBMC were also used to start $CD8^+$ T-cell lines. Autologous B-lymphoblastoid cell lines (BLCLs) were used as antigen-presenting cells. BLCLs were pulsed with 1 μM of the relevant SIV-specific peptide for 1 to 2 h at 37°C, washed twice, and irradiated (9,000 rads). BLCLs were then mixed with either whole or CD8-enriched PBMC at a ratio of 1:1 in RPMI 1640 (Cambrex, Walkersville, MD) supplemented with L-glutamine (Mediatech, Herndon, VA), antibiotic-antimycotic solution (Mediatech), and 15% fetal bovine serum (R15; HyClone, Logan, UT) with 10 ng/ml of recombinant human interleukin-7 (Sigma-Aldrich, St. Louis, MO) and incubated for 48 h. The cells were cultured with R15 containing 100 units of interleukin-2/ml (NIH AIDS Research and Reference Reagent Program, Germantown, MD) every 3 to 5 days thereafter. The $CD8^+$ T-cell lines were restimulated using peptide-pulsed, irradiated BLCL every 7 to 14 days. $CD8^+$ T-cell lines were routinely tested for epitope specificity after >14 days in culture by either ICS or MHC class I tetramer assays. MHC class I tetramer staining was performed as previously described (38).

VSA. We performed in vitro VSAs as previously described (36). Briefly, the target population consisted of CD8-depleted, phytohemagglutinin-stimulated lymphocytes that were infected with $SIV_{mac}239$ (28), or an engineered $CD8^+$ T-cell escape variant virus based on $SIV_{mac}239$ (17), at a multiplicity of infection of 5×10^{-5}. We used in vitro-stimulated epitope-specific $CD8^+$ T-cell lines that were sorted or grown to a high specificity (>91%) as effector cells. All $CD8^+$ T-cell lines used were less than 6 months old, and more than half of the data were derived from primary cell lines that underwent in vitro stimulation for approximately 2 months. Under these conditions, the age of the $CD8^+$ T-cell line did not appear to impact its ability to suppress SIV replication (data not shown).

We added 5.0×10^5 CD8-depleted, phytohemagglutinin-stimulated lymphocytes (targets) to each well of a 24-well plate. The effector cells ($CD8^+$ T-cell lines) were added to wells at effector-to-target (E:T) ratios of 1:10 and 1:20. These E:T ratios reflect the maximum possible number of potentially infectible target cells. The final volume of cell culture medium was 2 ml and contained 50 U/ml of interleukin-2 (NIH AIDS Research and Reference Reagent Program, Germantown, MD). The cocultures were maintained for 8 days. Every 2 days, 0.5 ml supernatant was collected and replaced with fresh medium to determine

vRNA concentrations. At the end of the assay (day 8), intracellular Gag p27 staining was performed on the coculture to measure $SIV_{mac}239$ infection, as previously described (36).

We cocultured MHC-mismatched effector and target cells as controls in each assay (36). Using MHC-mismatched effector and target cells, we observed negligible reduction in SIV replication compared to wells that contained only infected target cells (data not shown).

ICS assay. SIV-specific $CD8^+$ T-cell lines were used in TNF-α and IFN-γ ICS assays as previously described (67). Briefly, each test contained 2×10^5 $CD8^+$ T cells and 0.5×10^5 to 1×10^5 autologous BLCLs. As a positive control, phorbol myristate acetate (1 μg/ml) with ionomycin (2 μg/ml; Sigma-Aldrich) was used. Individual Mamu-A*01- or Mamu-A*02-restricted minimal optimal peptides were used at a concentration of 5 μM or in serial 10-fold dilutions from 5 μM to 5 pM. Approximately 2×10^5 lymphocyte-gated events were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software 6.4.1 (TreeStar). All values were normalized by subtracting the background level staining (negative control of $CD8^+$ T-cell lines in media without stimulation).

IFN-γ ELISPOT assay. PBMC were separated from whole heparin- or EDTA-treated blood by Ficoll-Paque PLUS (GE Health Sciences) density centrifugation. The PBMC were used directly in precoated $ELISpot^{PLUS}$ kits (MABTECH Inc., Mariemont, OH) for the detection of monkey IFN-γ according to the manufacturer's protocols. Briefly, 1.0×10^5 PBMC were used per well and incubated for 14 to 18 h at 37°C in 5% CO_2. As a positive control, 5 μg/ml of concanavalin A (Sigma Chemical, St. Louis, MO) was added to the cells, and a negative control of no peptide was also included on each plate. Peptides were used at 10 μM or in serial 10-fold dilutions from 10 μM to 10 pM. All tests were performed in triplicate.

Wells were imaged with an AID ELISPOT reader (AID, Strassberg, Germany), counted with AID EliSpot Reader version 3.2.3, and analyzed as previously described (38). Spots were counted by an automated system with set parameters for size, intensity, and gradient. Background (the mean of wells without peptide stimulation) levels were subtracted from each well on the plate. A response was considered positive if the mean number of spot-forming cells (SFC) from triplicate sample wells exceeded background plus 2 standard deviations.

Assay results are shown as SFC per 1×10^6 cells. Responses of <50 SFC per 1×10^6 cells were not considered positive. Wells containing concanavalin A (positive control) were always greater than 1,000 SFC per 1×10^6 PBMC.

RESULTS

Characteristics of $CD8^+$ T-cell populations against seven $CD8^+$ T-cell epitopes in the viral-suppression assay. Previously, we developed an in vitro VSA to examine the antiviral efficacies of $CD8^+$ T-cell populations that recognized two immunodominant Mamu-A*01-restricted epitopes, $Tat_{28–35}$SL8 and $Gag_{181–189}$CM9 (36). We found that the $Tat_{28–35}$SL8-specific $CD8^+$ T-cell response was highly effective in suppressing SIV replication compared to $CD8^+$ T cells directed against the $Gag_{181–189}$CM9 epitope. This supported the previously suggested idea that $CD8^+$ T lymphocytes directed against early proteins are better at controlling viral replication than $CD8^+$ T lymphocytes directed against proteins expressed later in the viral replication cycle (1, 65, 66, 72). We sought to identify additional $CD8^+$ T-cell populations capable of effective virus suppression. *Mamu-A*01*$^+$ and *Mamu-A*02*$^+$ macaques are routinely studied in vaccine and pathogenesis experiments due to the high frequency of these alleles, the knowledge of several well-defined $CD8^+$ T-cell epitopes, and the availability of MHC class I tetrameric reagents (3–6, 9, 17, 18, 32, 36, 38, 45, 49, 51–53, 58, 60, 69).

We selected seven common SIV-specific $CD8^+$ T-cell epitopes restricted by Mamu-A*01 and Mamu-A*02 for our investigation (Table 1). These epitopes are located within two early-expressed SIV proteins (Tat and Nef) and three late-expressed viral proteins (Gag, Env, and Vif) (30, 31, 55, 57). Previous investigations detected viral sequence variation in all of these $CD8^+$ T-cell epitopes (3, 5, 6, 38, 45, 49–51, 67). The timing of viral escape ranged from 4 weeks postinfection, in the cases of the Tat and Nef epitopes, to approximately 1 year postinfection for selected Gag and Vif epitopes (Table 1).

TABLE 1. Characteristics of epitope-specific $CD8^+$ T cells studied in the VSA

Protein	Amino acid positions	Short name	Sequence	MHC class I restriction	Earliest detected viral variation (wk)
Tat	28–35	SL8	STPESANL	Mamu-A*01	4
Vif	97–104	WY8	WTDVTPNY	Mamu-A*02	57
Nef	159–167	YY9	YTSGPGIRY	Mamu-A*02	4
Gag	71–79	GY9	GSENLKSLY	Mamu-A*02	59
Gag	181–189	CM9	CTPYDINQM	Mamu-A*01	18
Env	788–795	RY8	RTLLSRVY	Mamu-A*02	32
Env	620–628	TL9	TVPWPNASL	Mamu-A*01	14

As a marker for disease progression status, SIV viral loads (vRNA copies/ml of plasma) at the time of $CD8^+$ T-cell line generation are indicated in Table 2 for each macaque. We generated $CD8^+$ T-cell lines from at least two animals for each of the seven SIV-specific $CD8^+$ T-cell epitopes (Table 2). With the exception of the $Env_{620–628}$TL9 epitope, each epitope-specific $CD8^+$ T-cell line was derived from at least one elite-controller (EC) macaque, an animal maintaining chronic-phase SIV viremia below 1,000 vRNA copies/ml (73). Some previously published data on $Tat_{28–35}$SL8- and $Gag_{181–189}$CM9-specific $CD8^+$ T-cell viral suppression was incorporated for the disease progression comparison (36).

Effective suppression of viral replication by two SIV-specific $CD8^+$ T-cell populations. Previous studies showed differences in antiviral efficacy between HIV-specific $CD8^+$ T-cell clones directed against early proteins versus late proteins (1, 65, 66, 72). Therefore, we hypothesized that $CD8^+$ T cells directed against SIV epitopes in early proteins might be more effective at suppressing viral replication than those directed against epitopes in late proteins. We generated $CD8^+$ T-cell lines that recognized two early-protein epitopes ($Tat_{28–35}$SL8 and $Nef_{159–167}$YY9) and five late-protein epitopes ($Gag_{181–189}$CM9, $Gag_{71–79}$GY9, $Env_{620–628}$TL9, $Env_{788–795}$RY8, and $Vif_{97–104}$WY8). Epitopes within Gag, Nef, and Env were of particular interest due to the inclusion of the genes in recent $CD8^+$ T-cell-based vaccines (4–6, 9, 19, 34, 41, 43, 45, 60, 69).

Our analysis included seven SIV epitopes restricted by Mamu-A*01 or Mamu-A*02 (Table 1). $Tat_{28–35}$SL8-specific $CD8^+$ T-cell populations were effective at viral suppression (Fig. 1 and Table 2), as we have described before (36). $Tat_{28–35}$SL8-specific $CD8^+$ T-cell lines from four of the five animals tested reduced viral production and Gag $p27^+$ target cells by >55% (Fig. 1). $CD8^+$ T cells directed against $Gag_{181–189}$CM9 demonstrated the second-highest levels of viral suppression, in the range of 32 to 90% reduction. $Env_{620–628}$TL9-specific $CD8^+$ T cells did not suppress viral replication. Moreover, none of the four Mamu-A*02-restricted $CD8^+$ T-cell responses consistently suppressed viral replication >50% in the VSA. These included four $CD8^+$ T-cell lines directed against $Nef_{159–167}$YY9, although Nef is an early SIV protein (Fig. 1). Effective suppression was not simply due to a higher frequency of epitope-specific $CD8^+$ T cells in the coculture, as the frequencies were typically comparable when tested at the end of each assay (data not shown).

TABLE 2. Disease statuses of SIV-infected macaques used to generate CD8+ T-cell lines and representative antiviral efficacies of the CD8+ T-cell lines on day 8 of the VSA at an E:T ratio of 1:10

Animal	SIV-specific CD8+ T-cell line	Parameters near time of CD8+ T-cell line generation		Percent reduction in SIV:	
		No. of wk infected	Viral load (no. of vRNA copies/ml)	vRNA copies/ml	Gag p27+ cells
95061[a]	Gag 181–189 CM9	253	<50	83	76
95061[a]	Tat 28–35 SL8	290	<50	77	55
95061[a]	Env 788–795 RY8	288	<50	61	0
95061[a]	Nef 159–167 YY9	288	<50	1	0
95061[a]	Gag 71–79 GY9	266	109	72	24
98014[a]	Nef 159–167 YY9	96	<50	0	0
98016[a]	Vif 97–104 WY8	111	<50	2	2
98016[a]	Gag 71–79 GY9	96	323	7	0
98016[a]	Nef 159–167 YY9	96	323	0	0
98016[a]	Env 788–795 RY8	136	353	0	0
2125	Tat 28–35 SL8	42	158,000	99	99
2125	Gag 181–189 CM9	42	158,000	90	84
2125	Env 622–630 TL9	42	158,000	47	0
2128	Tat 28–35 SL8	18	190,000	98	98
2128	Env 788–795 RY8	47	457,000	28	14
2128	Nef 159–167 YY9	47	457,000	10	0
2128	Gag 71–79 GY9	71	461,000	52	44
01008	Env 788–795 RY8	71	40,400	0	0
01008	Vif 97–104 WY8	71	40,400	0	0
01008	Env 622–630 TL9	53	89,300	0	0
97110	Gag 181–189 CM9	39	65,100	45	32
97110	Env 788–795 RY8	32	126,000	0	0
97110	Tat 28–35 SL8	16	569,000	38	0
01034	Env 622–630 TL9	54	837,000	44	14
97044	Env 788–795 RY8	66	1,610,000	64	28
2095	Tat 28–35 SL8	172	2,580,000	98	90

[a] Elite-controller macaque (maintaining chronic-phase viral load of <1,000 viral RNA copies/ml).

Of the five CD8+ T-cell populations directed against late-expressed proteins, effector cells recognizing $Gag_{181–189}$CM9 displayed the highest levels of viral suppression (Fig. 1 and Table 2). Interestingly, the CD8+ T-cell response directed against $Gag_{71–79}$GY9 was not as effective as the $Gag_{181–189}$CM9-specific CD8+ T-cell population. These results suggest that antiviral efficacy may be independent of the viral protein from which the epitope is derived. Rather, antiviral efficacy should be eval-

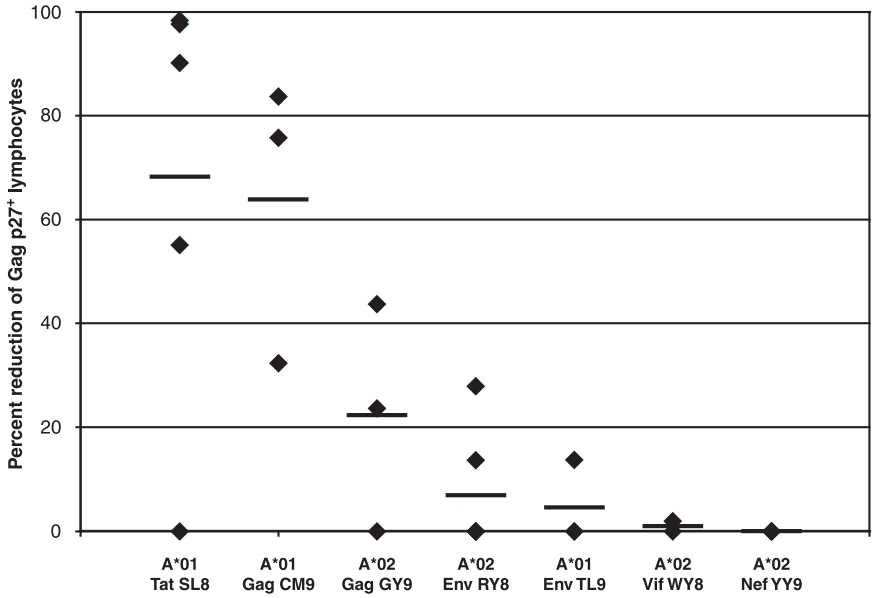

FIG. 1. Percent reduction of Gag p27+ target cells following an 8-day coculture with SIV-specific CD8+ T-cell lines at an E:T ratio of 1:10. Twenty-six epitope-specific CD8+ T-cell lines directed against seven SIV epitopes ($Tat_{28–35}$SL8, $Gag_{181–189}$CM9, $Gag_{71–79}$GY9, $Env_{788–795}$RY8, $Env_{620–628}$TL9, $Vif_{97–104}$WY8, and $Nef_{159–167}$YY9) were used in the VSA. These CD8+ T-cell lines were derived from 10 SIV-infected Indian rhesus macaques. The black bars indicate the average reduction for each SIV-specific CD8+ T-cell response. Exact values are listed in Table 2.

A) Binding (IC_{50}) of wildtype and variant peptides to Mamu-A*01

$Gag_{181-189}$CM9	CTPYDINQM	22nM	Tat_{28-35}SL8	STPESANL	43nM
escape variant	CAPYDINQM	354nM	escape variant	PTPESANQ	25,535nM

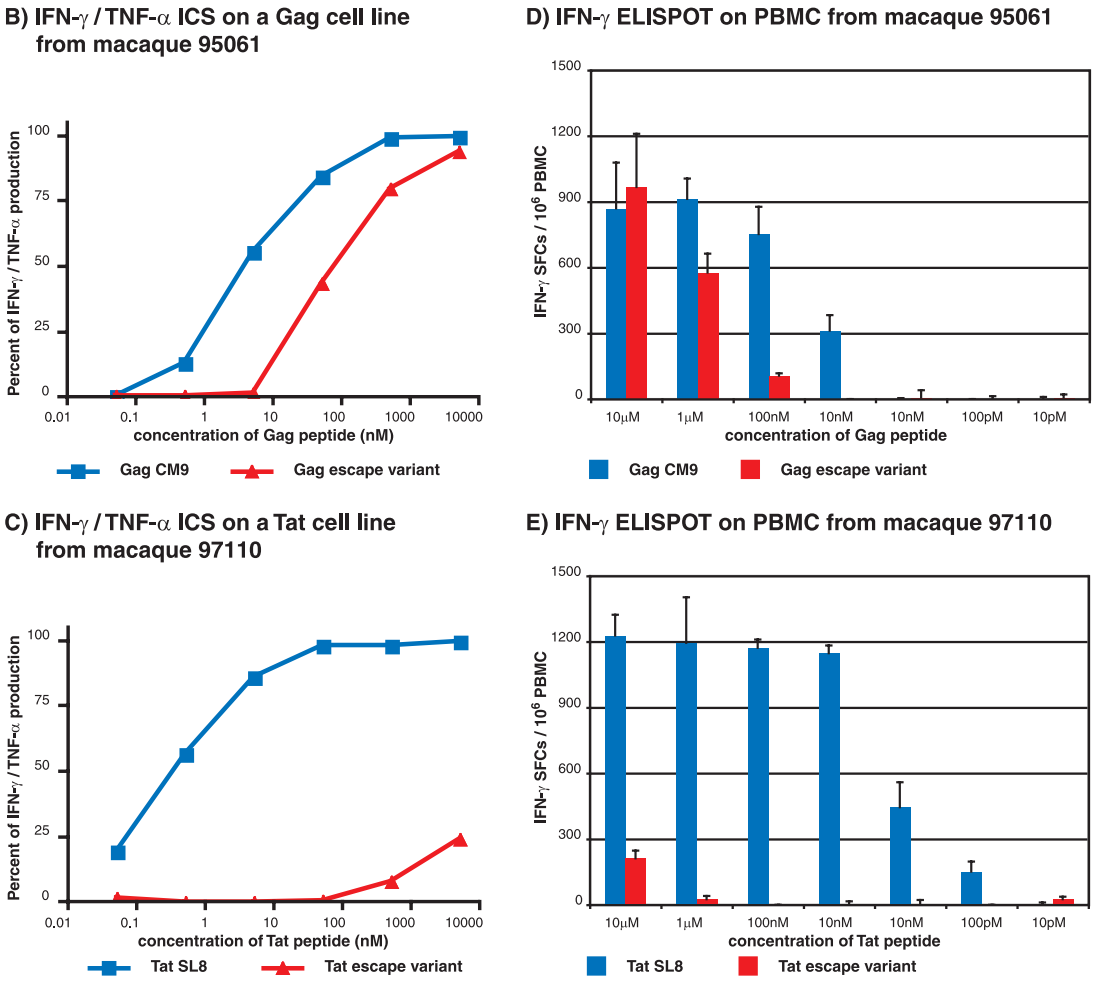

FIG. 2. Recognition of wild-type and mutant epitopes in Gag and Tat as measured by IFN-γ/TNF-α ICS and IFN-γ ELISPOT assays. (A) Binding (50% inhibitory concentrations [IC_{50}]) of the wild-type and variant peptides to Mamu-A*01 from a previous study (17). (B and C) IFN-γ/TNF-α ICS assay results for recognition of wild-type and escape variant peptides using $Gag_{181-189}$CM9- (B) and Tat_{28-35}SL8-specific (C) $CD8^+$ T-cell lines derived from macaques 95061 and 97110, respectively. The data were normalized against the cytokine production when the highest wild-type peptide concentration was used. This value was considered the maximal cytokine response and was labeled as 100%. (D and E) Ex vivo IFN-γ ELISPOT assay results for recognition of wild-type and escape variant peptides using freshly isolated PBMC from macaque 95061 to test Gag reactivity (D) and from macaque 97110 to test Tat reactivity (E). Mean values from triplicate wells were calculated for each assay. The background, the mean of wells without peptide, was subtracted from each well. Mean responses of <50 SFC per 1×10^6 cells were not considered positive. The error bars in panels D and E indicate standard deviations for the triplicate wells.

uated for each epitope of interest. The $Env_{620-628}$TL9-, $Env_{788-795}$RY8-, and Vif_{97-104}WY8-specific $CD8^+$ T-cell lines were ineffective at suppressing SIV replication (typically <20% reduction).

Disease status does not appear to predict the ability of $CD8^+$ T cells to suppress SIV replication. We also investigated the potential impact of viral replication (vRNA copies/ml) at the time the $CD8^+$ T-cell lines were generated on antiviral efficacy. We had previously observed some variability among animals in the ability of Tat_{28-35}SL8-specific $CD8^+$ T cells to suppress SIV replication (36). To address this question, we generated $CD8^+$ T-cell lines from macaques in various stages of disease progression, including three EC macaques, 95061, 98014, and 98016, that controlled SIV replication at <1,000 vRNA copies/ml (Table 2). We hypothesized that $CD8^+$ T-cell lines from EC macaques would be particularly effective at suppressing $SIV_{mac}239$ replication.

Remarkably, $CD8^+$ T-cell lines generated from elite-controller macaques were not always effective suppressors (Table 2). For instance, Tat_{28-35}SL8-specific $CD8^+$ T cells derived from animal 95061, with a viral load of <50 vRNA copies/ml, did not suppress SIV replication as effectively as Tat_{28-35}SL8-

specific CD8+ T-cell lines derived from progressor macaques 2125 and 2128. Despite the high plasma virus RNA concentrations at the time the CD8+ T-cell lines were generated, $Tat_{28–35}$SL8-specific cell lines from macaques 2125 and 2128 reduced vRNA concentrations and Gag p27+ target cells >90% (Table 2). None of the four Mamu-A*02-restricted responses had appreciable levels of viral suppression, despite the derivation of several SIV-specific CD8+ T-cell lines from elite controllers. These included CD8+ T-cell lines directed against the early protein $Nef_{159–167}$YY9. Similarly, no correlation was observed between CD4 counts at the time of cell line generation and the ability of CD8+ T cells to suppress SIV replication (data not shown).

Impact of viral escape on CD8+ T-cell antiviral efficacy. It is well established that CD8+ T lymphocytes select for viral escape variants (3, 5–7, 15, 23, 50, 54). Recent reports demonstrated that CD8+ T lymphocytes can mount responses to variant peptides in cytokine production assays, implying that HIV/SIV-specific CD8+ lymphocytes can recognize mutant epitopes (22, 24, 46, 64). Recent studies also quantified the cross-clade reactivities of cellular immune responses to HIV using the same assays (13, 21, 68). However, since they use PBMC or epitope-specific CD8+ T-cell lines pulsed with excessive amounts of synthetic peptide, these routine immunological assays do not reflect natural antigen processing and presentation. Therefore, we investigated the abilities of our two most effective CD8+ T-cell lines, $Tat_{28–35}$SL8 and $Gag_{181–189}$CM9, to recognize escape variants commonly generated in vivo (3, 5, 6, 17, 18, 50, 51, 53).

A previous study found that the two mutations at positions 1 and 8 of the Tat epitope (STPESANL to *P*TPESAN*Q*) essentially abrogated peptide binding to Mamu-A*01 (Fig. 2A) (17). However, while the position 2 amino acid substitution in the Gag epitope (CTPYDINQM to C*A*PYDINQM) diminished MHC class I binding, this variant peptide still bound at a biologically relevant affinity of 354 nM (50% inhibitory concentration).

Based upon the previous binding analysis of the escape variant epitopes, we hypothesized that we would observe some recognition of the Gag escape variant and none with the Tat escape variant. We first tested for peptide cross-reactivity in IFN-γ/TNF-α ICS assays using $Gag_{181–189}$CM9- and $Tat_{28–35}$SL8-specific CD8+ T-cell lines (Fig. 2B and C). The Gag variant peptide displayed cross-reactivity at a peptide concentration range of 10,000 nM to 100 nM, while the Tat escape variant was only weakly recognized at the highest peptide concentration (Fig. 2B and C). To confirm these results, we then tested the recognition of the variant peptides in ex vivo IFN-γ ELISPOT assays using freshly isolated PBMC. At a peptide concentration of 100 nM, the Gag escape variant induced cytokine secretion (Fig. 2D). Responses to the wild-type and variant Gag peptides were equal in magnitude at the highest peptide concentration. In contrast, the Tat escape variant showed minimal cytokine reactivity and only at the highest peptide concentration (Fig. 2E). The responses to variant peptides in IFN-γ ELISPOT were likely due to recognition of the variant peptide (cross-reactivity) and not the stimulation of a de novo response against the mutant peptides. When both peptides were tested in the same well, the number of SFC/10^6 PBMC did not exceed the number of SFC seen when either peptide was added alone (data not shown). Our results, in conjunction with previously published work (22, 24, 46, 64), suggested the possibility that the $Gag_{181–189}$CM9-specific CD8+ T-cell lines might be effective at suppressing an SIV escape variant.

To directly assess the abilities of epitope-specific CD8+ T cells to control the replication of the mutant virus, we used a variant of $SIV_{mac}239$ (17) that contained escape mutations in both $Tat_{28–35}$SL8 and $Gag_{181–189}$CM9 to infect target cells (Fig. 3). As a control to determine whether the CD8+ T cells used were still capable of suppressing viral replication, CD8− target cells were also infected with wild-type $SIV_{mac}239$. As expected, both the $Gag_{181–189}$CM9 and $Tat_{28–35}$SL8-specific CD8+ T cells effectively suppressed wild-type $SIV_{mac}239$ replication (Fig. 3). However, when the escape mutant SIV was used, neither of these CD8+ T-cell lines suppressed viral replication in four independent assays. We observed similar trends at an E:T ratio of 1:20 (data not shown). This poor suppression was particularly unexpected for the $Gag_{181–189}$CM9-specific CD8+ T cells. The variant epitope still bound with a biologically relevant affinity, and the variant peptide stimulated both SIV-specific CD8+ T-cell lines and PBMC in IFN-γ/TNF-α ICS and IFN-γ ELISPOT assays, respectively (Fig. 2). This inability of $Gag_{181–189}$CM9-specific CD8+ T cells to suppress the replication of the mutant virus stresses the importance of functional assays that incorporate natural antigen processing and presentation to evaluate the antiviral efficacies of epitope-specific CD8+ T-cell responses. These data also reemphasize the importance of viral escape in evading host immune responses.

DISCUSSION

Unlike studies involving antibody responses in which neutralization assays distinguish effective antibodies from ineffective ones, most current assays involving CD8+ T cells do not measure antiviral efficacy (70). We recently developed a functional in vitro VSA to assess the ability of CD8+ T cells to control SIV replication and to study the impact of viral variation (36). This new assay has enabled us to expand upon previous HIV studies, which hinted at differences in antiviral efficacy among various CD8+ T-cell populations (1, 62, 65, 66, 71, 72). We used our viral-suppression assay to examine the antiviral efficacies of seven common CD8+ T-cell responses against epitopes restricted by two high-frequency MHC class I alleles, Mamu-A*01 and Mamu-A*02 (Table 1). These CD8+ T cells recognized epitopes from both early- and late-expressed viral proteins. Twenty-six epitope-specific CD8+ T-cell lines were derived from 10 $SIV_{mac}239$-infected macaques with differing disease statuses, including three elite-controller macaques. We also examined the abilities of CD8+ T cells to suppress the replication of viruses containing common escape mutations in two immunodominant Mamu-A*01-restricted CD8+ T-cell epitopes.

We found that suppression of SIV replication varied, depending on the epitope specificity of the CD8+ T cell (Fig. 1 and Table 2). Furthermore, this suppressive ability was not related to whether the epitope was derived from an early- or late-expressed viral protein. Even in our broadened investigation, $Tat_{28–35}$SL8-specific CD8+ T cells remained the most

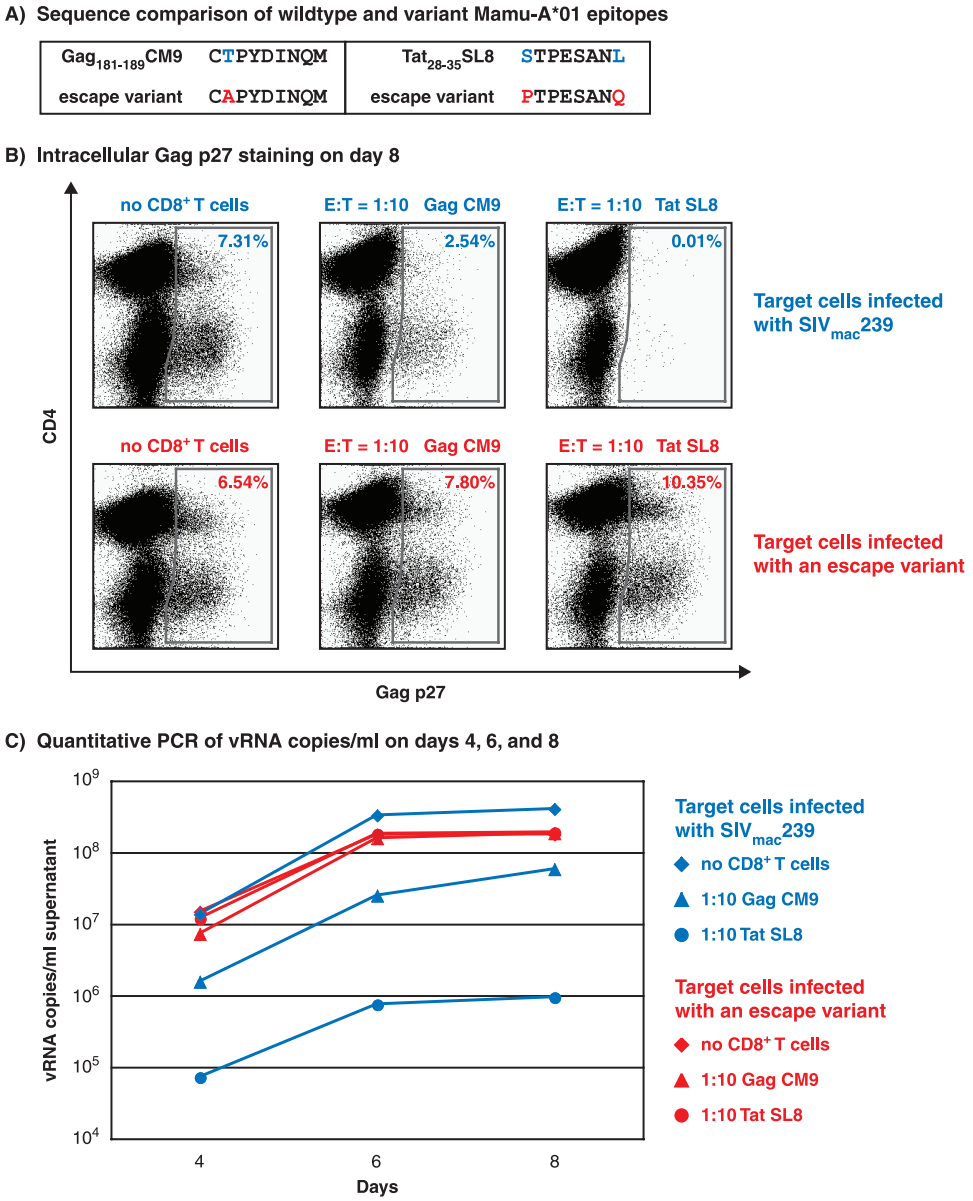

FIG. 3. $Gag_{181–189}$CM9- and $Tat_{28–35}$SL8-specific $CD8^+$ T lymphocytes were unable to suppress the replication of an $SIV_{mac}239$ escape variant. (A) Amino acid sequences of the wild-type and variant epitopes located in Gag and Tat. (B) Intracellular Gag p27 staining of representative day 8 VSA results using effector and target cells at 1:10 infected with either wild-type $SIV_{mac}239$ or an escape variant, $SIV_{mac}239$. (C) Quantitative PCR of viral RNA copies/ml on days 4, 6, and 8 of the same assay.

effective at suppressing viral replication, as we had shown in our initial studies (36). $Tat_{28–35}$SL8-specific $CD8^+$ T cells at an E:T ratio of 1:10 markedly reduced SIV replication (>55%) in four of the five $CD8^+$ T-cell lines tested. Epitope-specific $CD8^+$ T cells against $Gag_{181–189}$CM9 were also effective and exhibited levels of suppression in the range of 32 to 90%. While Gag is generally viewed as a late protein, recent findings have shown that Gag-specific epitopes can be recognized as early as 2 hours after SIV infection (58a). This early presentation advantage may contribute to the ability of $Gag_{181–189}$CM9 $CD8^+$ T cells to effectively suppress SIV replication. The remaining five SIV-specific $CD8^+$ T-cell responses, including $CD8^+$ T-cell lines against an early SIV epitope, $Nef_{159–167}$YY9, were largely ineffective at suppressing viral replication (Fig. 1). In vivo, $Nef_{159–167}$YY9 elicits an immunodominant $CD8^+$ T-cell response, with viral escape occurring as early as 4 weeks postinfection (50, 58, 67). However, $Nef_{159–167}$YY9-specific $CD8^+$ T-cell lines from four different macaques, including two elite controllers, failed to suppress viral replication. In addition, we found that $CD8^+$ T lymphocytes, which recognize two different epitopes within the same viral protein, did not exhibit similar suppressive properties. Both an immunodominant Mamu-A*01-restricted epitope, $Gag_{181–89}$CM9, and an immunodominant Mamu-A*02-restricted epitope, $Gag_{71–79}$GY9, are located in Gag. However, only $Gag_{181–189}$CM9-specific $CD8^+$ T-cell lines consistently suppressed SIV replication

>50% (Fig. 1 and Table 2). Similar to previous studies (11, 62, 72), the functional avidity of cytokine reactivity did not appear to correlate with antiviral efficacy in vitro (data not shown).

We also examined the impact that disease progression may have on the ability of $CD8^+$ T cells to effectively suppress viral replication. While Tat_{28-35}SL8-specific $CD8^+$ T cells were more effective at suppressing SIV replication, we previously observed animal-to-animal variability (36). However, this variability did not appear to directly reflect differences in the disease statuses or viral loads of macaques at the time of cell line generation (Table 2).

The effect of viral escape from $CD8^+$ T-cell responses is an important consideration for vaccine development. Certain high-frequency $CD8^+$ T-cell responses rapidly select for viral escape, driving $CD8^+$ T-cell-susceptible viral sequences to extinction within 4 weeks of infection (3, 50, 58, 67). Recent studies have also addressed the influence of escape on viral fitness (17, 27, 33, 40, 41). To explore the role of viral escape in antiviral efficacy, we generated a clone of $SIV_{mac}239$ that contained escape mutations in two Mamu-A*01-restricted $CD8^+$ T-cell epitopes, Tat_{28-35}SL8 and $Gag_{181-189}$CM9 (17). Tat_{28-35}SL8- and $Gag_{181-189}$CM9-specific $CD8^+$ T cells did not suppress the replication of this escape variant virus (Fig. 3). This loss of antiviral efficacy was somewhat surprising for $Gag_{181-189}$CM9-specific $CD8^+$ T cells, because the variant of $Gag_{181-189}$CM9 was well recognized in both IFN-γ/TNF-α ICS and IFN-γ ELISPOT assays (Fig. 2). Our data suggest that the currently employed routine immunological assays neither accurately predict the antiviral efficacy of $CD8^+$ T cells nor measure the impact of viral escape on the ability of these cells to suppress viral replication. Furthermore, similar cytokine production readouts are currently being employed to detect cross-clade $CD8^+$ T-cell responses induced by HIV infection and vaccine immunogens (13, 21, 68). Our results suggest that the correlation between cytokine production and antiviral function is neither direct nor consistent. Therefore, while $CD8^+$ T cells may release IFN-γ in response to peptides representing several HIV clades, this does not necessarily indicate that they can effectively suppress the replication of viruses in these clades.

In this study, we used epitope-specific $CD8^+$ T-cell lines rather than $CD8^+$ T-cell clones to reduce potential bias that could result from studying cell populations with limited or no T-cell receptor (TCR) diversity. Epitope-specific $CD8^+$ T-cell populations with diverse clonotypic repertoires are observed in HIV and SIV infection; indeed, TCR diversity may be beneficial (10, 14, 29, 56). Complementary studies in our laboratory using $CD8^+$ T-cell clones have shown that different clones specific for a single peptide can vary in their abilities to suppress virus replication, underscoring the importance of TCR diversity (11).

Previous studies of HIV and SIV demonstrated that epitope-specific $CD8^+$ T lymphocytes suppress viral replication primarily through direct cytolytic activity (20, 71, 72). In our investigation, we did not directly measure the cytolytic activities of our highly specific $CD8^+$ T-cell lines. While we cannot account for potential variability in the cytolytic activity and its effect on our VSA readouts, results from VSA and ICS assays displayed similar trends over time (data not shown).

It is plausible that findings using our in vitro viral-suppression assay might be extrapolated to the ability to suppress HIV/SIV replication in vivo. However, future investigations of $CD8^+$ T-cell antiviral efficacy might benefit from modifications of this assay. These include using higher multiplicities of infection to mimic the massive infection and rapid loss of $CD4^+$ T cells at mucosal surfaces (35, 44). Emerging evidence also suggests that $CD8^+$ T cells reactive to subdominant epitopes may contribute significantly to the control of HIV and SIV replication in vivo (16, 18a). More extensive evaluation of these T-cell populations will be necessary.

Overall, our results outline clear differences in $CD8^+$ T-cell efficacy and suggest that the antiviral efficacy of $CD8^+$ T cells should be measured for individual epitopes and not generalized by protein. We found no supporting evidence for the idea that $CD8^+$ T cells that recognize epitopes in early-expressed viral proteins were more efficacious than those that recognize epitopes in late-expressed proteins. However, recent findings regarding Gag epitope expression show that these timing designations might not be as appropriate as was once thought (58a). Furthermore, our findings indicate that routine immunological assays are both indirect and inadequate for assessing T-cell function and cross-reactivity. While ELISPOT and ICS assays are widely accepted means to track $CD8^+$ T-cell frequencies, these assays fail to differentiate among T-cell populations in terms of antiviral efficacy. The use of functional assays in addition to standard immunogenicity assays should enable us to more thoroughly evaluate candidate epitopes for inclusion in future HIV vaccines.

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