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NOTES

CD8$^+$ T Cell Escape Mutations in Simian Immunodeficiency Virus SIVmac239 Cause Fitness Defects *In Vivo*, and Many Revert after Transmission$^{\nabla}$†

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Virus-specific CD8$^+$ T lymphocytes select for escape mutations in human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV). To assess the effects of these mutations on viral fitness, we introduced escape mutations into 30 epitopes (bound by five major histocompatibility complex class I [MHC-I] molecules) in three different viruses. Two of these MHC-I alleles are associated with elite control. Two of the three viruses demonstrated reduced fitness *in vivo*, and 27% of the introduced mutations reverted. These findings suggest that T cell epitope diversity may not be such a daunting problem for the development of an HIV vaccine.

Human immunodeficiency virus (HIV) sequence diversity may pose a significant problem for vaccine development. The majority of viral variation outside of the envelope protein is selected for by CD8$^+$ T lymphocytes (1, 36). This sequence variation can either be maintained (18) or revert (2, 20, 21, 31, 41) after transmission to a new host with a disparate major histocompatibility complex class I (MHC-I) haplotype. Unfortunately, reversion has been examined only for a small number of previously studied and well-characterized CD8$^+$ T cell epitopes.

Our group has characterized the peptide-binding properties of several MHC-I molecules (5, 11, 39), mapped CD8$^+$ T lymphocyte epitopes (3, 12, 26, 27, 33, 38), and described viral escape (4, 13, 35, 36) in the Indian rhesus macaque model of immunodeficiency virus infection. We have now systematically identified all of the CD8$^+$ T cell epitopes located within the cloned simian immunodeficiency virus (SIV) SIVmac239 that are restricted by 5 different rhesus macaque MHC-I alleles: *Mamu-A1*00101* (3), *Mamu-A1*00201* (27), *Mamu-A1*01101* (38), *Mamu-B*00801* (26), and *Mamu-B*01701* (33). We previously infected rhesus macaques with a mutant SIVmac239 virus bearing escape mutations in three of these epitopes (14). The Mamu-A1*00101-restricted $Tat_{28\text{-}35}$SL8 escape mutant was maintained in Mamu-A1*00101-negative macaques. Conversely, escape mutations within two other immunodominant epitopes, the Mamu-A1*00101-restricted $Gag_{181\text{-}189}$CM9 epitope and the Mamu-B*01701-restricted $Nef_{165\text{-}173}$IW9 epitope, reverted to the wild-type sequence in macaques that did not express the selecting alleles (14). In the present study, we sought to broaden these observations in a systematic fashion by examining the reversion characteristics of 30 separate CD8$^+$ T lymphocyte escape mutations in epitopes restricted by five different MHC-I alleles (Table 1).

Selection of CD8$^+$ T lymphocyte escape mutations. We began by identifying escape mutations selected for by CD8$^+$ T lymphocytes that recognize epitopes bound by Mamu-A1*00101, Mamu-A1*00201, Mamu-A1*01101, Mamu-B*00801, and Mamu-B*01701. To do this, we utilized previously collected and published viral sequence data obtained from plasma samples taken at the time of death from a large cohort of MHC-I-typed, SIVmac239-infected macaques (36). We also obtained viral sequence data from 19 additional MHC-I-typed, SIVmac239-infected animals to increase the number of *Mamu-A1*01101*-postive macaques in our analysis. We have included the new sequencing data from these 19 animals in File S1 in the supplemental material. We have also provided a list of all the animals in this study, together with MHC-I typing information, in Table S1 in the supplemental material. We compiled the data to evaluate MHC-I-associated viral mutations within all identified CD8$^+$ T lymphocyte epitopes for the five MHC-I alleles. Putative processing mutations within 3 amino acids of the described minimal epitopes were also included in our analysis. We categorized sequence data into two groups for all 54 animals: animals with and animals without the relevant MHC-I allele. We chose muta-

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TABLE 1. $CD8^+$ T lymphocyte escape mutations engineered into three separate mutant SIVmac239 viruses

MHC-I allele	Mutant virus	Epitope	SIVmac239 sequence	Mutant sequence[a]	Comment code(s)[b]
*Mamu-A1*00101*	14x-SIVmac239	$Gag_{372-379}$LF8	LAPVPIPF	---G----	1
		$Pol_{625-633}$SV9	STPPLVRLV	--------A	1
		$Vif_{100-107}$VI8	VTPNYADI	-------T	1
		Tat_{28-35}SL8	STPESANL	P------P	1
		$Env_{233-241}$CL9	CAPPGYALL	-----FV--	1
		$Env_{620-628}$TL9	TVPWPNASL	--------I	1
		$Env_{726-735}$ST10	SSPPSYFQQT	---------I	1
*Mamu-A1*00201*	14x-SIVmac239	Gag_{71-79}GY9	GSENLKSLY	S-D------	1
		$Env_{788-795}$RY8	RTLLSRVY	----L---	1
		$Nef_{159-167}$YY9	YTSGPGIRY	--------F	1
		$Nef_{221-229}$YY9	YTYEAYVRY	H--------	1
*Mamu-A1*01101*	14x-SIVmac239	Pol_{92-100}AL9	AERKQREAL	-G-------	1
		$Env_{495-502}$GI8	GDYKLVEI	----V---	1
		$Nef_{124-132}$KI9	KEKGGLEGI	-Q-------	1
*Mamu-B*00801*	11x-SIVmac239	$Vif_{123-131}$RL9	RRAIRGEQL	-K-------	2
		$Vif_{172-179}$RL8	RRDNRRGL	-G------	2
		Vpr_{62-70}IF9	IRILQRALF	-K-------	3
		Rev_{12-20}KL9	KRLRLIHLL	-K-------	3
		Rev_{44-51}RL8	RRRWQQLL	-K----I-	2, 3
		$Env_{573-581}$KL9	KRQQELLRL	--------M	2
		$Env_{868-876}$RL9	RRIRQGLEL	--------R	4
		Nef_{8-16}RL9	RRSRPSGDL	----Q----	2
		$Nef_{137-146}$RL10	(A)RRHRILDIYL	(P)----------	2, 5
		$Nef_{245-253}$RL9	RRRLTARGL	KK------I	2, 3
		$Nef_{246-254}$RL9	RRLTARGLL	K------II	2
*Mamu-B*01701*	5x-SIVmac239	Vif_{66-73}HW8	HLEVQGYW	Y-------	6
		cRW9	RHLAFKCLW	--------R	7
		$Env_{830-838}$FW9	FHEAVQAVW	-Y-------	6
		$Nef_{165-173}$IW9	IRYPKTFGW	T----I---	8
		$Nef_{195-203}$MW9	MHPAQTSQW	V--------	6

[a] Dashes indicate residues identical to the wild-type sequence.

[b] Comments: 1, MHC-I allele-specific variation observed in sequences published in reference 36 and in sequences found in File S1 in the supplemental material; 2, variant published in reference 26; 3, previously unpublished variant discovered during chronic SIVmac239 infection in *Mamu-B*00801*-positive macaques; 4, variation in Mamu-B*00801 anchor residue observed in an HIV-2 sequence from the Los Alamos National Laboratory online HIV sequence database (www.hiv.lanl.gov); 5, putative processing mutation (extraepitopic variant published in references 22 and 24); 6, variant published in reference 29; 7, cryptic reading frame variant published in reference 28; 8, variant published in reference 16.

tions seen exclusively in animals with the MHC-I allele that restricts the T cell response directed against the epitope of interest and not in animals without the selecting allele. In many of the studied epitopes, more than one mutation occurred within the epitope in different animals. For the construction of mutant viruses, we chose to limit the number of mutations to only a single variant per T cell epitope. Some of these variants contained more than one amino acid change, if they were naturally observed in that form within the same animal in our viral sequence data. We selected a single variant for each epitope that exhibited T cell escape based upon the frequency of the variant in animals with the selecting MHC-I allele and/or the presence of a mutation within a key MHC-I anchor residue.

Construction of mutant SIVmac239 viruses. We created two different viruses bearing mutations in epitopes bound by alleles that are associated with elite control. We developed 11x-SIVmac239, which included mutations found in epitopes bound by Mamu-B*00801 (25), and 5x-SIVmac239, which included mutations found in epitopes bound by Mamu-B*01701 (43) (Table 1). We then created a third virus, 14x-SIVmac239, containing mutations discovered within T cell epitopes restricted by the other three MHC-I alleles, *Mamu-A1*00101*, *Mamu-A1*00201*, and *Mamu-A1*01101*, which are not associated with elite control (Table 1). We did not include an escape mutation within the Mamu-A1*00101-restricted $Gag_{181-189}$CM9 epitope in the 14x-SIVmac239 virus because of the fitness cost associated with an escape mutation in this epitope (14, 15). We have also already shown that an escape mutation in this epitope reverts after transmission (14).

We built 11x-SIVmac239 in an 8x-SIVmac239 virus backbone (40) using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and primers designed to incorporate the appropriate mutations (see Table S2 in the supplemental material). We used 5x-SIVmac239 virus that had been developed for a previous study (42). We had the 14x-SIVmac239 virus synthesized *de novo* by GeneArt (Regensburg, Germany). We generated each virus by ligation of two plasmids, one encoding the 5′ half of the SIVmac239 provirus and the other encoding the 3′ half. We cotransfected Vero cells with the ligated plasmids containing escape mutations and propagated the resulting virus using a previously

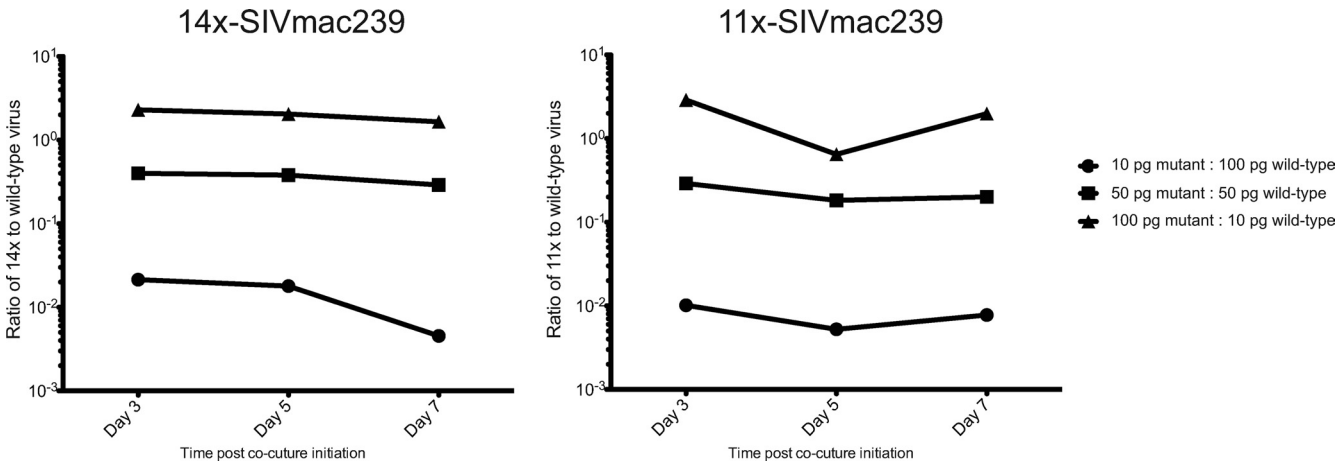

FIG. 1. *In vitro* viral fitness of 14x-SIVmac239 and 11x-SIVmac239 mutant viruses. We inoculated each mutant virus and wild-type SIVmac239 into cultures of concanavalin A-activated CD8-depleted peripheral blood mononuclear cell (PBMC) targets at the indicated ratio of mutant virus to wild-type virus (1:10, 1:1, or 10:1). We then harvested virus-containing supernatant at each time point indicated and quantified the proportion of each viral species by quantitative reverse transcription-PCR. The complete method, including primers used for quantitative PCR, has been described previously (40). Both mutant-inoculated cultures displayed relatively consistent ratios of wild-type to mutant virus over the 7 days of the assay, suggesting no *in vitro* fitness deficit or advantage for the introduced mutations. *In vitro* analysis of the 5x-SIVmac239 virus has been published previously (42).

described method (14, 40). We amplified each viral stock to high titer in CEMx174 cells. We sequenced each virus following production and verified that the correct mutations were incorporated.

We tested the *in vitro* fitness of the 11x-SIVmac239 and 14x-SIVmac239 viruses relative to wild-type SIVmac239 using a previously described competition assay (40). The 11x-SIVmac239 and 14x-SIVmac239 viruses both demonstrated fitness similar to the wild-type virus in a 7-day *in vitro* competition assay (Fig. 1). An *in vitro* competition assay using 5x-SIVmac239 also did not reveal a fitness deficit (42).

Infection of macaques with mutant SIVmac239 viruses. We infected three separate groups of four Indian rhesus macaques intravenously with each of our mutant SIVmac239 viruses. None of these animals expressed the relevant MHC-I alleles that had been involved in the selection of the escape mutants (see Table S3 in the supplemental material). We monitored viral loads in these animals using a previously published method (23) that can reproducibly detect viral loads of $\geq$30 viral RNA copies per ml of plasma. None of the introduced mutations affected the region of the virus detected by the primers in our viral load assay.

To our surprise we discovered that, despite apparent fitness *in vitro*, the viruses 14x-SIVmac239 (containing Mamu-A1*00101, Mamu-A1*00201, and Mamu-A1*01101 escape mutations) and 11x-SIVmac239 (containing Mamu-B*00801 escape mutations) did not replicate as well as wild-type SIVmac239 *in vivo* (Fig. 2C and A). Geometric mean viral loads during chronic infection in animals infected with these two viruses were more than 2 $\log_{10}$ lower than the geometric mean viral load of SIVmac239-infected historical controls (Fig. 2D). In contrast, viral loads in animals infected with 5x-SIVmac239 (containing Mamu-B*01701 escape mutations) (Fig. 2B) were indistinguishable from those in wild-type SIVmac239-infected animals (Fig. 2D). The discrepancy between apparent fitness levels of the 14x-SIVmac239 and 11x-SIVmac239 viruses *in vitro* and *in vivo* underscores the limitations of short-term *in vitro* viral fitness assays, including growth competition assays. Such short-term assays (7 days in this case) cannot be relied upon to quantify the potential impact mutations may have upon viral fitness over the course of many months in a host organism.

We next evaluated the stability of the introduced mutations. We sequenced plasma virus samples from two animals in each group at approximately 26 weeks postinfection. We discovered that 3 of 14 epitopes (21%) with mutations in 14x-SIVmac239, 4 of 11 epitopes (36%) with mutations in 11x-SIVmac239, and 1 of 5 epitopes (20%) with mutations in 5x-SIVmac239 reverted, at least in part, to the wild-type sequence (Fig. 3). It is possible that the proportion of these epitopes that revert may actually be higher than 8 of 30 (27%); however, our experiment was limited by low viral loads in two of the 11x-SIVmac239- and two of the 14x-SIVmac239-infected animals. In addition, permitting the animals to progress beyond 6 months may have allowed for the detection of additional reversion. Therefore, the measured rate of reversion (27%) in our experiment is likely a conservative estimate of the actual rate of reversion for these 30 variant epitopes were the experiment continued until pathological sequelae developed. Taken together, these data suggest that a substantial proportion of all $CD8^+$ T lymphocyte escape mutations which develop in SIVmac239, as represented by the 30 selected by these five MHC-I alleles, revert *in vivo* in the absence of the selecting allele.

To understand the potential relevance of our model system to the study of reversion in HIV isolates, it is important to discuss the origins of the SIVmac239 clonal virus. SIVmac239 (34) was cloned from infected cells that had been inoculated with various tissues from a rhesus macaque, Mm239-82, with simian AIDS (10). Prior to the infection of Mm239-82, the ancestral virus from which SIVmac239 originated was passaged through at least 7 other rhesus macaque hosts (17, 30). Therefore, SIVmac239 may be most analogous to a clonal HIV type

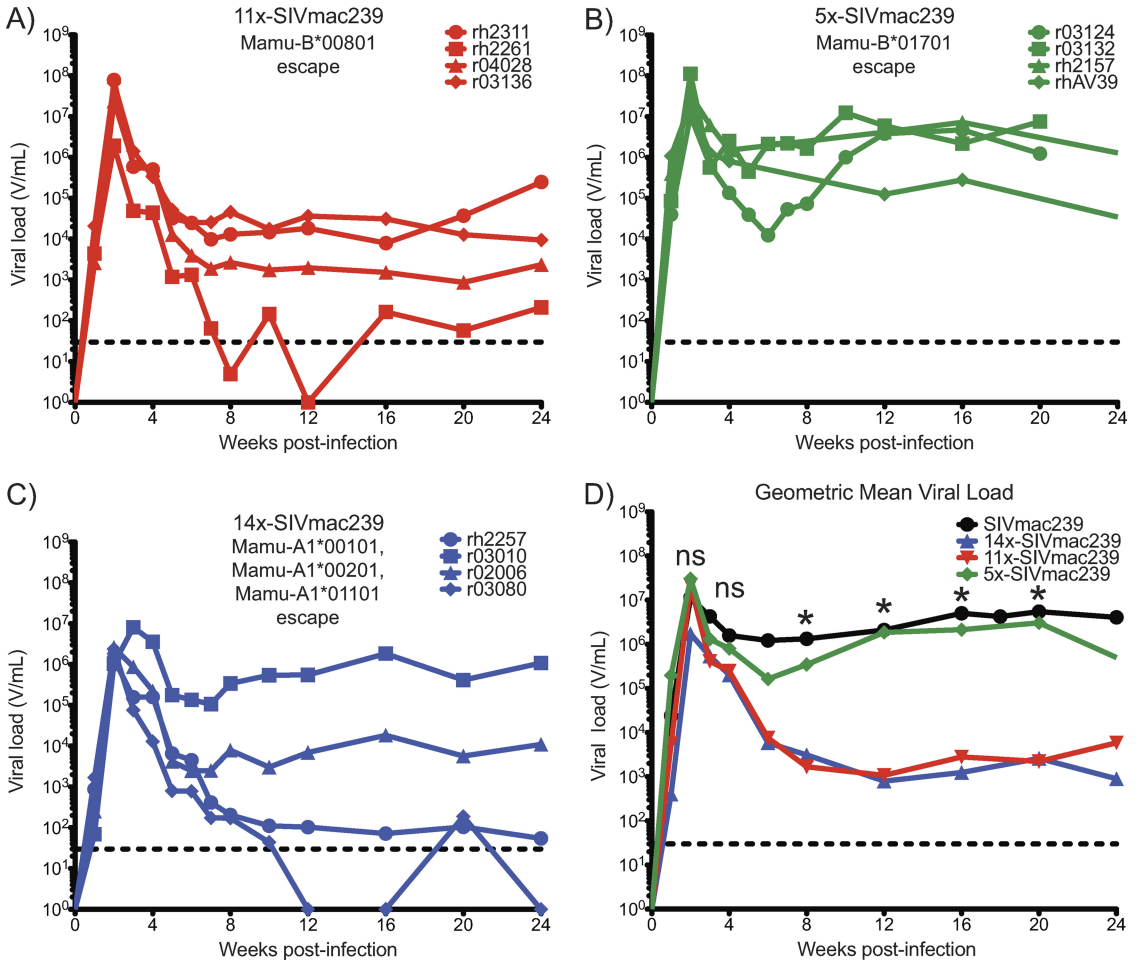

FIG. 2. Viral loads following mutant SIVmac239 virus infection. We infected four rhesus macaques with each of the indicated viruses. We measured viral loads with a previously described method (23). Viral loads of ≥30 viral RNA copies per ml of plasma (V/ml; dashed lines) can be reliably quantified using this method. (A to D) 11x-SIVmac239 (carrying Mamu-B*00801-restricted T cell epitope mutations) (A) and 14x-SIVmac239 (carrying Mamu-A1*00101-, Mamu-A1*00201-, and Mamu-A1*01101-restricted T cell epitope mutations) (C) do not replicate *in vivo* as well as 5x-SIVmac239 (carrying Mamu-B*01701-restricted T cell epitope mutations) (B) and wild-type SIVmac239 (D). (D) Geometric mean viral loads were calculated for each group of infected animals ($n = 4$) and wild-type SIVmac239-infected historical controls ($n = 33$). A Kruskal-Wallis test was performed with Dunn's multiple-comparison posttest comparing viral loads at the indicated time points postinfection among all wild-type SIVmac239-infected animals and each of the three mutant virus-infected groups of animals. Significant differences were discovered at weeks 8, 12, 16, and 20 postinfection in comparisons of 11x-SIVmac239-infected animals to wild-type SIVmac239-infected animals and of 14x-SIVmac239-infected animals to wild-type SIVmac239-infected animals (ns, not significant; *, $P < 0.05$). No significant differences were detected between 5x-SIVmac239- and wild-type SIVmac239-infected animals.

1 (HIV-1) isolate obtained early in the HIV-1 pandemic. Indeed, the immunodominant Tat_{28-35}SL8 epitope is intact in clonal SIVmac239, yet this epitope quickly escapes in animals expressing the high-frequency *Mamu-A1*00101* allele (expressed in 27% of captive rhesus macaques) via mutations that are maintained after transmission.

The relatively high frequency of escape mutation reversion that we detected in this experiment suggests that some $CD8^+$ T cell populations can target regions of the virus where mutations affect viral fitness. Because SIVmac239 likely represents a very early isolate after cross-species transmission of an immunodeficiency virus from its natural to a nonnatural host, many epitopes are still intact. This may not be the case in individuals infected with HIV today. Previous passage of today's viral isolates through many different individuals that express various HLA class I alleles may have left us with a preponderance of reverting epitopes rather than epitopes that do not revert. However, the creation of *de novo* epitopes from the buildup of nonreverting mutations and the selection of disparate escape mutations at the same residue by different MHC-I alleles may limit the frequency of reverting epitopes at the population level (6). Nevertheless, we suggest that the frequency of $CD8^+$ T lymphocyte escape mutation reversion relative to maintenance in currently circulating HIV-1 isolates that have undergone many transmission cycles may be higher than our conservative measurement of 27%.

Compensatory mutations can promote replication of viruses bearing T cell escape mutations that, in isolation, diminish viral replication capacity (8, 9, 15, 19, 32, 37). Unfortunately, associations between potential compensa-

14x-SIVmac239

Mamu-A1*00101-restricted epitopes

	$Gag_{372-379}$LF8	$Pol_{625-633}$SV9	$Vif_{100-107}$VI8	Tat_{28-35}SL8	$Env_{233-241}$CL9	$Env_{620-628}$TL9	$Env_{726-735}$ST10
SIVmac239	LAPVPIPF	STPPLVRLV	VTPNYADI	STPESANL	CAPPGYALL	TVPWPNASL	SSPPSYFQQT
14x	---G----	--------A	-------T	P------P	-----FV--	--------I	---------I
r02006	---G----	--------A	-------T	P---L--P	---------	--------I	---------I
r03010	---G----	--------A	-------T	P-----sP	-----F---	--------I	---------I

Mamu-A1*00201-restricted epitopes

	Gag_{71-79}GY9	$Env_{788-795}$RY8	$Nef_{159-167}$YY9	$Nef_{221-229}$YY9
SIVmac239	GSENLKSLY	RTLLSRVY	YTSGPGIRY	YTYEAYVRY
14x	S-D------	----L---	--------F	H--------
r02006	--D------	----L---	--------F	---------
r03010	---------	----L---	--------F	H--------

Mamu-A1*01101-restricted epitopes

	Pol_{92-100}AL9	$Env_{495-502}$GI8	$Nef_{124-132}$KI9
SIVmac239	AERKQREAL	GDYKLVEI	KEKGGLEGI
14x	-G-------	----V---	-Q-------
r02006	-G-------	----V---	-Q-------
r03010	-G-------	----V---	-Q-------

11x-SIVmac239

Mamu-B*00801-restricted epitopes

	$Vif_{123-131}$RL9	$Vif_{172-179}$RL8	Vpr_{62-70}IF9	Rev_{12-20}KL9	Rev_{44-51}RL8
SIVmac239	RRAIRGEQL	RRDNRRGL	IRILQRALF	KRLRLIHLL	RRRWQQLL
11x	-K-------	-G------	-K-------	-K-------	-K----I-
r03136	---------	-G------	-K-------	-k-------	-K----I-
rh2311	---------	-G------	-K-------	---------	-K----I-

	$Env_{573-581}$KL9	$Env_{868-876}$RL9	Nef_{8-16}RL9	$Nef_{137-146}$RL10	$Nef_{245-254}$RL9b/c
SIVmac239	KRQQELLRL	RRIRQGLEL	RRSRPSGDL	(A)RRHRILDIYL	RRRLTARGLL
11x	--------M	--------R	----Q----	(P)----------	KK------II
r03136	--------M	--------R	----QP---	(P)----------	----------
rh2311	--------M	--------R	----Q----	(P)----------	----------

5x-SIVmac239

Mamu-B*01701-restricted epitopes

	Vif_{66-73}HW8	cRW9	$Env_{830-838}$FW9	$Nef_{165-173}$IW9	$Nef_{195-203}$MW9
SIVmac239	HLEVQGYW	RHLAFKCLW	FHEAVQAVW	IRYPKTFGW	MHPAQTSQW
5x	Y-------	--------R	-Y-------	T----I---	V--------
rh2157	Y-------	--------R	-Y-------	t-f------	V--------
rhAV39	Y-------	--------R	-Y-------	T--------	V--------

FIG. 3. Plasma virus sequence. We selected two animals with high viral loads from each group for viral sequencing. Plasma virus samples from r02006, r03010, r03136, and rh2311 were sequenced approximately 26 weeks postinfection using whole-SIVmac239-genome Roche 454 sequencing (7). Mixed-base annotation indicates viral mutations encompassing more than 30% of total viral quasispecies. Capital-letter annotation indicates a mutation encompassing more than 70% of viral quasispecies. rh2157 and rhAV39 samples were sequenced using standard viral Sanger sequencing methods (22) at approximately 28 weeks postinfection. Reverting mutations are noted in yellow boxes. The Mamu-B*00801-restricted $Nef_{245-254}$RL9 epitope encompasses two overlapping T cell epitopes (Table 1), both containing mutations that reverted. Dashes indicate residues identical to the wild-type sequence.

tory mutations and specific T lymphocyte escape mutations are difficult to establish in the setting of limited sequence data (15, 19). For most mutations characterized in this study, only 1 to 5 individual animals harbored viruses that contained the mutation. This makes the establishment of connections between viral escape patterns and specific compensatory mutations impossible or nearly impossible. Only a single rhesus macaque T cell epitope in SIVmac239 with known compensatory mutations exists (15). It is possible that some of the mutations we have characterized as revert-

ing in the present study may have associated compensatory mutations that remain undefined at this time.

It is notable that reverting $CD8^+$ T lymphocyte escape mutations were selected by the two MHC-I alleles associated with elite control of SIVmac239 replication, *Mamu-B*00801* and *Mamu-B*01701*. Four of 11 Mamu-B*00801-restricted T cell epitopes in the 11x-SIVmac239 virus and 1 of 5 Mamu-B*01701-restricted T cell epitopes in the 5x-SIVmac239 virus reverted to the wild-type sequence after inoculation of the viruses into animals that did not express these alleles. In addition, we had previously observed reversion of the mutation introduced into the Mamu-B*00801-restricted epitope $Env_{573\text{-}581}$KL9 in a single Mamu-B*00801-negative animal in a separate study (40). Therefore, in total, 38% of mutations (6 of 16) introduced into epitopes restricted by these two rhesus macaque MHC-I alleles reverted in the absence of the selecting MHC-I allele.

None of the viruses in this study were designed to completely mimic naturally occurring escape mutant viruses in SIVmac239-infected rhesus macaques. Instead, we desired to create a model system to measure the effects of escape mutations within epitopes restricted by these five MHC-I alleles. Therefore, it is not surprising that two of the three viruses developed for this study did not appear to be fit *in vivo*. Of interest, the 5x-SIVmac239 virus containing Mamu-B*01701-restricted T cell epitope escape mutations was the only mutant virus in this study with fitness comparable to that of wild-type SIVmac239 *in vivo*. Mutations in 11 or 14 T cell epitopes, regardless of the restricting MHC-I allele, affected *in vivo* viral fitness more than the 5 mutations restricted by the protective MHC-I allele *Mamu-B*01701*. This suggests an additive effect of multiple $CD8^+$ T lymphocyte escape mutations upon viral fitness in the absence of compensatory mutations. In addition, at least with regard to *Mamu-B*01701*, escape mutations found in T cell epitopes restricted by a rhesus macaque allele associated with elite control do not affect viral fitness.

To summarize, in our study 27% of $CD8^+$ T lymphocyte escape mutations found in SIVmac239-infected rhesus macaques (32% if mutations in $Gag_{181\text{-}189}$CM9 [14] and $Env_{573\text{-}581}$KL9 [40] are included) reverted after transmission to hosts without the selecting MHC-I allele. The measured frequency of reverting mutations may increase with multiple viral transmission cycles through hosts with diverse MHC-I alleles, as nonreverting T cell epitope variants become the new consensus sequence. This suggests that $CD8^+$ T lymphocyte-selected HIV diversity may be limited by substitutions that revert in the absence of selecting pressure.

One of the major concerns in developing an HIV vaccine is the immense diversity and seemingly endless evolution of viral sequences. Our data from the SIV-infected Indian rhesus macaque model suggest that almost a third of T cell epitopes will be naturally preserved as vaccine targets. These mutations revert in the absence of selecting pressure after transmission to a new host. Therefore, vaccines containing consensus sequences that span the epitopes in which reversion is observed should be effective in inducing useful $CD8^+$ T cell responses. A carefully designed vaccine containing such targets may overcome the problem of T cell-selected HIV-1 diversity.

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