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Differential patterns of immune escape at Tat-specific cytotoxic T cell epitopes in pigtail macaques

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ABSTRACT

Cytotoxic T lymphocyte responses to conserved proteins such as Gag within HIV- or SIV-infected hosts can facilitate partial control of viremia. However, the utility of targeting variable viral proteins by CTL responses is unclear. We studied CTL responses to regulatory and accessory proteins of SIV in pigtail macaques. The regulatory and accessory proteins were the most commonly targeted proteins by CTL responses from pigtail macaques. We identified 2 novel Tat-specific CTL responses that were both restricted by the *Mane-A*10* allele. Viral escape at one of the Tat epitopes, KSA10, was slower in comparison to another Tat epitope KVA10. The kinetics of escape of the KSA10 Tat epitope were more similar to an immunodominant KP9 Gag epitope also restricted by *Mane-A*10*. Our results suggest that some regulatory or accessory CTL epitopes may be useful targets for vaccination against HIV.

Introduction

The quest for an HIV vaccine continues as scientists struggle to understand how to generate effective, long-lasting immunity against HIV. Despite decades of research several fundamental questions remain unanswered. The HIV proteins to target in order to provide optimal protection against HIV remain unclear. $CD8^+$ T cell responses clearly facilitate immune control of HIV/SIV (Jin et al., 1999; Koup et al., 1994; Kuroda et al., 1999) but did not protect humans from HIV-1 infection in a recent Adenovirus vector vaccine trial (Buchbinder et al., 2008; McElrath et al., 2008). It has been widely viewed that an effective HIV vaccine would need to generate broad anti-HIV $CD8^+$ T cell responses likely targeting as many proteins as possible (Betts et al., 1999; Chouquet et al., 2002). However, recent studies suggest that there are significant differences in the efficacy of T cell immune responses to individual HIV antigens with the occurrence of both useful and potentially detrimental virus-specific T cell responses (Kiepiela et al., 2007; Ngumbela et al., 2008). Gag-specific T cell responses have been shown to be particularly efficacious in controlling HIV viremia while Env-specific T cell responses appear to be less helpful (Kiepiela et al., 2007; Masemola et al., 2004; Zhuang et al., 2008). Similarly, differences in suppressive efficacy of individual SIV epitope-specific T cell lines have also been documented (Loffredo et al., 2007; Loffredo et al., 2005). As yet, however, there is no clear consensus regarding the optimal specificity and range of proteins that need to be targeted in order to establish long-term protection against HIV. While there is general agreement on the usefulness of targeting Gag antigens (Edwards et al., 2002; Masemola et al., 2004; Novitsky et al., 2003), the utility of $CD8^+$ T cells specific for other viral antigens remains unclear.

Non-structural proteins of SIV and HIV-1 are also frequent targets of CTL responses (Addo et al., 2001; Mothe et al., 2002). In the well-characterized Indian rhesus macaque model of SIV infection, the *Mamu-A*01* allele restricts both an immunodominant Tat and Gag CTL epitopes. However, the SL8 Tat epitope undergoes immune escape early and appears to contribute little to immune control of SIV, whereas the CM9 Gag tends to escape much later and is clearly associated with enhanced immune control of SIV (Allen et al., 2002; Allen et al., 2000; O'Connor et al., 2002b). Tat is a variable protein and whether all Tat epitopes are unhelpful or only a subset is not clear.

In order to study the utility of $CD8^+$ T cell responses against individual viral proteins, we examined $CD8^+$ T cell responses to SIV in a cohort of 32 pigtail macaques from a recent peptide-pulsed blood cell immunotherapy study (De Rose et al., 2008b). In that study we found that inducing $CD8^+$ T cell responses to SIV Gag alone was as effective at controlling viremia as inducing T cell responses to all SIV antigens. Furthermore, SIV Gag-specific $CD8^+$ T cell responses were superior to Env-specific $CD8^+$ T cell responses at delaying SIV disease progression (Peut and Kent, 2009). However, we now show that a large proportion of animals recognized a pool of SIV regulatory and accessory peptides. The majority of these responses were directed against SIV Nef and Tat. SIV Tat-specific $CD8^+$ T cell responses were mapped to 2 novel epitopes restricted by the MHC class I *Mane-A*10* allele. Sequencing across these Tat epitopes in the SIV infected animals

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revealed several mutations conferring escape from CTL in both Tat epitopes. There was a clear difference in the rate of CTL escape between the 2 Tat epitopes suggesting constraints in immune escape at one of the Tat epitopes. Our results suggest vaccinating against particular epitopes within some HIV regulatory/accessory proteins may help contribute to subsequent viral control.

Results

Robust CD8$^+$ T cell responses to SIV accessory and regulatory proteins following acute infection

CD8 T cell responses to HIV-1 accessory and regulatory proteins are common in HIV-infected humans (Addo et al., 2002; Altfeld et al., 2001) but the frequency of responses to similar accessory and regulatory proteins in outbred pigtail macaques infected with SIV is not known. When assessed across a large study of SIV-infected pigtail macaques we found a combined pool of peptides spanning the 6 regulatory/accessory proteins were more commonly targeted than Gag, Env or Pol proteins (Fig 1a). Eighteen out of thirty-two SIV_{mac251}-infected animals generated CD8$^+$ T cell responses to a combined SIV accessory and regulatory protein (RTNVVV) peptide pool 4 weeks post infection (p.i.) (De Rose et al., 2008a). By comparison, SIV Gag-, Pol- and Env-specific CD8$^+$ T cell responses at the same timepoint were observed in only 4, 1 and 7 animals, respectively. By 14 weeks p.i., 16, 4, 9 and 28 out of 32 animals had SIV Gag-, Pol-, Env- and RTNVVV-specific CD8$^+$ T cell responses, respectively. The SIV RTNVVV-specific IFNγ CD8$^+$ T cell responses ranged from 1–12% of total CD3$^+$CD8$^+$ T cells. Responses to SIV proteins present in the vaccines (either Gag or all SIV proteins) were overall higher in animals receiving those vaccinations (Fig. 1a shows a breakdown of responses by vaccine group), but the responses to the regulatory/accessory proteins were common across all groups.

To determine which of the 6 regulatory/accessory proteins were most commonly targeted by CD8 T cell responses, we mapped responses to peptide pools spanning individual proteins in the 13 animals with responses to the combined pool exceeding 0.5% of CD8 T cells (Fig. 1b). The frequency of responses to SIV regulatory/accessory proteins was Tat>Nef>Vif/Vpr>Vpx/Rev. Intriguingly, we noted that most animals targeting SIV Tat shared the MHC class I allele *Mane-A*10* suggesting the possibility that *Mane-A*10*-restricted a SIV Tat CD8$^+$ T cell epitope.

Identification of SIV Tat CD8$^+$ T cell epitopes

To facilitate further study of common SIV regulatory/accessory protein specific CD8$^+$ T cells we mapped SIV Nef- and Tat-specific responses in multiple animals, first to individual 15-mer peptides within the overlapping peptides. Mapping of CD8$^+$ T cell responses in 5 of the Nef responders revealed that the animals recognized 5 separate epitopes within 15-mer Nef peptides (Table 1). By contrast, parallel epitope mapping of SIV Tat-specific CD8$^+$ T cell responses revealed that CTL responses to Tat commonly mapped to 2 epitopes. Of the 7 animals studied, 3 animals recognized the 15-mer peptide Tat 5429 (TPKKAKNTSSASNK), 2 animals recognized an epitope overlapping 15-mer peptides Tat 5435-6 (QPEKAKKETVEKAVA and AKKETVEKAVATAPG) and one animal recognized both these epitopes. Animal 8346 responded to an adjacent epitope within Tat peptide 5437 (TVEKAVATAPGLGR).

Since MHC class I molecules generally bind peptides between 8 and 11 amino acids long, we tested dilutions of overlapping peptides of varying length by IFNγ ICS to determine the minimal optimal SIV

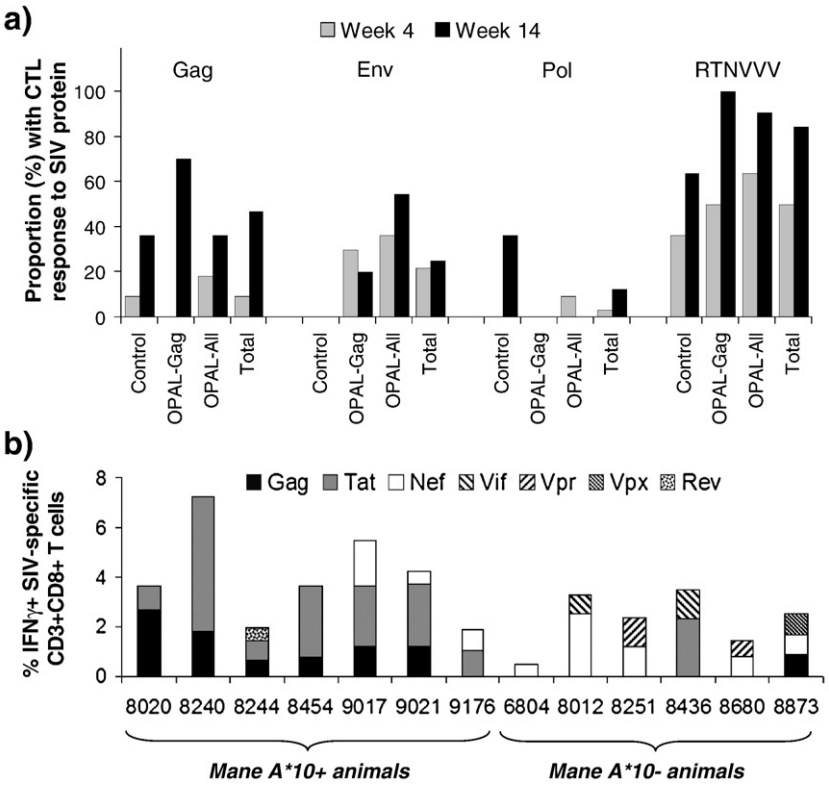

Fig. 1. Specificity of CD8$^+$ T cell responses directed against SIV. a) Proportion of SIV-infected pigtail macaques with IFN-γ production by CD8$^+$ T cells in response to SIV Gag, Env, Pol or a combined pool of the 6 regulatory/accessory proteins at 4 (black) and 14 (grey) weeks post-SIV infection. b) CD8 T cell responses to individual accessory/regulatory proteins following 6-h stimulation of whole blood with pools of overlapping peptides spanning Rev, Tat, Nef, Vif, Vpr and Vpx in individual pigtail macaques from 14 to 22 weeks p.i.. Animals expressing the *Mane-A*10* allele are noted below the graph.

Table 1
SIV Nef and Tat peptides targeted by CD8$^+$ T cells in SIV-infected pigtail macaques.

Animal	Peptide	SIV peptide sequence	MHC class I Mane allele					
			A*03	A*10	B*02	B*10	B*11/22	B*12
8012	Nef 8606	RVPLRTMSYKLAIDM	−	−	−	−	−	−
8251	Nef 8611	EKGGLEGIYYSARRH	−	−	−	−	−	−
8680	Nef 8621	IRYPKTFGWLWKLVP	−	−	−	−	−	−
9017	Nef 8631	DPWGEVLAWKFDPTL	−	+	−	−	−	−
	Nef 8632	EVLAWKFDPTLAYTY						
9176	Nef 8639	SGLSEEEVRRRLTAR	−	+	−	−	−	−
8020	Tat 5429	TPKKAKANTSSASNK		+	+	+	+	
8454	Tat 5429	TPKKAKANTSSASNK		+		+	+	
9017	Tat 5429	TPKKAKANTSSASNK		+	+	+	+	
9021	Tat 5429	TPKKAKANTSSASNK	+	+				+
8240	Tat 5435	QPEKAKKETVEKAVA	−	+	−	−	−	+
	Tat 5436	AKKETVEKAVATAPG						
9021	Tat 5435	QPEKAKKETVEKAVA	+	+	−	−	−	+
	Tat 5436	AKKETVEKAVATAPG						
9176	Tat 5435	QPEKAKKETVEKAVA	−	+	−	−	−	−
	Tat 5436	AKKETVEKAVATAPG						
8436	Tat 5437	TVEKAVATAPGLGR	−	−	−	−	−	−

Tat epitopes within the Tat 5429 and Tat 5435-6 15-mer peptides. The 10mer peptide KKETVEKAVA ($Tat_{114–123}$, KVA10) optimally stimulated IFNγ production by CD8$^+$ T cells in an SIV Tat 5435-6 responder (Fig. 2a). Based on the sequence similarity between Tat KVA10 (KKETVEKAVA) and the $Tat_{87–96}$ sequence KKAKANTSSA (KSA10) within Tat 5429, we compared Tat KSA10 and additional smaller peptides against the 15-mer peptide Tat 5429 at varying concentrations by IFNγ ICS (Fig. 2b). As expected, Tat KSA10 stimulated IFNγ production at lower concentrations than the other putative epitope peptides tested as well as the 15-mer peptide Tat 5429 suggesting that KKAKANTSSA ($Tat_{87–96}$, KSA10) was the minimal optimal epitope recognized by Tat 5429 responders.

*SIV Tat KSA10 and KVA10 CD8$^+$ T cell epitopes are presented by Mane-A*10*

To determine the MHC restriction of SIV Tat KSA10- and KVA10-specific CD8$^+$ T cells, we compared MHC class I genotyping of pigtail macaques responding to each epitope. Of the alleles typed for, only a single shared MHC class I allele, *Mane-A*10*, was common to SIV Tat KSA10 and KVA10 responders suggesting that both responses were likely restricted by *Mane-A*10* (Table 1). Further, we have previously identified an immunodominant *Mane-A*10*-restricted SIV Gag CD8$^+$ T cell epitope KP9 ($Gag_{164–172}$, KKFGAEVVP) (Fernandez et al., 2005; Smith et al., 2005b; Smith et al., 2005c). The shared N-terminus lysine repeat sequence motif in SIV Gag KP9 and both SIV Tat KSA10 and KVA10 epitopes further suggested that the two Tat CD8$^+$ T cell epitopes were also *Mane-A*10*-restricted. Since *in vitro* folding of MHC I/peptide complexes is intolerant of extensions or truncations to the optimal minimal peptide, we folded Tat KSA10 and KVA10 peptides with *Mane-A*10* molecules *in vitro*. We successfully folded both *Mane-A*10*/Tat KSA10 and *Mane-A*10*/Tat KVA10 tetramers (not shown). We then used PE-labeled tetramers to stain PBMC obtained at week 3 and 14 after SIV infection of 12 of the 13 *Mane-A*10*$^+$ animals from within the SIV infection study, including the animals with strong responses to these peptides identified by the earlier IFNγ ICS study (Table 2). Tetramer staining with *Mane-A*10* folded around either SIV Tat KSA10 or SIV Tat KVA10 peptides

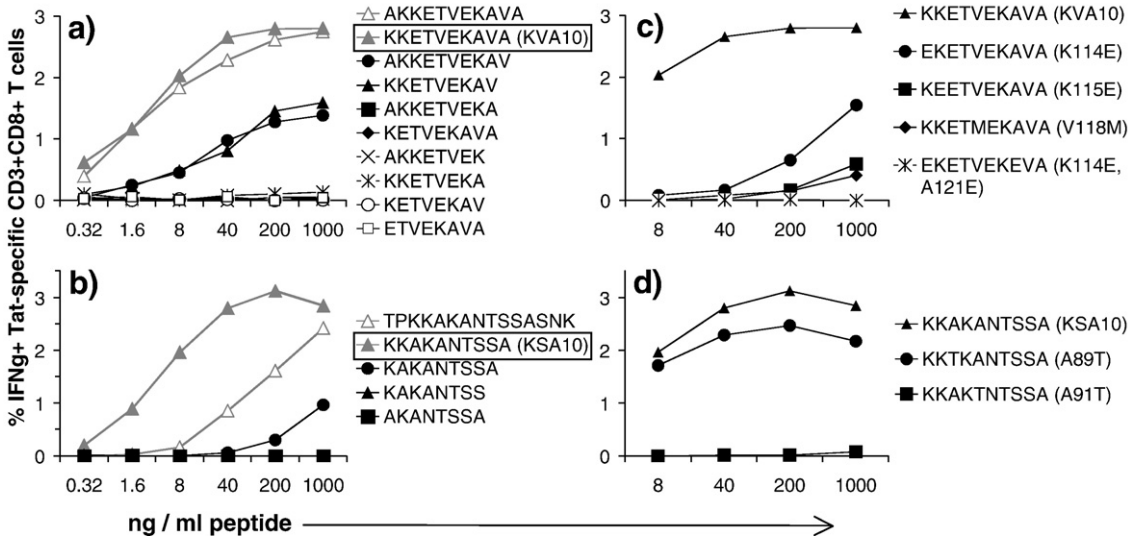

Fig. 2. Identification of minimal optimal CD8$^+$ T cell epitopes within SIV Tat and effect of escape mutations. Whole blood was stimulated with varying concentrations of overlapping 8-thru 11-mer synthetic peptides spanning amino acids (a) 114–123 and (b) 87–96 of SIV Tat and stained for intracellular IFNγ in order to identify the minimum sequence required for optimal stimulation of SIV Tat-specific CD8$^+$ T cells. The relative ability of synthetic peptides corresponding to (c) KVA10 and (d) KSA10 wild-type peptides and corresponding variant epitope sequences to stimulate CD8$^+$ T cell responses was measured by IFNγ ICS on whole blood following *in vitro* re-stimulation with a range of peptide concentrations.

Table 2
Frequency of tetramer$^+$CD3$^+$CD8$^+$ T cells in SIV-infected pigtail macaques 3 and 14 weeks post-infection.

Animal	Mane A*10 tetramer						Viral load (log10 RNA copies/ml)	
	SIV Gag KP9		SIV Tat KSA10		SIV Tat KVA10			
	Week		Week		Week		Week	
	3	14	3	14	3	14	14	36
1.3731	1.82*	1.21	0.07	1.04	2.81	0.27	3.11	4.42
8014	0.29	0.47	0.14	0.11	0.72	0.12	3.11	4.63
8020	1.38	4.53	0.07	0.43	1.46	0.19	3.80	4.94
8240	1.68	2.34	0.08	1.69	1.99	7.67	3.11	3.11
8241	0.05	0.60	0.03	2.22	1.47	0.14	5.19	6.38
8244	0.64	2.94	0.07	0.08	2.77	0.11	3.72	4.56
8454	1.23	2.27	0.36	1.47	0.57	0.21	3.11	3.11
9017	1.12	6.11	0.13	0.65	1.38	0.45	3.23	3.58
9020	1.12	1.22	0.05	0.22	0.85	0.28	3.11	3.11
9021	0.98	3.11	0.05	2.37	0.66	0.93	4.99	3.86
9175	0.30	0.75	0.01	0.08	1.14	0.30	3.48	4.91
9176	0.50	0.26	0.06	0.05	0.18	0.06	3.40	3.60

* Frequency of tetramer$^+$ cells are reported as a percentage of total CD3$^+$CD8$^+$ T cells with background tetramer FMO values subtracted. Shaded numbers are >0.15%.

confirmed that the majority of the *Mane-A*10*$^+$ animals responded (>0.15% frequency of CD8 T cells) to either or both Tat epitopes by week 14. All 12 of the SIV infected animals had tetramer$^+$ CD8 T cell responses specific for the SIV Gag KP9 epitope by week 14. In contrast, at 3 weeks after SIV infection, responses to KVA10 and KP9 were much more common than responses to KSA10, suggesting that this epitope is subdominant early in infection. We also studied the avidity of the KSA10 and KVA10 tetramer binding, in comparison to the KP9 tetramer, to CD8 T cells obtained at week 14 from animal 8240 which had robust responses to all 3 epitopes. The avidity of CD8 T cells to all 3 epitopes was similar (Fig. 3).

CD8$^+$ T cells specific for SIV Tat KVA10 but not KSA10 select for escape mutations during acute SIV infection

Rhesus macaques that express the *Mamu-A*01* MHC class I allele also respond to both a Gag (CM9) and a Tat (SL8) CTL epitope (Allen et al., 2000). The Tat SL8 epitope, but generally not the Gag CM9 epitope, undergoes immune escape during acute SIV infection (Price et al., 2004). Having identified 2 novel SIV Tat *Mane-A*10*-restricted epitopes, we sought to determine whether SIV Tat KSA10- and KVA10-specific CTL selected for escape mutations at these epitopes and compared them to the SIV Gag KP9 epitope. To assess putative CTL escape mutations within SIV Tat KSA10 and KVA10 we amplified Tat from plasma viral RNA and sequenced across both epitopes for all *Mane-A*10*$^+$ animals at multiple timepoints from 2 through to 36 weeks p.i. (Table 3). Multiple non-synonymous mutations within Tat KVA10 were detected in most *Mane-A*10*$^+$ animals as early as 3 weeks p.i. whereas mutations in Tat KSA10 were not detectable until at least 12 weeks p.i.. For comparison we also sequenced the same animals across the immunodominant SIV Gag KP9 epitope for which we have previously described selection of a specific CTL escape mutation (K165R) that exacts a significant fitness cost to the virus (Fernandez et al., 2005). Similar to SIV Tat KSA10, no mutations were present within SIV Gag KP9 3 weeks p.i., but escape, predominantly to the K165R variant, was present in most animals by week 20 (Fig. 4). Several mutations evolved within both SIV Tat KSA10 and KVA10 that were common to multiple animals although unlike the Gag KP9 epitope there was no single dominant pattern of mutation in either Tat epitope. Nonetheless, it appeared that mutations were somewhat more uniform within SIV Tat KSA10 than in Tat KVA10. Three mutations appeared in multiple animals within Tat KSA10 (A89T, A91T and S95P) whereas 5 mutations were detected in Tat KVA10 (K114E, K115E, V118M, E116K and V122M) in multiple animals.

We next examined whether common mutations identified within the SIV Tat KSA10 and KVA10 epitopes were in fact CTL escape mutations. We selected putative CTL escape mutations in both epitopes which were shared by multiple animals and compared T cell recognition of variant peptides incorporating these mutations with wild-type peptides by IFNγ ICS on fresh blood. For SIV Tat KVA10 variant peptides tested there was a significant difference in T cell recognition even at the highest peptide concentration tested between wild-type KVA10 and variant peptides incorporating common mutations observed in sequencing across the epitope (Fig. 2c). CD8$^+$ T cell response to wild-type KVA10 peptide was sustained even at low peptide concentrations (down to 8 ng/ml) while recognition of variant peptides was completely abrogated indicating that the single K114E, K115E, V118M and dual K114E and A121E substitutions all confer escape from SIV Tat KVA10-specific CD8$^+$ T cells. Analysis of T cell responses to wild-type SIV Tat KSA10 and variant peptides incorporating single A89T and A91T substitutions showed that A91T completely abrogated T cell recognition even at the highest peptide concentration tested while the A89T substitution had minimal effect on T cell recognition for all peptide concentrations tested (Fig. 2d). This suggests that for SIV Tat KSA10, A91T but not A89T confers escape from SIV Tat KSA-specific CD8$^+$ T cells. We did not detect any sequence variation within SIV Tat KSA10 and KVA10 epitopes in several animals not expressing the *Mane-A*10* allele when plasma RNA was sequenced 16 to 20 weeks p.i. (data not shown), suggesting that the mutations observed in *Mane-A*10*$^+$ animals evolved due to immune selection pressure by SIV Tat-specific CD8$^+$ T cells.

Complete escape from SIV Tat KVA10-specific CD8$^+$ T cells during acute infection

It has been suggested that the rate of CTL escape may be linked to functional avidity of the CTL response wherein CTL responses which rapidly select for escape mutations (i.e. acute-phase CTL escape) are of higher functional avidity than those which select for escape mutations later in infection (O'Connor et al., 2002b). We therefore assessed the kinetics and extent of CTL escape within SIV Tat KSA10 and KVA10 in *Mane-A*10*$^+$ macaque 9021 by cloning SIV Tat from plasma viral RNA and sequencing individual clones (Table 4). There was a clear difference between both the rate and extent of CTL escape within SIV Tat KSA10 and KVA10 epitopes. There was no evidence of CTL escape in SIV Tat KSA10 until 18 weeks p.i. and even after the emergence of CTL escape mutations at 18 weeks p.i. wild-type SIV Tat KSA10 sequence still accounted for at least 25% of the viral quasispecies out to 36 weeks p.i., the last timepoint tested. By contrast, CTL escape mutations in SIV Tat KVA10 were detected as

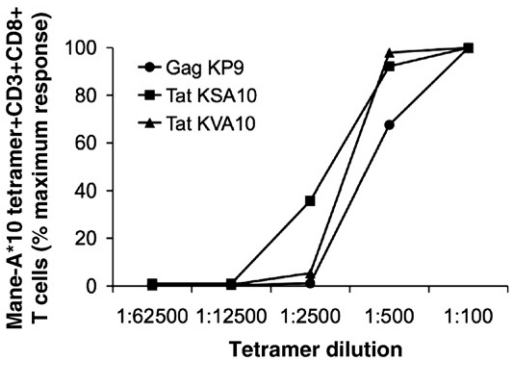

Fig. 3. Avidity of CD8 T cells for KSA10, KVA10, and KP9 epitopes. PBMC from animal 8240 obtained 14 weeks after SIV infection was stained with various dilutions of the *Mane-A*10* tetramers. Previous titration series showed that the optimal dilution of tetramer staining for each tetramer was ~1:500 (not shown).

Table 3
Sequence changes across SIV Tat KSA10, Tat KVA10 and Gag KP9 epitopes in *Mane-A*10*$^{+}$ animals.

Animal	Week	KSA10 Tat epitope	Animal	Week	KVA10 Tat epitope	Animal	Week	KP9 Gag epitope
1.3731	2	KKAKANTSSA	1.3731	2	KKETVEKAVA	1.3731	2	KKFGAEVVP
	3	————————		3	-EK—————		3	————————
	16	———————P-		24	————————		20	-R———————
	24	———————P-		36	————————			
	36	–T———P-						
8014	2	KKAKANTSSA	8014	2	KKETVEKAVA	8014	2	KKFGAEVVP
	3	ND[a]		3	——M———		3	————————
	16	————————		28	-E———————		20	-R———————
	28	———————P-		36	-E———————			
	36	———————P-						
8020	2	KKAKANTSSA	8020	2	KKETVEKAVA	8020	2	ND[a]
	3	————————		3	————————		3	————————
	16	————————		18	-T———————		20	-R———————
	28	–T-T———		28	-T———————			
	36	–T-T———		36	-T———————			
8240	2	KKAKANTSSA	8240	2	KKETVEKAVA	8240	2	KKFGAEVVP
	3	————————		3	–K-M—M-		3	————————
	12	————————		18	E————————		20	ND[a]
	28	———————T-		36	E————————			
	36	———————PT-						
8241	2	KKAKANTSSA	8241	2	KKETVEKAVA	8241	2	KKFGAEVVP
	3	ND[a]		3	-EK-M———		3	————————
	16	——T—P-		18	E————————		20	————————S
	28	———————P-		36	E————————			
	36	———————P-						
8244	2	KKAKANTSSA	8244	2	KKETVEKAVA	8244	2	KKFGAEVVP
	12	————————		3	——M—M-		3	————————
	28	–T-T———		18	E————————		20	-R———————
	36	–T-T———		28	-E———————			
				36	-E———————			
8454	2	KKAKANTSSA	8454	2	KKETVEKAVA	8454	2	KKFGAEVVP
	12	————————		3	-E———————		3	————————
	24	——T———		12	-E———————		20	ND[a]
	28	ND[a]		18	-E———————			
9017	2	KKAKANTSSA	9017	2	KKETVEKAVA	9017	2	KKFGAEVVP
	3	————————		3	–KTA———		3	ND
	28	————————		12	——A———		20	ND
	36	————————		18	—————E–			
				36	E————E–			
9020	2	KKAKANTSSA	9020	2	KKETVEKAVA	9020	2	KKFGAEVVP
	3	————————		3	ND[a]		3	————————
	28	————————		14	-E———————		20	ND[a]
	36	–T—————		36	ND[a]			
9021	2	KKAKANTSSA	9021	2	KKETVEKAVA	9021	2	KKFGAEVVP
	3	————————		3	-E———————		3	ND
	14	————————		14	-E———————		20	-R———————
	16	–T—————		16	-E———————			
	20	——T———		18	-E————M-			
	36	——T———		28	E—————M-			
9175	2	KKAKANTSSA	9175	2	KKETVEKAVA	9175	2	KKFGAEVVP
	12	————————		28	————————		3	————————
	28	–T-T———		36	————————		20	-R———————
	36	–V————V						
9176	2	KKAKANTSSA	9176	2	KKETVEKAVA	9176	2	KKFGAEVVP
	3	ND[a]		3	————————		3	————————
	12	————————		18	————————		20	————————
	36	————————		36	E————————			
9183	2	KKAKANTSSA	9183	2	KKETVEKAVA	9183	2	KKFGAEVVP
	3	————————		3	ND[a]		3	————————
	28	————————		16	E————————		20	-R———————
	36	———H–P-		36	E————————			

Results of bulk PCR sequence analysis including mixed bases.
[a] Not determined.

early as 3 weeks p.i. and from 14 weeks p.i. onwards 100% of KVA10 clones sequenced contained CTL escape mutations indicating complete escape from KVA10-specific CTL.

Discussion

HIV/SIV-specific CD8^{+} T cells clearly play a crucial role in controlling viral replication and an effective HIV vaccine will likely require generation of CD8^{+} T cell responses with potent anti-viral activity (Borrow et al., 1994; Goulder and Watkins, 2008; Jin et al., 1999; Koup et al., 1994). Although numerous HIV epitopes have been identified there are clear differences in the efficacy of CD8^{+} T cells specific for different HIV/SIV protein antigens (Loffredo et al., 2005). Escape from HIV/SIV-specific CD8^{+} T cells frequently undermines effective control of viral replication (Allen et al., 2000; Barouch et al., 2002; Borrow et al., 1997; Goulder et al., 1997; O'Connor et al., 2002a; Phillips et al., 1991). Our group and others have shown that Gag-specific CD8^{+} T cells are more effective at controlling viral replication

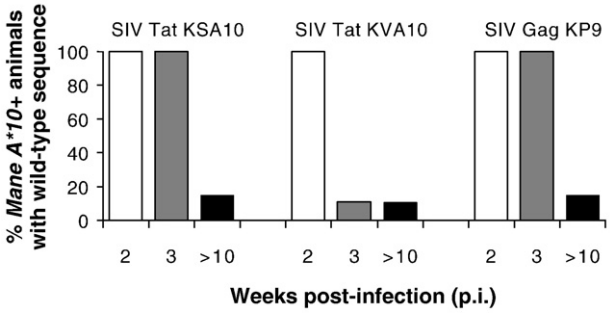

Fig. 4. Early immune escape at KVA10 Tat epitope. The proportion of 13 *Mane-A*10*$^+$ animals with wild-type sequence across the SIV Tat epitopes KSA10 and KVA10 and the SIV Gag epitope KP9 is shown over time after SIV infection. The data is derived from Table 3; at least 9 of the 13 animals yielded viable sequence at each time point. Week>10 refers to multiple time points sequenced between weeks 12–36 p.i..

than Env-specific CD8$^+$ T cells (Kiepiela et al., 2007; Peut and Kent, 2009). However, less is known about the utility of CD8$^+$ T cell responses to SIV regulatory/accessory proteins. We detected CD8$^+$ T cells targeting a pool of peptides spanning all SIV regulatory/accessory proteins in most SIV-infected pigtail macaques and found that Nef and Tat were the most commonly targeted proteins. Further investigation revealed that SIV Nef-specific CD8$^+$ T cells targeted a unique epitope in each Nef responder; however, multiple Tat responders recognized one of two SIV Tat regions which we mapped to SIV Tat_{87-96} (KSA10) and SIV $Tat_{114-123}$ (KVA10) epitopes. We subsequently showed that both Tat epitopes are restricted by a common pigtail macaque MHC class I molecule, *Mane-A*10*, which also restricts the immunodominant SIV $Gag_{164-172}$ KP9 CD8$^+$ T cell epitope (Smith et al., 2005a).

Viral sequencing across both SIV Tat KSA10 and KVA10 epitopes in *Mane-A*10*$^+$ animals identified multiple mutations that conferred escape from CTL recognition. There was a more rapid and complete shift away from wild-type sequence in SIV Tat KVA10 as compared to Tat KSA10 or Gag KP9. The occurrence of pigtail macaque SIV Tat and Gag epitopes restricted by the same high-frequency MHC class I molecule yet with widely differing escape kinetics mirrors to some extent the SIV Tat_{28-35} SL8 and $Gag_{181-189}$ CM9 responses observed in *Mamu-A*01*$^+$ rhesus macaques (Table 5) (Allen et al., 2000; Barouch et al., 2003; Friedrich et al., 2004a; Loffredo et al., 2007). Although both Tat SL8- and Gag CM9-specific CTLs are present at similar frequencies during acute SIV infection of rhesus macaques, only Tat SL8-specific CTL rapidly select for escape mutations during acute infection (Loffredo et al., 2007; Mothe et al., 2002). We speculate that the SIV Tat KVA10 response in pigtail macaques is analogous to SIV Tat SL8 response, whereas the SIV Tat KSA10 response is more analogous to the SIV Gag KP9 and CM9 responses.

Table 4
Cloning and sequencing across SIV Tat KSA10 and KVA10 epitopes in *Mane-A*10*$^+$ animal 9021.

Week	KKETVEKAVA	# Clones	Week	KKAKANTSSA	# Clones
2	————————	7/7	2	————————	7/7
3	————————	2/7	3	————————	25/25
3	-E———————	1/7			
3	———————M-	1/7			
3	——A———	2/7			
3	————E—	1/7			
12	————————	1/13	12	————————	2/2
12	—K————	1/13			
12	-K—————	11/13			
14	-E———————	13/14	14	————————	3/3
14	-EK—————	1/14			
16	-E———————	18/18	16	————————	1/1
			18	————————	10/14
				——T———	3/14
				———————P-	1/14
20	-E———————	15/17	20	————————	5/8
20	-E————M-	2/17	20	-T———————	1/8
			20	——T———	2/8
			36	————————	3/11
			36	———————T	2/11
			36	——T———	5/11
			36	——T——T	1/11

Table 5
Characteristics of rhesus and pigtail macaque CD8$^+$ T cell epitopes.

Species	MHC class I restriction	Protein (amino acid position)	Sequence	Viral escape detected (acute/chronic)
Rhesus	*Mamu-A*01*	Tat (28–35)	STPESANL	Acute
	*Mamu-A*01*	Gag (181–189)	CTPYDINQM	Chronic
Pigtail	*Mane-A*10*	Tat (87–96)	KKAKANTSSA	Chronic
	*Mane-A*10*	Tat (114–123)	KKETVEKAVA	Acute
	*Mane-A*10*	Gag (164–172)	KKFGAEVVP	Chronic

In *Mamu-A*01*$^+$ rhesus macaques, the difference in timing of escape of CTL epitopes has been linked to the "functional avidity" of the CTL response wherein CTL responses that drive rapid escape during acute infection are more potent and effective than those which select for escape mutations more slowly (O'Connor et al., 2002a). We found similar avidity for all 3 *Mane-A*10* restricted epitopes studied. Acute-phase CD8$^+$ T cell responses rapidly kill virus-infected cells and facilitate control of virus but emergence of CTL escape mutations in these epitopes enables virus to persist. Indeed, in rhesus macaques, Tat SL8-specific CTLs have been shown to have potent antiviral activity during acute infection and *in vitro* studies confirm that Tat SL8-specific CTLs are more effective at suppressing viral replication than Gag CM9-specific CTL (Loffredo et al., 2005; Mandl et al., 2007). However, there is little or no fitness cost associated with escape mutations in Tat SL8 and these mutations persist even in *Mamu-A*01*-animals (Friedrich et al., 2004a; Mandl et al., 2007). By contrast, mutations in SIV Gag CM9 develop more slowly and are often accompanied by compensatory mutations in the flanking sequence and completely revert to wild-type in *Mamu-A*01*-animals (Friedrich et al., 2004a; Friedrich et al., 2004b; Peyerl et al., 2003). Thus, it appears that while SIV Tat SL8-specific CTLs are potent inhibitors of viral replication during acute infection, Gag CM9-specific CTLs facilitate control of viral replication during chronic infection by targeting a constrained epitope less able to tolerate escape mutations. The comparative functional profiles of these SIV CD8 T cells (either ability to express multiple cytokines/chemokines or kill virus infected targets) could also impact upon their ability to select escape mutations but remains to be determined.

The rapid and complete selection of mutations in SIV Tat KVA10, which persists through chronic infection, suggests that KVA10 mutations may be well tolerated by the virus and that SIV Tat KVA10-specific CD8$^+$ T cells are only effective in controlling wild-type virus during acute-phase viremia. The KVA10-specific CTLs commonly declined later after acute infection, consistent with the early emergence of escape. By contrast, the slower generation of KSA10-specific CTLs and the slow emergence of mutations in SIV Tat KSA10 suggest that this epitope may be subdominant during early infection but potentially exact a greater fitness cost to viral replication. The persistence of the A89T mutation at later timepoints despite lack of significant escape from Tat KSA10-specific CD8$^+$ T cells is also notable. Additional studies testing virus with mutated SIV Tat KSA10 or KVA10 sequence in *in vitro* replication fitness assays or *in vivo* reversion studies in *Mane-A*10* negative animals should further assess the utility of SIV Tat KSA10- and KVA10-specific CD8$^+$ T cells. A prediction from our studies is that the region in Tat targeted by the KSA10 response is more functionally constrained (either within Tat or in the overlapping reading frames) compared to the KVA10 or SL8 regions of Tat. This can now be directly analyzed in functional studies by

expressing mutated Tat proteins *in vitro* and assessing their effects on transcription and other functions of Tat.

The identification of 2 novel *Mane-A*10*-restricted SIV Tat epitopes with distinct CTL escape kinetics will help in evaluating the effectiveness of individual epitope-specific $CD8^+$ T cell responses in HIV/SIV infection. Since the same MHC class I molecule restricts both epitopes and are located in the same protein, they can be studied without the confounding effects of MHC restriction, timing and/or quantity of protein expressed. This is a significant advance in studying CTL responses in pigtail macaques and should provide further insights into the utility and limitations of SIV-specific CTLs. We have previously shown *Mane-A*10*$^+$ macaques have lower viral loads and delayed SIV disease progression (De Rose et al., 2008a; Smith et al., 2005b; Smith et al., 2005c) which we postulated was associated with the immunodominant *Mane-A*10*-restricted SIV Gag KP9 response. However, since it is now clear that concomitant SIV Tat KSA10- and KVA10-specific $CD8^+$ T cell responses are present in most if not all SIV Gag KP9 responders, and that the mutation patterns are reasonably uniform, we cannot be certain which of these responses, or combination or responses, are ultimately responsible for the improved control of viral replication in *Mane-A*10*$^+$ positive pigtail macaques. We are currently undertaking additional studies in *Mane-A*10*$^+$ macaques with viral vector vaccines expressing only individual SIV Gag KP9, Tat KSA10 and Tat KVA10 epitopes to delineate the protective efficacy of $CD8^+$ T cells specific for each of these epitopes.

Materials and methods

Animals

Thirty two pigtail macaques (*Macaca nemestrina*) with SIV_{mac251} infection examined in this study were part of a peptide-pulsed blood cell vaccine study (De Rose et al., 2008a). Briefly, the animals were infected with SIV_{mac251}, placed on antiretroviral therapy from weeks 3–10 and vaccinated with autologous PBMC pulsed with overlapping 15-mer SIV Gag peptides (10 animals), overlapping 15-mer peptides spanning all SIV proteins (11 animals) at weeks 4, 6, 8 and 10 or unvaccinated controls (11 animals). The peptides were supplied by the NIH AIDS reagent repository. All animals were followed for viral load and disease progression. The SIV_{mac251} was kindly supplied by Drs N. Miller and R. Pal (Pal et al., 2002) and injected intravenously at 40 TCID50 as previously described (Batten et al., 2006). Plasma SIV RNA levels were determined by real-time PCR as previously described (Dale et al., 2004). All animals in this study had MHC typing for common MHC I alleles by RSCA as previously described (Pratt et al., 2006; Smith et al., 2005b). The MHC typing included evaluating animals for the presence of the *Mane-A*10* allele which restricts the immunodominant Gag $CD8^+$ T cell epitope KP9.

Immunological assays

SIV-specific $CD8^+$ T cell responses were evaluated using intracellular cytokine staining (ICS) for SIV-specific expression of IFNγ following *in vitro* peptide re-stimulation as previously described (De Rose et al., 2008a). SIV_{mac239} and SIV_{mac251} complete peptide sets (#6204, 6205, 6207, 6443, 6448, 6449, 6450, 6883 and 8680) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Briefly, 230 μl whole blood was incubated with 1 μg/ml/peptide overlapping 15-mer SIV_{mac239} peptides or dimethyl sufloxide (DMSO) control in the presence of co-stimulatory antibodies anti-CD28 and anti-CD49d (both from BD Biosciences/Pharmingen, San Diego CA, USA) and 10 μg/ml Brefeldin A (Sigma, St. Louis MO, USA) for 6 h. Cells were labeled with anti-CD3 PE (clone SP34-2) and anti-CD8 PerCP (clone SK1), red blood cells lysed with BD FACS Lysing Solution and remaining leukocytes permeabilized with BD Perm-2 then incubated with anti-human IFNγ APC (clone B27) (all from BD Biosciences) and fixed with 1% formaldehyde (Polysciences, Inc Warrington PA, USA) for FACS analysis.

Tetramer staining

Cryopreserved PBMC were used to stain $CD8^+$ T cells with tetrameric complexes of the MHC class I protein *Mane-A*10* folded around either SIV Gag KP9, SIV Tat KSA10 or SIV Tat KVA10 peptides, constructed as previously described (Smith et al., 2005c) and kindly provided by Dr Andrew Brooks. Briefly, PE-conjugated tetramer was added to 1×10^6 PBMC and incubated for 30 min at room temperature before addition of anti-CD3 Pacific blue (clone SP34-2) and anti-CD8 PerCP (clone SK1) (both from BS Biosciences) and a further incubation for 30 min at room temperature. Excess antibody was removed by washing cells first with FACS wash buffer followed by PBS before fixing cells with 1% formaldehyde.

Amplification of vRNA from plasma

Viral RNA was extracted from cell-plasma using the QIAmp viral RNA Mini kit (Qiagen) and cDNA prepared from each RNA sample using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Hot Start High-Fidelity DNA Polymerase (Phusion) was used to amplify Gag and Tat viral sequences from cDNA by PCR according to manufacturer's protocol. The sense and antisense oligonucleotides used for PCR amplification were 5′-GAAAGGGGAAGGAGCAG-3′ and 5′-GAAAGGGGAAGGAGCAG-3′ for the *Mane-A*10*-restricted Tat KSA10 epitope, 5′-GGCAATTGGTTTGACCTTGCT-3′ and 5′-CTTGTGGAAAGTCCCTGCTG-3′ for the *Mane-A*10*-restricted Tat KVA10 epitope and 5′-CACGCAGAA-GAGAAAGTGAA-3′ and 5′-GTTCCTGCAATRTCKGAT-3′ for *Mane-A*10*-restricted Gag KP9 epitope. The following cycling conditions were used for each amplification: 98 °C for 30 s followed by 40 cycles of 98 °C for 10 s, 58 °C for 15 s, and 72 °C for 1 min, with a final extension of 72°C for 10 min. The final PCR products were analyzed by 1% agarose gel electrophoresis and corresponding bands excised and purified using Qiagen gel purification.

Viral sequencing

SIV Gag and Tat PCR fragments were gel purified (Qiagen) and used for sequencing of bulk PCR products on an ABI3130xl genetic analyzer automated sequencer as previously described (Loh et al., 2007). For analysis of individual cloned viral sequences, gel purified amplicons were cloned into pGEM-T Easy Vector (Promega) and direct sequencing of individual bacterial colonies was performed. Briefly, a single bacterial colony (~2 mm) was transferred to 15 μl distilled water and resuspended by pipeting. The cell suspension was then heated at 92 °C for 15 min and used as template for direct sequencing (Peut and Kent, 2007). Chromatograms were carefully inspected using ContigExpress (Invitrogen).

Data analysis

Flow cytometric data was analyzed with FloJo software version 7.2.2 (Tree Star Inc, Ashland OR, USA). Background DMSO or tetramer FMO subtraction was performed for all SIV-specific responses reported.

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