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Rapid Communication

Substantial envelope-specific CD8 T-cell immunity fails to control siv disease

Viv Peut, Stephen J. Kent *

Department of Microbiology and Immunology, University of Melbourne, Parkville Victoria, 3010 Australia

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ABSTRACT

It is unknown which HIV proteins to target by vaccination in order to generate the most effective CD8 T-cell immunity. We recently immunized SIV_{mac251}-infected pigtail macaques with Gag peptides or a cocktail of peptides spanning all SIV proteins, including SIV Env. High-level SIV Env-specific CD8 T-cell responses were generated and 7 novel Env-specific CD8 T-cell epitopes in 10 animals were mapped. Env-specific CD8 T-cell responses were significantly inferior to Gag-specific responses, and no better than unvaccinated control animals, in the control of SIV replication and prevention of disease. Escape mutations emerged within several Env-specific CTL epitopes, suggesting at least some pressure imparted by the Env CTL responses, but this did not correlate with significantly reduced SIV replication. We conclude Env-specific CTL may not be the most effective response to induce by vaccination.

Introduction

Structural proteins appeal as CD8 T-cell vaccine targets due to their potentially more conserved genome and tighter restraint on mutation, ostensibly to maintain structure and function. Gag is a frequent component of HIV vaccine candidates progressing through clinical trials and Env is frequently included to broaden T-cell immunity and induce antibody responses (Catanzaro et al., 2007).

Gag-specific T-cell immunity has been the focus of several recent human and macaque studies. Broad Gag-specific CD8 T-cell responses have been shown to correlate with reduced viremia compared to other HIV-1 antigens including Env in humans (Kiepiela et al., 2007; Masemola et al., 2004). Gag is presented and available for CTL-mediated killing of SIV-infected PBMCs much earlier in the viral life cycle than Env (Sacha et al., 2007). Compelling studies have shown that immune escape mutations in Gag commonly result in impaired viral fitness in both human studies of acute and chronic HIV-1 infection (Allen et al., 2005; Fernandez et al., 2005; Friedrich et al., 2004; Leslie et al., 2004), and in SHIV/SIV-infected macaques (Allen et al., 2005; Fernandez et al., 2005; Friedrich et al., 2004; Leslie et al., 2004).

Env, however, is the only HIV protein targeted by both the cell-mediated and neutralizing antibody (NAb) responses. Should Env require multiple mutations to evade both CTL and NAb, we speculated that the resultant fitness cost to the virus may slow progression to disease and limit transmission (Peut and Kent, 2007). Several macaque vaccine studies have suggested vaccines expressing multiple proteins, including Env, contribute to enhanced control of chimeric SHIV strains; however, these studies may be confounded by induction of NAb to the closely homologous challenge virus (Amara et al., 2002; Polacino et al., 1999). We recently reported that HIV-1 Env-specific CD8 T-cell responses were not effective in controlling SHIV challenge after vaccination that could not induce NAb (Peut and Kent, 2007). However, that study elicited only low levels of Env-specific CTL, the vaccination and challenge Env strains were from different subtypes, and the challenge SHIV was a CXCR-4 utilizing strain, rapidly depleting CD4 T-cells. The X4–SHIV systems do not accurately model human infection with CCR5-tropic HIV-1, the most frequently transmitted strains. The role of high levels of Env-specific CD8 T-cells in controlling a CCR5-utilizing non-human primate lentivirus requires further study.

We recently reported a therapeutic vaccine study of overlapping SIV peptides in pigtail macaques previously infected with the R5-tropic SIV_{mac251} (De Rose et al., 2008). The immunizations induced high levels of SIV-specific T-cells without inducing antibodies. This study presented an ideal opportunity to prospectively identify and map SIV Env-specific CD8 T-cell responses, and then compare the impact of Env- and Gag-specific CD8 T-cell responses upon the outcome of an R5-SIV infection.

Results

SIV Env CTL responses

To investigate Env- and Gag-specific CD8 T-cell immunity in controlling a CCR5-utilizing virus, CTL responses were tracked in all 32 SIV_{mac251}-infected pigtail macaques participating in a peptide-based

* Corresponding author. Fax: +61383443846.
E-mail address: skent@unimelb.edu.au (S.J. Kent).

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therapeutic vaccine study (De Rose et al., 2007). The vaccine strategy involved using Overlapping Peptide-pulsed Autologous peripheral blood cells (termed OPAL). We measured the proportion of CD8 T-cells specifically expressing intracellular IFNγ in response to stimulation with SIV peptide pools. Substantial T-cell immunity was induced to the relevant antigens across the vaccine groups, with mean Gag-specific CD8 T-cells of 1.58% in the OPAL-Gag group and 0.37% in the OPAL-All group, 2 weeks after the last vaccination. At this same time point, mean Env-specific responses in the OPAL-Gag and OPAL-All group were 0.15% and 11.76% respectively (Table 1). After the antiretrovirals were withdrawn at week 10, there was an overall 10-fold reduction in plasma SIV viral load (VL) in both treatment groups compared to unimmunized controls during the 1 year follow up (De Rose et al., 2008).

To determine the utility of Env-specific CTLs, we undertook an extensive process of identifying, confirming and mapping Env-specific CD8 T-cell responses. Antigen-specific CD8 T-cells releasing IFNγ were quantified by intracellular cytokine staining (ICS) as previously described (Dale et al., 2004), using 15mer SIV peptides overlapping by 11 amino acids (kindly supplied by the NIH AIDS reagent repository). Responses were mapped to individual 15mers and minimal CD8 T-cell epitopes were identified using ICS titration assays of candidate peptides as shown in Fig. 1 (Peut and Kent, 2007). Seven Env-specific CD8 T-cell epitopes were identified in 10 animals. Three epitopes, $HF8_{226–233}$, $SL10_{524–533}$ and $DL9_{679–687}$ were identified in multiple animals (Table 1). The 3 animals recognising the epitope SL10 had remarkably strong peak responses of 12–54% of all CD8 T-cells at week 12 post infection. The substantial Env-specific CD8 T-cell response in animal 8676 is illustrated in Figs. 1A–D, where very high levels of CD8 T-cells responding to the pool of 25 15mer Env peptides numbered 125–149 at week 14 post infection and mapped to a single 15mer peptide 131 (containing the SL10 10mer epitope) at week 16 post infection.

Role of Env CTL in controlling SIV

We then assessed the role of Env-specific CD8 T-cell responses in controlling SIV replication. We first compared VL and peripheral CD4 levels in Env and Gag CTL responders, since Gag-specific cellular immunity has been clearly linked to improved outcomes (Kiepiela et al., 2007). Our analysis was conducted on animals within the vaccine groups to assess the effect of enhancing Env- or Gag-specific T-cells by therapeutic immunization. Our primary analysis excluded *Mane*-A*10 positive animals, given these animals all mount a dominant Gag-specific CTL responses to the KP9 epitope associated with improved control of SIV (Fernandez et al., 2005, 2007; Smith et al., 2005a). Interestingly, the VL of the Env non-responders including the A*10 animals is slightly higher (0.57 $\log_{10}$ copies/mL) than the VL of the Env non-responders excluding the A*10 animals (Supplementary Table 1), perhaps reflecting immune escape at the dominant Gag KP9 epitope (data not shown) being detrimental as recently described (Fernandez et al., 2007). Our

Table 1
Env and Gag CD8 T-cell immunity

Vaccine regime	*n*	Animal no.	*Mane*-A[a]10 status	Week 12[d] % CD8 Env resp[a]	Week 12 % CD8 Gag resp[a]	Week 12 VL (ART ceased wk 10)	Euthanised week #	Mapped Env epitope	Location on Env protein (aa #)
Controls	11	6169	Neg	0.00	**0.79**	3.94			
		7992	Neg	0.05	**0.26**	5.58	44		
		8014	Pos	0.09	0.12	4.55			
		8252	Neg	0.00	0.00	5.43	48		
		8436	Neg	0.03	0.00	4.72	46		
		8868	Neg	0.14	**0.48**	4.72	46		
		8883	Neg	0.00	0.03	5.24	46		
		9017	Pos	0.00	**0.97**	3.91			
		9019	Neg	0.08	0.03	4.62	46		
		9176	Pos	0.00	0.11	3.94			
		9183	Pos	0.03	**0.39**	3.43			
		MEAN		0.04	0.29	4.55			
OPAL-Gag	10	9196	Neg	**3.12**	**0.94**	3.76		HYWDTIRF (HF8)	226–233 (C2)
		6597	Neg	**0.44**	**2.73**	4.26	44	DVFGNWFDL (DL9)	679–687 (gp41)
		8012	Neg	**0.30**	**1.00**	5.07	43	HYWDTIRF (HF8)	226–233 (C2)
		6804	Neg	0.00	**1.36**	4.97			
		8020	Pos	0.00	**4.00**	5.12			
		8241	Pos	0.10	**0.35**	5.55	44		
		8244	Pos	0.09	**0.29**	3.83			
		8454	Pos	0.08	**2.59**	3.67			
		8673	Neg	0.10	**0.79**	4.67			
		8873	Neg	[b]0.32	**1.71**	4.32			
		MEAN		0.15	1.58	4.06			
OPAL-All	11	8676	Neg	**54.20**	0.13	5.43	44	SRNKRGVFVL (SL10)	524–533 (C5)
		8682	Neg	**50.11**	0.04	4.11		SRNKRGVFVL (SL10)	524–533 (C5)
		8247	Neg	**12.57**	0.05	5.59	41	VLPVTIMSGLV (VV11)	322–335 (C2)
								SRNKRGVFVL (SL10)	524–533 (C5)
		2.3308	Neg	**5.46**	0.18	4.78	44	SRVYQILQP (SP9)	795–803 (gp41)
		8680	Neg	**3.92**	0.05	3.80		TMSAEVAELY (TY10)	484–493 (C5)
		8251	Neg	**1.76**	0.16	4.66		MNWFLNWVED (MD10)	407–416 (V4)
		8240	Pos	**0.48**	**1.05**	3.11		DVFGNWFDL (DL9)	679–687 (gp41)
		9020	Pos	0.07	**0.38**	3.11			
		9021	Pos	[b]0.29	**1.21**	3.82			
		9175	Pos	[c]0.18	**0.17**	3.50			
		1.3731	Pos	[b]0.28	**0.66**	4.48			
		MEAN		11.76	0.37	3.86			

[a] CD8 T-cell responses <0.20% were too low to sustain the fine mapping procedure or to be considered to have an antigen-specific response.
[b] Exceptions were 8873, 1.3731 and 9021 when the Env-specific responses dropped dramatically to 0.06%, 0.13% and 0.09% at week 14 post infection, precluding them from the fine mapping procedure and
[c] 9175, whose Gag-specific CD8 T-cell response more than doubled to 0.39% at week 14 post infection and was then classified as having a positive response.
[d] Week 12 post infection was 2 weeks after last vaccination.

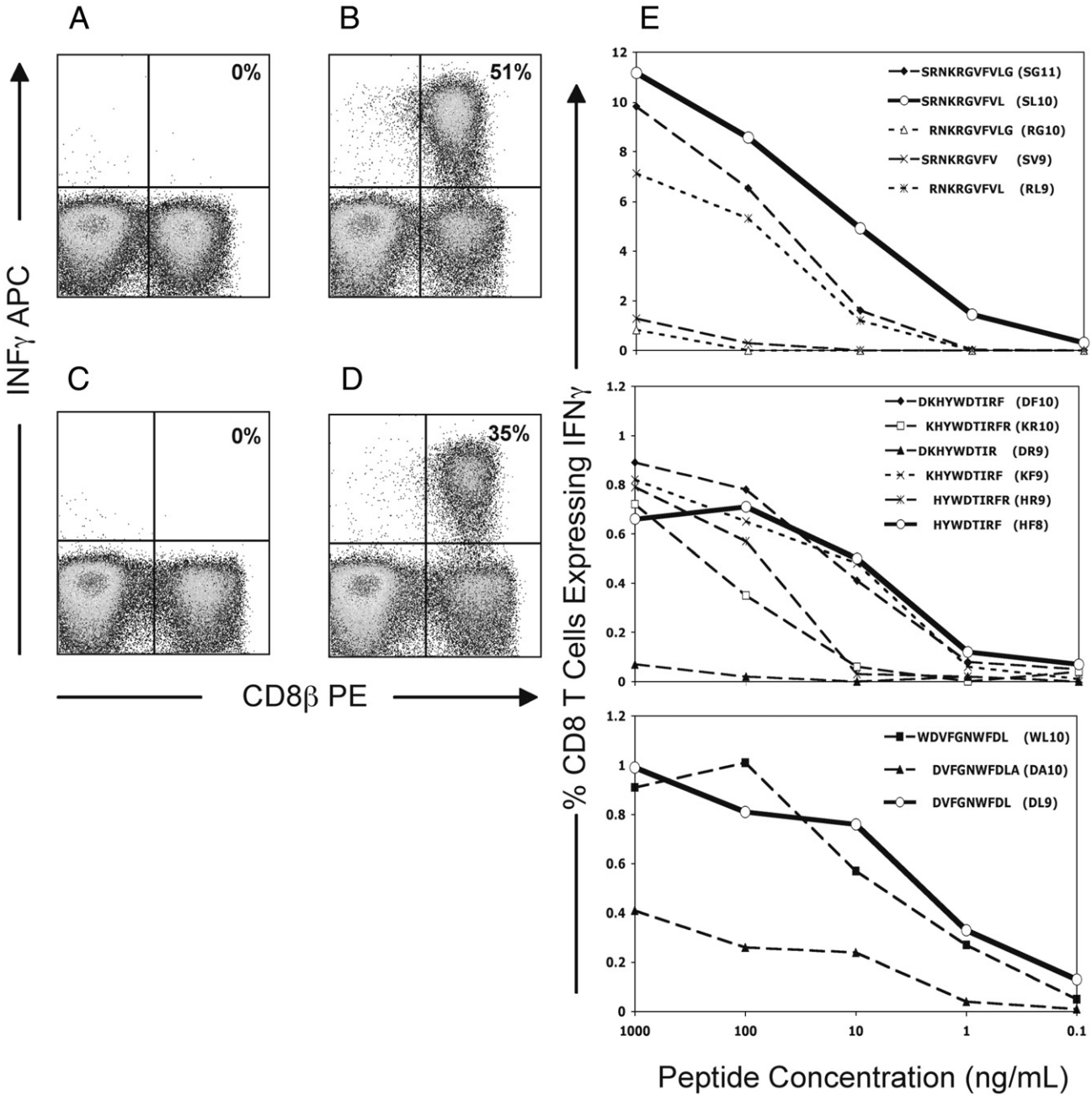

Fig. 1. Identifying minimal Env CD8 T-cell epitopes. Large Env-specific CD8 T-cell responses were mapped to individual minimal epitopes. Panels A–D illustrate FACS plots of control responses (A, C) and Env-specific responses (B, D) in 2 macaques. (A) Shows a FACS plot of a negative response to a non-responding Env peptide pool by animal 8676 at week 14 post infection. (B) 51% of all CD8 T-cells react to the peptide pool containing 25 15mer Env peptides numbered 125–149 for animal 8676 at week 14 post infection. (C) Negative control DMSO for animal 8676 at week 16 post infection. (D) 35% of all CD8 T-cells react to the single 15mer peptide number 131 at 16 weeks post infection. (E) To identify minimal SIV Env CD8 T-cell epitopes, ICS assays were performed on fresh whole blood with titration curves to ascertain the smallest candidate peptide that elicited the largest or equal largest proportion of CD8 T-cells expressing IFNγ down a concentration gradient. Examples of 3 epitopes are shown — with the minimal epitopes being SL10, HF8 and DL9 (bold lines with open circles).

results should however be interpreted cautiously given the limited numbers in each sample.

Env-only CTL responders maintained a significantly higher average VL between weeks 12–64 post infection than did animals with Gag-only CD8 T-cell responses excluding the *Mane*-A*10+ animals (5.05±0.38 $\log_{10}$ copies/mL versus 3.65±0.24 $\log_{10}$ copies/mL respectively. $P=0.039$, Fig. 2A). The average VL of the 6 Env-only responders was similar to the 7 *Mane*-A*10 negative, unvaccinated, controls (5.05±0.38 $\log_{10}$ copies/mL and 5.49±0.35 $\log_{10}$ copies/mL respectively. $P=0.445$, Fig. 2A).

To address speculation that a broad, multi-protein response could be beneficial, those animals with CD8 T-cell responses to both Env and Gag were then included in this analysis. VL for the 3 animals with both Env- and Gag-specific responses averaged 5.01±0.56 $\log_{10}$ copies/mL between weeks 12–64 post infection. This was significantly higher than the Gag-only responders ($P=0.049$) and no better than the Env-only response.

For completeness, the 9 *Mane*-A*10 positive animals in the vaccinated groups were then included and characterised on the basis of having an Env-specific CTL response or not (Supplementary Table 1). Eight of these *Mane*-A*10 positive animals had no Env response and 1 *Mane*-A*10 positive animal had both an Env and a Gag response. A similar trend was observed where having an Env-only or an Env- and Gag-specific response is no more beneficial for viremic

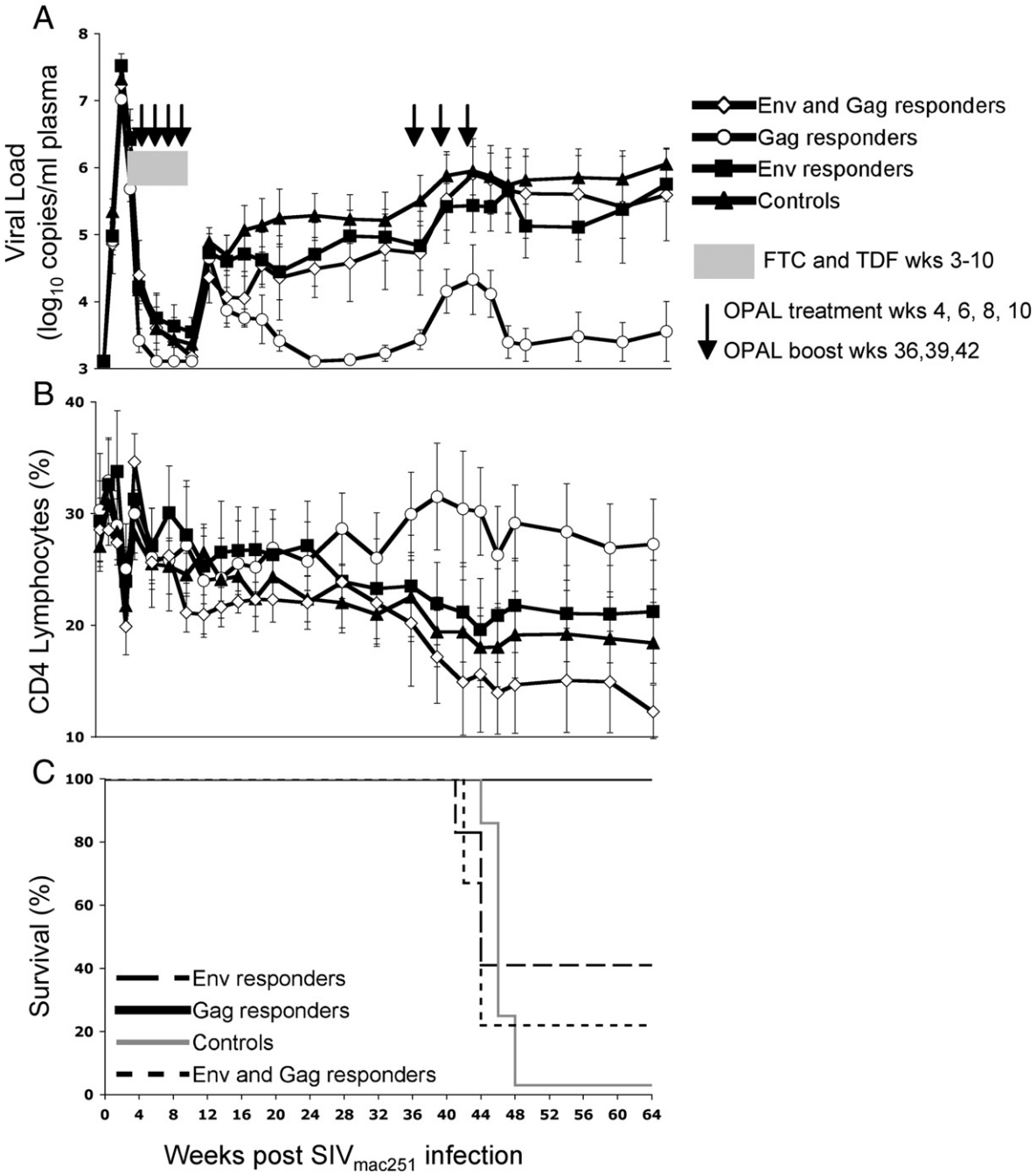

Fig. 2. CD8 T-Cell Env responders versus Gag responders. Six Env-only responders, 3 Gag-only responders, 3 animals with both Env- and Gag-specific CD8 T-cell responses and 7 unvaccinated controls were studied for – (A) Viral load. ART treatment of tenofovir (TDF) and emtricitabine (FTC) was given from weeks 3 to 10 (gray block) – daily from weeks 3–5 and 3 times per week from weeks 6–10. The OPAL vaccination therapy was given as single applications at weeks 4, 6, 8 and 10 post infection (black arrows). A further OPAL boost was given at weeks 36, 39 and 42. (B) Peripheral CD4 T-cell levels. (C) Survival graph showing 2 of 6 Env-only responders euthanised by week 44, 0 of 3 Gag responders euthanised by week 64, 2 of 3 animals with both Env- and Gag-specific responses euthanised by week 44 and 7 of 11 control animals euthanised by week 64 post infection. No *Mane*-A*10 positive animals included. A last observation carried forward analysis was used for VL and CD4 T-cell counts where animals were euthanised prior to week 64.

control than having no Env-specific response, and is significantly inferior to having a Gag-only response.

We followed the animals in this trial for just over 1 year after the last vaccination and removal of ART. This enabled an analysis of survival in animals responding to Env or Gag. During follow up, a total of 6 vaccinated animals and 6 controls of the 32 animals were euthanised with incipient AIDS, including weight loss, CD4 T-cell depletion and thrombocytopenia (De Rose et al., 2008). Animals responding to Env-only CD8 T-cell epitopes progressed to AIDS more frequently than animals with CTL responses to Gag alone (Fig. 2C).

Immune escape at SIV Env CTLs

One characteristic of an effective CTL response is its ability to force viral mutation. To identify mutations within Env-specific CTL epitopes, we sequenced multiple viral clones from plasma SIV RNA at several time-points as described (Peut and Kent, 2007) using SIV Env primers and PCR conditions shown in Supplementary Table 2. Viral mutation was identified in the 3 common Env CTL epitopes (HF8, SP9 and SL10) in 5 animals (Supplementary Table 3). The timing and rates of mutations within the CTL epitopes varied markedly (Fig. 3A). The dominant mutations within SL10 epitope, N526D and N526T, were

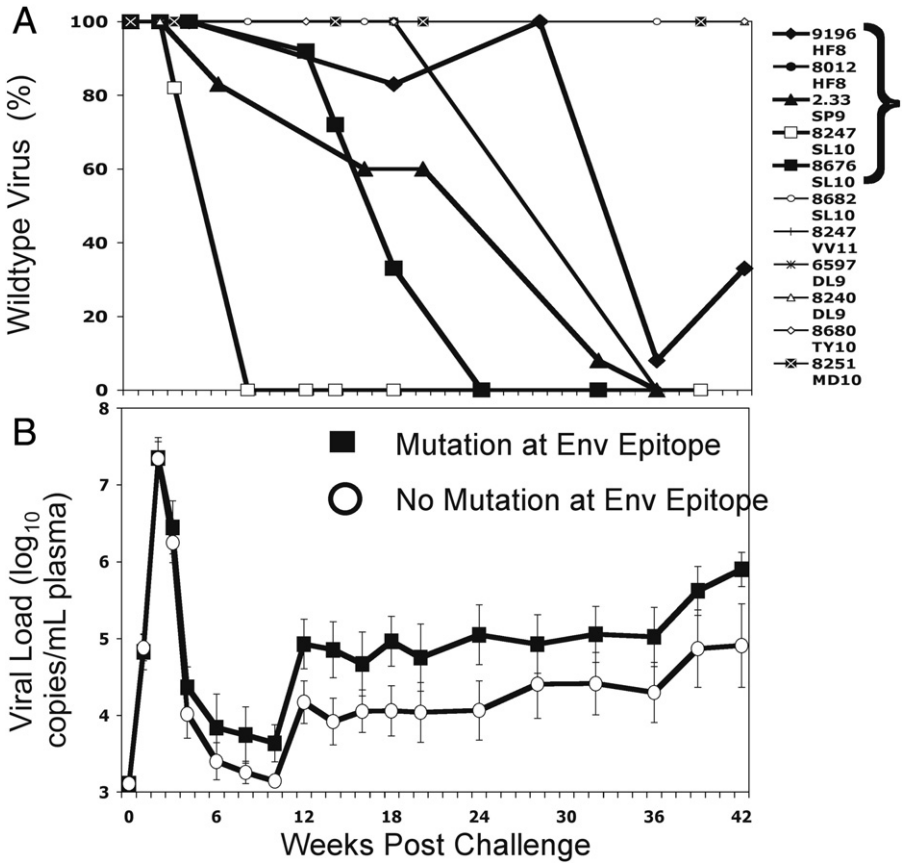

Fig. 3. Mutations within Env-specific CD8 T-cell epitopes. (A) Mutation occurred in 5 animals, representing 3 Env-specific CD8 T-cell epitopes (bracketed). (B) Comparison of average VL between animals with mutated virus at the identified CD8 T-cell epitope, and those without mutation.

indeed immune escape mutations, with 42.92% of CD8 T-cells responding to the wildtype SL10 peptide, but only 0.04% and 0.01% of CD8 T-cells specific to the mutants N526D and N526T SL10 peptides respectively. No single, common, dominant mutational motif arose in the same SIV Env-specific CD8 T-cell epitope in different animals (Supplementary Table 3), similar to our observations in immune escape from HIV-1 Env CTL epitopes in macaques (Peut and Kent, 2007). There was a non-significantly lower average VL in the 6 animals that exhibited no viral mutation at their Env-specific CD8 T-cell epitopes to week 42 post infection (including one *Mane*-A*10 animal), compared to the 5 animals that exhibited viral mutation within their defined epitopes (4.29 ± 0.39 $\log_{10}$ copies/mL and 5.07 ± 0.36 $\log_{10}$ copies/mL respectively $P = 0.428$, Fig. 3B).

Discussion

In summary, this SIV_{mac251}-macaque study highlights three concerns regarding the utility of Env as a CD8 T-cell vaccine target. First, the SIV Env-specific CD8 T-cell responses identified were unable to significantly impact viral replication or disease progression. Second, Env responses may potentially inhibit more effective CD8 T-cell responses, highlighted by the observation that a Gag-specific CTL response correlated with better control of SIV replication, yet animals with both Env- and Gag-specific CD8 T-cell responses fared no better than Env-only responders or unvaccinated controls. Third, there was no discernable effect of SIV Env-specific CD8 T-cell responses forcing common escape mutations, potentially indicating minimal constraints on mutation and implying that there may be modest fitness costs of escape from Env CTL responses.

Despite the large size of this randomized SIV-macaque study, several limitations are apparent. CD8 T-cell responses to SIV antigens other than Env or Gag were low, variable and of limited frequency. Mean Pol and combined regulatory protein-specific CD8 responses were 0.3% and 2.4%, respectively, in the OPAL-All group compared to ≤0.4% for CD8 responses to non-Gag antigens in control and OPAL-Gag groups. Their potential effect on VL and peripheral CD4 levels are likely to be important (Kiepiela et al., 2007). Further, low animal numbers in some responder groups (3–6) reduced the robustness of the findings, particularly when *Mane*-A*10 positive animals were excluded to remove a pro-Gag bias. Having now identified common Env-specific CTL epitopes in pigtail macaques, we can begin to MHC-restrict the responses, develop MHC tetramers to more sensitively detect responses and randomize future studies based on the ability to respond to these epitopes. An additional limitation is the study of only IFNγ expression as a marker of the functionality of the SIV-specific CTLs. It is becoming clearer that expression of multiple effector molecules as well as *in vitro* killing function of CTLs are likely more accurate measures of CTL effectiveness (Betts et al., 2006; Sacha et al., 2007) and future studies should assess a wider variety of functions of Env-specific CTLs.

SIV Env is a potent stimulant to the cell-mediated immune system. Unfortunately, the resultant CD8 T-cell response was of limited value in controlling SIV replication or preventing SIV disease. Our results are consistent with human observational cohorts (Kiepiela et al., 2007) and our previous work on HIV-1 Env CTL responses in macaque vaccine studies (Peut and Kent, 2007). Worse, large Env CTL responses may hinder other subdominant protein-specific CD8 T-cell responses more adept at restraining viral growth (Frahm et al., 2006; Friedrich et al., 2007). The large Env responses identified in 3 animals correlated with poor non-Env responses and very poor outcomes for the animals. Although there may be Env epitopes that are indeed useful, to date our data suggest that Env-specific CD8 T-cell responses are comparatively ineffective.

Materials and methods

Animals and vaccinations

The animals analyzed were part of a recently reported vaccination study (De Rose et al., 2008). In brief, the study involved 36 macaques injected intravenously with 40 $TCID_{50}$ of SIV_{mac251} directly as supplied by Dr R. Pal (Pal et al., 2002) as previously described (Batten et al., 2006; Smith et al., 2005a). The macaques were randomized into 3 groups of 12 animals, stratified for peak SIV viral load at week 2, weight, gender and the MHC I gene *Mane*-A*10 (known to enhance immune control of SIV (Pratt et al., 2006; Smith et al., 2005b)). Animals received subcutaneous injections of dual anti-retroviral therapy (ART) with tenofovir and emtricitibine (Gilead; both 30 mg/kg/animal) for 7 weeks from week 3 (Hel et al., 2000; Lisziewicz et al., 2005; Lori et al., 2000; Shen et al., 2003; Villinger et al., 2002). Four animals developed lethal, acute SIV infection prior to the first immunization with diarrhoea and rapid weight loss, leaving 32 animals remaining in the trial (Table 1). The two vaccine groups were immunized with PBMC pulsed with either a pool of 125 SIV_{mac239} Gag peptides or 823 peptides spanning all 9 SIV_{mac239} proteins at 10 μg/ml of each peptide within the pool (De Rose et al., 2008). The peptide-pulsed PBMC were held for 1 h at 37°C and then reinfused IV into the autologous animal. The first 4 immunizations were administered while the macaques were on ART at weeks 4, 6, 8 and 10 after infection. A second set of immunizations was undertaken at weeks 36, 39 and 42 without ART cover. Control macaques did not receive vaccine treatment.

Immunologic and virologic evaluations

T-cell immunity was studied by IFNγ intracellular cytokine staining (ICS) as previously described (Peut and Kent, 2007). Briefly, fresh blood samples (200 μl) were stimulated with overlapping SIV peptide pools for 6 h then the red cells lysed, and the remaining cells fixed and permeabilized. Cells were stained with antibodies to CD3, CD4, CD8 and IFNγ as previously described. Gated CD4+ or CD8+ T lymphocytes expressing IFNγ were analyzed by flow cytometry. To identify individual Env epitopes we performed the IFNγ ICS assay progressively smaller peptide pools and then purchased minimal using peptide epitopes and performed titration series as shown in Fig. 1 (Peut and Kent, 2007).

SIV viral load in plasma and CD4 T-cell depletion was monitored as previously described (De Rose et al., 2008). To analyze viral escape at Env CD8 T-cell epitopes, we cloned and sequenced plasma SIV RNA from animals responding at Env epitopes at multiple times during the course of infection as previously described (Peut and Kent, 2007). SIV Env primers and PCR conditions are shown in Supplementary Table 2. Differences in SIV viral load between groups of animals used a time-weighted area under the curve analysis as previously described (Peut and Kent, 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.11.030.

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