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Tat protein vaccination of cynomolgus macaques influences SHIV-89.6P$_{cy243}$ epitope variability

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Abstract In a previous study we showed that vaccination with the native Tat protein controlled virus replication in five out of seven monkeys against challenge with the simian human immunodeficiency virus (SHIV)-89.6P$_{cy243}$ and that this protection correlated with T helper (Th)-1 response and cytotoxic T lymphocyte (CTL) activity. To address the evolution of the SHIV-89.6P$_{cy243}$ both in control and vaccinated infected monkeys, the sequence of the human immunodeficiency virus (HIV)-1 Tat protein and the C2-V3 Env region of the proviral-DNA-derived clones were analyzed in both control and vaccinated but unprotected animals. We also performed analysis of the T cell epitope using a predictive epitope model taking into consideration the phylogeny of the variants. Our results suggest that even though the viral evolution observed in both groups of monkeys was directed toward variations in the major histocompatibility complex (MHC)-I epitopes, in the control animals it was associated with mutational escape of such epitopes. On the contrary, it is possible that viral evolution in the vaccinated monkeys was linked to mutations that arose to keep high the viral fitness. In the vaccinated animals the reduction of epitope variability, obtained prompting the immune system by vaccination and inducing a specific immunological response against virus, was able to reduce the emergence of escape mutants. Thus the intervention of host's selective forces in driving CTL escape mutants and in modulating viral fitness appeared to be different in the two groups of monkeys. We concluded that in the vaccinated unprotected animals, vaccination with the Tat protein induced a broad antiviral response, as demonstrated by the reduced ability to develop escape mutants, which is known to help in the control of viral replication.

Keywords SHIV · Sequence · Variability · Computer analysis · Prediction · CTL

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Introduction

Cytotoxic T lymphocyte (CTL) responses against human immunodeficiency virus (HIV)-1 or simian immunodeficiency virus (SIV) have been shown to greatly contribute to the control of viremia during primary infection [1]. Accordingly, virus escape from CTL surveillance is believed to be critical to promote progression to disease [1, 2]. Escape mutants arise during viral infection because CTL exert pressure on virus replication in vivo and contribute to the persistence of the virus [2–5]. Viral escape from CTL responses occurs through amino acid mutations close to or within an epitope essential for major histocompatibility complex (MHC) binding or T cell receptor recognition. Identification of early escape mutants as well as of wild-type variants is critical for a CTL-based vaccine and virus pathogenesis [6]. To be effective a CTL-based vaccine should target epitopes so critical that any change in their sequences would result in a major decrease of the replicative fitness of the virus [4, 7]. It has been suggested that CTL responses against epitopes expressed early upon infection, such as the Tat protein, may be important for the immune control of HIV-1 infection [8, 9]. In fact, Tat is

frequently a target of CTL in natural HIV-1 infection [8–10]. However, information on immune response during primary infection is very limited because most patients are diagnosed at much later times. In this regard non-human primate models have been essential to determine the importance of CD8 CTL in containing SIV and SHIV replication especially during primary infection [11]. In particular during SIV infection, Tat specific CTL have been shown to select for viral escape mutants within an immunodominant Tat epitope by 8 weeks post infection [12, 13].

Tat is produced early upon infection to promote and expand HIV replication and transmission [14]. In addition, Tat protein is released from infected cells and has paracrine and pleiotropic activities within the host such as the capability of modulating the generation of CTL epitopes [15]. In fact, Tat affects the subunit composition of immunoproteasomes and increases all three major proteolityc activities [15]. In this regard, alterations in proteosomal cleavage have been described as a mechanism underlying the viral escape process [2]. This may have an impact on both the control of infected cells during the natural course of the disease and the use of Tat for vaccination strategies. Our previous studies indicated that a Tat-based vaccine controlled virus replication in five out of seven monkeys after challenge with SHIV-89.6P_{cy243}, and this protection correlated with an induction of Th1 response and a CTL activity [16, 17]. Since during viral infection escape from CTL response arises in targeted epitopes of the virus, we studied the sequence evolution of the HIV-1 Tat protein and the C2-V3 Env region in controls and vaccinated unprotected monkeys starting from week 2 up to week 71 post-infection. In contrast, the vaccinated and protected animals could not be studied since each attempt to isolate virus failed. The analysis of the Tat protein, characterized by a low variability, provided indications on the selection pressure derived from the vaccination, whereas the C2-V3 Env region was chosen because it is highly variable and rapidly evolving. Our results, obtained by using computer programs which allowed the prediction of CTL epitopes, indicate that the immunological pressure exerted by the Tat vaccine was able to contain the emergence of escape mutants by the reduction of positive variability obtained by priming the immune system of the vaccinees. In contrast in the control monkeys, viral replication without the constraints of an antiviral immunoresponse may exploit changes of the viral fitness. Although the Tat vaccination in the unprotected monkeys was unable to suppress viremia and/or to eradicate the infection, still these animals showed a more effective immune response as compared to the control monkeys, as indirectly demonstrated by their ability to contain the development of escape mutants.

Materials and methods

Monkeys, virus stock, and sample collection

Adult cynomolgus monkeys of Mauritian origins were used in this study. The vaccination protocols and schedules have been reported elsewhere [16]. All animals were challenged intravenously with 10 MID_{50} of the SHIV-89.6P_{cy243} that was derived by infecting a cynomolgus macaque with SHIV-89.6P as previously described [18]. Briefly, five out of seven monkeys vaccinated with the Tat protein were protected from the challenge. In fact, p27 and viral RNA were always undetectable in plasma for up to 68 weeks post-challenge. In addition, SHIV proviral DNA was undetectable in all the protected monkeys or was only sporadically detected in a few animals and at very low copy number. In contrast, peripheral blood mononuclear cells (PBMCs) from the controls and the two vaccinated and infected animals had a very high proviral copy number from week 2 after infection throughout the follow-up period. Virus isolation with CD8-depleted PBMCs stimulated with PHA and IL-2 resulted always negative in the five protected macaques vaccinated with Tat. Low anti-SIV titers were detected in the five protected monkeys and they became negative over time. Anti-HIV-1 Env antibodies were detected only in sera from the infected animals. Two out of seven vaccinated monkeys were overtly infected with values of plasma viremia (range: 1×10^4–1×10^6) and $CD4^+$ T cell decline in the acute phase of infection comparable to those of the controls [16]. In the protected animals active viral replication and $CD4^+$ T cell decline were not observed and this correlated with the presence of a Tat-specific Th-1 response and anti-Tat CTL. The vaccinated unprotected monkey 54963 and monkey 55396, and the controls, monkey 55123 and monkey 55129, were selected for this study. DNAs from PBMCs were analyzed at weeks 2, 10, or 14 and 41 weeks post-infection (p.i.), whereas DNAs from lymph nodes were analyzed at 71 weeks p.i.

Detection of antibodies against Tat

Microtiter plates were coated with Tat (100 ng/200 µl per well of 0.05 M carbonate buffer, pH 9.6) for 12 h (4°C) and extensively washed with PBS without Ca^{2+} and Mg^{2+} containing 0.05% Tween 20 (PBS/Tween). Plasma, 200 µl diluted in buffer, was then added to each well in duplicate. Plates were incubated for 90 min (37°C), washed five times with PBS/Tween, and 100 µl horseradish peroxidase-conjugated secondary antibody (Sigma) diluted 1:1,000 in PBS/Tween (containing 1% BSA) was added for 90 min (37°C). After extensive washing of the plates, 100 µl

peroxidase substrate (ABTS 1 mM, Amersham Pharmacia Biotech, Milan, Italy) was added and the absorbance at 405 nm was measured with a spectrophotometer. A rabbit polyclonal antiserum against Tat, used at serial twofold dilutions (1:200–1:6,400), was the positive control. Monkey preimmune plasma (diluted 1:50 and 1:100) was the negative control. The mean of the negative controls +3 standard deviations represented the cut-off value. The minimal plasma dilution used was 1:50.

T-cell proliferation assays

To evaluate the proliferative response to Tat and the recall antigen tetanus toxoid (TT), Ficoll-purified PBMCs (2×10^5 per well) were seeded in flat-bottomed 96-well microtiter culture plates in triplicates in a final volume of 200 μl of RPMI containing 10% FCS (medium) and cultured either alone or in the presence of PHA (2 μg/ml), TT (5 μg/ml, Connaught, Ontario, Canada), Tat protein (5 mg/ml), or buffer [phosphate-buffered saline (PBS) containing 0.1% BSA]. After five days of incubation at 37°C in 5% CO_2, cell cultures were pulsed with 1 μCi/well of [^{3}H]thymidine (Amersham Life Science, Buckingamshire, UK) and the incorporated radioactivity was measured 18 h later, as previously described [16]. A stimulation index (SI) ≥ 3 was considered positive.

Interferon (IFN)-γ ELISpot assay

The number of IFN-γ producing cells (spot forming cells, sfc) was measured by a commercial kit (Human IFN-γ ELISpot, Euroclone, Paignton, UK), following the manufacturer's instructions. Briefly, PBMCs (2×10^5 per well, in duplicate) were cultured with medium alone or in the presence of PHA (2 μg/ml), TT (5 μg/ml) or Tat protein (5 μg/ml), or buffer in flat-bottomed 96-well plates previously coated with a mAb to IFN-γ. After 18 h, cells were removed and locally produced IFN-γ was revealed by an immunoenzymatic reaction into a gel matrix as coloured spots. The spots, a measure of IFN-γ producing cells, were counted under a light microscope and expressed as number of sfc per 10^6 PBMCs upon subtraction of background counts. Based on data obtained with PBMCs from 24 naive monkeys, a number of sfc greater than 20×10^6 cells were considered as a positive response to the antigen.

PCR amplification, cloning and sequencing

DNAs were extracted from whole blood using QIAamp DNA Blood Kit (QIAGEN) according to the manufacturer's protocol. For the amplification of the *tat* and C2-V3 *env* coding region, primers were designed for direct or nested PCR using the GenBank sequence of SHIV89.6P (accession number U89134) [19]. Specifically, for amplification of first exon of Tat the following primers were used: (5778–5797) 5′-AATCGTCACGGAGACACT CT-3′ and (6336–6316) 5′-CCACTGTCTTCTGCTCTT TCT-3′ (direct PCR); (5924–5944) 5′-TAGAAGCAT GCTGTAGAGCAA-3′ and (6219–6197) 5′-GCTACTACTAGTGCTACTATTGC-3′ (nested PCR). For amplification of the second exon of Tat the following primers were used: (7642–7663) 5′-CCATCAC AGGACA AATTAGATG-3′ and (8695–8675) 5′-CTGC GTCCCA GAAGTTCCACA-3′ (direct PCR); (8459–8480) 5′-GCAG GGATATTCACCATTATCG-3′ (nested PCR). Primers used for C2-V3 Env amplification were: (6934–6956) 5′-GTAACACCTCAGTCATTACACAG-3′ and (7891–7872) 5′-AGGAACACAGCTCCTATTCC-3′ (direct PCR); (6961–6982) 5′-GTCCAAAGGTATCCTTTCAGCC-3′ and (7444–7425) 5′-TCCCCTCCTGAGGATTGATT-3′ (nested PCR). In the first round, ~0.2 μg of DNA was added to the mixture and thermocycled with an initial denaturation step at 95°C for 12 min, followed by 35 amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s) and 1 cycle at 72°C for 10 min. Ten microliters of each amplified product in the first round was then used for the second PCR round. Control amplifications with no template were included in each experiment to monitor for carry over contamination. Amplified DNA fragments were cloned into pCR2.1 using TA cloning kit (INVITROGEN). All clones containing an insert of the expected size upon digestion with *Eco*RI restriction enzyme were submitted to sequencing using M13 forward (−20) and M13 reverse primers. Sequences were aligned by using the BIOEDIT package and were then manually adjusted. The phylogenetic tree was made by the neighbor-joining substitution model utilizing the software MEGA 3. The scale bar indicates distances.

Computer-assisted epitope prediction

The prediction of T-cell epitopes was performed following the method of Yusim et al. [20] with minor modifications. In particular, the absence of a fully reconstructed database for experimentally defined SIV/HIV-1 Mafa epitopes was overcome by means of T-cell epitope prediction using the online software SYFPEITHI (version 1.0), a database of MHC ligands and peptide motifs (http://www.syfpeithi.de/). The Mafa aplotype system is at the moment poorly investigated; as a consequence the Mamu aplotype system was used as a model in epitope prediction analysis. SYFPEITHI is a database comprising peptide sequences known to bind class I and class II MHC molecules compiled from

published reports only [21, 22]. The prediction of T-cell epitopes was also performed by using the website "MHCPathway—Macaque" (http://www.mamu.liai.org) and utilizing a cut-off of (IC50) <1000 for predicted MHC affinity. This website provides access to predictions of peptide binding to Macaque MHC class I molecules [23].

Results

Variability and evolution of the virus population in control and vaccinated unprotected monkeys

In a previous study, seven animals were vaccinated with the Tat protein. Two out of seven vaccinees (mk 54963 and mk 55396) were overtly infected with values of plasma viremia and $CD4^+$ T cell decline comparable to those of the control monkeys (mk 55123 and mk 55129) [16, 17]. Tat-specific immune responses were evaluated in these animals at several time points during the follow-up by detection of antibodies, lymphoproliferative response, and IFN-γ ELISpot against Tat. After challenge with SHIV-89.6P_{cy243}, in the vaccinees anti-Tat IgG titers were stable up to week 14, declined thereafter, and remained detectable up to week 71; conversely no anti-Tat antibodies were found in the controls. Thus, exposure to the challenge virus did not result in the induction of anti-Tat antibodies [16, 17]. In the vaccinees, proliferative responses to Tat were detected before and after challenge whereas in the controls a sporadic proliferative response was detected after challenge [16, 17]. IFN-γ ELISpot to Tat was positive in mk 54963. Of note, in this macaques a strong response to Tat correlated with negative virus isolation. In contrast, in mk 55396, from which virus was frequently isolated, no production of IFN-γ in response to Tat was detected. Similarly, none of the control animals produced IFN-γ in response to Tat. In order to evaluate whether and to what extent nucleotide sequence variability and accumulation rates of synonymous and non-synonymous substitutions varied during the follow-up period, the differences in the sequences of the *tat* gene and the C2-V3 *env* region in vaccinees and controls were investigated. Sequential samples of proviral DNA were collected from monkey PBMCs spanning a period of 41 weeks after challenge. At week 71 DNA samples were obtained after a lymph node biopsy. Five clones per each clinical sample were analyzed at each time point. All clones were compared to prototype SHIV-89.6P_{cy243} challenge stock (Fig. 1a and b). Overall, the entire follow-up period, starting from week 2 up to week 71 post infection (p.i.), was characterized by relative homogeneity of the *tat* gene since the total number of nucleotide changes in this region did not vary appreciably among all monkeys (Fig. 1a). Conversely, the C2-V3 *env* region that was analyzed at week 2 and at week 71 p.i., showed mutations in a greater number of clones compared to the *tat* gene in both control and vaccinated monkeys (Fig. 1b). No nucleotide differences were found when the controls were compared to the vaccinees. This result was emphasized by the comparison of the amino acid variability of Tat and C2-V3 Env region of each single clone obtained from control and vaccinated monkeys with the sequences from SHIV-89.6P_{cy243} (Fig. 2). In all animals, analysis of the amino acid sequence of Tat revealed a high percentage of similarity between the prototype virus and the single clones (Fig. 2a). In fact, in the vaccinated infected monkeys only a few amino acid mutations appeared in one out of five clones analyzed (mk 54963: mutation D5/G at week 2 and A21/T, P81/L at week 10; mk 55396 M1/L at week 2). Also in the two control monkeys the Tat sequence was quite homogeneous (Fig. 2a). Potential inactivating mutations were observed in some clones from both vaccinees and controls. In particular, at week 71 one clone of the vaccinated mk 54963 showed one in frame stop codon in the first *tat* exon, while at week 2 one clone of vaccinated mk 55396 had a mutation in the *tat* start codon and at week 71 the same monkey presented a frameshift in the second *tat* exon. In the control mk 55129 one clone showed a frameshift in the first *tat* exon at week 41 (Fig. 2a). When the C2-V3 Env region was analyzed, a greater variability than in Tat sequence was observed since in both the vaccinated monkeys and in the control monkey 55129, more than one clone showed amino acid changes from the prototype. These mutations were accumulating over time in both the controls and in the vaccinees. Then we analyzed the amino acid/nucleotide mutation ratio of the mutant clones with respect to the parental virus (Table 1). In this analysis the presence of values ≥ 1 indicates a higher concentration of the non-synonymous respect to the synonymous mutations. This analysis better correlates our data compared to the canonical synonymous/non-synonymous mutation ratio analysis. Different from Tat coding region, the C2-V3 Env region presented a substantially higher concentration of non-synonymous mutations.

Phylogenetic and epitope analysis of viral sequences in the control and vaccinated monkeys

To determine the evolutionary relationships of the viral variants over time, a phylogenetic analysis of the Tat protein and the C2-V3 Env region in each monkey was performed. Figures 3 and 4 show a schematic representation of the phylogenetic relationship and amino acid variations between the isolates and the parental virus SHIV-89.6P_{cy243}. We observed that in the control mk

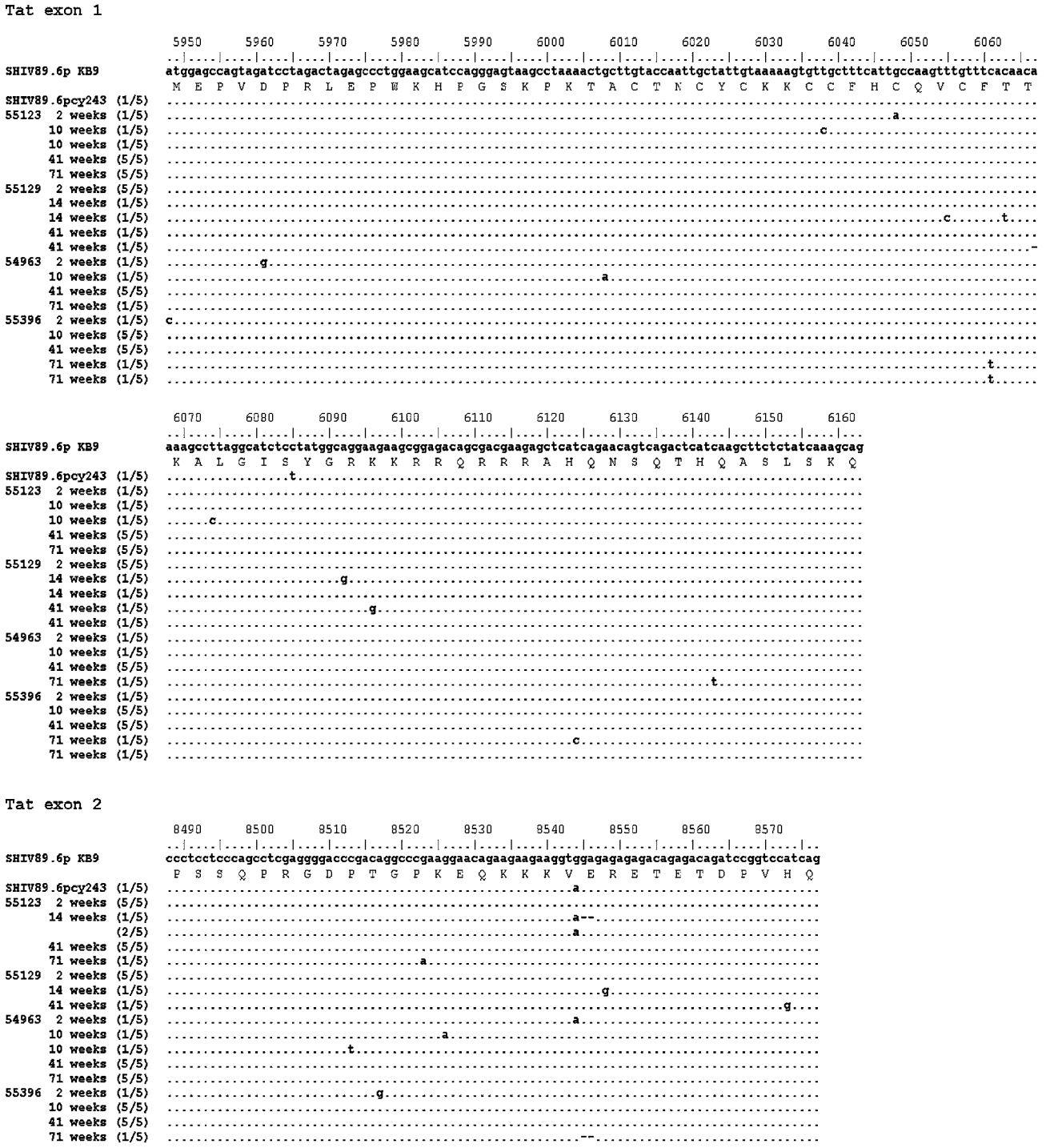

Fig. 1 Tat (**a**) and C2-V3 env (**b**) nucleotide alignments. SHIV-89.6P KB9 is used as reference sequence. Only nucleotide changes different from the consensus sequence are shown for each clone. In brackets the number of clones bearing the mutations is shown. Deleted nucleotides are indicated with dashed line (-). Mk 55123 and mk 55129 are controls, whereas mk 54963 and mk 55396 are vaccinees

55129 the first exon of *tat* in the viral population changed more than in the control mk 55123 and in the vaccinated mk 54963, whereas in the vaccinated mk 55396 no further evolution could be clearly described after 2 week p.i. (Fig. 3). Interestingly, in the controls the major selective pressure was directed to the amino acid stretch 31–50, whereas in the vaccinees selective pressure was exerted on the amino-terminal part of the protein. Moreover, a higher evolution of the C2-V3 Env region compared to the Tat protein could be observed especially in the vaccinees (Fig. 4). In fact, the viral populations in mk 55396 and in mk 54963 were characterized by three and two new different lineages, respectively, as compared to that of mk 55129 that produced only two lineages and mk 55123 that showed only 1 lineage. Interestingly, a non-conservative mutation E308K arose in both controls but not in the

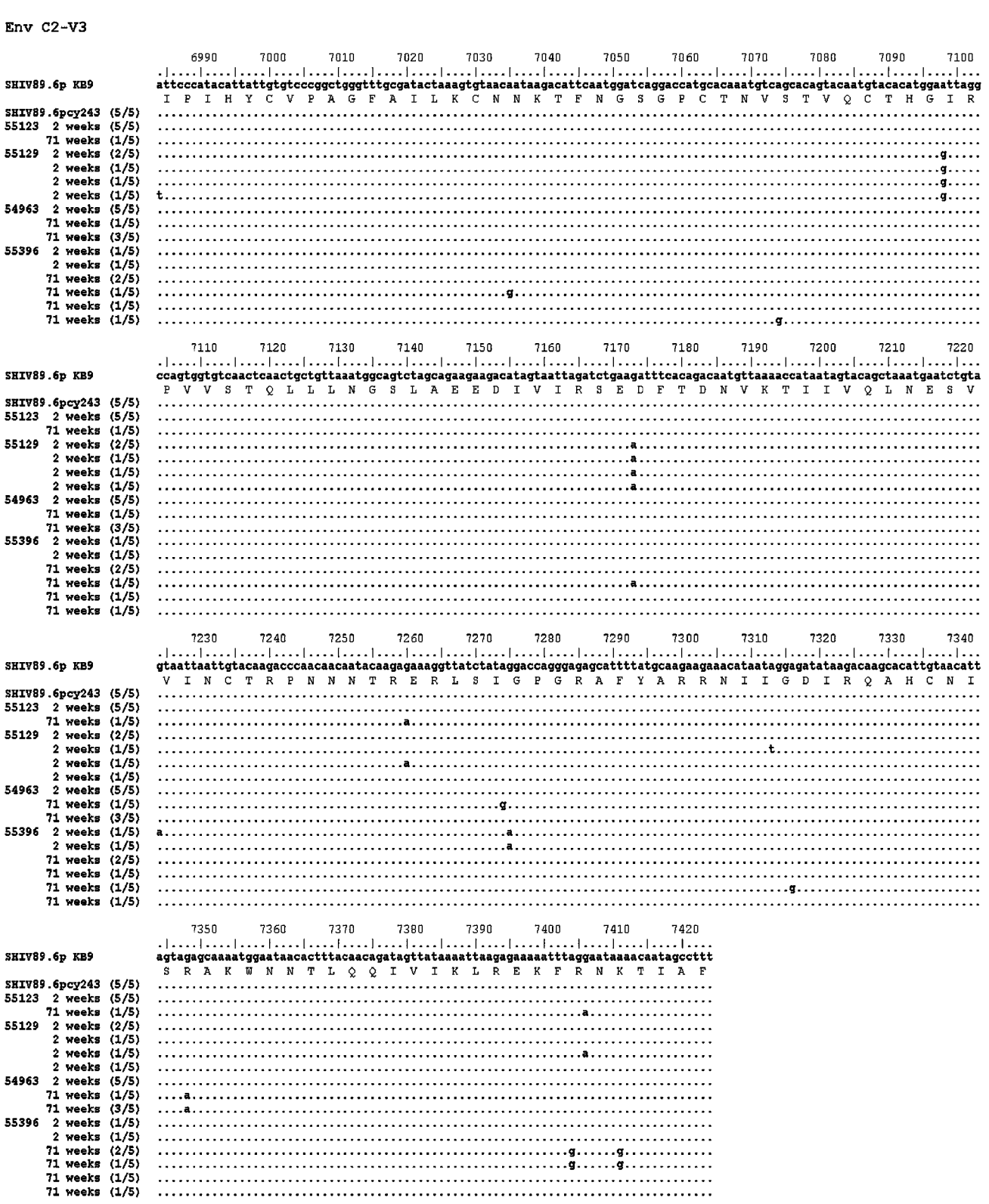

Fig. 1 continued

vaccinees. These results confirm that the differences in evolution of Tat and of C2-V3 Env region in both groups of monkeys could reflect the different selection pressure driving viral escape from CTL recognition. The MHC-I binding motifs recognized by the MHC molecules of rhesus macaques binding known $CD8^+$ T lymphocyte epitopes, analyzed by means of the data available in the SYFPEITHI database, showed some interesting features. The evolution of predicted epitopes in Tat and C2-V3 regions of all analyzed clones displayed more changes in the controls as

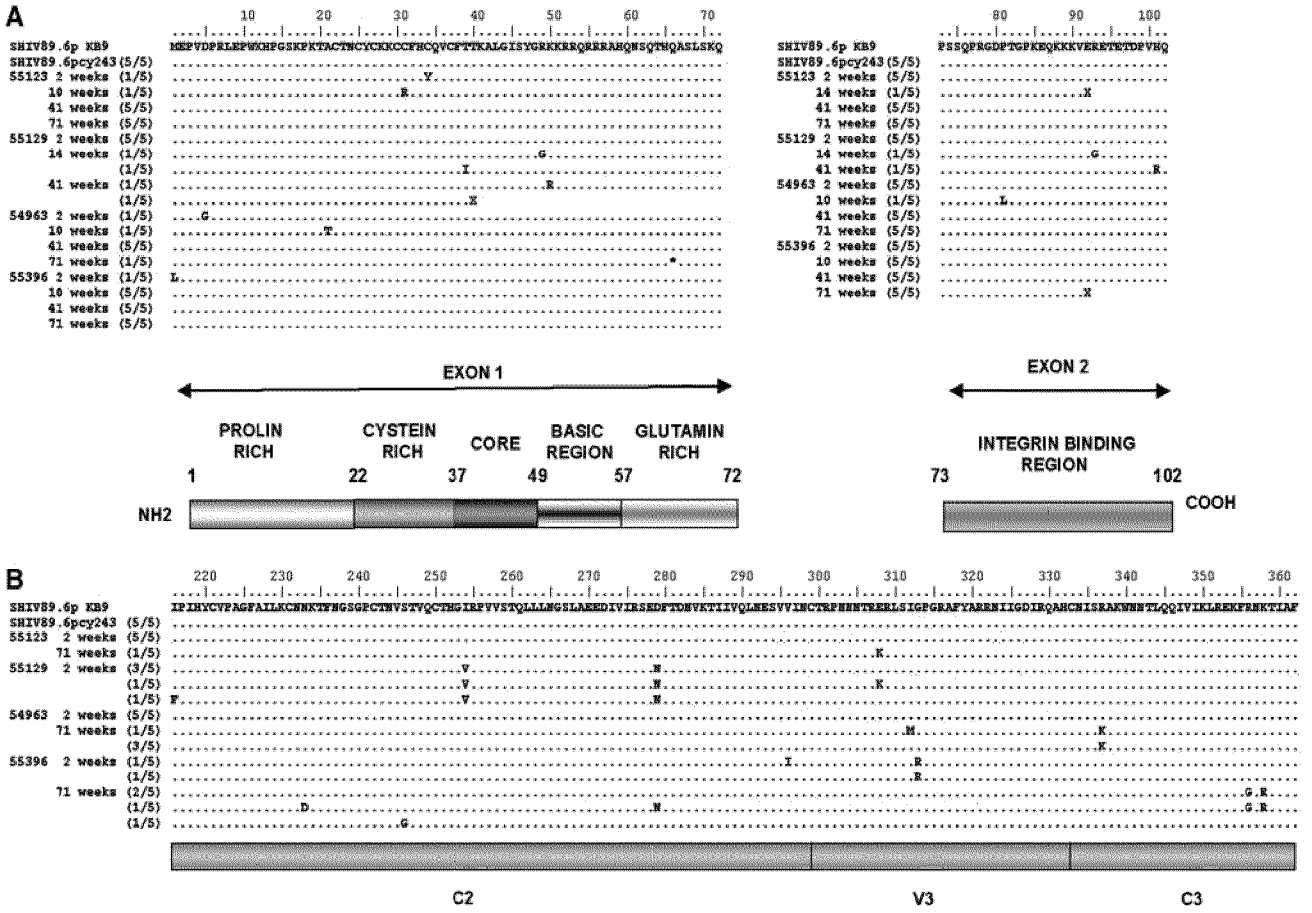

Fig. 2 Tat (**a**) and C2-V3 Env (**b**) amino acid alignments. SHIV-89.6P KB9 is used as the reference sequence. Only amino acid changes different from the consensus sequence are shown for each clone. In brackets the number of clones bearing the mutation is shown. The asterisk represents a stop codon. The "X" indicates a deletion that modifies the reading frame sequences. Mk 55123 and mk 55129 are controls, whereas mk 54963 and mk 55396 are vaccinees

Table 1 Amino acid/nucleotide mutation ratio

Animal	Week 2	Week 10/14	Week 41	Week 71
Tat exon 1				
55123	0.63	0.63	0	0
55129	0	0.83	0.63	Nd
54963	0.63	0.63	0	0
55396	0.63	0	0	0
C2-V3 Env region				
55123	0	Nd	Nd	1.48
55129	2.69	Nd	Nd	0
54963	0	Nd	Nd	3.03
55396	3.03	Nd	Nd	3.48

All sequences were compared to SHIV-89.6P$_{cy243}$ challenge virus Mk 55123 and mk 55129 are controls whereas mk 54963 and mk 55396 are vaccinees

compared to the vaccinees. In particular, mk 55123 presented eight epitope changes in the first exon of *tat*, three of which were acquired and five were lost. Conversely, mk 54963 showed four epitope variations, three of which were acquired and one lost. No evolution in Tat epitopes was detected in mk 55129 and mk 55396 (Table 2). When the C2-V3 Env region was analyzed, a total of 49 epitope variations, 24 of which were newly acquired epitopes, were found. Of note, 36 out of 49 epitope variations occurred in the control animals. In fact, the prediction analysis revealed 18 acquired epitopes and 12 lost epitopes in mk 55129 and three acquired epitopes in mk 55123. In the vaccinated animals only 13 epitope changes were found, in particular five variations in mk 55396, four of which represent lost epitopes and one acquired epitope and eight lost epitopes in mk 54963 (Table 3). Very similar results were obtained from the prediction analysis performed by using the web-site "MHCPathway—Macaque" (http://www.mamu.liai.org) [23 and data not shown].

Discussion

During the course of natural infection there is successful viral clearance when the host resolves the infection, while in the majority of cases persistent infection is established because of adaptation of the virus population to the

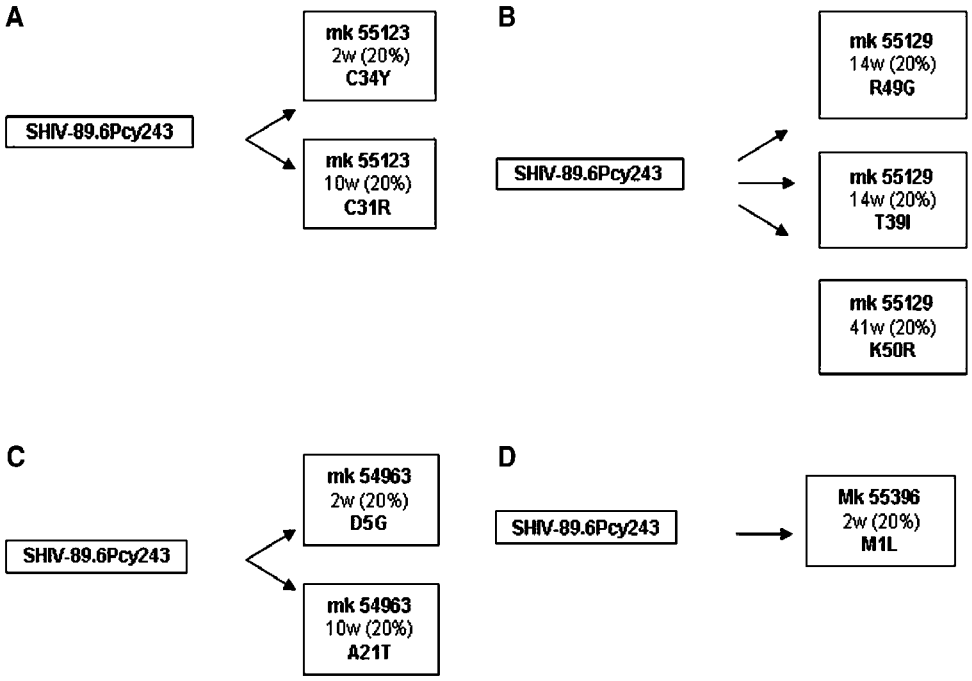

Fig. 3 Evolution of the Tat protein from the challenge virus SHIV-89.6P$_{cy243}$. Virus evolution is shown for control mk 55123 (part A), control mk 55129 (part B), vaccinated mk 54963 (part C), and vaccinated mk 55396 (part D). In brackets the percentage of clones representing those variants is shown

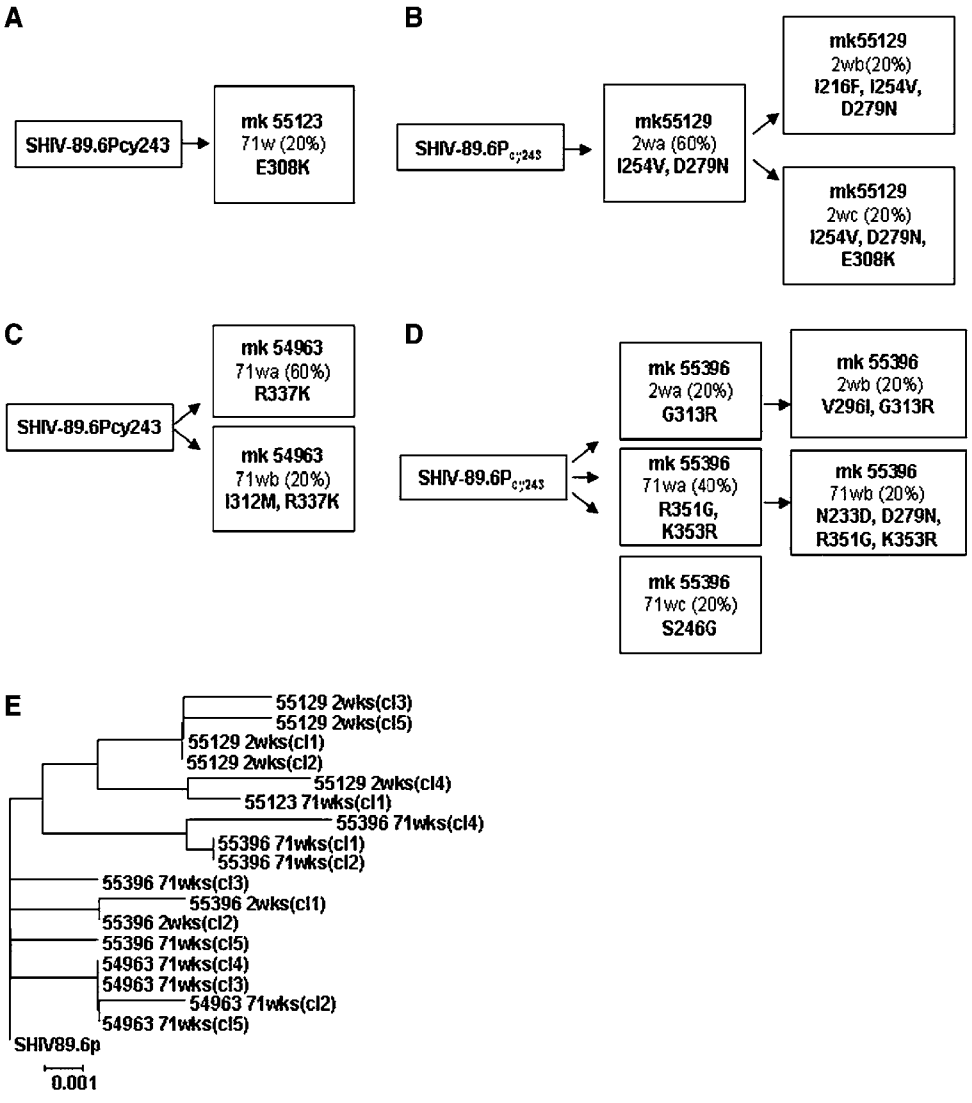

Fig. 4 Evolution of the C2-V3 Env region from the challenge virus SHIV-89.6P$_{cy243}$ to main SHIV variants. Cartoons depicting virus evolution are shown for control mk 55123 (part A), control mk 55129 (part B), vaccinated mk 54963 (part C) and vaccinated mk 55396 (part D). In brackets the percentage of clones representing those variants is shown. For mutations the amino acid changes are shown with the residue number. The phylogenetic tree was made by the neighbor-joining substitution model utilizing the software MEGA 3. The scale bar indicates distances (part E)

Table 2 Summary of the Tat epitope modifications in relation to the mutant viruses

Isolate	Tat exon 1			
	D5G	A21T	C31R	C34Y
55123 2w				A*02 11mer, ACQU
				A*11 9mer, LOST
55123 10w			A*01 9mer, LOST	
			A*01 10mer, ACQU	
			A*01 11mer, LOST	
			A*02 9mer, ACQU	
			A*02 10mer, LOST	
			A*11 9mer, LOST	
54963 2w	A*01 9mer, ACQU			
	A*01 11mer, ACQU			
54963 10w		A*01 9mer, ACQU		
		A*01 11mer, LOST		

The Mamu haplotype system was used as model in the prediction. LOST indicates unrecognized epitopes; ACQU indicates newly acquired epitopes. Mk 55123 and mk 55129 are controls whereas mk 54963 and mk 55396 are vaccinees

Table 3 Summary of the C2-V3 epitope modifications in relation to the mutant viruses

Isolate	C2-V3 Env region					
	N233D	I254V	D279N	E308K	G313R	R337K
55123 71w				A*01 11mer, ACQU		
				A*02 8mer, ACQU		
				A*02 9mer, ACQU		
55129 2w a[#], b		A*01 10mer, LOST	A*11 10mer, LOST			
		A*01 11mer, LOST				
		A*02 9mer, ACQU				
		A*02 10mer, ACQU				
		A*02 11mer, ACQU				
55129 2w c		A*01 10mer, LOST	A*11 10mer, LOST			
		A*01 11mer, LOST		A*01 11mer, ACQU		
		A*02 9mer, ACQU		A*02 8mer, ACQU		
		A*02 10mer, ACQU		A*02 9mer, ACQU		
		A*02 11mer, ACQU				
54963 71w a[#], b						A*01 9mer, LOST
						A*02 10mer, LOST
55396 2w a					A*01 9mer, LOST	
					A*01 10mer, LOST	
					A*11 8mer, ACQU	
55396 71w b	A*01 11mer, LOST		A*11 10mer, LOST			

The Mamu haplotype system was used as model in the prediction. LOST indicates unrecognized epitopes; ACQU indicates newly acquired epitopes. Mk 55123 and mk 55129 are controls, whereas mk 54963 and mk 55396 are vaccines

[#] Three clones were considered for these variants

immune selection [24]. For example, persistence of HCV may be helped by viral evolution during infection, enabling escape from prominent CTL responses [25]. In HCV infection, a tight association between viral persistence and the development of escape mutants has been demonstrated in the chimpanzee model [26]. In HIV-1 infection, the high rate of virus replication and mutation renders its genome susceptible to changes in epitopes recognized by the humoral system or within and flanking HLA-restricted epitopes. Thus, to ensure viral persistence HIV-1 might use the high genetic variability and accumulate mutations within CTL epitopes to impair CTL recognition. However,

the variability that a virus genome can allow is limited; the error threshold is in fact a critical point beyond which genetic information is lost and is directly related to the fitness of the virus. Although the escaping mechanism is in relationship with the maintenance of a high fitness, mutations may appear at the expense of viral fitness [6, 11]. In the present work, we evaluated the sequence evolution of the HIV-1 Tat protein and the C2-V3 Env region from control and vaccinated unprotected monkeys challenged with the SHIV-89.6P_{cy243}. The influence of immune activation on sequence evolution was estimated by phylogenetic analysis of the viral quasispecies developed during the immune responses initiated through experimental immunizations with the native Tat antigen. Although in both groups of monkeys nucleotide and amino acid variability of the Tat protein and C2-V3 Env region was similar, virus populations in vaccinees did show a slightly more differentiated dynamics with evolutive lineages arising from the prototype. A possible explanation for the evolutive difference in Tat and C2-V3 Env proteins, in both groups of animals, could be due to the selective pressure that was increased following vaccination. In fact, selection pressure exerted by the humoral and cellular immune responses to the virus fixes mutations that provide advantage, ignores the neutral ones, and eliminates the deleterious changes, thus acting on the virus evolution. In the vaccinees we characterized the results obtained from the interaction between a composite immune response consequent to both Tat vaccination and to the replication of the virus. However, we should consider that this study was conducted by using proviral DNA, thus providing only partial information on the real fitness of the genomes that we have identified. Recently, a series of databases have been constructed to investigate amino acid sequences and to predict epitopes [21, 22, 27]. The database contains epitopes mapped in experimental results and attempts to consider as many as possible factors involved in the process of presentation. We have chosen SYFPEITHI, one of the most used databases [21]. The analysis is based on a computer prediction of epitopes based on the known data on anchoring residues associated with the restriction of epitopes to MHC haplotype. Cynomolgus macaques from Mauritius Islands, as the monkeys enrolled in this study, share a high degree of MHC I allele combinations suggesting that these animals are particularly valuable in studies of cellular immunity [28, 29]. In particular, our analysis was mainly based on the prediction of appearing or disappearing of T-cell epitopes, using the Macaca mulatta (Mamu) system as a model, and considering the phylogeny of the viral variants. In our animals the epitope predictive analysis of the Tat protein and the C2-V3 Env region showed that the controls presented a variety of viral epitope changes that did not appear in the vaccinees: in particular in the Tat protein for mk 55123 and in C2-V3 Env region for mk 55129. These results could be focused on the effects that mutations have on the viral fitness. Viral fitness refers to the relative ability of a virus to replicate under particular conditions, such as those that occur when the immune response or other factors are acting on the virus. The selection pressure exerted by the immune response generates mutations in targeted epitopes of the virus. If the major prerequisite of the virus is to survive, the evolution will exclude all variants controlled by the immune system. As a consequence, epitope variation found in the two groups of animals may be quantitatively and qualitatively different. The amino acid variations observed in vaccinated animals, in which the immune system was primed by vaccination and in which the immunological control immediately recognize a wide spectrum of antigens, were directed to keep high the fitness and not to fall in error threshold. On the contrary, the mutations observed in the control animals were directed toward the development of escape mutants. Our results have shown that mutations in Tat and Env accumulate preferentially in regions containing potential CTL epitopes. Such a prediction-based approach could be criticized as it lacks the actual immunological verification. Unfortunately, the ability of circulating CTL to recognize the predicted epitopes could not be identified because of lack of information on cynomolgus monkeys' MHC profile. However, there are a growing number of data indicating that prediction of epitopes based on their anchoring motif to the MHC molecule is accurate, and such approaches have even led to the discovery of new epitopes [25, 26]. Our results contribute to the description of the consequences of vaccination on the virus dynamics: harnessing of the immune response against the Tat protein led to a diminishing of epitope variability and to an apparent reduction in the emergence of escape mutants. The Tat vaccine evaluated in this study was able to induce a broad antiviral response and our results are in agreement with the data collected in vaccination studies for chronic diseases. This implies that Tat-specific CTLs may be significantly involved in controlling wild-type virus replication and suggests that responses against viral proteins that are expressed early during the viral life cycle might be attractive targets for HIV vaccine development.

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References

1. D.H. Barouch, N.L. Letvin, Lancet **364**, 10–11 (2004)
2. P.J. Goulder, D.I. Watkins, Nat. Rev. Immunol. **4**, 630–640 (2004)
3. P. Klenerman, Y. Wu, R. Phillips, Curr. Opin. Microbiol. **5**, 408–413 (2002)
4. N.L. Letvin, B.D. Walker, Nat. Med. **9**, 861–866 (2003)
5. B. Li, A.D. Gladden, M. Altfeld, J.M. Kaldor, D.A. Cooper, A.D. Kelleher, T.M. Allen, J. Virol. **81**, 193–201 (2007)
6. F.W. Peyerl, H.S. Bazick, M.H. Newberg, D.H. Barouch, J. Sodroski, N.L. Letvin, J. Virol. **78**, 13901–13910 (2004)
7. T. de Oliveira, M. Salemi, M. Gordon, A.M. Vandamme, E.J. van Rensburg, S. Engelbrecht, H.M. Coovadia, S. Cassol, Genetics **167**, 1047–1058 (2004)
8. M.M. Addo, M. Altfeld, E.S. Rosenberg, R.L. Eldridge, M.N. Philips, K. Habeeb, A. Khatri, C. Brander, G.K. Robbins, G.P. Mazzara, P.J. Goulder, B.D. Walker, (2001) Proc. Natl. Acad. Sci. USA **98**, 1781–1786
9. C.A. van Baalen, O. Pontesilli, R.C. Huisman, A.M. Geretti, M.R. Klein, W.F. de, F. Miedema, R.A. Gruters, A.D. Osterhaus, J. Gen. Virol. **78**(Pt 8), 1913–1918 (1997)
10. N.A. Jones, X. Wei, D.R. Flower, M. Wong, F. Michor, M.S. Saag, B.H. Hahn, M.A. Nowak, G.M. Shaw, P. Borrow, J. Exp. Med. **200**, 1243–1256 (2004)
11. T. Matano, M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D.H. O'Connor, D.I. Watkins, Y. Nagai, J. Exp. Med. **199**, 1709–1718 (2004)
12. T.M. Allen, D.H. O'Connor, P. Jing, J.L. Dzuris, B.R. Mothe, T.U. Vogel, E. Dunphy, M.E. Liebl, C. Emerson, N. Wilson, K.J. Kunstman, X. Wang, D.B. Allison, A.L. Hughes, R.C. Desrosiers, J.D. Altman, S.M. Wolinsky, A. Sette, D.I. Watkins, Nature **407**, 386–390 (2000)
13. T.C. Friedrich, E.J. Dodds, L.J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D.T. Evans, R.C. Desrosiers, B.R. Mothe, J. Sidney, A. Sette, K. Kunstman, S. Wolinsky, M. Piatak, J. Lifson, A.L. Hughes, N. Wilson, D.H. O'Connor, D.I. Watkins, Nat. Med. **10**, 275–281 (2004)
14. Y. Wu, J.W. Marsh, Microbes Infect. **5**, 1023–1027 (2003)
15. R. Gavioli, E. Gallerani, C. Fortini, M. Fabris, A. Bottoni, A. Canella, A. Bonaccorsi, M. Marastoni, F. Micheletti, A. Cafaro, P. Rimessi, A. Caputo, B. Ensoli, J. Immunol. **173**, 3838–3843 (2004)
16. A. Cafaro, A. Caputo, C. Fracasso, M.T. Maggiorella, D. Goletti, S. Baroncelli, M. Pace, L. Sernicola, M.L. Koanga-Mogtomo, M. Betti, A. Borsetti, R. Belli, L. Akerblom, F. Corrias, S. Buttò, J. Heeney, P. Verani, F. Titti, B. Ensoli, Nat. Med. **5**, 643–650 (1999)
17. M.T. Maggiorella, S. Baroncelli, Z. Michelini, E. Fanales-Belasio, S. Moretti, L. Sernicola, A. Cara, D.R. Negri, S. Butto, V. Fiorelli, A. Tripiciano, A. Scoglio, A. Caputo, A. Borsetti, B. Ridolfi, R. Bona, H.P. ten Haaft, I. Macchia, P. Leone, M.R. Pavone-Cossut, F. Nappi, M. Ciccozzi, J. Heeney, F. Titti, A. Cafaro, B. Ensoli, Vaccine **22**, 3258–3269 (2004)
18. A. Cafaro, A. Caputo, M.T. Maggiorella, S. Baroncelli, C. Fracasso, M. Pace, A. Borsetti, L. Sernicola, D.R. Negri, H.P. ten Haaft, M. Betti, Z. Michelini, I. Macchia, E. Fanales-Belasio, R. Belli, F. Corrias, S. Butto, P. Verani, F. Titti, B. Ensoli, J. Med. Primatol. **29**, 193–208 (2000)
19. G.B. Karlsson, M. Halloran, J. Li, I.W. Park, R. Gomila, K.A. Reimann, M.K. Axthelm, S.A. Iliff, N.L. Letvin, J. Sodroski, J. Virol. **71**, 4218–4225 (1997)
20. K. Yusim, C. Kesmir, B. Gaschen, M.M. Addo, M. Altfeld, S. Brunak, A. Chigaev, V. Detours, B.T. Korber, J. Virol. **76**, 8757–8768 (2002)
21. H. Rammensee, J. Bachmann, N.P. Emmerich, O.A. Bachor, S. Stevanovic, Immunogenetics **50**, 213–219 (1999)
22. H. Rammensee J. Bachmann N. P. Emmerich O. A. Bachor S. Stevanovic, *MHC Ligands and Peptide Motifs*. (Landes Bioscience Pbl., Georgetown, 2007)
23. B. Peters, H.H. Bui, J. Sidney, Z. Weng, J.T. Loffredo, D.I. Watkins, B.R. Mothe, A. Sette, Vaccine **23**, 5212–5224 (2005)
24. J. Timm, G.M. Lauer, D.G. Kavanagh, I. Sheridan, A.Y. Kim, M. Lucas, T. Pillay, K. Ouchi, L.L. Reyor, W.J. Schulze zur, R.T. Gandhi, R.T. Chung, N. Bhardwaj, P. Klenerman, B.D. Walker, T.M. Allen, J. Exp. Med. **200**, 1593–1604 (2004)
25. D. Genovese, S. Dettori, C. Argentini, U. Villano, P. Chionne, M. Angelico, M. Rapicetta, J. Clin. Microbiol. **43**, 1902–1909 (2005)
26. A. Weiner, A.L. Erickson, J. Kansopon, K. Crawford, E. Muchmore, A.L. Hughes, M. Houghton, C.M. Walker, Proc. Natl. Acad. Sci. USA **92**, 2755–2759 (1995)
27. N. Jojic, M. Reyes-Gomez, D. Heckerman, C. Kadie, O. Schueler-Furman, Bioinformatics **22**, e227-e235 (2006)
28. K.C. Krebs, Z. Jin, R. Rudersdorf, A.L. Hughes, D.H. O'Connor, J. Immunol. **175**, 5230–5239 (2005)
29. R.W. Wiseman, J.A. Wojcechowskyj, J.M. Greene, A.J. Blasky, T. Gopon, T. Soma, T.C. Friedrich, S.L. O'Connor, D.H. O'Connor, J. Virol. **81**, 349–361 (2007)