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Research paper

Potential immune escape mutations under inferred selection pressure in HIV-1 strains circulating in Medellín, Colombia

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ABSTRACT

The introduction of highly active antiretroviral therapy (HAART) has significantly improved life expectancy of HIV-infected patients; nevertheless, it does not eliminate the virus from hosts, so a cure for this infection is crucial. Some strategies have employed the induction of anti-HIV $CD8^+$ T cells. However, the high genetic variability of HIV-1 represents the biggest obstacle for these strategies, since immune escape mutations within epitopes restricted by Human Leukocyte Antigen class I molecules (HLA-I) abrogate the antiviral activity of these cells. We used a bioinformatics pipeline for the determination of such mutations, based on selection pressure and docking/refinement analyses. Fifty HIV-1 infected patients were recruited; HLA-A and HLA-B alleles were typified using sequence-specific oligonucleotide approach, and viral RNA was extracted for the amplification of HIV-1 *gag*, which was bulk sequenced and aligned to perform selection pressure analysis, using Single Likelihood Ancestor Counting (SLAC) and Fast Unconstrained Bayesian Approximation (FUBAR) algorithms. Positively selected sites were mapped into HLA-I-specific epitopes, and both mutated and wild type epitopes were modelled using PEP-FOLD. Molecular docking and refinement assays were carried out using AutoDock Vina 4 and FlexPepDock. Five positively selected sites were found: S54 at HLA-A*02 GC9, T84 at HLA-A*02 SL9, S125 at HLA-B*35 HY9, S173 at HLA-A*02/B*57 KS12 and I223 at HLA-B*35 HA9. Although some mutations have been previously described as immune escape mutations, the majority of them have not been reported. Molecular docking/refinement analysis showed that one combination of mutations at GC9, one at SL9, and eight at HY9 epitopes could act as immune escape mutations. Moreover, HLA-A*02-positive patients harbouring mutations at KS12, and HLA-B*35-positive patients with mutations at HY9 have significantly higher plasma viral loads than patients lacking such mutations. Thus, HLA-A and -B alleles could be shaping the genetic diversity of HIV-1 through the selection of potential immune escape mutations.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), still represents one of the major health problems worldwide. Since the beginning of the pandemic until 2015, > 70 million people have been infected throughout the globe, and around 35 million people have died of AIDS-related illnesses (World Health Organization, 2016). This infection has a higher impact in low-income countries, being the fifth cause of death according to the World Health Organization (World Health

Abbreviations: HAART, Highly active antiretroviral therapy; HLA-I, Human Leukocyte Antigen class I molecules; SLAC, Single Likelihood Ancestor Counting; FUBAR, Fast Unconstrained Bayesian Approximation; HIV-1, Human immunodeficiency virus type 1; AIDS, Acquired immunodeficiency syndrome; pVL, Plasma viral load; PBMCs, Peripheral blood mononuclear cells; SSO, Sequence-specific oligonucleotide; CRFs, Circulating recombinant forms; d_N, Non-synonymous substitutions per non-synonymous site; d_S, Synonymous substitutions per synonymous site; TCR, T cell receptor

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Organization, 2015). The introduction of HAART has improved the life expectancy of HIV-infected patients (Maartens et al., 2014), and has played a crucial role in the gradual decrease of the global incidence of the infection (UNAIDS, 2017). However, the HAART strategy has various disadvantages, as it does not eliminate the virus from the host, and an infected patient must take the therapy over the whole course of the infection in order to control viral replication (Arts & Hazuda, 2012). In addition, the global coverage of HAART is insufficient, as only 46% of infected patients have access to the drugs (UNAIDS, 2017). Finally, due the high genetic variability of HIV-1, viral variants with mutations that confer resistance to one or more antiretroviral drugs can be selected within hosts (Shafer & Schapiro, 2008). All these problems point to the need for further investigations in new strategies, such as vaccines, to control the global epidemic of HIV-1.

Some vaccine strategies employed so far have focused on the induction of specific $CD8^+$ T cell responses, as these cells not only participate but also play a crucial role in the control of HIV-1 replication *in vivo* (Koup et al., 1994; Borrow et al., 1994). These cells are expanded since the acute phase of the infection, their antiviral response has been associated with the drastic decline in plasma viraemia observed in infected patients (Moir et al., 2011; Ferrari et al., 2011), and their direct effector function can be observed throughout the chronic phase of the disease (Walker & McMichael, 2012). Their fundamental role in the control of viral replication has been evidenced in rhesus macaques (*Macaca mulatta*) infected with simian immunodeficiency virus, where a depletion of peripheral blood $CD8^+$ T cells led to a dramatic increase in viraemia, and the recovery of this cell population was associated with a decrease in plasma viral load in the infected animals (Jin et al., 1999). However, the anti-HIV activity of $CD8^+$ T cells does not completely eliminate the virus, as HIV-1 can evade $CD8^+$ T cell responses through the selection of mutations within HLA-I restricted epitopes or at their flanking sequences (Cao et al., 2003; Jones et al., 2004). The appearance of such mutations, known as immune escape mutations, has been related with a decline of $CD8^+$ T cell responses (Jamieson et al., 2003), impairing the ability of these cells to clear the virus. These mutations are the result of the high genetic variability of the virus, which is mediated by several factors including mainly the error-prone activity of viral reverse transcriptase (Abram et al., 2010), and their location in HIV-1 proteome is highly dependent on the immunogenicity of viral proteins, as these mutations are preferentially selected in epitopes derived from Gag, Pol and Nef (Wang et al., 2009). It has been previously established that HIV-1 immune escape mutations can be positively selected in epitopes presented by the most frequent HLA-I alleles from a population (Moore et al., 2002), indicating the preponderant role of the HLA-I-restricted $CD8^+$ T cell responses in the adaptation of HIV-1 at the population level. Specifically, this phenomenon has been described in Caribbean (Kawashima et al., 2009) and South American populations (Acevedo-Saenz et al., 2015), where escape mutations have been positively selected at the population level in specific epitopes presented by the predominant HLA-I alleles in the corresponding populations. The evaluation of these mutations in a population gives insights into the evolutionary processes that are shaping the genetic diversity of HIV-1 around the globe, and large-scale analysis of escape mutations are necessary to rationally develop $CD8^+$ T cell-based strategies of vaccination (Arcia et al., 2017). This study was aimed to determine the presence of positively selected mutations and their association with a potential immune escape phenotype in epitopes derived from HIV-1 *gag* and presented by HLA-I alleles in the population from Medellin, Colombia.

2. Materials and methods

2.1. Study population

Fifty HIV-1 positive HAART-naïve adult patients from Medellin, Colombia, with plasma viral load (pVL) higher than 5000 copies/mL, were recruited. Patients were selected based on a confirmed HIV-1 infection, according to Colombian guidelines of diagnosis (Ministerio de Salud y Protección Social, 2014). This study was carried out in accordance with the recommendations of the ethics committee of the medical investigations institute from Universidad de Antioquia. All subjects gave written informed consent after a clear explanation of the study in accordance with the Declaration of Helsinki. The protocol was approved by the aforementioned committee.

2.2. Plasma viral load, $CD4^+$ T cell count and HLA-I typing

The measurement of pVL was performed using the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test, v2.0 assay (Roche, Indianapolis, IN, USA), which has a detection range between 20 and 10.000.000 RNA copies/mL. T-cell counts were performed in peripheral blood by flow cytometry using the following monoclonal antibodies: anti-CD3 APC-Cy7, anti-CD4 Pacific Blue and anti-CD8 APC (BD Pharmigen, San Diego, CA, USA). Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and data was analysed using the FACSDiva software v 6.1.3 (BD Biosciences). HLA-I typification was performed in genomic DNA extracted from peripheral blood mononuclear cells (PBMCs) through phenol/chloroform DNA extraction protocol. HLA-A and HLA-B alleles were typified by the sequence-specific oligonucleotide (SSO) technology, using the Lifecodes® HLA-SSO Typing Kit (Immucor Transplant Diagnostics, Inc., Stamford, CT, USA) and measured using the Luminex® 100/200™ instrument (Luminex, Austin, TX, USA).

2.3. HIV-1 gag amplification and sequencing

Viral RNA was extracted from plasma using the QIAamp® Viral RNA kit (Qiagen, Germantown, MD, USA), according to manufacturer instructions. The cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE, USA), employing the HIV-1 specific primer UNINEF 7′ (Nadai et al., 2008). Using the synthesized cDNA as template, a nested PCR was done to amplify the whole *gag* gene of HIV-1. The length and quality of the PCR product was verified by an agarose gel electrophoresis at a 1% concentration, and PCR products of the adequate length and quality were sequenced by Sanger sequencing methodology using the ABI3730XL system (Applied Biosystems, Foster City, CA, USA) at Macrogen Inc. (Geumcheon-gu, Seoul, South Korea). The information about the primers and PCR conditions are detailed in Supplementary Tables 1 and 2. The *gag* sequences were edited using the SeqMan Pro® software (DNASTAR, Madison, WI, USA), with the HXB2 sequence as reference (GenBank access number: K03455.1). After edition, the quality of each sequence was compared against HXB2 using the Needleman-Wunsch global alignment algorithm. Sequences were then aligned using the ClustalW algorithm implemented in MEGA7 software, employing the IUB DNA weight matrix with a gap open penalty of 15 and a gag extension penalty of 6.66 (Kumar et al., 2016). Once aligned, sequences were analysed for the presence of recombination breakpoints using the GARD algorithm in the Datamonkey server. Sequences derived from this study are deposited in GenBank under the accession numbers MF190472 to MF190518.

2.4. Phylogenetic analysis, epitope searching and identification

The sequences in the dataset were subject of phylogenetic analyses by Neighbour-Joining, Maximum Likelihood and Maximum Parsimony methods using the MEGA7 package. The general-time-reversible model of nucleotide substitution was used in the first two methods, whereas for the latter one hundred bootstrap replicates were performed, rooting the tree using non-B sequences as outgroup. Homologous subtype B, non-B and circulating recombinant forms (CRFs) sequences from South America were also included in the analyses. The epitopes presented by

the HLA-I alleles of the studied population were identified using the "HIV Databases" of Los Alamos National Laboratory. Each epitope was located in the HXB2 sequence as well as in the sequence from the patients, and the frequency of amino acid substitutions was calculated and classified as previously reported escape mutations, as described in the HIV Molecular Immunology Database (HIV Molecular Immunology, 2009). Any mutation found that was not included in the previously cited database was classified as an "unknown" mutation.

2.5. Selection pressure analysis

To determine evidence of positive selection in the sequences, two different strategies were used; both based on the ratio of the estimated non-synonymous to synonymous substitutions rates (d_N/d_S). SLAC is a maximum-likelihood-based counting method, where a global Muse-Gaut substitution model for codons is fitted to the alignment to generate a maximum likelihood reconstruction of ancestral codons using the GTR nucleotide substitution model (Kosakovsky Pond & Frost, 2005). On the other hand, FUBAR is a hierarchical Bayesian approach using Markov chain Monte Carlo to estimate the distribution of the rates in each codon position without enforcing a predefined substitution model (Murrell et al., 2013). The selection pressure analyses were carried on the Datamonkey server (Pond & Frost, 2005). Positive selection was defined when the d_N/d_S ratio was > 1 and when the null hypothesis of neutral evolution ($d_N = d_S$) was rejected with a $p < .05$ in SLAC, and when the Bayesian posterior probability of $d_N > d_S$ ($Pr[d_N > d_S]$) was ≥ 0.95 in FUBAR. Positively selected mutations were mapped on the resulting phylogenetic trees using the most parsimonious reconstruction of ancestral states.

2.6. Molecular docking

For molecular docking simulations, the identified epitopes with positively selected mutations were modelled using the PEP-FOLD server (Shen et al., 2014), and the respective HLA-I crystalized molecules were accessed through the Protein Data Bank database (Berman et al., 2000); for HLA-A*02 the PDB ID: 1T1Z (Martinez-Hackert et al., 2006) was employed, whereas for HLA-B*35 the PDB ID: 1ZHK was used (Tynan et al., 2005). Both the epitopes and the HLA-I molecules were parameterized to remove crystal water molecules and to add partial charges to each atom using AutoDockTools-1.5.6 (The Scripps Research Institute, La Joya, CA, USA). Molecular docking between the epitope and its corresponding HLA-I was done using AutoDock Vina (Trott & Olson, 2010). Then, refinement of the molecular interactions for the best docking models was made through the FlexPepDock protocol (Raveh et al., 2010). This refinement step was performed three times for each docking model, and for each refinement repetition, the best three models were selected according to previous knowledge of what should be the position of the epitope respective to the HLA-I molecule. Binding affinity is expressed as Rosetta energy units, where the more negative the value, the more affinity the epitope has for the HLA-I molecule. Hydrogen bonds between epitopes and HLA-I molecules was visualized and annotated using the LigPlus software.

2.7. Statistical analysis

For all the evaluated variables, Shapiro-Wilk test was performed in order to verify the normality of the data. For normally distributed data, a one-way ANOVA with Dunnett multiple comparison tests or Student's *t*-test were done, whereas for data with a non-normal distribution, a Mann-Whitney *U* test was applied. Statistical significance was considered with *p* values lower than 0.05. All the statistical analyses were performed with the GraphPad Prism 6 software (GraphPad Software, Inc., La Joya, CA, USA).

3. Results

3.1. Patient clinical data and HLA-I typification

Demographic and clinical characteristic of the patients are summarized in Supplementary Table 3; in general, the population had a pVL median of 63,702 copies/mL, and a $CD4^+$ T cell count median of 390 cells/μL. The allelic frequency revealed that the most prevalent HLA-A and HLA-B alleles in the studied population were HLA-A*02 (22%), HLA-A*24 (20%) and HLA-B*35 (20%). Patient HLA-A and –B data, as well as epitope variants and GenBank accession numbers for each patient are detailed in Supplementary Table 4.

3.2. HIV-1 subtype B gag sequences harbour positively selected mutations in epitopes presented by the most prevalent HLA-I alleles

In order to evaluate the presence of positively selected mutations in *gag* epitopes, Neighbour-Joining, Maximum Likelihood and Maximum Parsimony phylogenetic analysis were performed, in order to determine the HIV-1 subtype of the obtained *gag* sequences. The trees generated by the different phylogenetic methods had considerable differences in the topology and showed low levels of support in bootstrap analyses. Nevertheless, all sequences obtained in this study clustered in the same clade with other subtype B sequences from South America. Moreover, GARD analysis showed no recombination breakpoints in the sequences. The maximum parsimony tree is shown in Supplementary Fig. 1, and sequences employed for the phylogenetic tree are listed in Supplementary Table 5.

Next, selection pressure analyses were carried out using SLAC and FUBAR algorithms. This analysis showed five codons with evidence of positively selected mutations: codons S54 (TCA), T84 (ACC), S125 (AGC), S173 (TCA) and I223 (ATT). Interestingly, all these codons codify for amino acids within epitopes presented by the most prevalent HLA-I alleles in the studied population (Table 1), and these mutations were also accompanied by other mutations within the same epitope with a d_N/d_S ratio > 1, but without reaching statistical significance (Fig. 1). Some combinations of mutations have been previously described as immune escape mutations, *e.g.* the V82I/T84V mutation in SLYNTVATL epitope (SL9, residues: 77–85), as this mutation decreases the affinity of the epitope towards HLA-A*02 (Tenzer et al., 2009; Bennett et al., 2010), indicating that the methodological approach used in this study is able to correctly identify sites involved in immune escape mechanisms, as positively selected. Nevertheless, most of the positively selected mutations detected in the analysis have not been yet associated with an escape phenomenon. Mutations in codons with evidence of positive selection appeared repeatedly in different branches of the tree, indicating the presence of multiple reverse and parallel mutations. Between 16 and 27 substitutions were inferred in each of the five codons. Most, 76/103 (73.8%) of these mutations mapped on the terminal branches of the tree.

We then correlated the presence/absence of positively selected mutations in all patients with their respective HLA-I. Remarkably, only for the HLA-A*02-restricted epitope SL9, all the patients that had the mutations expressed at least one of the HLA-I alleles specific for the epitope, and in the case of the HLA-A*02 and –B*57-restricted epitope KAFSPEVIPMFS (KS12, Residues: 162–173), around 70% of the patients expressing the specific HLA-I harbour these mutations. Interestingly, for the HLA-A*02-restricted epitope GLLETSEGC (GC9, Residues: 49–57), and for the HLA-B*35-restricted epitopes HSNQVSQNY (HY9, Residues: 124–132) and HPVHAGPIA (HA9, Residues: 216–224) more than half of the patients with the mutations did not express the respective HLA-I (Fig. 2).

Table 1
Frequency of positively selected mutations in HIV-1 *gag* with no previous association with immune escape.

Viral protein	Codon	WT epitope	Mutated epitope	Frequency (%)	HLA-I	FUBAR		SLAC	
						d_N/d_S	$Pr[d_N > d_S]$	d_N-d_S	*p* value
p17	54	GLLET**S**EGC (GC9, residues: 49–57)	GLLET**A**DGC	6	A2	6.27	0.98	4.94	0.002
			GLLET**A**EGC	16					
			GLLET**A**GGC	2					
			GLLET**A**TGC	2					
			GLLET**T**EGC	6					
			SLLET**A**EGC	4					
	84	SLYNTVA**T**L (SL9, residues: 77–85)	SLYNT**V**AVL	12	A*02:01	5.26	> 0.99	5.61	0.004
			SLYNT**I**AVL	16	A24				
			SLFNT**V**AVF	2	B44				
			SLYNA**V**AVL	8	B58				
			SVFNT**V**AVI	2					
	125	H**S**NQVSQNY (HY9, residues: 124–132)	H**D**SPVSQNY	2	B*35:01	4.45	0.97	4.29	0.04
			H**R**KQVSQNY	2					
			H**R**NQVSQNY	2					
			K**D**SPVSQNY	2					
			K**N**NPVSQNY	2					
			N**G**SQVSQNY	2					
			N**K**NQVSQNY	2					
			N**K**SQVSQNY	2					
			N**N**SKVSQNY	4					
			N**N**SQVSQNY	14					
			N**R**SNVSQNY	2					
			S**G**SKVSQNY	2					
			S**N**SQVSQNY	2					
p24	173	KAFSPEVIPMF**S** (KS12, residues: 162–173)	KNFSPEVIPMF**T**	2	A*02:01	4.18	0.97	4.49	0.003
			KAFSPEVIPMF**T**	22	B*57:01				
			KAFSPEVIPMF**A**	8	B*58:01				
	223	HPVHAGP**I**A (HA9, residues: 216–224)	HPAQAGP**V**A	6	B*35:01	8.00	> 0.99	4.83	0.003
			HPPQAGP**V**A	2					
			HPVHAGP**A**A	6					
			HPVHAGP**T**A	2					
			HPVHAGP**V**A	24					
			HPVPAGP**V**A	2					

The amino acids in bold correspond to those where positively selected mutations where found. FUBAR: Fast-unconstrained bayesian approximation, SLAC: single likelihood ancestor counting, d_N: non-synonymous mutations per non-synonymous site, d_s: synonymous mutations per synonymous site.

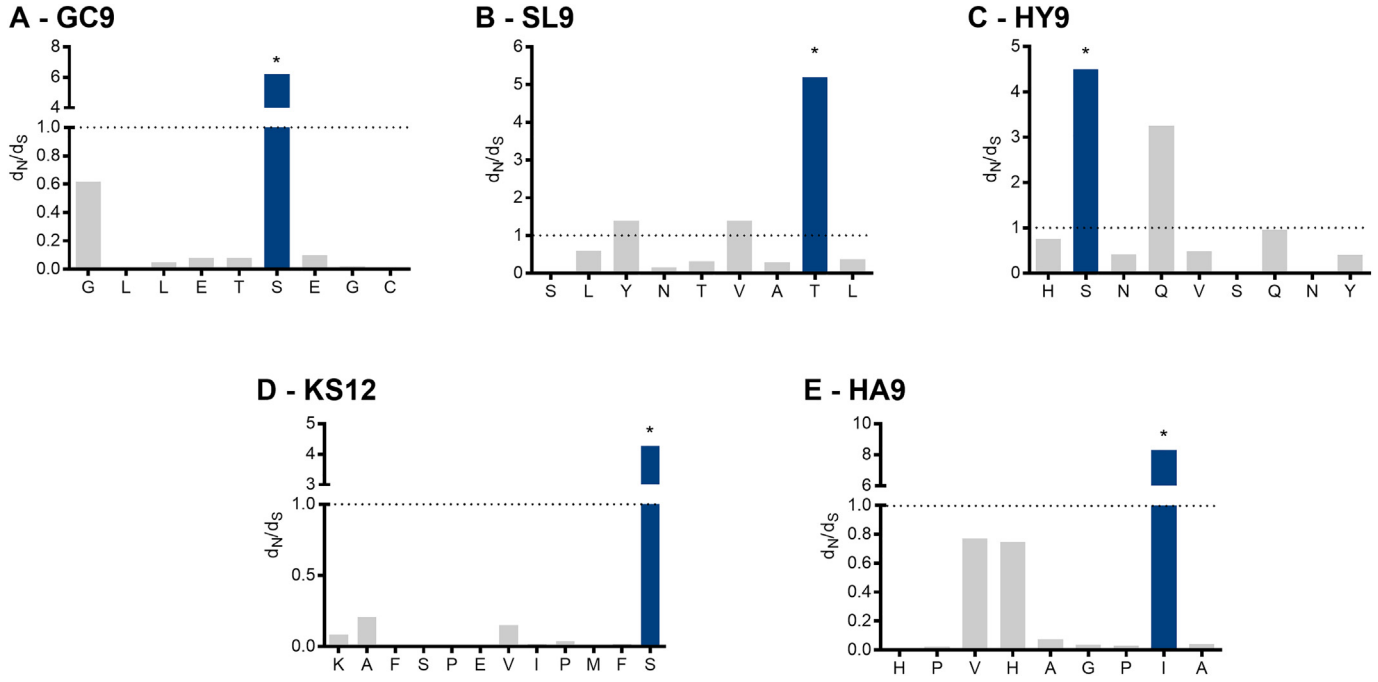

Fig. 1. d_N/d_S patterns across all the amino acids in the five epitopes where positive selection was observed. Amino acids above the dotted line and marked with an asterisk represent those with statistical significant evidence of positive selection, whereas those above the dotted line, but not marked with an asterisk, are amino acids under no statistical significant positive selection.

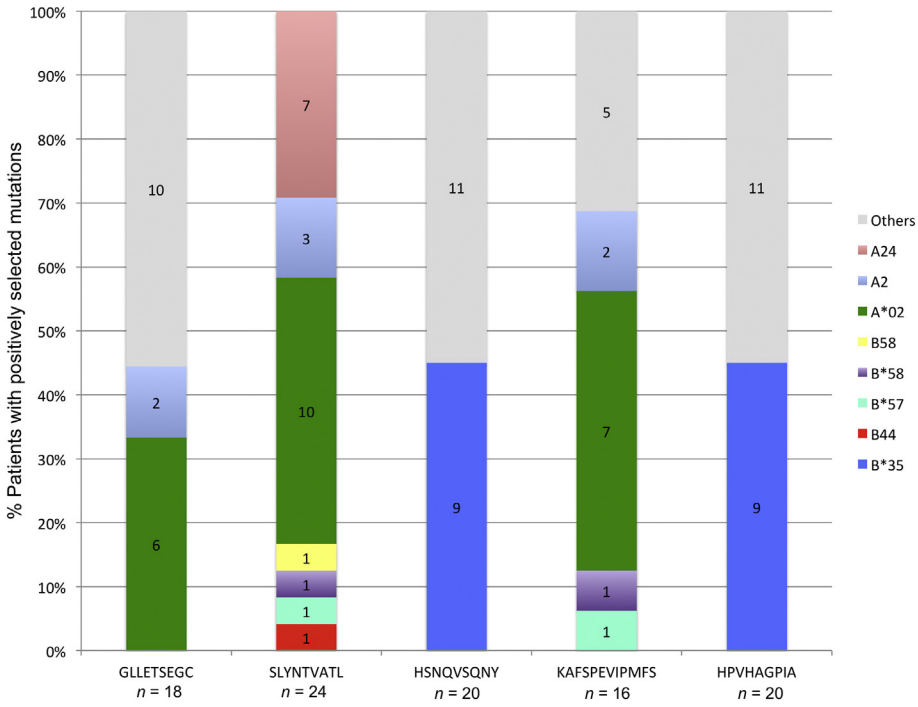

Fig. 2. Distribution of positively selected mutations among patients expressing different HLA-I alleles. Each colour represents the number of patients expressing the HLA allele that restricts the epitope. The grey bar depicts the number of patients that harbours positively selected mutations in the respective epitopes, but without expressing the epitope-specific HLA-I allele.

*3.3. Positively selected mutations at HLA-A*02-restricted epitopes act as potential immune escape mutations*

To further explore if positively selected mutations in HLA-A*02-restricted epitopes could act as potential immune escape mutations, molecular docking and refinement assays were carried out between the mutated and WT epitopes and the corresponding HLA-I molecule. The analysis demonstrated that three different combinations of mutations at the SL9 epitope significantly decreased the binding affinity of the epitope towards HLA-A*02 compared to the WT epitope, *i.e.* Y79F/T84V, Y79H/T81A/T84V and Y79F/T84V/L85F (Fig. 3A). With regard to mutations at GC9 epitope, different patterns of affinity were found. The S54A/E55G combination reduced the binding affinity of the GC9 epitope towards HLA-A*02; however, the S54A and S54T mutations significantly increased the binding affinity of the epitope towards this HLA molecule (Fig. 3B). Most of the mutations found reducing HLA binding affinity were present in one (2%) of the HIV-1 *gag* sequences evaluated, whereas the S54A and S54T mutations in GC9 expressed a frequency of 6% (Table 1). Finally, molecular docking and refinement analysis were performed in the last nine amino acids of the KS12 peptide, as the full-length peptide displayed non-biological relevant structures in docking assays. In this regard, the S173T mutation in the SPEVIPMFS peptide, which is observed in 24% of the patients, had a significantly higher binding affinity towards HLA-A*02 (Fig. 3C). Altogether, these results suggest that two different combinations of mutations (SL9 Y79F/T84V/L85F and GC9 S54A/E55G) could act as potential immune escape mutations, decreasing the binding affinity of the epitopes to HLA-A*02.

*3.4. Positively selected mutations at HLA-B*35-restricted epitopes have mixed phenotypes*

Similar docking/refinement analyses were performed for positively

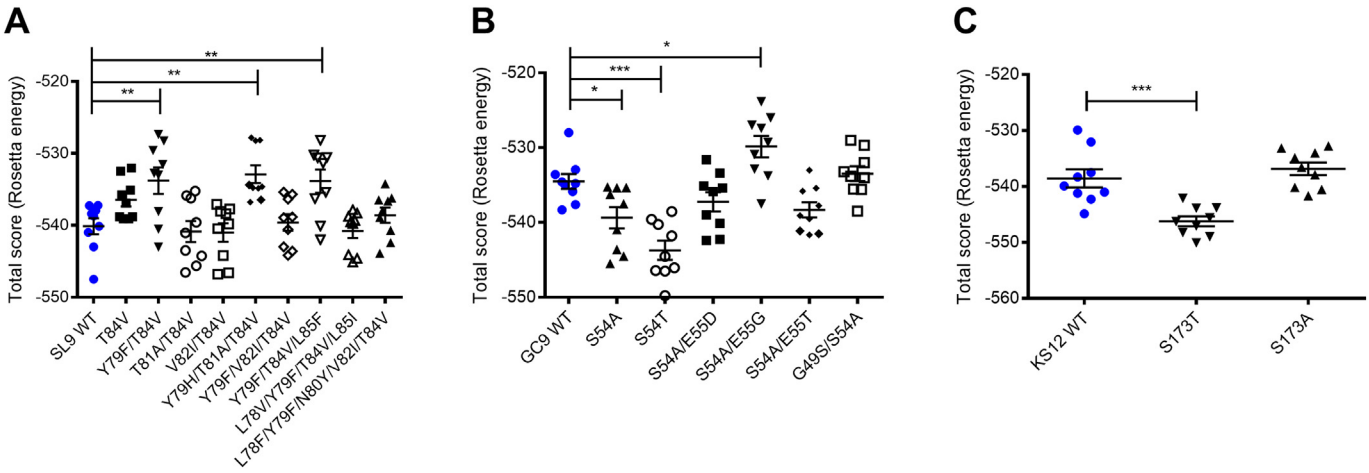

Fig. 3. Four different combinations of mutations at HLA-A*02-restricted epitopes act as potential immune escape mutations. The Y79F/T84V, Y79H/T81A/T84V and Y79F/T84V/L85F combinations in SL9 epitope (A), and S54A/E55G in GC9 epitope (B) significantly reduce the epitope binding affinity towards HLA-A*02, as compared with the WT epitopes (Blue dots). The binding affinity is expressed as the total score of FlexPepDock, where the more negative the value, the more affinity the epitope have for the HLA-I molecule. Data are represented as mean ± standard deviation; $n = 9$; * $p < .05$, ** $p < .01$, *** $p < .001$, one-way ANOVA with Dunnett's multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

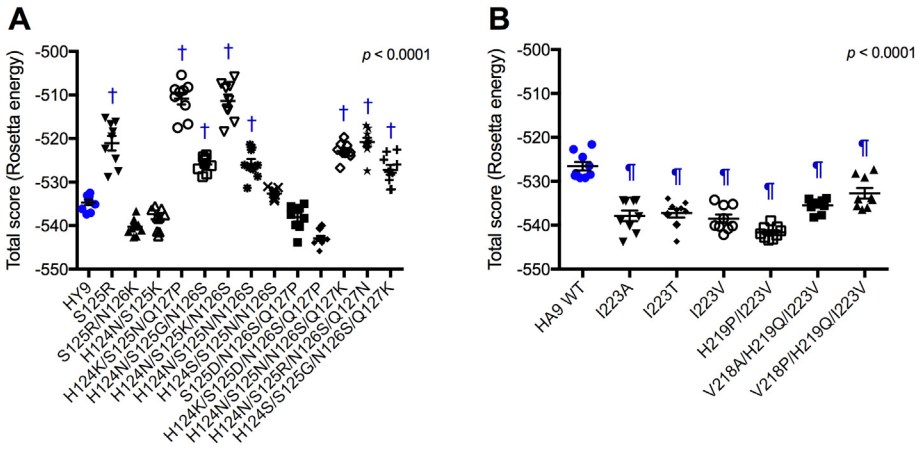

Fig. 4. Positively selected mutations at HLA-B*35-restricted epitopes have mixed phenotypes. Eight different combinations of mutations at HY9 epitope significantly reduce the epitope binding affinity to the HLA-B*35 molecule (A), whereas all the mutations found at HA9 epitope significantly increase the epitope binding affinity towards HLA-B*35 (B). †: Mutated HY9 epitope that has a significantly reduced binding affinity to HLA-B*35 compared with WT HY9; ¶: mutated HA9 epitope that has a significantly higher binding affinity to HLA-B*35 compared with WT HA9. Data are represented as mean ± standard deviation; $n = 9$; one-way ANOVA with Dunnett's multiple comparison test.

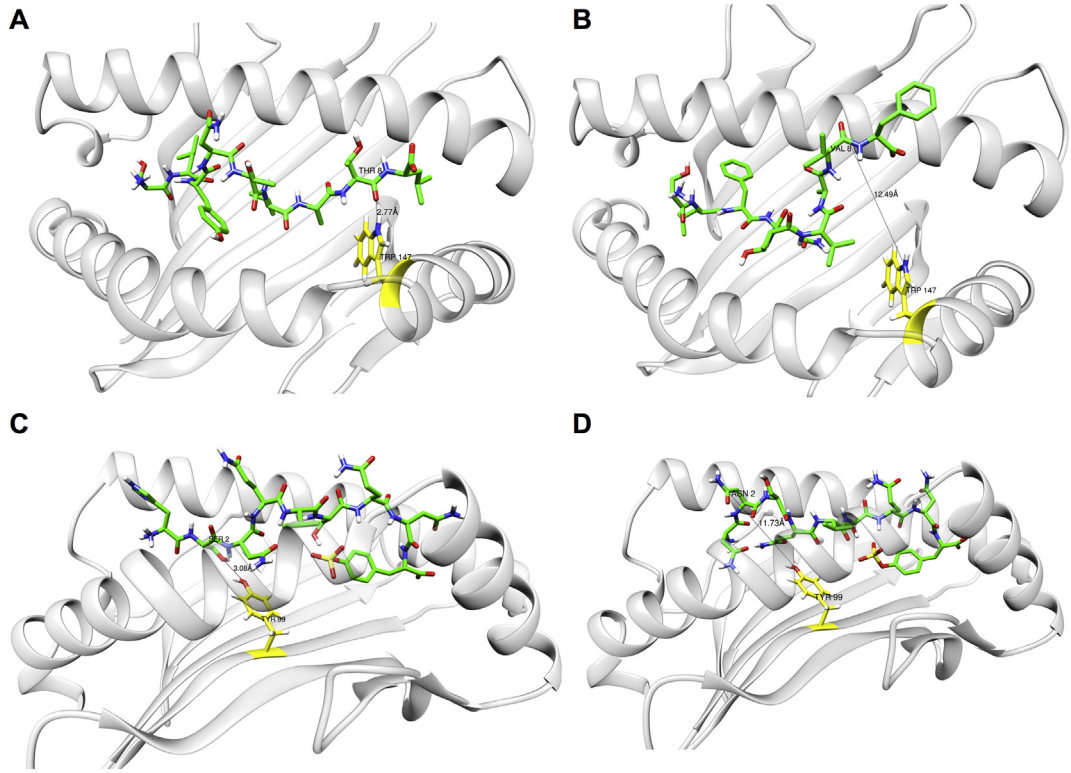

Fig. 5. Positively selected mutations that decrease the epitope binding affinity affect the interaction towards key HLA-I residues. Graphical representation of the interaction between HLA-A*02 with WT SL9 epitope (A), or with Y79F/T84 V/L85F mutations (B); as well as the interaction between HLA-B*35 with WT HY9 epitope (C) or with H124N/S125 N/N126S mutations (D). In each graph, the residues that are likely to contact amino acids in the epitope are coloured yellow, and the distance between both amino acids is represented as a bold line measured in Armstrong (Å). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

selected mutations at HLA-B*35-restricted epitopes. Remarkably, eight different combination of mutations found in the HY9 epitope significantly reduced the binding affinity of the epitope to HLA-B*35. These mutations are as follows: S125R, H124K/S125 N/Q127P, H124N/S125G/N126S, H124N/S125 K/N126S, H124N/S125 N/N126S, H124N/S125 N/N126S/Q127K, H124N/S125R/N126S/Q127N and H124S/S125G/N126S/Q127K (Fig. 4A). Conversely, all mutations found at the HA9 epitope significantly increased the epitope binding affinity towards HLA-B*35 (Fig. 4B).

Regarding the frequency of the combinations of mutations at HY9, the most prevalent combination was H124N/S125 N/N126S, which was present in 14% of HIV-1 *gag* sequences, whereas the rest of the combinations were found at low percentages (between 2% - 4%) (Table 1). It is noteworthy that none of the mutations found either in HY9 or HA9 epitopes has been previously reported as immune escape mutations. Together, these results show that positively selected mutations at HY9 epitope may represent novel immune escape mutations.

3.5. Chemical and molecular characteristics of epitopes with potential immune escape mutations

In order to explore why some positively selected mutations decreased the HLA-I binding affinity, the three-dimensional complexes of HLA-I mutated epitopes obtained by molecular docking were inspected. It is well established that residues at B and F pockets of any HLA-I molecule form hydrogen bonds with amino acids at the second and eighth/ninth position of the epitope, respectively, being these interactions the main responsible for the epitope orientation and affinity

towards the HLA-I molecule and dictating the chemical specificity of different HLA-I for certain epitopes (Matsumura et al., 1992). In HLA-A*02-restricted epitopes SL9 and GC9, the mutations found to reduce HLA binding affinity induced a drastic change in the orientation of the epitope within the HLA-I pockets. As seen in Fig. 5B, mutations in these epitopes block the expected interaction of the F pocket of HLA-A*02 with the C-terminal portion of the epitopes, and in the case of Y79H/T81A/T84 V and Y79F/T84 V/L85F combinations of mutations at SL9 epitope, this could be explained by a lack of hydrogen bonds between amino acids in the F pocket of HLA-A*02 and valine found at the eighth position of these mutated epitopes (Supplementary Table 6).

For mutations found at HY9 that decreased the predicted binding affinity of the epitope towards HLA-B*35, it was observed a defective interaction between the second position of mutated epitopes and the B pocket of this HLA-I molecule (Fig. 5D), which is accompanied by a lack of hydrogen bonds between these residues (Supplementary Table 7). These results show that the decreased binding affinity of mutated epitopes towards their respective HLA-I could be explained by a lack of hydrogen bonds between key residues of the epitope and HLA-I pockets, due to an alteration in the chemical composition of the mutated epitopes.

3.6. Role of positively selected mutations on the clinical course of the infection

Finally, as it has been reported that some HIV-1 immune escape mutations selected in $CD8^+$ T cell epitopes under the context of a specific HLA-I molecule lead to a higher replication of the virus, evidenced with an increase in pVL (Feeney et al., 2004), an exploration of whether positively selected mutations could affect the virologic and immunologic course in HIV-infected patients was performed. For this, patients expressing each of the HLA-I alleles specific for the epitopes with positively selected mutations were divided into different groups: patients harbouring the positively selected mutation in the specific HLA-I epitope (Mutated), and patients lacking the mutations (WT), to further compare the pVL and $CD4^+$ T cell count between these groups.

No significant differences were observed in the pVL between individuals expressing HLA-A*02 and harbouring mutations in GC9 (Fig. 6A) or SL9 (Fig. 6B) epitopes, neither in those individuals expressing HLA-B*35 and harbouring mutations in HA9 epitope (Fig. 6E) compared to those that do not have the mutation. However, it was found that patients with the S173 T or S173A mutations in the KAFSPEVIPMFS epitope (KS12, Residues: 162–173) have higher pVL than patients with the WT epitope (Fig. 6C). In addition, patients with S125X mutations in HY9 epitope have significantly higher viral loads than patients not having such mutations (Fig. 6D). An evaluation of the behaviour of pVL from patients harbouring or not these mutations in a one-year retrospective analysis was done, with measurements obtained each three to four months. Interestingly, this inquiry into historical pVL measurements revealed that patients with mutations at KS12 or HY9 epitopes consistently exhibited higher viral loads, compared to patients with WT epitopes (Fig. 7C and D). Regarding $CD4^+$ T cell counts, no significant differences were found between patients harbouring positively selected mutations in the different epitopes and patients without mutations (Fig. 8). However, it was found that patients with positively selected mutations at GC9 epitope presented significantly lower $CD4^+$ T cell counts in the one-year historical analysis compared to patients without such mutations (Fig. 9A). Altogether, these results highlight the potential role of mutations found at KS12 and HY9 epitopes as potential immune escape mutations, as they are associated with higher pVL in patients presenting such mutations.

As different combination of mutations showed dissimilar HLA-I affinity patterns, we compared pVL and $CD4^+$ T cell count only in patients harbouring mutations with predicted high or low affinity. In this regard, we observed that patients with the S173 T mutations in the KS12 epitope had significantly higher pVL than patients with WT epitope (Supplementary Fig. 2C), whereas patients with mutations that decrease the binding affinity of the HY9 epitope towards HLA-B*35 exhibit significantly lower $CD4^+$ T cell counts (Supplementary Fig. 3D).

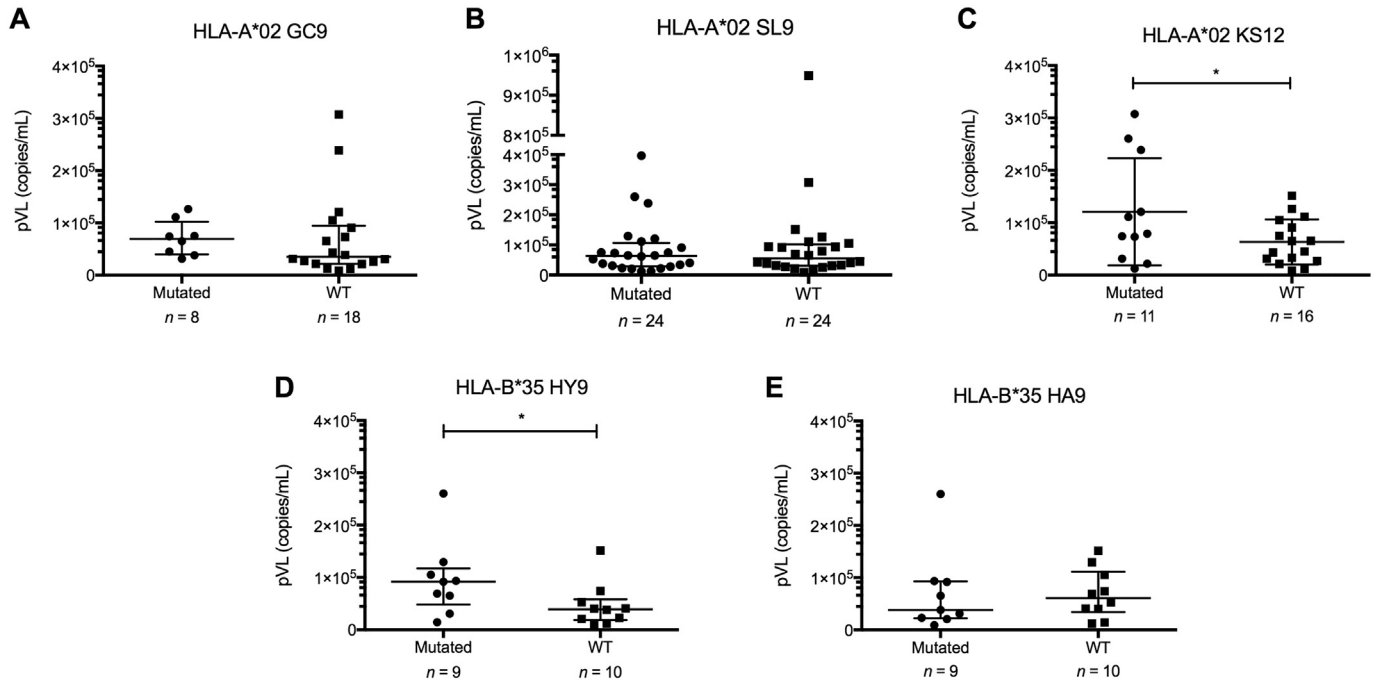

Fig. 6. Positively selected mutations at KS12 and HY9 epitopes are associated with higher pVL. Comparison of pVL of patients expressing the respective HLA-I for the epitopes and harbouring the positively selected mutations (Mutated) and patients expressing the respective HLA-I but not having the mutations (WT). Data are expressed as median and IQR, except for Fig. C, where mean ± SD is presented. Mann-Whitney *U* test (except for HLA-A*02-restricted KS12 where a Student's *t*-test was applied); * $p < .05$.

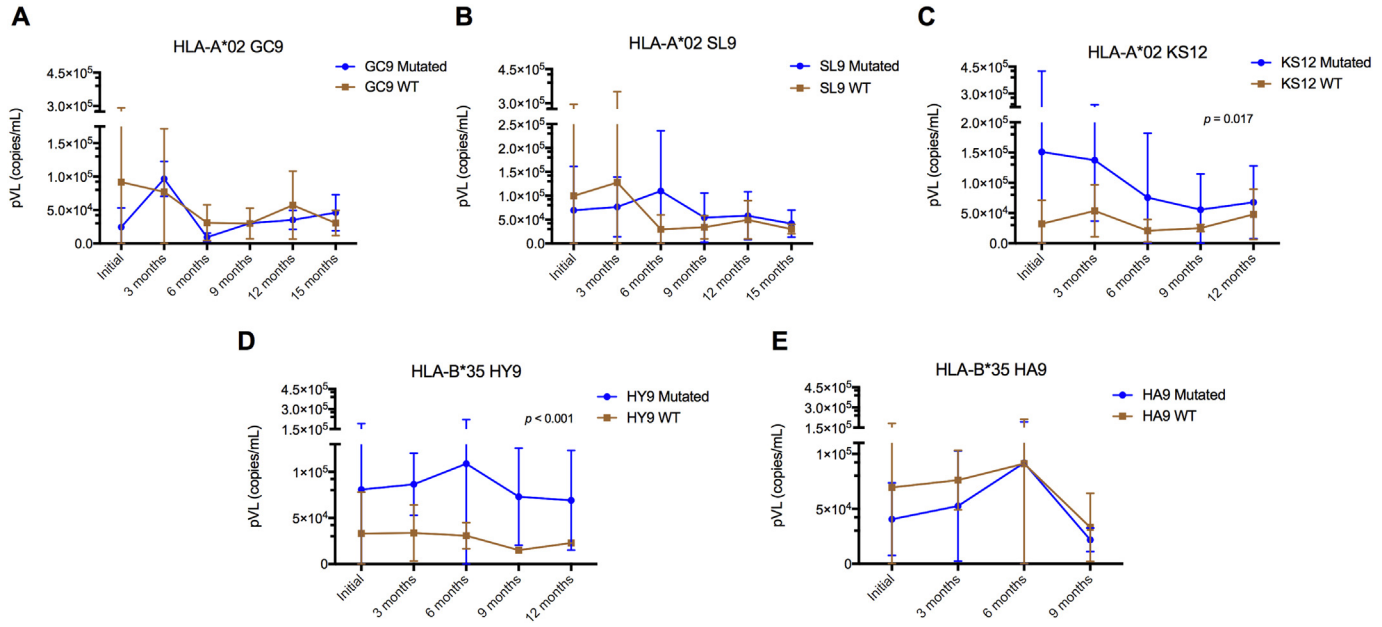

Fig. 7. Patients harbouring positively selected mutations at KS12 and HY9 epitopes have higher pVL over time. Comparison of pVL of patients expressing the respective HLA-I for the epitopes and harbouring the positively selected mutations (Mutated) and patients expressing the respective HLA-I but not having the mutations (WT) in a one-year historical evaluation. Each point at the X-axis represents pVL measurements performed each three to four months, and are depicted as mean ± SD. The *p* value is depicted in the cases where statistical significance was observed; unpaired *t*-test.

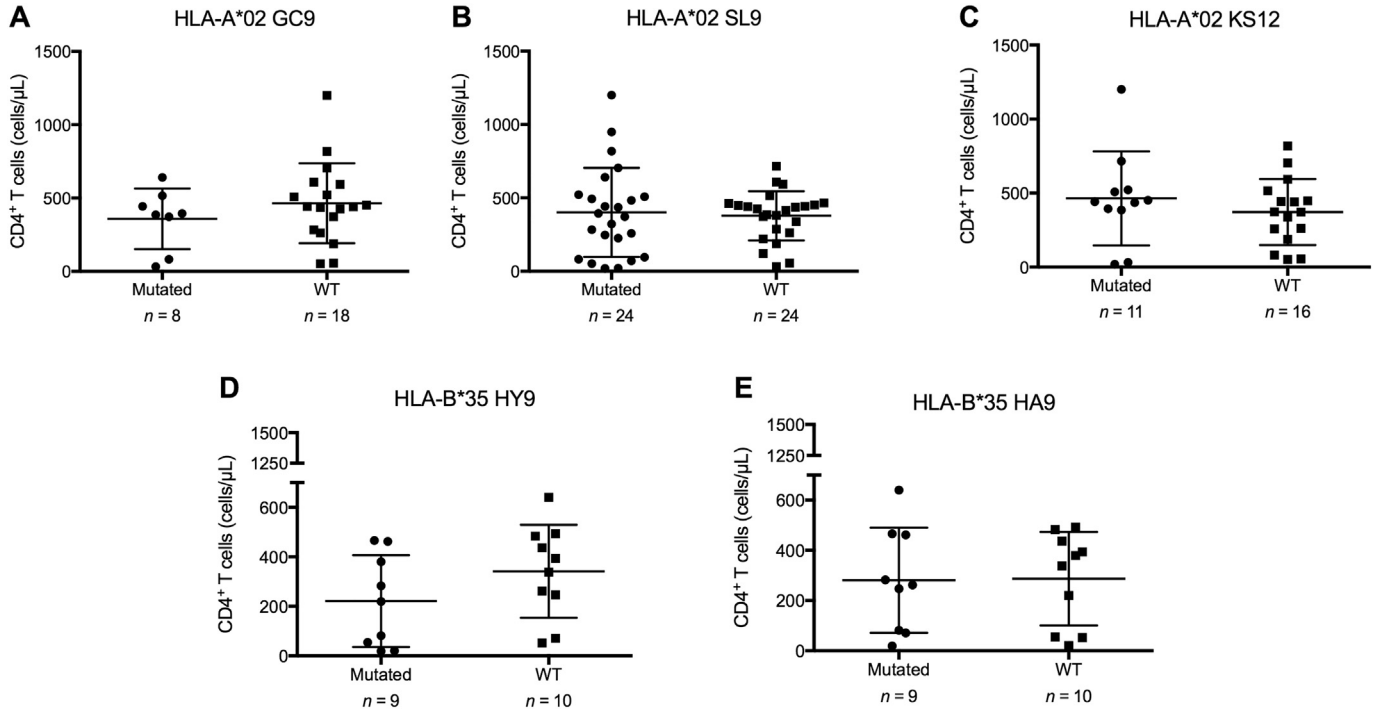

Fig. 8. Positively selected mutations do not affect the CD4$^+$ T cell count. Comparison of CD4$^+$ T cell counts of patients expressing the respective HLA-I for the epitopes and harbouring the positively selected mutations (Mutated) and patients expressing the respective HLA-I but not having the mutations (WT). Data are represented as mean ± SD, Student's *t*-test.

4. Discussion

CD8$^+$ T cells play a crucial role in the control of HIV-1 replication (Koup et al., 1994; Schmitz et al., 1999), and the evidence of a durable control of HIV-1 infection in some patients with higher cytotoxic responses restricted to specific protective HLA-I alleles (O'Brien et al., 2001) highlight the potential benefit of targeting these cells for novel therapeutic strategies, such as vaccines. However, the selection of HIV-1 escape mutations in specific CD8$^+$ T cell epitopes restricted by HLA-I molecules represents the main hindrance for the effectiveness of this strategy (Carlson et al., 2015). This study was focused on the determination of potential HIV-1 escape mutations restricted by HLA-I molecules in the Gag protein of HIV-1 from chronically infected patients of Medellin, as it has been reported that epitopes derived from this protein have high immunodominance patterns (Sacha et al., 2007).

First, a phylogenetic analysis of the sequences was performed.

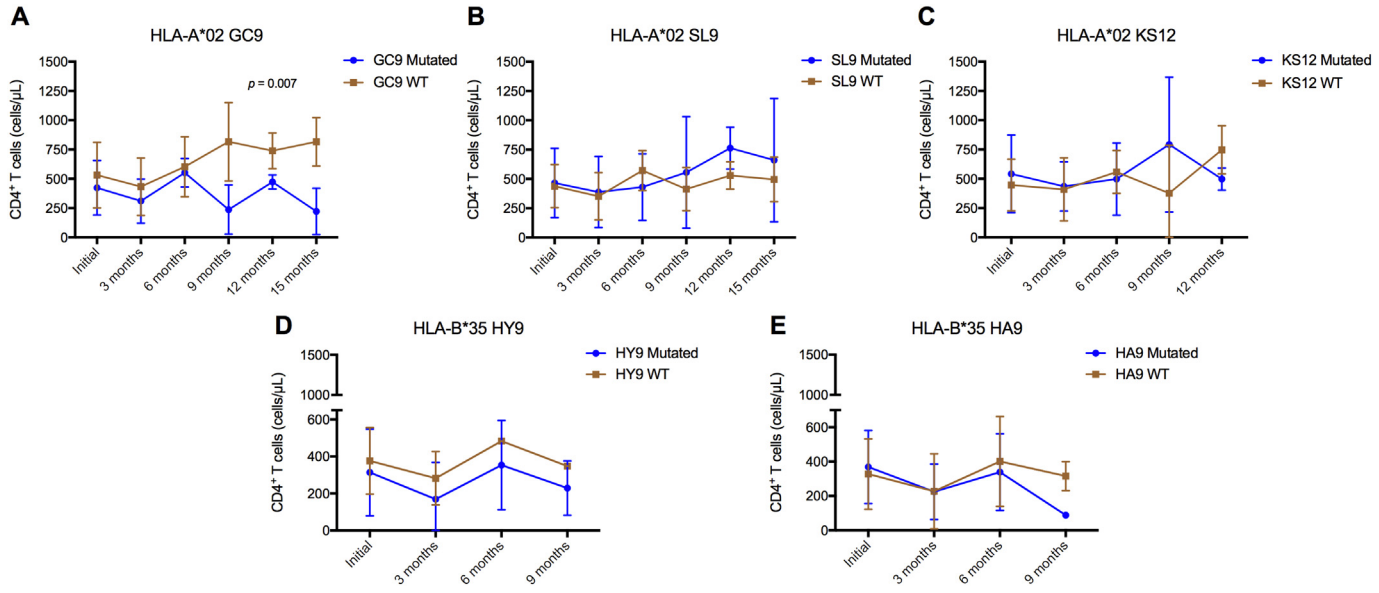

Fig. 9. Positively selected mutations at GC9 epitope are related with decreased CD4$^+$ T cell count over a one-year period. Comparison of CD4$^+$ T cell counts of patients expressing the respective HLA-I for the epitopes and harbouring the positively selected mutations (Mutated) and patients expressing the respective HLA-I but not having the mutations (WT) in a one-year historical evaluation. Each point at the X-axis represents pVL measurements performed each three to four months, and are depicted as mean ± SD. The *p* value is depicted in the cases where statistical significance was observed; unpaired *t*-test.

Although the analysed dataset included 413 parsimony-informative sites, phylogenetic analyses produced trees with low statistical support (Supplementary Fig. 1). This is probably due to the high degree of homoplasy in the dataset, as evidenced by the multiple hits inferred from mapping the selected mutations and by the low consistency index (0,208) and retention index (0,362) of the parsimony analysis. Despite the low support, the coincidence of the monophyletic clustering of all sequences of this study with other subtype B sequences in all analyses indicates that this is the predominant subtype in the sampled population. To detect the presence of positively selected sites in HIV-1 *gag*, a maximum-likelihood based method (SLAC) and a Bayesian method (FUBAR) were applied. SLAC method has been widely employed in different studies addressing HIV evolution (Rolland et al., 2010; Poon et al., 2007; Snoeck et al., 2011); however, these studies have employed larger datasets, comprising > 1000 sequences on average. The sample size of our study could represent a hindrance for the detection of positively selected sites across HIV-1 *gag* sequences, nevertheless, it has been proven that SLAC method is reliable for datasets with > 40 sequences, as this results in a consistent determination of positively selected sites with a low probability of type I and type II errors (Kosakovsky Pond & Frost, 2005). On the other hand, FUBAR method performs the determination of positive selection unconstraining the datasets in a predefined evolution model, therefore it is able to identify positive selection in much smaller sample sizes (Murrell et al., 2013). The reason for using two different approaches was to improve the reliability of the results, since a codon identified as positively selected by two methods based on different statistical frameworks is less likely to represent a false positive. Five positively selected sites were found in the dataset, positions S54, T84, S125, S173 and I223, being all these positions located within epitopes presented by the most frequent HLA-I alleles. Three of these epitopes are recognized as optimal epitopes for the corresponding HLA-I, namely SL9 for HLA-A*02, and HY9 and HA9 for HLA-B*35 (Llano et al., 2013). It has been previously described that positively selected sites are enriched in CD8$^+$ T cell epitopes presented by the most frequent HLA-I alleles in the respective populations, irrespective of the circulating subtype or recombinant form of HIV-1, a phenomenon referred as HLA imprinting effect (Yoshida et al., 2011; Brumme et al., 2007; Leslie et al., 2006). Importantly, this HLA imprinting is often described in critical HLA anchor residues of the epitope (Poon et al., 2007), which was observed in HLA-A*02-restricted SL9 epitope, and HLA-B*35-restricted HY9 and HA9 epitopes.

Two interesting results from this study are the finding of positively selected mutations within HLA-I-specific epitopes in patients lacking such HLA-I alleles, and a few cases of patients carrying viruses without mutations in epitopes recognized by the patient HLA-I alleles (Fig. 2). In the first case, it is likely that these mutated viruses were transmitted from a host that expressed the corresponding HLA-I for the epitope, and that upon transmission this mutation did not revert to a WT phenotype. The rate of reversion depends on whether the mutation was selected by a protective HLA-I allele, such as HLA-B*57/58:01 or -*27, as transmission of mutations in epitopes restricted by such alleles to a HLA-mismatched host leads to a rapid reversion towards a WT phenotype (Leslie et al., 2004; Goulder et al., 2001). Remarkably, various studies have reported stable transmission of mutations selected in patients expressing HLA-A*02 or HLA-B*35 to patients lacking such alleles (Kawashima et al., 2009; Gijsbers et al., 2014; Navis et al., 2008; Duda et al., 2009; Eriksson et al., 2014). The issue that a fraction of patients does not harbour positively selected mutations, despite the expression of the specific HLA-I molecule, could be explained by the sequencing methodology used in this study. Bulk sequencing is insensitive to variants representing < 30% of the viral population by itself (Davenport et al., 2008), unless cloning is previously performed in order to detect minority variants in the viral population. Various studies have employed cloning and sequencing of different viral variants to overcome this limitation (Kearney et al., 2009; Novitsky et al., 2010), and it is necessary to determine if positively selected mutations found in this study could be present in low-frequency variants.

Molecular docking/refinement analysis showed that in HLA-A*02-restricted epitopes, three combinations of mutations at SL9 epitope (Y79F/T84 V, Y79H/T81A/T84 V and Y79F/T84 V/L85F), and one combination at GC9 epitope (S54A/E55G), significantly decreased the binding affinity of the epitopes towards HLA-A*02. Previously, it has been described that the Y79F/T84 V combination reduces the epitope binding affinity towards HLA-A*02 (Tenzer et al., 2009), while the Y79H/T81A/T84 V combination confers immune escape, although its exact mechanism is unclear (Goulder et al., 1997a; Goulder et al., 1997b). Based on molecular docking analysis, it is plausible to infer that the Y79H/T81A/T84 V combination could act as an immune escape

mutation decreasing the epitope binding affinity towards HLA-A*02. Of the four previously described immune escape mutations identified as positively selected at SL9 epitope, two of them did not alter the epitope binding affinity for the HLA-I molecule in docking/refinement assays: T84 V and V82I/T84 V. The latter has been confirmed to act as a TCR-related mutation (Bennett et al., 2010), whilst for the former no mechanism has been established (Jamieson et al., 2003; Tenzer et al., 2009). According to the results obtained in this study, a decrease in the binding affinity towards HLA-A*02 seems unlikely. In HLA-B*35-restricted epitopes, eight different combinations of mutations at HY9 epitope showed a significant decrease in the epitope binding affinity towards HLA-B*35. Altogether, these results demonstrate the sensibility of the selection pressure analysis to find different previously reported immune escape mutations, and the specificity of the employed docking/refinement strategy to suggest HLA-associated mutations that could act as immune escape mutations. Similar studies using selective pressure and docking analyses have been employed to evaluate the possible role of unusual mutations in HIV-1 *pol*, leading to the identification of novel immune escape mutations (Acevedo-Saenz et al., 2015) and drug-resistance mutations (Mata-Munguia et al., 2014), highlighting the potential benefit of using such strategies for the fast determination of different phenotypes related to mutations in the HIV-1 genome.

Surprisingly, it was also observed that the S173 T mutation at KS12 epitope, two combinations of mutations at GC9 epitope (S54A and S54 T/E55T) and all mutations found at the HA9 epitope significantly increased the epitope binding affinity towards its corresponding HLA-I molecule. Mutations with such behaviours have been previously detected using artificial neural network analysis in $CD8^+$ T cell epitopes (Smidt, 2013; Sundaramurthi et al., 2012), and it has been reported that some mutations at HLA-I non-anchor positions can increase the epitope binding affinity (Pogue et al., 1995). Such high affinity epitopes have been described in both $CD4^+$ T cell- (Ahlers et al., 2001) and $CD8^+$ T cell-epitopes, leading to an induction of a Th1 cytokine profile and an augmented $CD8^+$ T cell response (Sundaramurthi et al., 2012; Zarling & Lee, 1998). However, the reason why such epitopes are being selected at the population level in Colombian strains remains elusive, since a higher affinity for the HLA-I molecule could be inducing $CD8^+$ T cell responses that would have a deleterious effect on the virus. The fact that patients harbouring the S173 T mutation at KS12 have significantly higher pVL than patients with the WT epitope suggests that this mutation can act as an immune escape mutation. It is plausible that this mutation along confer non-HLA related immune escape, which are unable to be identified by this methodological approach, for which further experimental analyses are needed to determine if these mutations could affect the cellular processing of the peptides or their recognition by the TCR of $CD8^+$ T cells.

Finally, it was observed that some positively selected mutations found in patients expressing the respective HLA-I allele are associated with a negative outcome of the infection. Specifically, mutations at HLA-B*57 and -A*02-restricted KS12 and HLA-B*35-restricted HY9 epitopes are associated with higher pVL in patients expressing the respective HLA-I (Figs. 6C and D, and Supplementary Fig. 2C). Remarkably, these associations were consistent over time, as revealed by the analysis of clinical data spanning one year of the infection (Fig. 7C and D). It is noteworthy the association of mutations at HLA-B*35-HY9 with higher pVL, as docking/refinement analyses showed that these mutations significantly decrease the epitope binding affinity towards HLA-B*35, indicating that these mutations could confer immune escape. Several studies have reported an increase in pVL upon selection of immune escape mutations, both in HLA-I protective (Crawford et al., 2009; Ammaranond et al., 2011) and non-protective alleles (Brumme et al., 2008). This is consistent with the loss of the immune control mediated by immune escape mutations, leading to an increased replication of the virus within infected hosts. Altogether, these results further support our previous docking/refinement results, suggesting that some amino acid variations at positively selected sites could act as potential immune escape mutations, decreasing epitope binding affinity towards its respective HLA-I, resulting in an increased replicative rate of the mutated virus.

5. Concluding remarks

The most frequent HLA-I alleles in Medellin, specifically HLA-A*02 and HLA-B*35, could be shaping the genetic diversity of HIV-1 *gag* gene through the selection of potential immune escape mutations. Due the association of these mutations with pVL and $CD4^+$ T cell counts, the understanding of positively selected mutations in epitopes restricted by most prevalent HLA-I alleles is fundamental for the understanding of the clinical progression of infected patients. Yet, as this study is an *in silico* approximation for the determination of potential immune escape mutations, further *in vitro* analyses must be performed in order to confirm these results.

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Declaration of interest

None.

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