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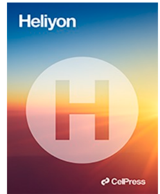

Research article

CD8$^+$T-cell response to mutated HLA-B*35-restricted Gag HY9 and HA9 epitopes from HIV-1 variants from Medellin, Colombia

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ABSTRACT

The HLA-B*35 alleles have been associated with a slow or rapid progression of HIV-1 infection. However, the mechanisms related to HIV-1 progression have yet to be entirely understood. Several reports indicate that the binding affinity between the HLA-I molecule and peptides could be associated with an increased CD8$^+$ T-cell response. Novel HLA-B*35-restricted mutated variants have been described from HSNQVSQNY (HY9) and HPVHAGPIA (HA9) epitopes. Bioinformatic analysis has indicated that these mutated epitopes show low and high binding affinity towards HLA-B*35, respectively. However, the polyfunctionality of CD8$^+$ T-cells stimulated with these mutated and wild-type epitopes has yet to be reported. The results suggest that the low-binding affinity H124 N/S125 N/N126S mutated peptide in the HY9 epitope induced a lower percentage of CD107a$^+$CD8$^+$ T-cells than the wild-type epitope. Instead, the high-binding affinity peptides I223V and I223A in the HA9 epitope induced a significantly higher frequency of polyfunctional CD8$^+$ T-cells. Also, a higher proportion of CD8$^+$ T-cells with two functions, with Granzyme B$^+$ Perforin$^+$ being the predominant profile, was observed after stimulation with mutated peptides associated with high binding affinity in the HA9 epitope. These results suggest that the high-affinity mutated peptides induced a more polyfunctional CD8$^+$ T-cell response, which could be related to the control of viral replication.

1. Introduction

During the last 40 years since HIV/AIDS was discovered, different prophylactic and therapeutic vaccine strategies have been evaluated; however, they have been unsuccessful [1,2]. Currently, the standard treatment for HIV$^+$ individuals is cART (combined antiretroviral therapy), which can control viremia and transmission. Still, it cannot cure HIV-1 infection, and its poor adherence can result in viral rebound [3]. Therefore, pursuing a therapeutic vaccine capable of sustaining viral suppression without the need for cART, also known as a “functional cure,” is a priority [4]. In addition, CD8$^+$ T-cells are crucial in controlling HIV by killing infected cells and thus clearing infection [5,6]. T-cell receptors (TCRs) on CD8$^+$ T-cells recognize viral antigens processed by the infected host

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cells, which are presented as short peptides, mainly with lengths of 8–10 amino acids, by Human Leukocytes Antigen class I molecules (HLA-I) [7]. Hence, vaccines promoting $CD8^+$ T-cell activation are considered a primary aim, especially targeting highly conserved HIV proteins such as Gag, since Gag-specific $CD8^+$ T-cells have been inversely correlated with viral loads and positively related to $CD4^+$ T-cell counts [8–11].

The HLA-I is the most polymorphic allelic system in humans, and HLA-I molecules display a diverse peptide array to the $CD8^+$ T-cells for the immune recognition [12], which influences peptide binding specificity, TCR interactions with the peptide-HLA-I complex, and the nature of the antiviral $CD8^+$ T-cell mediated response. Thus, peptide-based vaccine strategies must consider the HLA-I diversity, targeting conserved epitopes presented by the most frequent HLA-I molecules. Among the HLA-I alleles, the HLA-B locus has the highest allelic diversity, which increases the repertoire of peptides that $CD8^+$ T-cells can present [12]. In Colombia, the most frequent HLA-B allele is HLA-B*35 [13], an allele associated with rapid, but also with delayed progression of AIDS depending on the HLA-B*35 specific proteins and the circulating HIV-1 strains [14–18]. Interestingly, HLA-B*35 alleles are thought to display a greater diversity of self-peptides, leading to a lower probability of recognizing mutated viral epitopes compared to a $CD8^+$ T-cell restricted by other HLA-I molecules [19]. For example, the Gag p24 NY10 epitope presented by HLA-B*35 is associated with the lack of immunogenicity of $CD8^+$ T-cells. However, the mutated epitope with a change of aspartic acid (-D) for glutamic acid (E) in the eighth position (P8) critically increases the binding affinity of the peptide-HLA-I interaction. Also, it leads to a robust $CD8^+$ T-cell response, indicating that a single change in a peptide residue is essential in achieving immune control of HIV infection [16]. Consequently, *in silico* prediction of the peptide-HLA-I binding affinity can expedite the screening of immunogenic peptides and facilitate the development of peptide-based vaccines.

Previously, we identified mutations mapped into HLA-I-specific Gag p17 HY9 and Gag p24 HA9 epitopes associated with decreased or increased binding affinity towards the HLA-B*35 molecule of HIV-1 variants from Medellin, Colombia [20]. Our analysis showed that two HLA-B*35-restricted Gag p17 HY9 epitope mutations were associated with low binding to the HLA-B*35 molecule. Instead, three Gag p24 HA9 epitope mutations showed high HLA-binding affinity to HLA-B*35. Nevertheless, experimental assessment of the peptide immunogenicity is required to validate the peptide and HLA-I binding affinity prediction since it has been demonstrated that the binding affinity of the epitope to HLA-I is not the only determinant of immunogenicity [21]. Therefore, we aimed to evaluate the polyfunctionality of $CD8^+$ T-cells exposed to HLA-B*35-restricted mutations from HIV-1 variants prevalent in our region.

2. Materials and methods

2.1. Study participants

HIV-1^+ individuals possessing at least one allele belonging to the HLA-B*35 subtype were identified. Inclusion and exclusion criteria for this study were established as previously reported [22]. The Bioethics Committee of the Faculty of Medicine, Universidad de Antioquia, reviewed and approved this study. Before participation, all participants provided written informed consent.

3. T-cell counts

T-cell counts were determined by flow cytometry. Briefly, 100 μL of whole anticoagulated peripheral blood was incubated with anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies (BD Pharmigen) for 30 min. Then, red blood cell lysis was performed using 2 mL of BD FACS Lysing Solution 1X (BD Bioscience). Finally, 100.000 events in the lymphocyte region were counted using BD LSR Fortessa flow cytometer and FACSDiva software v8.0.1 (BD Biosciences). The results were expressed as the percentage and absolute $CD4^+$ and $CD8^+$ T-cell counts.

3.1. HLA-I typing and peptides synthesis

As previously reported, HLA-I typing was performed by sequence-specific oligonucleotide (SSO) technology [22]. Peptides were provided by Núcleo de Biotecnología Curauma, Pontificia Universidad Católica de Valparaíso, Chile. Peptide synthesis was performed as previously described [22].

3.2. $CD8^+$ T-cells stimulation

The functional profile of $CD8^+$ T-cells was analyzed on cryopreserved peripheral blood mononuclear cells (PBMCs). Purified PBMCs were cultured with a density of 4×10^6 cells/mL in complete RPMI-1640 media (10 % heat-inactivated fetal bovine serum (FBS)) in 96-well V bottom plates (Costar, Corning). All cells were cultured with 2 μM monensin, 10 μg/mL brefeldin A (both from Thermo Fisher Scientific), 1 μg/mL anti-CD28 (clone CD28.2), 1 μg/mL anti-CD49d (clone 9F10) (both from eBioscience) and 5 μL anti-CD107a-APC (clone H4A3, BD Pharmigen) for 12 h at 37 °C in 5 % CO_2. The cells were incubated with 10 μg/mL of each peptide. Cells incubated only with anti-CD28 and anti-CD49d served as a negative control. PBMCs were treated with 10 μg/mL Staphylococcus Enterotoxin B (SEB) or 50 ng/mL phorbol 12-myristate 13-acetate (PMA) plus 500 ng/ml ionomycin (Sigma-Aldrich) as positive controls.

3.3. Intracellular cytokine staining

After PBMCs incubation, cells were stained with surface antibodies: CD3 (clone UCHT1) and CD8 (clone RPA-T8) and incubated

with the Fixable Viability Dye eFluor 506 (all from eBioscience). Then, cells were fixed/permeabilized using the Foxp3 staining buffer kit (eBioscience), and stained with the following intracellular antibodies: Granzyme B (clone GB11), IL-2 (clone 5344.111), all from BD Bioscience, Perforin (clone B-D48) and IFN-γ (clone 4S.B3) from Biolegend, and IL-10 (clone JES3-9D3 and TNFα (clone Mab 11) from eBioscience. A minimum of 100,000 CD3^{+} events were acquired using the BD LSR Fortessa flow cytometer and FACSDiva software v 8.0.1 (BD Biosciences).

3.4. Flow cytometry analysis

Flow cytometry data were analyzed using FlowJo version X (TreeStar). Background signal was removed, subtracting the negative control values. Gate defining the positive production of cytokines and cytotoxic molecules were combined by the boolean gating strategy as indicated in the FlowJo software. The polyfunctional profile was defined as the capacity of CD8^{+} T-cells to produce multiple markers simultaneously [23,24]. The percentage of cells exhibiting from one to six functions was analyzed as previously described using the SPICE v5.35 software [22,25].

3.5. Statistical analysis

Statistical analysis was conducted using GraphPad Prism v7.0. Shapiro-Wilk test was used to evaluate normality. Additionally, all numerical data were presented as a median and interquartile range. Mann-Whitney test or Wilcoxon signed-rank tests were used to compare groups. P-value <0.05 was considered significant.

4. Results

*4.1. Clinical characteristics of HLA-B*35^{+} HIV-1^{+} participants*

Eleven HIV-1^{+} participants were identified as expressing the HLA-B*35 allele (Table 2). Notably, 82 % of the participants were men, and the median age was 31 years. The median CD4^{+} T-cell count and CD4^{+}/CD8^{+} T-cell ratio were 762 cells/μL and 0.82, respectively. Six out of eleven participants were under combined antiretroviral therapy (cART), with a median of 54 months under treatment and viral loads under the limits of detection. Five out of eleven participants were not receiving treatment (cART-naïve). These participants had a median of viral loads of 25,500 copies/mL and a median diagnosis time of 27 months.

4.2. CD8^{+} T-cell functional capacity is maintained in the study population

First, we aimed to determine the functional competence of the CD8^{+} T-cells obtained from HLA-B*35^{+} HIV-1^{+} participants. Thus, we used antigen-independent stimulation (SEB and PMA/Ionomycin) to assess the CD8^{+} T-cell production of cytokines (IFN-γ, TNF-α, IL-2, and IL-10) and cytotoxic molecules (CD107a, granzyme B and perforin). Both SEB and PMA/ionomycin induced all measured cytokines and cytotoxic molecules production and CD107a expression in CD8^{+} T-cells from most participants (Fig. 1).

Alterations in the functional capacity of CD8^{+} T-cells have been found in cART-naïve participants with chronic infection [26]. However, previous studies had reported a partial re-establishment of CD8^{+} T-cells polyfunctionality in cART-receiving participants [27]. Therefore, we analyzed the differences in CD8^{+} T-cell functional capacity in both populations. The percentage of CD107a^{+-} Granzyme B^{+} CD8^{+} T-cells after PMA/Ionomycin stimulation was higher in naïve-cART participants in contrast to participants on cART (Supplementary Fig. 1). However, no differences were observed in the expression of the other functional markers evaluated (data not shown).

Additionally, we assessed the CD8^{+} T-cell polyfunctionality in HLA-B*35^{+} HIV-1^{+} participants. PMA/Ionomycin promoted an increased polyfunctional profile compared to SEB (Fig. 2). SEB stimulation was characterized by a monofunctional response dominated by TNF-α (purple arc), whereas PMA/Ionomycin induced an IFN-γ-mediated monofunctional response (pink arc). TNF-α and IFN-γ predominated in the bifunctional response to both polyclonal stimuli (Fig. 2A). Stimulation with PMA/Ionomycin significantly increased the percentage of CD8^{+} T-cells with two, three, four, and five functions compared to SEB (Fig. 2C–F). Thus, these results indicate that CD8^{+} T-cells in HLA-B*35^{+} HIV-1^{+} participants produce cytokines and cytotoxic molecules after polyclonal activation.

Lower percentage of CD107a^{+}CD8^{+} T-cells is found after stimulation by the HLA-B*35 low-binding affinity peptide HY9 H124 N/S125 N/N126S.

Table 1
List of wild-type (WT) and mutated peptides located in HY9 and HA9 Gag epitopes.

HIV-1 Protein	Position in Gag	WT epitope	WT epitope sequence	Mutated sequence	Abbreviation Mutated sequence	Peptide affinity relative to WT
Gag-p17	124–132	HY9	HSNQVSQNY	NNSQVSQNY	H124 N/S125 N/N126S	Decrease
				NNSKVSQNY	H124 N/S125 N/N126S/Q127K	
Gag-p24	216–224	HA9	HPVHAGPIA	HPVHAGPVA	I223V	Increase
				HPVHAGPAA	I223A	
				HPAQAGPVA	V218A/H219Q/I223V	

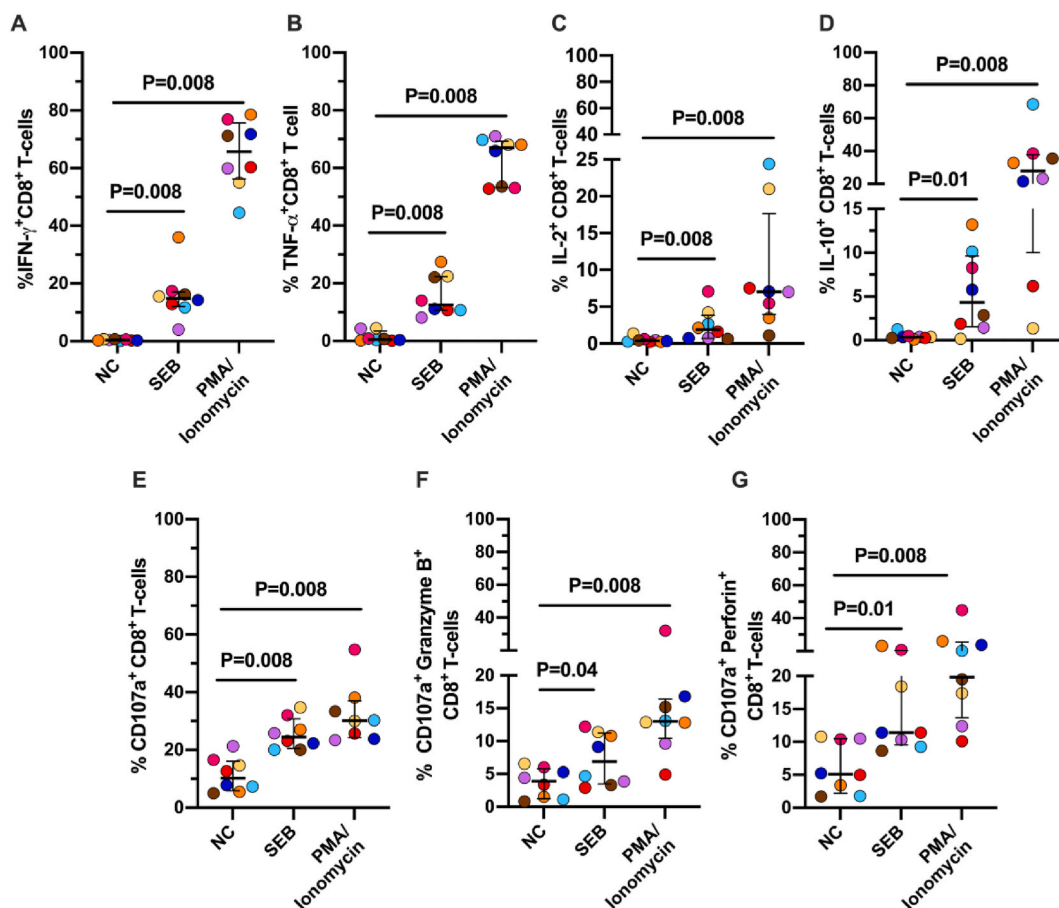


Fig. 1. CD8$^+$ T-cells after antigen-independent stimulation. Percentage of CD8$^+$ T-cells producing IFN-γ **(A)**, TNF-α **(B)**, IL-2 **(C)**, IL-10 **(D)**, expressing CD107a **(E)**, and co-expressing of CD107a with granzyme B **(F)** or perforin **(G)**. CD8$^+$ T-cells in the negative control (NC) were incubated with the costimulatory molecules anti-CD28 and anti-CD49d. CD8$^+$ T-cells were treated with the polyclonal stimuli (SEB and PMA/Ionomycin) for 12 h. Comparison was performed with Wilcoxon signed-rank test. Plots depict median and IQR, n = 8.

The percentage of CD8$^+$ T-cells producing the different cytokines and cytotoxic molecules was evaluated to characterize the functional capacity after antigen-dependent stimulation. CD8$^+$ T-cells were stimulated with the wild-type (HY9 WT) and two mutated peptides of HIV-1 Gag p17 protein (H124 N/S125 N/N126S and H124 N/S125 N/N126S/Q127K) located in the HY9 epitope (Table 1). HY9 WT and the two mutated peptides that had been associated with a lower HLA-B*35-binding affinity induced the production of the measured cytokines and cytotoxic molecules by CD8$^+$ T-cells in most participants (Fig. 3). Notably, the percentage of CD107a$^+$CD8$^+$ T-cells was significantly lower after stimulation with H124 N/S125 N/N126S compared to HY9 WT (Fig. 3E). No differences were observed between HY9 WT and the mutated peptides in the other parameters evaluated (Fig. 3). In addition, no differences were observed in the functional capacity of CD8$^+$ T-cells obtained from naïve-cART participants compared to participants on cART.

Also, we evaluated the CD8$^+$ T-cell polyfunctionality after stimulation by HY9 WT and the mutated peptides. All peptides in the HY9 epitope promoted a monofunctional response characterized by the production of TNF-α (purple arc) (Fig. 4A). Furthermore, HY9 WT bifunctional response was represented by the production of TNF-α and IL-2. Instead, both mutated peptides H124 N/S125 N/N126S and H124 N/S125 N/N126S/Q127K induced a bifunctional response characterized by granzyme B and perforin release. Overall, a similar distribution of the polyfunctional profile was observed with each peptide (Fig. 4).

Higher CD8$^+$ T-cells polyfunctionality is observed after stimulation by the HLA-B*35 high-binding affinity peptides HA9 I223V and HA9 I223A.

The functional capacity of CD8$^+$ T-cells stimulated with the wild-type (HA9 WT) and three HLA-B*35 high-binding affinity mutated peptides of HIV-1 Gag p24 protein (I223V, I223A, and V218A/H219Q/I223V) was evaluated (Table 1). No differences were observed between HA9 WT and the mutated peptides in the parameters evaluated (Fig. 5).

Additionally, the polyfunctionality of CD8$^+$ T-cells was assessed after stimulation by HA9 WT and the mutated peptides. TNF-α, granzyme B, or perforin production characterized each monofunctional response. The bifunctional response was dominated by granzyme B and perforin. The three mutations, I223V, I223A, and V218A/H219Q/I223V, had a higher bifunctional proportion of cells in contrast to HA9 WT (Fig. 6A). Interestingly, the responses of four and six functions were significantly higher for I223A in contrast to

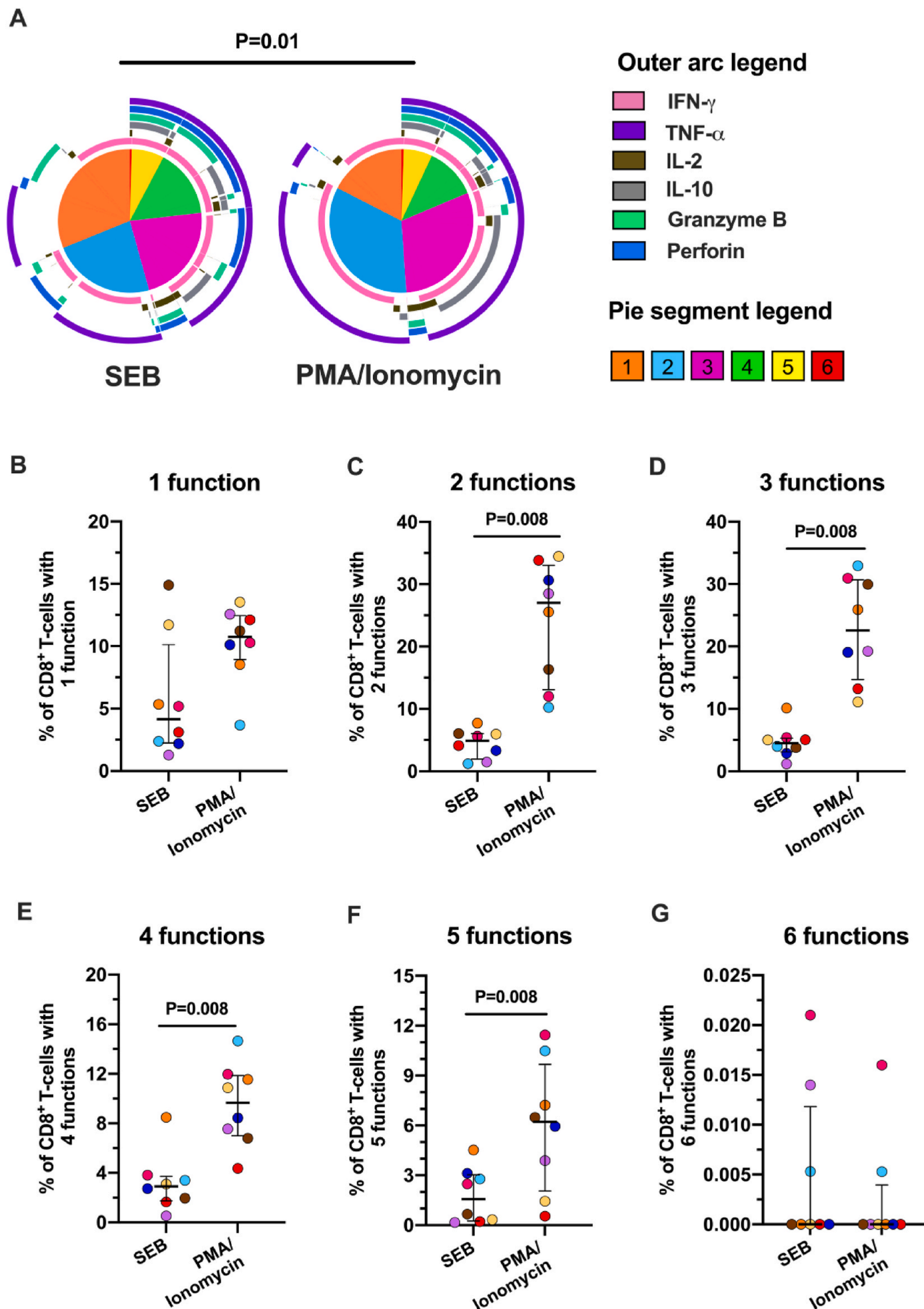


Fig. 2. CD8$^+$ T-cells polyfunctionality after antigen-independent stimulation. **(A)** Pie charts depict the proportion of cells with one to six functions defined according to the production of cytokines and cytotoxic molecules. Outer arc legends represent the molecules produced by each proportion of cells under the arc. Percentage of cells with one **(B)**, two **(C)**, three **(D)**, four **(E)**, five **(F)**, and six **(G)** functions. Comparison was performed with Wilcoxon signed-rank test. Median and IQR are shown; n = 8.

Table 2
Clinical characteristics of the study cohort.

Parameter	Value
Age, years, median (IQR)	31 (25–51)
Gender, Male/Female, number	9 (82 %)/2 (18 %)
$CD4^+$ T-cell counts, cells/mL, median (IQR)	762 (400–1120)
$CD8^+$ T-cell counts, cells/mL, median (IQR)	898 (426–1228)
$CD4^+/CD8^+$ T-cell ratio, median (IQR)	0.82 (0.46–1.62)
Receiving cART, number	6 (55 %)
Time on cART, months, median (IQR)	54 (44–55)
Time between diagnosis and cART initiation, months, median (IQR)	15 (0–26)
Viral load at the time of cART initiation, copies/mL, median (IQR)	685 (144-44,592)
Time since diagnosis cART-naïve individuals	27 (7–31)
Viral load cART-naïve, copies/mL, median (IQR)	25,500 (4535–80,521)

IQR, interquartile range; cART, combination antiretroviral therapy.

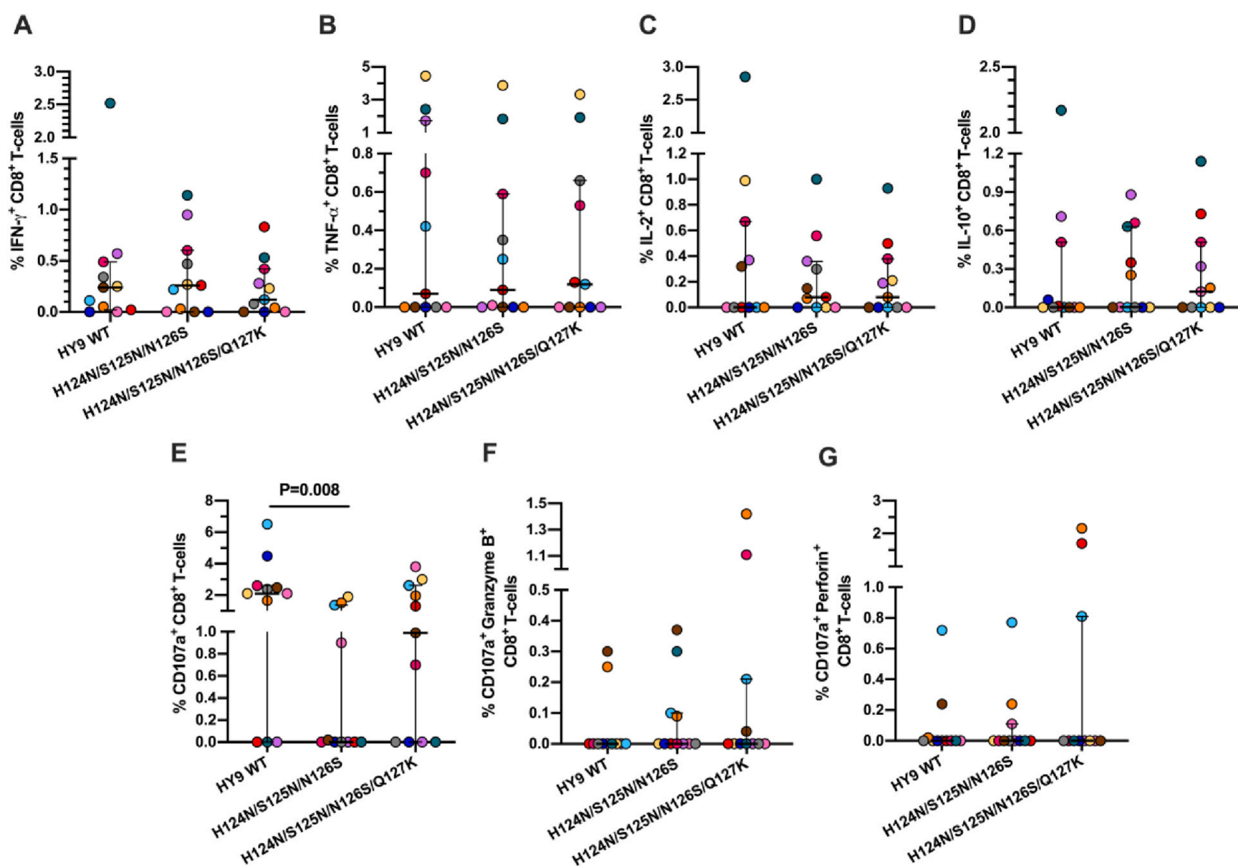
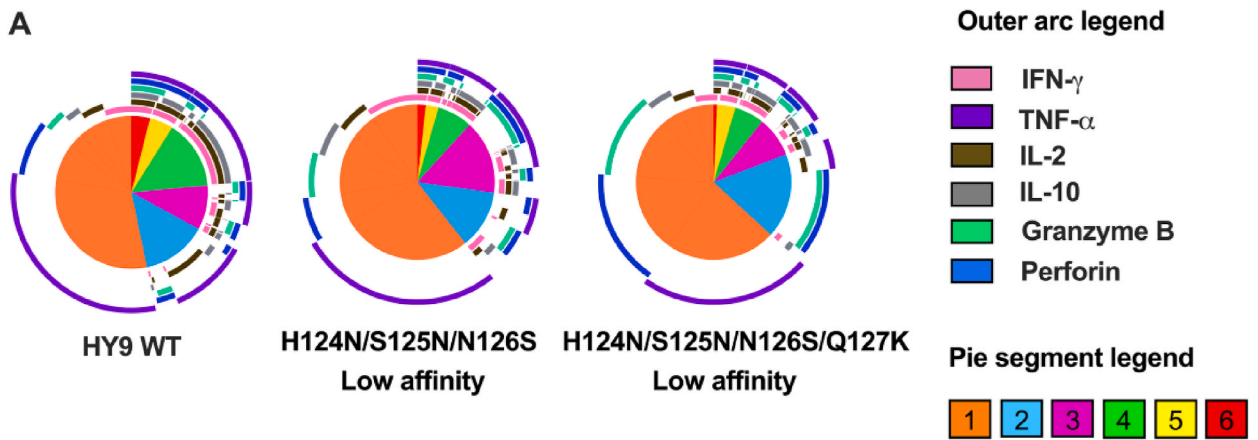


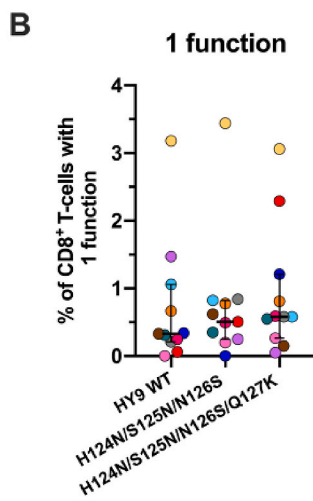
Fig. 3. Cytokine and cytotoxic molecules production in $CD8^+$ T-cell stimulated with HY9 WT, H124 N/S125 N/N126S and H124 N/S125 N/N126S/Q127K peptides. Percentage of $CD8^+$ T-cells producing IFN-γ **(A)**, TNF-α **(B)**, IL-2 **(C)**, IL-10 **(D)**, expressing CD107a **(E)**, and co-expressing CD107a with granzyme B **(F)** or perforin **(G)**. Comparison was performed with Wilcoxon signed-rank test. Graphs show median and IQR, n = 11.

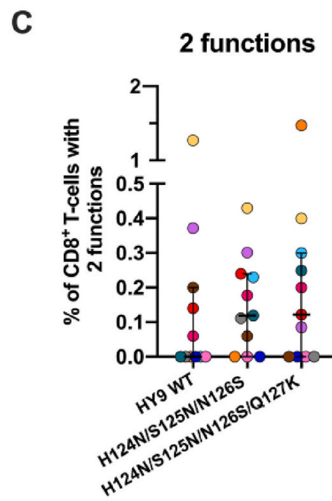
HA9 WT (Fig. 6E and G). Also, the response of five functions was significantly higher for I223V than HA9 WT (Fig. 6F). No differences were found regarding the percentage of $CD8^+$ T-cells with one, two, or three functions (Fig. 6B, C, and 6D).

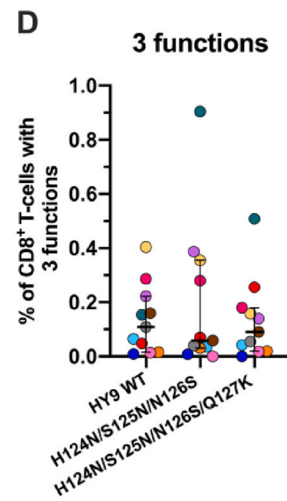
5. Discussion

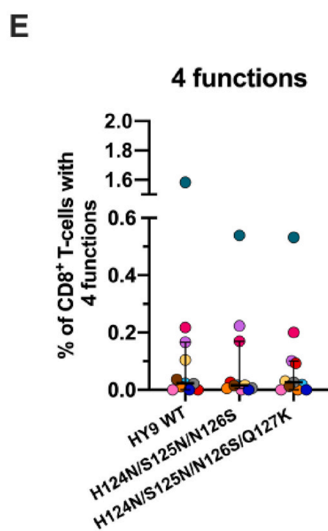
HLA-I molecules are responsible for the binding and presentation of peptides known as epitopes to $CD8^+$ T-cells. Interestingly, HLA-I genes are the most polymorphic in the human genome; thus, they exhibit a variable ability to bind and establish the interaction and affinity of the HLA-I-peptide-TCR complex. The association of HLA-I alleles with the progression of HIV-1 infection is thought to be due to the peptides presented in the HLA-I molecule and the immune response that the antigenic recognition triggers. Multiple studies have

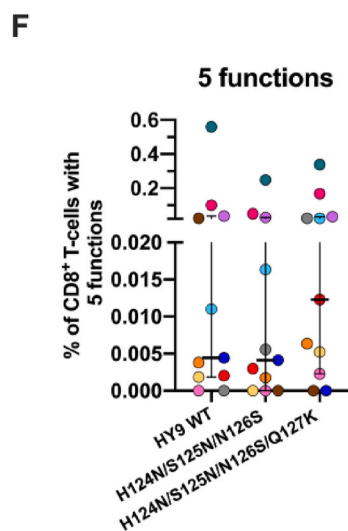












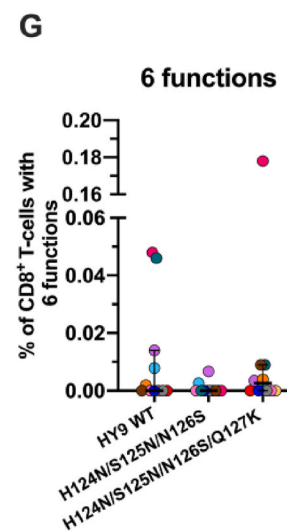


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Fig. 4. CD8$^+$ T-cells polyfunctionality stimulated with HY9 WT, H124 N/S125 N/N126S and H124 N/S125 N/N126S/Q127K. **(A)** Pie charts depict the proportion of cells with one to six functions defined according to the production of cytokines and cytotoxic molecules. Outer arc legends represent the molecules produced by each proportion of cells under the arc. Percentage of cells with one **(B)**, two **(C)**, three **(D)**, four **(E)**, five **(F)**, and six **(G)** functions. Comparison was performed with Wilcoxon signed-rank test. Plots depict the median and IQR, n = 11.

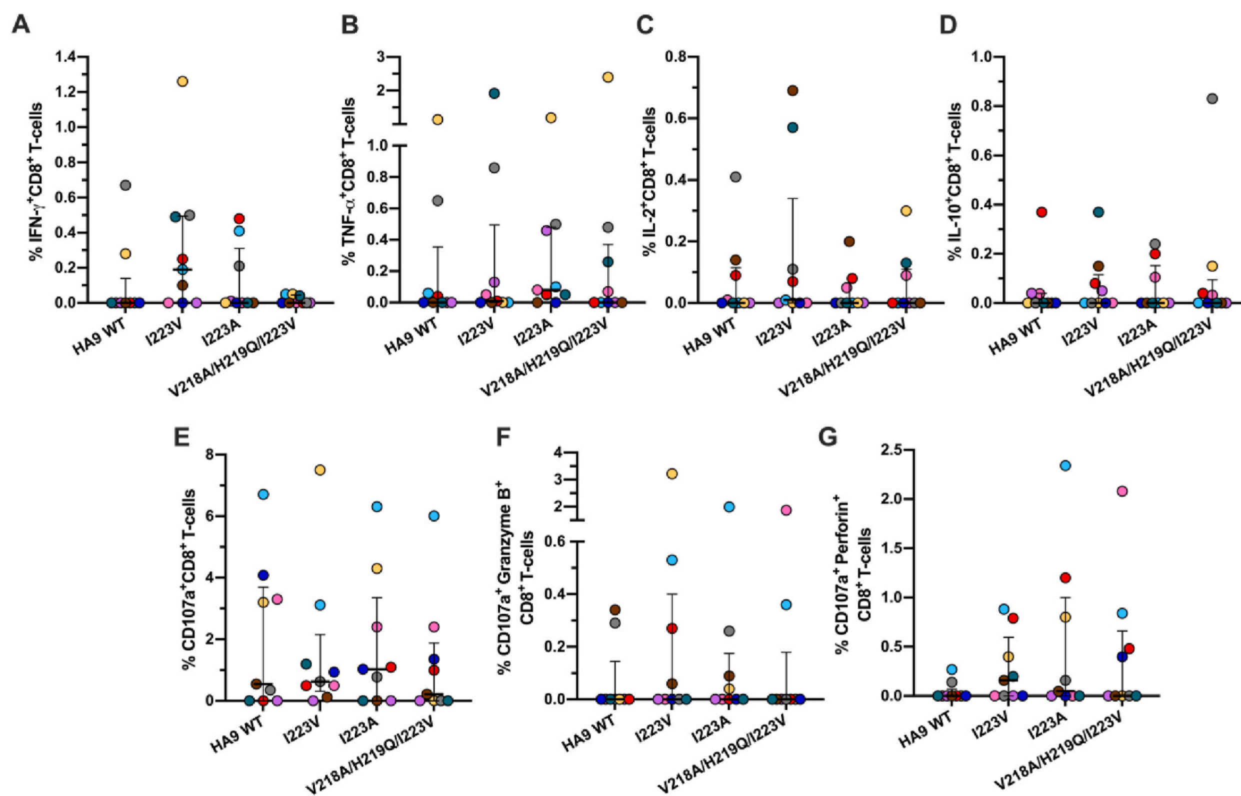

Fig. 5. Cytokine and cytotoxic molecules production in CD8$^+$ T-cell stimulated with HA9 WT, I223V, I223A, and V218A/H219Q/I223V peptides. Percentage of CD8$^+$ T-cells producing IFN-γ **(A)**, TNF-α **(B)**, IL-2 **(C)**, IL-10 **(D)**, expressing CD107a **(E)**, and co-expressing CD107a with granzyme B **(F)** or perforin **(G)**. Comparison was performed with Wilcoxon signed-rank test. Graphs show median and IQR, n = 9.

demonstrated the critical role of CD8$^+$ T-cells in controlling HIV-1 replication [28–30], attributing the control of viremia and slower progression of HIV-1 infection to CD8$^+$ T-cell responses targeting peptides derived from HIV-1 Gag. Previously, we determined the CD8$^+$ T-cell response to Gag peptides restricted to the HLA-A*02 allele and could associate the peptide binding affinity with CD8$^+$ T-cells polyfunctionality [22]. Importantly, HLA-I allotype restriction is a limitation for developing effective peptide-based vaccines; therefore, identifying immunodominant Gag peptides restricted to the most prevalent HLA-I molecules makes peptide-based vaccines accessible to larger patient cohorts [31,32]. Thus, this study evaluated the response of CD8$^+$ T-cells stimulated with the wild-type and mutated peptides from HA9 and HY9 Gag-epitopes restricted to the HLA-B*35 allele. Interestingly, the CD8$^+$ T-cell polyfunctional response was also associated with the binding affinity to the HLA-B*35 allele.

The most polymorphic HLA-I molecule is HLA-B, with HLA-B*35:01 as the most prevalent allele in many populations [33]. For example, the reported HLA-B*35 frequency in Colombia is 22.7 %, with HLA-B*35:01 as the most common allele [34,35]. Interestingly, HLA-B*35 alleles are classified into B*35-Py and -Px alleles. HLA-B*35-Py molecules (B*35:01/08) preferably bind peptides with a proline (P) at anchor residue in position 2 (P2) and a tyrosine (Y) at position 9 (P9). HLA-B*35-Px molecules (B*35:02/03/04), which are associated with a more rapid disease progression, also bind peptides with a proline at P2 but differ in their F-pocket anchor residue P9, with a more promiscuous peptide binding in this site [36]. CD8$^+$ T-cell failure in HLA-B*35-Px$^+$ individuals to control viral replication has also been addressed to selective upregulation of CTLA-4 rather than the peptide specificity [37]. However, in a Peruvian cohort of HIV-1$^+$ individuals, HLA-B*35-Px and HLA-B*35-Py differentiation did not correlate with the HIV-1 progression [38]. Our study included 11 HIV-1 chronically infected participants, of which only 25 % expressed HLA-B*35-Px alleles. The mutated peptide-specific CD8$^+$ T-cells expressed a heterogeneous profile without significant differences between individuals with HLA-B*35-Px, HLA-B*35-Py, or unclassified alleles.

We evaluated the response of CD8$^+$ T-cells to polyclonal stimuli and Gag-specific peptides to determine the functional response of cells obtained from chronically HIV-1$^+$ participants with and without cART. The results demonstrated the functional competence of the CD8$^+$ T-cell population in all participants, possibly associated with the early start of therapy after the diagnosis and because patients

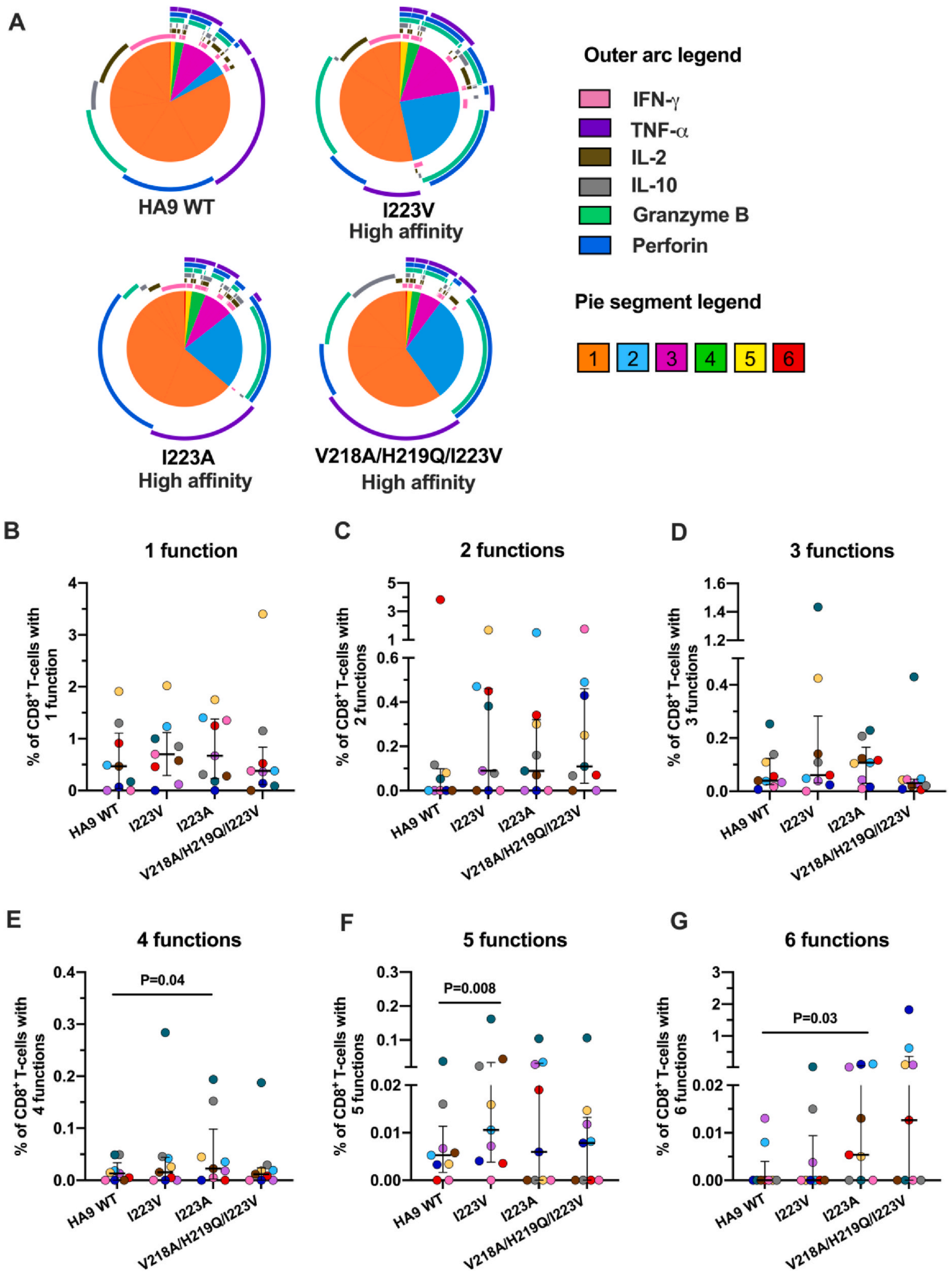

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Fig. 6. $CD8^+$ T-cell in stimulated with HA9 WT, I223V, I223A and V218A/H219Q/I223V. **(A)** Pie charts depict the proportion of cells with one to six functions defined according to the production of cytokines and cytotoxic molecules. Outer arc legends represent the molecules produced by each proportion of cells under the arc. Percentage of cells with one **(B)**, two **(C)**, three **(D)**, four **(E)**, five **(F)**, and six **(G)** functions. Comparison was performed with Wilcoxon signed-rank test. Graphs show median and IQR, n = 9.

without treatment did not show marked clinical or immunological deterioration. Nevertheless, during the acute phase of HIV-1 infection, individuals under treatment have shown an expansion of Nef- and Gag-specific $CD8^+$ T-cells, along with a highly polyfunctional response [39]. Conversely, proliferative capacity, cytokine production, and degranulation of cytotoxic molecules in $CD8^+$ T-cells were reduced in chronically infected patients with therapy [40]. However, other studies have suggested that cART partially restores $CD8^+$ T-cell functions, increases the percentage of HIV-specific $CD8^+$ T-cells with a polyfunctional response, and decreases the expression of the PD-1 molecule associated with immune exhaustion [41,42].

The HSNQVSQNY (HY9) epitope is located in the p17 protein, close to the p17/p24 protease cleavage site, and is a variant observed in HIV-1 sequences belonging to subtype B [43]. NSSKVSQNY (NY9) epitope is known as an optimal HIV-1 subtype B epitope associated with IL-2 and IFN-γ production by $CD8^+$ T-cells [21,44]. Luo M et al. evaluated the recognition of variants belonging to subtypes A, B, and C of the HY9 epitope in patients expressing the protective allele HLA-A*01:01. Notably, the variants HY9, H124 N/S125 N/N126S, and H124 N/S125 N/N126S/Q127K showed comparable affinities, off-rates and IFN-γ release [32]. Our study is the first report describing that more than 1 % of $CD8^+$ T-cells stimulated with the wild-type and the mutated variants of the HY9 epitope in HLA-B^*35^+ participants induce a functional response and can secrete IFN-γ, TNF-α, IL-2, IL-10, granzyme B, and perforin. The NNS pattern has been widely recognized as the typical consensus sequence for glycosylation [45]. Glycopeptides could impact HLA class I binding, interaction with the T-cell receptor, or proteasome digestion [46]. Studies on HIV have examined the effect of glycopeptides and the immune response in gp120-derived epitopes, where N-glycosylation induced $CD4^+$ T-cell responses and altering glycans impaired this response [47]. In cancer research, mice immunized with MUC1 glycoproteins demonstrated a robust $CD8^+$ T cell response and high affinity to H 2K b [48]. Analysis of Gag, a matrix/capsid protein in HIV, revealed numerous sites with β O glycosylation patterns within optimal $CD8^+$ T cell epitopes [46]; however, no information was available regarding N-glycosylation. Additionally, it is noted that our in vitro study utilized synthetic peptides lacking specific glycan patterns. Further research is necessary to confirm whether these epitopes possess significant glycosylation patterns capable of eliciting specific T-cell responses.

The HPVHAGPIA epitope (HA9) is located in the p24 protein and is an optimal epitope, observed in sequences belonging to the B, C, and D subtypes [43] and is thought to be restricted to HLA-B*07, -B*35, and -B*55 alleles [49,50]. $CD8^+$ T-cells stimulated with the HA9 peptide induced high IFN-γ production in patients expressing the HLA-B*35:01 allele, which has been correlated with high binding affinity and stability [49,51]. However, cells restricted to that epitope possibly lack the proliferative potential in chronically infected individuals since IL-2 production has been observed in lower levels [49]. Unlike the HPVHAGPAA (I223A) and HPAQAGPVA (V218A/H219Q/I223V) mutated variants, the HPVHAGPVA mutated variant (I223V) was previously reported by Jessen H et al. [50]; however, the $CD8^+$ T-cells polyfunctionality restricted to this mutated variant has yet to be reported. This is the first study evaluating the production of cytokines and cytotoxic molecules, detecting IL-2^+CD8^+ T-cells in 50 % of the patients stimulated with HA9. Moreover, we observed that IL-2 production was not limited to the wild-type peptide. Cells producing this cytokine were also quantified upon stimulation with the mutated variants derived from the HA9 epitope.

Moreover, we found a predominance of bifunctional Granzyme B^+ $Perforin^+$ $CD8^+$ T-cells after stimulation with high-affinity peptides in the HA9 epitope. The ability of $CD8^+$ T-cells to synthesize cytotoxic molecules such as granzyme B and perforin, following an encounter with a specific epitope, plays a vital role in the elimination of virus-infected cells [52]. Remarkably, the expression of granzyme B, one of the most potent proapoptotic granzymes, by HIV-specific $CD8^+$ T-cells is associated with replication control [53] and the ability to rapidly mediate the elimination of HIV-infected $CD4^+$ T-cells [42]. Interestingly, granzyme B is a cytotoxic molecule expressed at higher levels in HIV-1^+ individuals with uncontrolled viremia, leading to rapid death of infected $CD4^+$ T-cells [54]. The percentage of Granzyme B^+ $CD8^+$ T-cells decreased when the $CD4^+$ T-cell counts improved [55]. Conversely, perforin expression has been observed in HIV-specific $CD8^+$ T-cells from elite controllers/long-term non-progressors, showing an inverse correlation between virus replication and proliferation of this cell subpopulation [55,56].

Notably, the binding affinity of peptides to HLA molecules tends to be associated with higher immune responses [22,57], where amino acid substitutions at critical HIV-1 epitope sites can affect antigenic presentation, reducing the binding affinity and weaken the TCR-peptide-HLA complex interaction [58,59]. Previously, Lund and collaborators reported that peptides with proline in P2 and tyrosine in P9 of the peptide could bind to B7 supertype alleles [60]. Also, it has already been suggested that a proline in P2 contributes to the stability and immunogenicity of the HIV-1 peptide-HLA-B*35 complex [61]. In our study, high-binding affinity mutated peptides HPVHAGPVA (I223V), HPVHAGPAA (I223A), and HPAQAGPVA (V218A/H219Q/I223V) conserved the same amino acid in residue P2. However, only I223V and I223A, which had changes solely in position 8 (P8) but conserving hydrophobic side chains, exhibited increased polyfunctionality. This implies that variations in the amino acid sequence at the C-terminal may contribute to a reduction in the dissociation rate of these epitopes, thereby prolonging the stability of their presentation. As a result, specific $CD8^+$ T-cell clones can properly recognize and activate through T-cell receptor (TCR) recognition [62,63]. These results suggest that the binding peptide affinity predicted *in silico* to HLA-I could be associated with the polyfunctional profile of $CD8^+$ T-cells, which validates previous assumptions implying that a high binding affinity to the HLA-I molecule could contribute to the peptide immunogenicity [64].

6. Conclusions

The results of this study indicate that previously reported mutated variants derived from the HA9 and HY9 epitopes [20] induced a response in $CD8^+$ T-cells from HIV-1^+ participants expressing the HLA-B*35 allele. In addition, high-affinity mutated variants derived from the HPVHAGPIA (HA9) epitope induced a higher percentage of $CD8^+$ T-cells with a polyfunctional profile. Furthermore, these findings highlight the necessity to identify mutations within epitopes restricted to HLA-B*35 to discover potential peptides that contribute to developing an antiviral immune response and designing an accessible peptide-based vaccine for a larger HIV-1^+ cohort.

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Ethical approval

This study was approved by the Bioethical Committee of the Universidad de Antioquia (Act No. 10, May 22, 2017 – Instituto de Investigaciones Médicas). In addition, all individuals signed an informed consent based on Resolution 008430 from 1993 of the Colombian Legislation.

Data availability statement

Data will be made available on request.

Consent statement

Written informed consent was obtained from all the participants prior to enrollment in this study.

CRediT authorship contribution statement

Alexandra Sánchez-Martínez: Methodology, Investigation, Formal analysis, Conceptualization. **Sofía Giraldo Hoyos:** Investigation. **Juan Carlos Alzate-Ángel:** Methodology, Investigation. **Fanny Guzmán:** Writing – review & editing, Methodology, Investigation. **Tanya Roman:** Methodology, Investigation. **Paula A. Velilla:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Liliana Acevedo-Sáenz:** Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paula Velilla-Hernandez reports financial support was provided by Colombia Ministry of Science Technology and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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