CTL Escape Mediated by Proteasomal Destruction of an HIV-1 Cryptic Epitope
Sylvain Cardinaud, Gesa Consiglieri, Romain Bouziat, Alejandra Urrutia, Stéphanie Graff-Dubois, Slim Fourati, Isabelle Malet, Julien Guergnon, Amélie Guihot, Christine Katlama, et al.

To cite this version:
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Abstract
Cytotoxic CD8+ T cells (CTLs) play a critical role in controlling viral infections. HIV-infected individuals develop CTL responses against epitopes derived from viral proteins, but also against cryptic epitopes encoded by viral alternative reading frames (ARF). We studied here the mechanisms of HIV-1 escape from CTLs targeting one such cryptic epitope, Q9VF, encoded by an HIV gag ARF and presented by HLA-B*07. Using PBMCs of HIV-infected patients, we first cloned and sequenced proviral DNA encoding for Q9VF. We identified several polymorphisms with a minority of proviruses encoding at position 5 an aspartic acid (Q9VF/5D) and a majority encoding an asparagine (Q9VF/5N). We compared the prevalence of each variant in PBMCs of HLA-B*07+ and HLA-B*07- patients. Proviruses encoding Q9VF/5D were significantly less represented in HLA-B*07+ than in HLA-B*07- patients, suggesting that Q9VF/5D encoding viruses might be under selective pressure in HLA-B*07+ individuals. We thus analyzed ex vivo CTL responses directed against Q9VF/5D and Q9VF/5N. Around 16% of HLA-B*07+ patients exhibited CTL responses targeting Q9VF epitopes. The frequency and the magnitude of CTL responses induced with Q9VF/5D or Q9VF/5N peptides were almost equal indicating a possible cross-reactivity of the same CTLs on the two peptides. We then dissected the cellular mechanisms involved in the presentation of Q9VF variants. As expected, cells infected with HIV strains encoding for Q9VF/5D were recognized by Q9VF/5D-specific CTLs. In contrast, Q9VF/5N-encoding strains were neither recognized by Q9VF/5N- nor by Q9VF/5D-specific CTLs. Using in vitro proteasomal digestions and MS/MS analysis, we demonstrate that the 5N variation introduces a strong proteasomal cleavage site within the epitope, leading to a dramatic reduction of Q9VF epitope production. Our results strongly suggest that HIV-1 escapes CTL surveillance by introducing mutations leading to HIV ARF–epitope destruction by proteasomes.

Introduction

Multiple lines of evidence suggest that CD8+ cytotoxic T lymphocytes (CTLs) play a critical role in controlling HIV-1 replication. During acute infection, expansion of HIV-specific CD8+ T cells (HS-CTL), before appearance of neutralizing antibodies, is associated with decreased viremia [1] and most likely determines the viral set point during chronic infection [2,3]. Resistance to disease progression correlates with the detection of Gag-specific CTLs and with the presence of particular HLA alleles, such as HLA-B*57 and –B*27 [4,5]. HIV rapidly mutates to evade virus-specific CD8+ T lymphocyte responses, underlining the selection pressure exerted by Gags [6,7,11]. In large part due to its error prone reverse transcriptase activity, HIV possesses a unique capacity to mutate and evade CTL responses. During acute and chronic HIV infection, CTL escape mutations have been well documented [9,12,13]. In most cases, these mutations are intraepitopic and affect HLA binding and/or alter TCR interactions leading to loss of CTL activation or more subtle effects [14]. However, interference with antigen processing may also lead to a reduced generation of precursor peptides and consequently peptide/MHC-I complex formation and T cell activation. This could occur at any stage of the processing pathway. Mutations in epitope-flanking regions might affect proteasomal processing or N-terminal trimming leading to escape from CTL recognition [15–20].

CTLs recognize peptides originating from proteasomal processing of viral proteins or truncated misfolded viral polypeptides, also called DRiPS (for defective ribosomal products) [21–23]. These viral polypeptides are typically derived from the fifteen HIV-1 viral proteins encoded by the nine primary open reading frames.
**Author Summary**

In addition to the classical open reading frames encoding for the well-characterized HIV proteins, HIV exhibits a vast number of alternative reading frames that have the potential to encode proteins or polypeptides. We have previously shown that such reading frames within gag, pol and env genes express T cell epitopes. In the present work, we further characterized the role of T-cell responses targeting the gag-overlapping reading frame in the selection of HIV variants in vivo. We demonstrate that under CD8+ T cell immune pressure, HIV escapes by introducing mutation that affects T-cell recognition of HIV-infected cells. We characterized the mechanism of CTL-escape and demonstrate that HIV manipulates antigen processing and presentation. Our results highlight the importance of CTL targeting these alternative reading frame-encoded antigens in the control of HIV replication.

[24]. However CTLs also target peptides translated from alternative reading frames or ARFs (also called cryptic epitopes). ARF-derived peptides (ARFPs) result from a differential usage of the three-letter codon alphabet during protein synthesis. How this change of reading frame occurs remains elusive but various mechanisms have been proposed. Ribosomes can initiate translation at an internal initiation codon (Met or Cys), change reading frame by shifting, or translate alternatively spliced mRNA. Nonetheless, ARF polypeptides are processed in cells and thus constitute an important source of cryptic epitopes for MHC-I presentation [25]. CTL responses directed against these cryptic epitopes have been detected in autoimmune disease [26], in tumors [27,28] but also in several infectious diseases, including influenza virus [29], murine AIDS [30], SIV [31] and importantly HIV infections [32–35].

We previously described six ARFPs presented by HLA-B*0702 overlapping the alternative reading frames of HIV-1 gag, pol or env genes [32]. CTL responses specific for these ARF-derived peptides were detected in the blood of HIV+ patients. In addition, HIV-infected cells were recognized by CTLs specific for the gag-overlapping ARF epitope (so called Q9VF/5D epitope). Importantly, we showed that the introduction of a stop codon within gag-ARF abrogated Q9VF/5D epitope generation and Q9VF/5D-specific CTL activation [32]. Recent studies further highlighted the in vivo relevance of ARFP-specific CTL responses [33,34,36]. In two independent cohorts studies, Bansal et al. and Berger et al. investigated the association between specific HLA alleles and HIV sequence polymorphisms within ARFs. This “HLA class I footprint approach” allowed the prediction of numerous ARFPs within the HIV-1 genome, both from sense and antisense transcripts. On a restricted number of ARFPs, they also demonstrated that these cryptic epitopes induced CTL responses during natural infection that might contribute to viral control in vivo [33,34].

In the present work, we bring to light a novel mechanism of CTL escape altering the processing and presentation of the Q9VF epitope encoded by the gag-overlapping ARF. In PBMCs of HLA-B*07+ and HLA-B*07- HIV-infected individuals, we first compared the prevalence of QPRSNTYVF (Q9VF/5N) and QPRSDTTHVF (Q9VF/5D) variants of the gag-ARFP. To this end, we PCR amplified and sequenced twenty HIV proviral genomes per individuals. We noticed that the proportion of proviruses encoding Q9VF/5D was significantly lower in HLA-B*07+ than in HLA-B*07- patients, suggesting that Q9VF/5D encoding viruses might be under selective pressure in HLA-B*07+ individuals. In HLA-B*07+ and HLA-B*07- patients, we analyzed ex vivo CTL responses directed against Q9VF/5D and Q9VF/5N and we dissected the immunogenicity of Q9VF variants. We observed that cells infected with HIV-1 strains encoding Q9VF/5N were neither recognized by Q9VF/5N nor Q9VF/5D-specific CTLs. We demonstrate that this single amino acid (AA) variation is responsible for the lack of CD8+ T cell recognition. We show that HIV can escape CTL surveillance by introducing mutations leading to epitope destruction by proteasomes.

**Results**

Analysis of Q9VF gag proviral sequences and Q9VF-specific CTL responses in HLA-B*07+ patients

Q9VF was originally predicted from the sequence of the consensus HIV_HxB2 (HIVLAI) isolate [32]. HIV_LAI bears an asparagine (N) to aspartic acid (D) substitution at position 5 (Q9VF/5D) representing less than 5% of HIV-1 clade B strains retrieved from Genbank. We decided to extend these observations by sequencing HIV proviral sequences isolated from 10 HLA-B*07+ and 10 HLA-B*07- patients. HLA-typing, virological and clinical characteristics of these patients are presented in Table 1. Both groups were age-matched and did not present any significant differences in terms of CD4 counts, viral loads or treatments (not shown). From the PBMCs of each patient, we cloned and sequenced at least 20 HIV-proviral sequences encompassing the gag-ARF DNA region (Figure 1A and Supplementary Figure S1). The isolated HIV sequences encoded either Q9VF/5N (present in 16 out of 20 patients, representing 62% of all isolates), Q9VF/5N variants (exhibiting within the epitope an additional AA difference from the consensus sequence, 9 out of 20 patients, 14% of all isolates) or Q9VF/5D (7 out of 20 patients, 15% of all isolates) and Q9VF/5D variants (2 out of 20 patients, 1% of all isolates) (Table 2). Between Q9VF/5N and Q9VF/5N-variants, Q9VF/5N was the major variant representing 80% of proviral sequences in this group. Q9VF/5D was the major sequence representing 94% of proviral sequences among Q9VF/5D and Q9VF/5D-variants. Note that these mutations did not impact the translation of classical gag ORF (Supplementary Figure S1 and not shown). In contrast, HIV proviruses harboring a STOP codon prior to Q9VF (8% of all isolates) that most likely abolishes Q9VF translation were also identified (Figure 1A). HIV proviral sequences encoding Q9VF/5N and Q9VF/5N-variants were predominant in both HLA-B*07+ and HLA-B*07- patients. Q9VF/5D or Q9VF/5D-variant HIV proviral sequences could be retrieved in two out of the ten HLA-B*07+ patients and in six out of the ten HLA-B*07- donors. Taking into consideration the diversity of HIV sequences per donor with regard to their HLA-B7 status, we observe a significant lower proportion of Q9VF/5D+ HIV strains in HLA-B*07+ than in HLA-B*07- donors (p=0.04, mean value 3% vs 29% of proviral sequences in HLA-B*07+ and HLA-B*07- donors, respectively, Figure 2B). Altogether, these results suggested that Q9VF/5D-encoding HIV strains might be under negative selective pressure in HLA-B*07+ donors. We thus analyzed CTL responses directed against Q9VF/5D and Q9VF/5N epitopes in PBMCs of patients including the 10 HLA-B*07+ patients used for the analysis of HIV proviral sequences.

PBMCs from 31 HLA-B*07+ patients were loaded with various peptides and submitted to IFNγ-ELISPOT (Figure 1C and not shown). Incubations with peptides corresponding to well-characterized HLA-B*0702-restricted immunodominant epitopes from HIV-1 Gag classical ORF (SPRTLNAWV, TPQDLNTML, YPLASLRSLF) induced a significant IFNγ-release, demonstrating that in the course of natural infection the donors mounted CTL
responses to HIV-1 antigens. Five out of the 31 HLA-B*07+ donors showed a low but significant activation with Q9VF/5D and Q9VF/5N peptides (Figure 1G). Note that donors reacted to both peptides or reacted to none and that the frequencies of CTL responding to Q9VF/5D and Q9VF/5N peptides were in the same order of magnitude (from 150 to 300 CTL per million of PBMCs), suggesting that the reactivity to one or the other peptide might be due to cross reactivity. We previously demonstrated that the reactivity to one or the other peptide peptide immunization of HLA-B*0702 transgenic mice and in vitro restimulations previously reported, HIV-infected cells induced a robust affects neither MHC nor TCR binding of the peptide. As we previously demonstrated that the Q9VF/5N variant cross-react on Q9VF/5D peptide (Supplementary Figure S2 and vice versa (32) and Supplementary Figure S2). Viruses encoding Q9VF/5D were not isolated from PBMCs of the five Q9VF responders (Figure 1), with the exception of patients P1 that harbored proviruses encoding a Q9VF/5D variant (QPRGDIHV, representing 16% of sequences in this donor). These data prompt us to study the immunogenicity of the Q9VF/5N- specific CTLs secreted high levels of IFN- 

Table 1. List of patients used in this study.

<table>
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<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>CD4 count (cells/mL)</th>
<th>Time since HIV infection (yr)</th>
<th>Viral load*</th>
<th>Antiretroviral therapyb</th>
<th>Duration of ART (yr)</th>
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<td>B*07</td>
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<td>491</td>
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<td>M</td>
<td>nd</td>
<td>B*07</td>
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<td>9</td>
<td>1776</td>
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<td>B*07</td>
<td>B*08</td>
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<td>1546</td>
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<td>&lt;20</td>
<td>TDF/FTC-DRV</td>
<td>22</td>
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<td>B*07</td>
<td>B*51</td>
<td>414</td>
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<td>B*07</td>
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<td>B*07</td>
<td>B*08</td>
<td>434</td>
<td>24</td>
<td>&lt;20</td>
<td>TDF/FTC-ETR/RAL</td>
<td>20</td>
</tr>
</tbody>
</table>

*Copies of HIV-1 RNA per milliliter of plasma at the time of study.

bTreatment at the time of study: d4T, stavudine; ddi, didanosine; TDF, Tenofovir; FTC, Emtricitabine; ATV, Atazanavir; r, ritonavir; DRV, Darunavir; ETR, Etravirine; LPV, Lopinavir; RAL, Raltegravir; 3TC, Lamivudine; ABC, Abacavir; EFV, Efavirenz; FPV, Fosamprenavir; NVP, Nevirapine; SQV, Saquinavir; AZT, Zidovudine; MVC, Maraviroc. ART, antiretroviral therapy; nd, not determined.

doi:10.1371/journal.ppat.1002049.t001
Figure 1. Q9VF/5D-specific CTLs exert a selection pressure on HIV Q9VF gag-overlapping ARF. (A) Analysis of Q9VF proviral sequences in HIV-infected donors. Using PBMCs, proviral DNA of 20 HIV+ individuals were extracted and the region corresponding to gag-ARF PCR-amplified and cloned. Twenty clones per donor were sequenced. Results are presented as percentage of provirus encoding for Q9VF/5D and 5D variants exhibiting within the epitope an additional AA difference from the consensus sequence, Q9VF/5N and 5N variants, and sequence harboring a stop codon prior the epitope (no epitope). Pies on the right represent percentage of provirus combined for all isolates. Top and bottom panels, results for HLA-B*07+ and HLA-B*07- donors, respectively. (B) Percentage of provirus encoding Q9VF/5D or 5D variants within HLA-B*07+ and HLA-B*07- patients. Each dot represents percentage within the PBMCs of one donor. In HLA-B*07+ patients, variants with 5D are under-represented (P=0.04). (C) Immunogenicity
to the incapacity of HIVNL-AD8-infected cells to activate HS-CTLs since CTL clones specific for an HLA-B*0702-restricted HIV-1 Nef epitope (F10LR), raised as a control in these experiments, were activated upon co-culture with HIVLAP- and HIVNL-AD8-infected cells.

To extend these observations to other HIV-1 isolates, HLA-B*0702+ cells were also infected with HIVMN that encodes for Q9VF/5N and used as target cells to activate Q9VF/5D- and Q9VF/5N-specific CTLs (Supplementary Figure S3). HIVNL-AD8- and HIVMN-infected cells did not induce Q9VF/5D- nor Q9VF/5N-specific CTL activity. Overall, these results suggested that HIV-infected cells did not present the Q9VF/5N peptide.

Epitope flanking regions have a direct impact on antigen processing and presentation [38]. Thereafter, to exclude the possibility that HIV sequence variations outside the Q9VF/5N peptide might be responsible for the lack of presentation, we introduced in HIVLAI a D to N mutation within the Q9VF epitope (so called HIVLAI-D3>D5N). This mutation did not affect the primary open reading frame of Gag (Supplementary Figure S1) and did not alter viral replication in T cell lines or primary CD4+ T cells (Figure 2B). However, cells infected with HIVLAI-D3>D5N could not activate Q9VF/5D- nor Q9VF/5N-specific CTLs (Figure 2C). Thereafter, this single amino acid substitution was sufficient to abrogate CTL recognition, thus indicating that this asparagine alters Q9VF MHC-I presentation. We then sought to dissect the mechanism responsible for the lack of Q9VF/5N MHC-I presentation.

Q9VF/5N binds TAP pumps and HLA-B*0702 molecules

The capacity of antigenic peptides to bind to a given HLA allele is determined by the so-called anchor residues [39]. Mutating an anchor residue abrogates peptide HLA-binding and subsequent T cell activation, a strategy often used by viruses to escape viral-specific T cell responses. The anchor residues of HLA-B*0702 reside at position 2 and 9 of the peptide-ligands. Thereafter, the D to N substitution at position 5 was not predicted to influence Q9VF peptide binding to HLA-B*0702 [40]. However, besides anchor residues, auxiliary residues might affect peptide binding, we thus compared the capacity of Q9VF/5D and Q9VF/5N peptides to bind HLA-B*0702. To this end, T2-HLA-B*0702 cells were loaded with Q9VF/5D or Q9VF/5N peptides and binding to HLA-B*0702 molecules at the cell surface monitored by FACS (Figure 3A, left panel). Q9VF/5D and Q9VF/5N peptides exhibited similar capacities to bind HLA-B*0702 with a relative affinity (RA, based on the reference peptide) of 2.6 and 1.5 respectively (Figure 3A, left panel). To further characterize the impact of the D to N substitution on peptide-MHC interactions, we compared the capacity of the peptides to stabilize HLA-B*0702 molecules at the cell surface of T2-HLA-B*0702 (Figure 3A, right panel). To this end, T2-HLA-B*0702 were cultured with O/N at 26°C to allow surface expression of peptide-receptive MHC molecules, loaded with a high concentration of peptides, shifted to 37°C and the stability of HLA-B*0702-peptide complexes monitored by FACs at various time points. An exponential regression of HLA-B*0702 mean fluorescence intensity (MFI) vs. time reveals that the stability (t1/2) of HLA-B*0702 pulsed with an irrelevant peptide (S9L) is 22 min while binding of Q9VF/5D and Q9VF/5N peptides prolongs the t1/2 to 211 and 641 min respectively (Figure 3A, right panel). Thereafter, Q9VF/5D and Q9VF/5N peptides are very good HLA-B*0702-binders and 5D to 5N substitution tends to prolong surface expression of HLA-B*0702.

Precursor peptides are transported by the TAP pumps (transporter associated with antigen processing) from the cytosol into the endoplasmic reticulum (ER), and then loaded on nascent MHC-I molecules [41]. N-terminally extended peptide precursors are also transported and further trimmed in the ER by the endoplasmic reticulum aminopeptidase ERAAP and bound to MHC-I molecules [42,43]. We asked whether the absence of Q9VF/5N peptide presentation by HLA-B*0702 within infected cells might be the result of inefficient ER-translocation of the Q9VF/5N epitope and/or Q9VF/5N-peptide precursors by TAP. Hence, we used a TAP-binding assay [44] to evaluate the affinities of Q9VF/5D and Q9VF/5N and their precursors with TAP. Q9VF/5D and Q9VF/5N exhibited a poor affinity for TAP (Figure 3B), most likely due to the presence of a proline at position 2 that negatively impacts on TAP-mediated peptide transport [44]. In contrast, their N-terminally extended peptide precursors EGF-Q9VF/5D and EGF-Q9VF/5N showed at least a two-log increased efficiency to compete for TAP with an equal 1/IC50 of 0.15. Whatever the precursor, Q9VF/5D and Q9VF/5N containing peptides did not show differences in their capacity to bind human TAP molecules.

Overall, these data demonstrated that the D to N substitution within Q9VF does not impact on TAP transport and HLA binding. In contrast, the 5N substitution might prolong epitope presentation on the cell surface.

Q9VF/5D epitope generation is dependent on proteasomal cleavages

The proteasomes, that are the major catalytic enzymes involved in antigen processing, generate the carboxyl termini of most MHC-bound peptides [38,45]. We thus asked whether the generation of Q9VF/5D was dependent on proteasomal processing. To this end, HLA-B*0702+ cells were infected with HIVLAI. Five days pi, infected cells were incubated with a potent and selective proteasome inhibitor, epoxomicin [46], treated with a citrate-phosphate buffer to remove residual MHC-peptide complexes, washed and cultured with Q9VF/5D-specific CTLs as previously described. Epoxomicin treatment abolished the capacity of HIVLAI-infected cells to activate Q9VF/5D-specific CTLs, as measured in IFNγ-ELISpot (Figure 3C, left panel). Note that epoxomicin inhibition affected neither MHC-density (as monitored by FACS, not shown) nor the capacity of treated cells to present exogenous peptide (at 0.1 μg/ml) [Figure 3C, right panel]. Thereafter, these results demonstrated that the generation of Q9VF epitope depends on proteasomal processing.

5N introduces an aberrant proteasomal cleavage site within Q9VF epitope

Proteasomes might also destroy CTL epitopes by generating aberrant cleavages within the epitope [47] or in epitope-flanking regions [19,48]. We thus asked whether aberrant proteasomal
Table 2. Frequencies of HIV-1 proviruses encoding Q9VF epitope variants in PBMCs of studied patients.

<table>
<thead>
<tr>
<th>Provirus encoding Q9VF variant</th>
<th>Patients HLA-B*07+</th>
<th>Frequency of provirus (%)b</th>
<th>Patients HLA-B*07-</th>
<th>Frequency of provirus (%)b</th>
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<td>QPRSDFTHVF</td>
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<tr>
<td>QPRSDFTHVF</td>
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<tr>
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**Notes:**

- Number of patients in which at least one proviral clone encodes the Q9VF variant epitope/total number of tested patients.
- Frequency of proviral clones encoding Q9VF variant epitope among the twenty clones sequenced per patient.
- Average frequency of proviruses among the ten studied patients (HLA-B*07+ or HLA-B*07-).

doi:10.1371/journal.ppat.1002049.t002
Figure 2. Q9VF/5D to Q9VF/5N substitution abrogates CTL recognition of HIV-infected cells. (A) T1-B7 cells were infected with HIVLAI and HIVNL-AD8 expressing Q9VF/5D and Q9VF/5N, respectively. Two days p.i., the percentage of HIV-infected cells was monitored by intracellular p24 staining and flow cytometry: 50 and 47% of the cells were infected with HIVLAI and HIVNL-AD8, respectively. In an IFN-γ-ELISpot assay, infected cells were then used to activate CTL lines specific for Q9VF/5D, Q9VF/5N or an HLA-B*07-restricted HIV-1 Nef epitope (FPVTPQVPLR, F10LR) used as control. For each peptide, specific CTL lines were generated in three different HLA-B*0702 transgenic mice and used in two independent experiments.
HIV-1 Escapes CTLs Specific for Cryptic Epitope

The three-letter codon alphabet allows protein synthesis in six possible overlapping reading frames. A vast number of ARFs have the potential to encode proteins or epitopic peptides (ARFPs). Using an “HLA class I footprint” approach, Bansal et al and Berger et al recently predicted the existence of numerous ARFPs within HIV-1 genome [33,34]. We have previously shown that ARFP-specific CTLs are induced during natural infection [32]. These CTL responses might contribute to viral control driving HIV evolution at the population level. ARFPs can mutate during the first year of infection, suggesting a possible selection of escapes variants [33,34]. Such a scenario has been highlighted in the macaque model of SIV infection [31]. Mamu-B*17+ macaques generate strong CTL responses against SIV ARF-encoded epitopes leading to ARF mutation affecting epitope binding to Mamu-B*17 molecules and subsequent SIV replication rebound [31]. In the present study, we characterized a novel mechanism of ARFP-specific CTL escape resulting from HIV epitope destruction by the proteasomes. We suggest that ARFP-specific CTLs exert a selection pressure leading to negative selection of targeted HIV strains. Overall, our work shows that CTL escape mutations are not limited to epitopes encoded by classical ORF, highlighting the role of ARFP-specific CTLs in the control of HIV infection.

We previously identified a panel of epitopes encoded by ARFs within HIV-1 gag, pol and env genes [32]. The gag-overlapping ARF encoding for the Q9VF epitope presented by HLA-B*0702 drew our attention due to its polymorphism. In a cross-sectional cohort study, we report that proviruses encoding the Q9VF/5D epitope (and 5D variants) are rare and significantly under-represented in PBMCs of HLA-B*0702+ patients, thus suggesting Q9VF/5D-specific CTLs might exert a negative selection pressure on HIV strains encoding Q9VF/5D variants. In HIV-1 gag ARF, the virus might escape CTL immune pressure by introducing a 5D to 5N substitution or Stop codons but prior the epitope. We thus analyzed CTL responses directed against Q9VF/5D and Q9VF/5N expressing Q9VF/5N was engineered by PCR mutagenesis of the HIV-1 strain. Whatever the viral input (1, 10 or 100 ng/ml), SN substitution did not alter the replication capacity of HIV-1 gag ARF. T1-B7 cell infection (left panel) was monitored using GFP expression (upon trans-activation of LTR-GFP). Data are representative of at least five independent experiments using various viral inputs. CD4+ T cells infection was monitored using p24-Elsa (right panel) and correspond to the mean values (±SD) of two infections using activated CD4+ T cells from two donors and are representative of two independent experiments (using various viral input). NI: not infected. (C) SN substitution is sufficient to abrogate CTL recognition of HIV-infected cells. As in (A) using T1-B7 cells infected with HIVLAI, HIVNL-ADA and HIVLAI-5D -infected rates were around 30% of p24+ cells.

doi:10.1371/journal.ppat.1002049.g002
Figure 3. Q9VF/5N binds TAP pumps and HLA-B*0702 molecules. (A) Q9VF/5N and Q9VF/5D peptides exhibit similar affinities for HLA-B*0702. (Left panel) Q9VF/5D, Q9VF/5N and their natural EGF Nt-extended precursors were loaded O/N at RT on T2-B7 cells. An HLA-B*07-restricted CMV-derived reference epitope (pp65 RPHERNGFTV, R10TV) and an HLA-A*02-restricted HIV-1-derived epitope (p17 SLYNTVATL, SL9) were also used as positive and negative control, respectively. HLA-B*0702 binding was monitored using ME-1 antibody and flow cytometry. Based on the reference peptide R10TV, a relative affinity (RA) was calculated. Data are representative of three different experiments (mean values of triplicates $\pm$SD). (Right panel) T2-B7 were cultured O/N at 26°C to increase peptide-receptive cell surface molecules, pulsed with the indicated peptides for 2 h in presence of β2-microglobulin and BFA to stop delivery of newly synthesized MHC-I molecules. Cells were then shifted to 37°C for 1 h, washed to remove unbound peptides and incubated at 37°C in presence of BFA (0.5 μg/ml) which is considered as time “zero”. At the indicated time points, samples were harvested and processed for flow cytometry.

(B) Inhibitory binding assay. The inhibition of 1/IC50 was calculated as the ratio of specific binding of the indicated peptide to the binding of the reference peptide R10TV (RA=1). The IC50 values were calculated using the Sigmoidal dose response fit (logistic). The inhibitory binding of peptides Q9VF/5N and Q9VF/5D to HLA-B*0702 was significantly different (p<0.05) from the binding of the reference peptide R10TV. (C) Analysis of IFNγ production in response to Q9VF/5D peptide stimulation in the absence or presence of HIV LAI. The IFNγ production was determined by ELISA and expressed as the number of IFNγ-producing cells per million cells. The induction of IFNγ production was significantly higher in the presence of the peptide Q9VF/5D compared to the mock control. The addition of Epoxomicin significantly reduced the IFNγ production.
were removed to 0°C, stained on ice using ME1 Ab and analyzed by FACS. Data are mean values of two independent experiments. The capacity of each peptide to stabilize HLA-B*0702 (t1/2) was compared using exponential regression. t1/2 of HLA-B*0702 pulsed with the irrelevant peptide (59L) was 22 min while binding of Q9VF/5D and Q9VF/5N peptides prolonged the t1/2 to 211 and 641 min respectively. t1/2 of CMV (pp65 TPRVTGGGAM, T10AM) and Gag (p24 TPQDLNTML, T9ML) peptides used as positive were 552 and 124 min respectively. T1/2 of HLA-B*0702 pulsed with the irrelevant peptide (S9L) was 22 min while binding of Q9VF/5D and Q9VF/5N peptides prolonged the t1/2 to 211 and 641 min respectively. T1/2 of CMV (pp65 TPRVTGGGAM, T10AM) and Gag (p24 TPQDLNTML, T9ML) peptides used as positive were 552 and 124 min respectively. T1/2 of HLA-B*0702 pulsed with the irrelevant peptide (S9L) was 22 min while binding of Q9VF/5D and Q9VF/5N peptides prolonged the t1/2 to 211 and 641 min respectively. T1/2 of CMV (pp65 TPRVTGGGAM, T10AM) and Gag (p24 TPQDLNTML, T9ML) peptides used as positive were 552 and 124 min respectively.

We dissected the immunogenicity of the Q9VF/5N epitope. We showed that cells infected with HIV-1 strains encoding Q9VF/5N (HIVNLAD and HIVMN) were not recognized by Q9VF/5N-specific CTLs. In contrast, Q9VF/5N- and Q9VF/5D-specific CTLs were activated by HIV-1 strains encoding Q9VF/5D (HIVLAI). We demonstrated that the single AA substitution from 3D to 5N in HIVLAI sequence is sufficient and required to abrogate CTL recognition of HIV-infected cells. Thereafter, the acquisition of this 5N mutation by HIV might help the virus to interfere with Q9VF epitope expression or processing and presentation. Viruses can interfere with antigen expression to escape CTL lysis [23]. Various mechanisms have been proposed for the biosynthesis of ARF-derived polypeptides. Ribosomes can scan through conventional initiation codons [29], initiate translation at an internal initiation non-AUG-codons (Leu or Cys) [34,32], change reading frame by shifting [33], or translate alternatively spliced mRNA (for review see [25]). We previously described the presence of a conserved slippery motif (UUUAAAU) upstream of gag-ARF start codon that may facilitate ribosomal slippage and thus Q9VF synthesis [32]. Interestingly, a structured region (hairpin) in HIV-1 RNA has been identified downstream of this slippery motif [33]. This highly structured RNA region might cause ribosomal pausing during gag translation thus facilitating ribosomal slippage and Q9VF expression. The D to N substitution within the Q9VF epitope is translated from a codon that is located in the flexible loop of the RNA hairpin structure [33]. Although it remains to be formally proven, this D to N substitution most likely does not impact the RNA structure and hence Q9VF expression.

Viruses also manipulate antigen processing and presentation to escape CTL responses. Interference with antigen presentation could arise at any stage in the pathway, including processing by proteasomes, binding of epitope-precursors to TAP, destruction of these precursors by peptidases in the ER or cytosol and peptide binding to the MHC-I molecule. HIV-specific CTL responses have been shown repeatedly to select for intra-epitope mutations that affect HLA-binding or TcR recognition. In addition, HIV escape mutations outside the epitope (extra-epitope mutations) can interfere with antigen processing by proteasomes [17–19,47,54,55] or by the ER aminopeptidase ERAAP [16]. To our knowledge, intra-epitope mutations affecting antigen processing have not been described thus far. Several studies proposed that intra-epitope variation might affect processing but did not provide a mechanism [34,30]. The only evidence that intra-epitope mutations might affect proteasomal processing of viral antigens comes from mouse models [47,36].

We provide several lines of evidence strongly suggesting that the D to N substitution within the Q9VF epitope impacts neither TcR recognition nor MHC binding: i) Q9VF/5N- and Q9VF/5D-specific CTLs can be generate upon peptide immunization of HIVLAI-transgenic mice and cross-react to the alternate peptide [[32] and Supplementary Figure S2]; and ii) Q9VF/5N and Q9VF/5D peptides bind HLA-B*0702 (Figure 3A). In addition, we show that Q9VF/5N and Q9VF/5D peptide and their precursors (elongated on the N-termini) efficiently bind TAP, thus demonstrating that the D to N substitution does not affect peptide translocation into the ER. As previously observed with peptides bearing a proline at position 2 [44], the optimal Q9VF/5N- and Q9VF/5D epitopes had a reduced capacity to bind TAP as compared to their Nt-extended precursors (Figure 3B), suggesting that in the ER peptide-trimming is required for proper HLA-B*0702 binding. The ER aminopeptidase ERAAP provides peptides for many MHC-I molecules but has been also implicated in the destruction of CTL epitopes [16]. However, ERAAP cannot process X-P motifs in peptide sequences [32]. Thereafter, though it cannot be formally excluded, a role of ERAAP in the destruction of Q9VF/5N is very unlikely. Overall, these data support the concept that the intra-epitope D to N substitution interferes with proteasomal processing. Using in vitro proteasomal digestions, we demonstrate that the D to N substitution introduces a major cleavage site within the Q9VF epitope (at position N15). Note that at 1 h-digestion time point we identify mainly primary cleavage products since less than 50% of the peptide substrates (the 27mer) have been digested (Figure 4A). To further highlight the potential impact of this N15 cleavage site in the generation of the Q9VF epitope, we performed kinetics of peptide digestion using IP. We observed that amounts of Q9VF/5N epitope and precursors produced were markedly reduced as compared to Q9VF/5D. These results strongly suggest that proteasome cleavages at
A. 

Q9VF/5D sequence

B. 

Q9VF/5D sequence
position N15 destroy the Q9VF/5N epitope and precursors resulting in the lack of MHC-I presentation and CTL activation. In conclusion, a single amino acid variation within HIV epitope can result in epitope destruction and absence of HIV-specific CTL activation.

Mutation in HIV-1 genome can be silent or can differentially impact the fitness of the virus. Due to the redundancy of the codon alphabet, the 5D to 5N substitution in Q9VF does not impact the primary gag-ORF and thus viral replication (Figure 2B). Nevertheless, considering the multitude of existing ARFs, some mutations within ARF encoding sequences most likely affect viral fitness and these ARF sequences might be unavoidably conserved throughout HIV-1 isolates. The great diversity of ARF epitopes produced during HIV infection offers a vast panel of therapeutic targets to stimulate CTL responses. It is interesting to note that ARF-specific CD8+ T cells can perform multiple functions [33,34] and control viral replication in vitro, characteristics that correlate with slow disease progression [57]. In addition, CTLs targeting ARF-derived epitopes can be induced upon vaccination [58] and tumor infiltrating CTLs specific for ARFPs have been also identified in various cancers, including melanoma and breast cancers [25]. Such responses against cryptic epitopes represent a great potential for future immunotherapeutic strategies.

Materials and Methods

Study population

HIV-1-infected peripheral blood mononuclear cells (PBMCs) were obtained from HCV (Hepatitis C virus) negative French ALT-ANRS-CO15 cohort patients [59]. The 31 HLA-B*07+ and 10 HLA-B*07- individuals were identified using the anti-HLA-B*07 antibody ME1. HLA status was further confirmed by genotyping using PCR [60] or using the Luminex xMAP technology [61]. HLA-typing, virological and clinical characteristics of the ten HLA-B*07+ and ten HLA-B*07- patients included in the study are presented in Table 1.

Ethics statement

Patient samples were collected according to French Ethical rules. Written informed consent and approval by institutional review Board at the Pitié-Salpêtrière Hospital were obtained. Animals were bred at the Pasteur Institute. The Office of Laboratory Animal Care at Pasteur Institute reviewed and approved protocols for compliance with the French and European regulations on Animal Welfare and with Public Health Service recommendations (Directive 2010/63/EU).

Human CTL assays

PBMCs were isolated by ficoll-centrifugation, pulsed with Q9VF peptides (1 μM, 1 h at 37°C), and submitted to IFNγ-ELISpot assays as previously described [46]. The HLA-B*0702-restricted peptides used were: HCV-derived epitope G9AT (GPRLGVRAT), CMV-derived epitope T10AM (pp65 417TPRTTGGGMAG26), used as negative and positive control respectively and a pool of known Gag HIV-1-derived epitopes (p24 15SPRTLNAWV24, p24 66TPQDLNTML65, p27/p19 57T1YPLASLRSLF25) as control for HIV reactivity [24]. Responses were considered positive when IFNγ production was superior to 50 spots/10⁶ PBMCs and at least threefold higher than background (measured with the HCV peptide).

Mouse CTL recognition of infected T1 cells

Mouse CTL lines were derived from splenocytes of peptide immunized HLA-B*07+transgenic mice. In brief, these mice express HLA-B*0702 heavy chain with a murine β2m domain and their H-2Kk and H-2Dd class la genes have been inactivated [37]. Cytolytic activity of splenocyte cultures was first assessed in a51Cr release assay [32]. Peptide specific CTL lines were stimulated in vitro (5 μg/mL of peptide) and cultured in RPMI 1640 medium supplemented with 10% FCS, 0.5 μM 2-mercaptoethanol (Sigma), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco-BRL). Ten days later, 2×10⁶, 400 and 80 CTLs in triplicates were stimulated by 10⁶ HIV-1-infected T1-B7 cells and IFNγ release was detected by ELISpot assay. Cross-reactivity of Q9VF/5D- and Q9VF/5N-specific CTLs was tested in IFNγ-ELISpot and Cr³¹-release assays [32] using T1-B7 peptide-loaded cells. Mouse CTL lines specific for the HLA-B*0702-restricted HIV-1 Nef-derived epitope F10LR (Nef68FPVTPQVPLR77; [22]) were used as controls. When stated, HIV-infected T1-B7 cells were treated with epoxomicin (6 h, 1 μg/mL, Calbiochem). To remove residual MHC-peptide complexes, epoxomicin-exposed cells were treated with a citrate-phosphate buffer (pH 3.3) containing 1% BSA and washed twice, prior co-culture with CTLs for an additional 8 h.

Virus and infections

HIVLAT 3D035N was generated by a single amino acid mutation in HIVLAT provirus. The GAT codon (D) of Gag-ARF (AA in position 15) was replaced by an AAT codon (N) without affecting the primary Gag AA coding sequence, using the following primer (5'-GGC TTT CAG CCC AGA AGT AAC CAT GTT TCC AGC) and Quikchange XL Site-directed Mutagenesis Kit (Stratagene). HIVLAT, HIVLAT 3D035N, HIVX6-LAD1 and HIVX63N were produced by transfection of 293T cells using routine procedures [62]. T1 cells (174xCEM, CCR5+LTR-GFP+) stably translected with the HIV-B v T1-B7 cells, [53]) were infected and used as antigen-presenting cells. 5×10⁶ T1-B7 cells were infected with 500 ng of p24 for 3 h in culture medium containing 10 mM Hesper and 4 μg/mL DEAE-dextran. 2 to 5 days p.i., infected T1-B7 cells were used as antigen-presenting cells in IFNγ-ELISpot assay. For the infection kinetics, T1-B7 cells were infected with the indicated viruses according to the same procedure using 1, 10 or 100 ng/mL of p24. Primary CD4+ T cells were isolated from the blood of healthy donors using ficoll centrifugation and magnetic beads (Miltenyi) and activated using PHA (1 μg/mL, PAA) and
rhIL-2 (50 IU/ml, Chiron) [62]. Seven days post activation, CD4+ PHA blasts were infected with various doses of HIV (from 1 to 100 ng/ml of p24). HIV infection was monitored by FACS (Becton Dickinson) using intracellular HIV p24 staining (KC57 Ab, Beckman Coulter) or p24-Elisa (PerkinElmer).

Sequencing of the Gag-ARF encoding region from clonal HIV-1 populations

Total DNA was extracted from PBMCs of HLA-B*0702+ and HLA-B*07- HIV+ patients using QIAamp blood DNA minikit (Qiagen). To analyze the diversity of HIV-1 proviruses in the PBMCs of patients, a 267-bp fragment encompassing the Gag-ARF coding sequence was amplified by nested PCRs as follows: 5 min of initial denaturation at 94°C, 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C for 30 cycles, followed by 7 min at 72°C. The outer primer pair used was (5’- ATC AAG CTT GCA GAG GAA GCA GCA GCT GAG) and (5’- CAG GAA GTA CTA GTA GCA CTC TTC AGG AAT TCG G), and the inner primer pair was (5’- TAC CCT ATA GTG CAG AAC ATC CAG GG) and (5’- GAT AGA GTG CAT CCA GTG CAT GCA). Samples were treated separately and negative controls were systematically included. Purified PCR products were cloned using a TOPO-TA cloning kit (Invitrogen). Twenty clones per patient were isolated and gag-ARF inserts from each clonal DNA plasmid were amplified by PCR using M13 primers and sequenced (Applied Biosystem).

HLA-B*0702-peptide binding and stabilization assays

The capacity of the peptides to bind HLA-B*0702 was determined using a classical HLA stabilization assays with the TAP-deficient cell line T2 HLA-B*0702+ [37]. Briefly, cells were incubated overnight with 100, 10, 1 and 0.1 μM of peptide in serum-free medium at room temperature. Cells were then stained with the anti-HLA-B*0702 ME.1 antibody and HLA-B*0702 surface expression analyzed by FACS (Becton Dickinson). The concentration needed to reach 50% of the maximal fluorescence (as defined with the R10TV peptide (CMV pp65265RPHERNGFTV274)) was calculated (IC50). The relative affinity (RA) is the IC50 ratio of the tested and R10TV reference peptide (the lower the relative affinity, the higher the ratio) is deduced from radioactive reporter peptide. TAP affinities were determined as the concentrations required to inhibit 50% of reporter peptide binding (IC50). Results are expressed as 1/IC50 ratios and are mean values from three independent experiments. The highest the 1/IC50 ratio, the highest the affinity.

In vitro proteasome digestions

Immuno-proteasomes were isolated from T2.27mp cells (that stably express all three immunosubunits) as previously described [51]. Purified proteasomes were analyzed by SDS-PAGE. The yield was calculated at 90-95%. The 27mer peptides encompassing Q9VF/5D or Q9VF/5N were synthesized using standard Fmoc method on an Applied Biosystems 433A automated synthesizer. The peptides were purified by HPLC and analyzed by mass spectrometry. Three nmol of peptides were digested in vitro using 1 μg of proteasomes (for 0.5, 1, 2, 4, 8 and 18 h) in 100 μl of buffer containing 20 mM Hepes/KOH, pH 7.8, 2 mM magnesium acetate and 2 mM dithiothreitol. Reactions were stopped by the addition of trifluoroacetic acid to a final concentration of 0.3%. The digestions were analyzed, by mass spectrometry (RP-HPLC ESI) and the products were identified by MS/MS.

Statistical analysis

A standard two-tailed nonparametric Mann-Whitney U-test (with P<0.05 considered significant) was used to perform statistical comparison of HIV-1 proviral sequences frequencies using statistical analysis Prism software (GraphPad).

Supporting Information

Figure S1 Amino acid and nucleotide sequences of Gag and Gag-ARF. A) Nucleotide and corresponding amino acid sequenc-es of Gag (frame 1) and Gag-ARF (frame 3, bold) are depicted. Nucleotide numbering is according to HIV_HXB2 sequence. ATG start and TGA stop codons of Gag-ARF are underlined. B) Nucleotide and amino acid sequences of Gag and Gag-ARF from HIV_LAI, HIV_NL-AD8, HIV_MN and HIV_LAI-5D-5N strains. (TIF)

Figure S2 Q9VF/5D and Q9VF/5N CTL cross-reactivity. The cross-reactivity of Q9VF/5D- and Q9VF/5N-specific CTLs (generated in HLA-B*0702 transgenic mice) was tested in IFN-γ-ELISPOT (A) and Cr51-release assays (B) using T1-B7 cells loaded with a single dose (1 μg/ml) (A) or a titration (B) of Q9VF/5D or Q9VF/5N peptides. A CMV-derived HLA-B*0702-restricted epi-top (RPHERNFTV, R10TV) was used as negative control. Q9VF/5D- and Q9VF/5N-specific CTLs displayed similar capacity to recognize cells loaded with their cognate peptides. CTLs were also equally activated by the alternate peptides. Data are mean values of triplicates ±SD and representative of at least three independent experiments. (TIF)

Figure S3 Q9VF/5N encoding HIV strains are not recognized by Q9VF-specific CTLs. As in Figure 2A using T1-B7 cells infected with HIV_LAI, HIV_NL-AD8 or HIV_MN (X4-tropic isolate encoding Q9VF/5N), infection rates were equivalent (around 30% of p24+ cells). Infected cells were then used in an IFN-γ-ELISPOT assay to activate Q9VF/5D- and Q9VF/5N-specific CTLs. For each peptide, specific CTL lines were generated in three different HLA-B*0702 transgenic mice and used in two independent experiments. One representative experiment with one CTL line is shown (mean values of triplicates ±SD). (TIF)
Acknowledgments

The authors are grateful to F. Guivel, A.Lehmann and K.Textoris-Taube for technical assistance, D. Duffy for critical reading of the manuscript, L. Weiss, A. Samiri and G. Carcelian for help and for providing reagents. We thank Zabrina Brumme for help in statistical analysis. We thank the ALT study group and all ALT patients for participating in the study.

Author Contributions

Conceived and designed the experiments: AM SC. Performed the experiments: SC GC RB AU SDF SF IM JG PVc AM. Analyzed the data: SC PVc PMK AM. Contributed reagents/materials/analysis tools: AG CK BA FAL VA OS. Wrote the paper: SC AM.

References


