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Gag sequences from HIV-1-infected individuals on highly active antiretroviral therapy differ from the consensus

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Abstract

The latent HIV-1 reservoir in HIV-1-infected individuals undergoing highly active antiretroviral therapy (HAART) is the major barrier to curing HIV-1 infection. Recent studies suggest that these latent HIV-1 reservoirs can be eliminated by HIV-1-specific $CD8^+$ cytotoxic T cells (CTLs) using a HIV-1-specific CTL-based vaccine. However, mutations in the latent HIV-1 reservoir form a major obstacle to this approach. Recent evidence suggests that vaccines based on CTLs stimulated with peptides of which the sequence matched that of the viruses infecting the individual were more effective than vaccines based on CTLS stimulated with consensus peptides. In addition, priming of naive $CD8^+$ T cells with autologous HIV-1 peptides by dendritic cells (DC) was suggested to further improve effectivity. Therefore, it is hypothesised that DC-primed HIV-1-specific naive $CD8^+$ T cells are more effective when primed with autologous Gag peptides than when primed with consensus Gag peptides. To address this hypothesis, the aim of our study was to assess the variation of HIV-1 Gag sequences in PBMCs obtained from long-term HAART-treated HIV-1-infected individuals. Nested PCR to amplify the *gag* gene in such individuals was successfully performed followed by Sanger sequencing. As expected, sequence differences compared to the consensus were found throughout the entire *gag* gene including some within the immunodominant SL9 CTL-epitope. In conclusion, we show using a newly established method for the sequencing of the *gag* gene, that Gag sequences from HAART-treated clade B HIV-1-infected individuals differ considerably from consensus. Our sequencing method could be used to design autologous Gag peptides. This method will therefore further help address the hypothesis that CTLs primed with autologous Gag peptides are more effective than when primed with consensus Gag peptides.

Content

Introduction

Human immunodeficiency virus (HIV) infection causes immunodeficiency, which ultimately leads to the development of acquired immune deficiency syndrome (AIDS) in the vast majority of infected persons if left untreated [1]. The virus currently infects more than 36.7 million people worldwide [2] and is spread mainly by sexual transmission [3], mother-to-child transmission, and contact with body fluids [4].

HIV replication and disease progression can be inhibited by highly active antiretroviral therapy (HAART) [5]. HAART has greatly improved the prognosis of HIV-1 infection [6], but the therapy is not curative. Instead, HIV persists as integrated proviruses in cellular reservoirs within HAART-treated individuals, allowing the virus to rapidly re-emerge if HAART is ceased [5][7]. The best characterised reservoir consists of latently infected memory $CD4^+$ T cells [8][9][10]. These reservoirs express little or no viral RNA and no viral proteins [11][12] making them 'invisible' to the immune system and therefore resistant to elimination [12]. Furthermore, the reservoirs are not susceptible to antiretroviral drugs, but can be induced to produce virus if the cell becomes activated [9].

One potential strategy for eliminating latent HIV reservoirs is to induce virus expression, which should enable killing of the host cell by activating the immune response against the virus [13]. This is sometimes referred to as an “activation-elimination” or "kick-and-kill" approach. This approach would ideally be performed under continuous HAART to prevent newly expressed virus from spreading to additional host cells and to minimise mutation rates [13]. These “activation-elimination” approaches aim to reactivate the latent HIV-1 reservoirs using latency reversing agents [14][15]. In addition, these approaches aim to eliminate reactivated latent HIV-1 reservoirs through induction of HIV-1-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses, which together with anti-HIV-1 $CD4^+$ T cell responses, decline during HAART [16][17]. HIV-specific CTLs are critical to early immune control of HIV-1 infection and can target viral epitopes displayed on the surface of infected cells by major histocompatibility complex class I (MHC-I) molecules [18]. The HIV-1 Gag protein is preferentially targeted by HIV-1-specific CTLs [19]. It has recently been shown that high numbers of HIV-specific CTLs are associated with reduced disease progression and with increased viral control [20].

Unfortunately, HIV can avoid elimination by CTLs through the development of escape mutations [21]. These mutations cause changes in the peptide sequence, which can abrogate binding to the MHC-molecule and/or inhibit recognition by the T cell receptor (TCR) [22][23]. It has been demonstrated in the past that although T cell responses could be detected in HIV-infected individuals using peptides based on conserved viral sequences [24], the number of responders and the magnitude of the responses increased when the sequence of the stimulating peptides matched that of the viruses infecting the individual [25]. Viral load was especially decreased when HIV-1-specific CTL responses were directed against Gag peptides associated with CTL escape mutations [26][27][28]. However, a study by Draenert and colleagues showed that HIV-1-infected cells were not effectively killed despite the presence of CTLs specific for autologous HIV-1 [29]. This finding suggests that these CTLs might be dysfunctional or suppressed. A recent study indicates that this problem could be resolved by priming naive $CD8^+$ T cells with autologous virus using mature dendritic cells (DC) instead of stimulating memory $CD8^+$ T cells [30].

Taking these findings together, it is hypothesised that DC-primed HIV-1-specific naive $CD8^+$ T cells induce a greater T cell response when primed with autologous Gag peptides than when primed with consensus Gag peptides. To address this hypothesis, the aim of our study was to assess the variation of HIV-1 Gag sequences in PBMCs obtained from long-term HAART-treated HIV-1-infected individuals. To this end, the *gag* gene in PBMCs obtained from such individuals was amplified by polymerase chain reaction and sequenced using Sanger sequencing.

Material and Methods

Study subjects

Buffy coats from healthy adults were provided by the German Red Cross in Berlin. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, USA). After washing to remove excess platelets, cells were resuspended in PBS at a concentration of 5×10^6 cells/ml. Frozen PBMCs from nine long-term HAART-treated clade B HIV-1-infected patients (designated as P1 to P9) were obtained from volunteers attending the AIDS outpatients' clinic at the Charité in Berlin. The age, sex, ethnicity and disease status of the patients were not provided to the lab. The study was approved by the Ethics Committee of the Charité and all subjects gave written informed consent.

Cell culture

Two T-cell lines were used in this study. ACH-2 (NIH Aids Reagent Program, Germantown, USA), an HIV-1 latent T-cell line with one integrated proviral copy per cell, was used as a positive control and C8166 (HPA Culture Collections, Salisbury, England), an HTLV-1-transformed T-cell line, was used as a negative control. Both cell lines were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (FCS), 200 mM glutamine and 100U/ml penicillin/streptomycin (all Gibco, Carlsbad, CA, USA). Cells were counted using a hemacytometer (Thermo Fisher Scientific, Waltham, MA, USA). All steps were performed in a safety cabinet and cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Infection of healthy PBMCs with different HIV-1 subtypes

A pool of human PBMCs purified from three buffy coats (German Red Cross) were stimulated with 2µg/ml PHA (Sigma-Aldrich, St. Louis, USA) and cultivated (37°C, 5% CO_2, humidified) for three days in 75 cm^2 flasks at 2×10^6 cells/ml in RPMI 1640 media supplemented with 20% FCS, 200 mM glutamine and 100U/ml penicillin/streptomycin (all Gibco, Carlsbad, CA, USA). The cells were then left uninfected or infected with HIV-1 clade A, clade B, clade C, clade D, clade F, or clade G (3.5 - 4.5 $TCID_{50}$/ml) and cultivated for a further 14 days in the presence of 100 IU/ml IL-2. Cells were then harvested and pellets containing 5×10^5 PBMC were frozen at -80°C until used for analysis.

Polymerase chain reaction

To allow sequencing of the *gag* gene, amplification of the complete *gag* of HIV-1 was performed using polymerase chain reaction (PCR). Sequencing focused on the *gag* gene because it is known to be an important target of the CTL response [31]. DNA was isolated from ACH-2, C8166, frozen PBMCs from HAART-treated clade B HIV-1-infected patients and PBMCs from healthy donors infected with clade A, B, C, D, F, or G using the QIAamp mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop ND-1000 Spectophotometer (Thermo Fisher Scientific). PCR amplification of the *gag* gene was performed using multiple primers (table 1 and figure S1). Primers GOPF and GOPR have been described previously [32]. Primers 793F, 1190F, 1621F, 144R, 2018R and 2280R were designed based on the sequence of the HIV-1 molecular clone HXB2 targeting conserved regions of the genome according to the Los Alamos National Laboratory HIV database. The PCR reaction mixture of 25µl contained 300ng DNA, 1x SuperFiBuffer (Invitrogen, Waltham, MA, USA), 2mM MgCL2, 0.2 mM dNTPs each (Thermo Fisher Scientific), 1 U of Platinum SuperFiDNA Polymerase (Invitrogen) and 0.5 µM of primer (table 1). DNA was amplified using a PTC-200 DNAEngine Peltier Thermal cycler (Bio-Rad, Hercules, USA). The thermocycler profile was: denaturation at 95°C for 5 min, followed by 35 cycles of

denaturation at 95°C for 1 min, annealing at 57-64°C (table 1) for 1 min and extension at 72°C for 1 min, with a final extension of at 72°C for 15 min. For the nested PCR, 30 cycles of denaturation, annealing and extension were used. The amplified products were electrophoresed on 1.5% agarose gel, stained by ethidium bromide and visualised under ultraviolet light using Gel Doc XR+ System (Bio-Rad). Analysis was performed using ImageJ software. PCR products were purified using QIAquick PCR Purification Kit (Qiagen). After DNA purification, DNA was quantified using Qubit 3.0 fluorometer (Thermo Fisher Scientific). Control amplifications with no template were included in every PCR experiment to test for carryover contamination.

Table 1. PCR primer name, sequence, binding location, expected fragment size and annealing temperature.

PCR Primers	Sequence 5'-3'	Location HXB2	Fragment size	Annealing temperature (°C)
GOPF	F: CTCTCGACGCAGGACTCGGCTTGC	683-706	1722	64
GOPR	R: CCAATTCCCCCTATCATTTTTGG	2382-2404		
793F	F: GGTGCGAGAGCGTCAGTATTAAGC	793-816	1488	57
2280R	R: GGGGTCGTTGCCAAAGAGTG	2261-2280		
793F	F: GGTGCGAGAGCGTCAGTATTAAGC	793-816	652	57
1444R	R: GCACTGGATGCACTCTATCCCATT	1421-1444		
1621F	F: AGCCCTACCAGCATTCTGGACATA	1621-1644	660	57
2280R	R: GGGGTCGTTGCCAAAGAGTG	2261-2280		
1190F	F: TAGTGCAGAACATCCAGGGGCAAA	1190-1213	829	57
2018R	R: TTCCTAGGGGCCCTGCAATTTCT	1996-2018		
1190F	F: TAGTGCAGAACATCCAGGGGCAAA	1190-1213	1091	57
2280R	R: GGGGTCGTTGCCAAAGAGTG	2261-2280		

Table 2. Sequencing primer name, sequence, binding location and annealing temperature.

Sequencing Primers	Sequence 5'-3'	Location HXB2	Annealing temperature (°C)
GOPF	F: CUCUCGACGCAGGACUCGGCUUGC	683-706	64
GIPF	F: GAGGCUAGAAGGAGAGAGAUGGG	772-794	58
793F	F: GGTGCGAGAGCGTCAGTATTAAGC	793-816	57
1190F	F: TAGTGCAGAACATCCAGGGGCAAA	1190-1213	57
GSP1	F: CCAUCAAUGAGGAAGCUGC	1400-1418	57
1444R	R: GCACTGGATGCACTCTATCCCATT	1421-1444	57
2018R	R: TTCCTAGGGGCCCTGCAATTTCT	1996-2018	57
2280R	R: GGGGTCGTTGCCAAAGAGTG	2261-2280	57
GIPR	R: GGCAACGACCCCUCGUCACAAUAA	2269-2292	58
GOPR	R: CCAAAAAUGAUAGGGGGAAUUGG	2382-2404	64

Sanger sequencing

PCR products of the *gag* gene were sequenced by Sanger sequencing using the previously described primers GOPF, GIPF, GSP1, GIPR and GOPR [32] (table 2 and figure S1) and primers 793F, 1190F, 144R and 2280R, whose design was based on HIV-1 HXB2 at conserved regions (table 2 and figure S1). The reaction mixture of 10 µl contained 20 ng of PCR product, 0.75x Sequencing Buffer (Thermo Fisher Scientific), 1x BigDye Terminator v3.1 Ready Reaction Mix (Thermo Fisher Scientific) and 0.5 µM of primer. The PCR product was prepared on a PTC-200 DNAEngine Peltier Thermal cycler (Bio-Rad) using the following cycling conditions: denaturation at 96°C for 1 min, followed by 25 cycles

of denaturation at 96°C for 30 sec, annealing at 57-64°C (table 2) for 5 sec and extension at 60°C for 4 min. The sequencing reaction was performed on ABI 3500xL Dx (Thermo Fisher Scientific). Analysis was performed using SeqMan Pro and MegAlign software (DNA Star/Lasergene, Madison, WI). For the analysis, nt 790-2292 of HXB2, was aligned with the sequences.

Duplex-qPCR HIV-1

A duplex-qPCR was performed to determine the HIV-1 copy number in HAART-treated clade B HIV-1-infected patient samples. The reaction mixture of 25 µl contained 300 ng DNA, 1x TaqMan®Universal Master Mix II (Invitrogen), 200 nM CCR5 Forward Primer (5' - ATGATTCCTGGGAGAGACGC-3'), 200 nM CCR5 Reverse Primer (5' - AGCCAGGACGGTCACCTT - 3'), 200 nM CCR5 probe (5' - VIC-AACACAGCCACCACCCAAGTGATCA - 3'), 300 nM HIV-1 Forward Primer (5' - TACTGACGCTCTCGCACC - 3'), 300 nM HIV-1 Reverse Primer (5' - TCTCGACGCAGGACTCG - 3') and 120 nM HIV-1 probe (5' - FAM-CTCTCTCCTTCTAGCCTC - 3'). The qPCR was run on the Stratagene Mx3005P System (Agilent Technologies, Santa Clara, USA). The thermocycler profile was: denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 30 sec and annealing and elongation at 60°C for 1 min. A standard curve was acquired using serial dilutions of pNL4-3 HIV-1 vector DNA (AF324493), ranging from 4 x 10^6 to 4 × 10^0 copies. The cycle threshold (Cq) values were determined using the quantitation analysis of the MxPro Software (Agilent Technologies). The reactions were performed in triplicate.

Results

Gag amplification in a HIV-1 positive cell line

To test the specificity and sensitivity of PCR amplification of the *gag* gene, PCR amplification was performed on ACH-2, an HIV-1 positive cell line, using GOPF and GOPR as PCR primers (table 1). The predicted 1.7 Kb fragment was visible for ACH-2 on the gel (figure 1). Moreover, no additional fragments were observed (figure 1). Thus, PCR amplification of the *gag* gene using GOPF and GOPR as PCR primers was successful in a HIV-1 positive cell line.

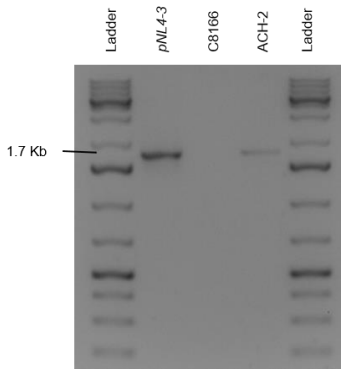

Figure 1. Ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification of the *gag* gene in an HIV-1 positive cell line. GOPF and GOPR were used as PCR primers. The amount of DNA used as template was 300 ng. Lanes one and five contain a 1 Kb plus DNA ladder (Invitrogen). The targeted 1.7 Kb fragment is labelled. The HIV-1 plasmid pNL4-3 (10^7 copies) was included as positive control and DNA from C8166 cells as negative control.

Gag sequencing in a HIV-1 positive cell line

Following successful PCR amplification of the *gag* gene in ACH-2 cells (figure 1), the PCR product was used to verify the sequencing method using the sequencing primers, GOPF, GIPF, GSP1, GIPR and GIPF (table 1). The complete *gag* gene in ACH-2 PCR product was sequenced using the five different sequencing primers and compared

to the known ACH-2 sequence (data not shown). Thus, sequencing of the *gag* gene using GOPF, GIPF, GSP1, GIPR and GIPF as sequencing primers was successful in an HIV-1 positive cell line.

Gag amplification in a HIV-1 positive patient sample

As amplification and sequencing of the *gag* gene from a HIV-1 positive cell line was successful we attempted the same using PBMC from a HAART-treated clade B HIV-1-infected patient (P1). PCR amplification of *gag* was carried out using the GOPF and GOPR primers (table 1). However, no 1.7 Kb fragment was visible for P1 on the gel (figure 2), indicating that the PCR amplification was not successful.

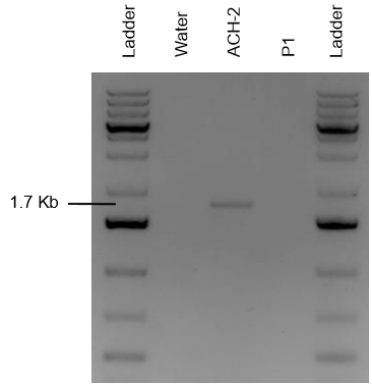

Figure 2. Ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification of the *gag* gene in a HAART-treated clade B HIV-1-infected patient sample (P1) using GOPF and GOPR primers. The amount of DNA used as template was 300 ng. Lanes one and five contain a 1 Kb plus DNA ladder (Invitrogen). The expected 1.7 Kb fragment is labelled. The HIV-1 positive cell line ACH-2 was included as positive control and sterile distilled water was used in place of template DNA as negative control.

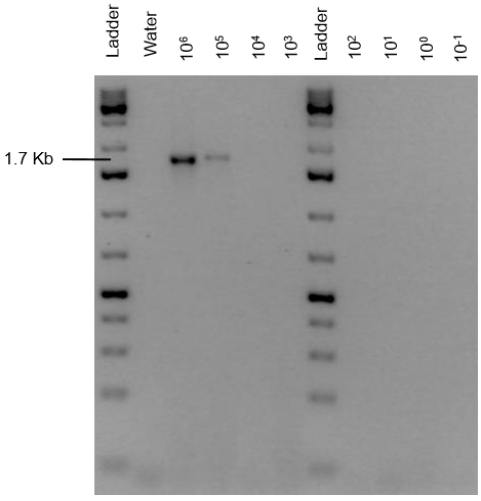

Figure 3. Ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification of the *gag* gene using copy numbers of the HIV-1 vector pNL4-3 ranging from 10^6 to 10^{-1} as template and GOPF and GOPR as primers. Lanes one and seven contain a 1 Kb plus DNA ladder (Invitrogen). The expected 1.7 Kb fragment is labelled. Sterile distilled water was used in place of template DNA as negative control.

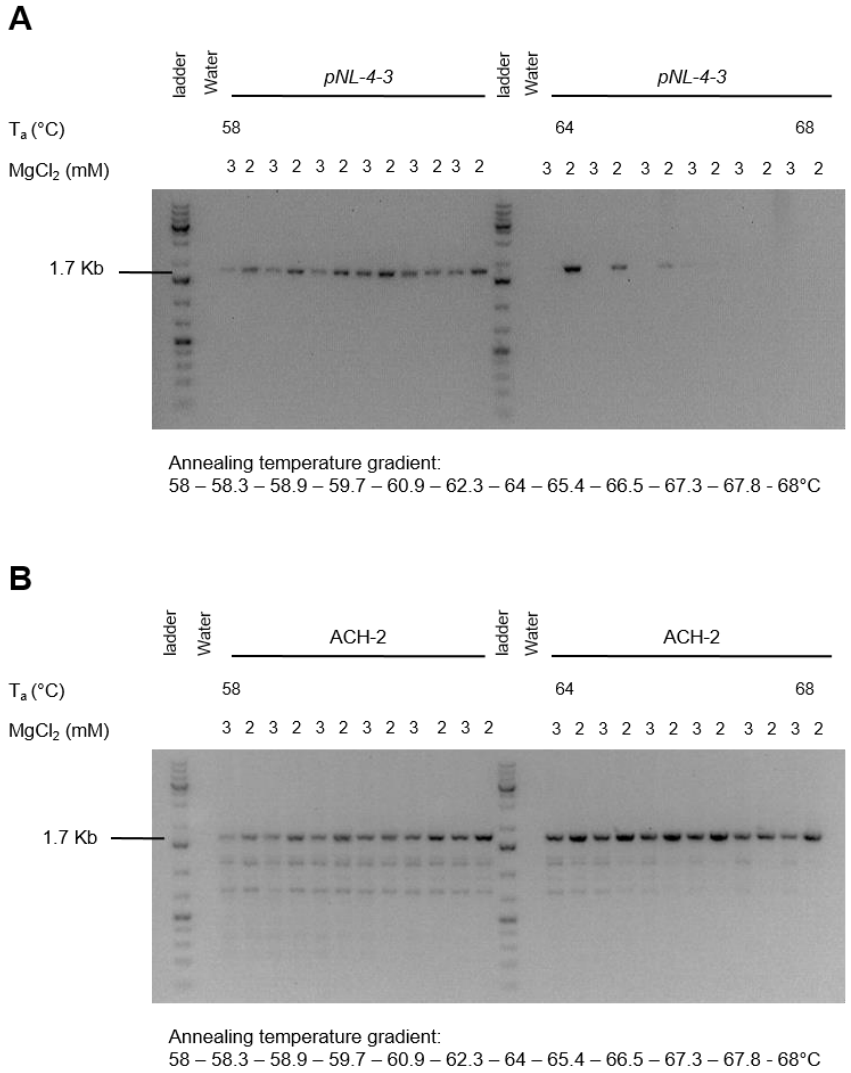

Figure 4. Ethidium bromide stained agarose gels showing DNA fragments produced by gradient PCR amplification of the *gag* gene from 10^4 copies of the HIV-1 vector pNL4-3 and the HIV-1 positive cell line ACH-2. (A) An agarose gel showing DNA fragments produced by gradient PCR amplification of the *gag* gene from the HIV-1 vector *pNL4-3* (10^4 copies). (B) An agarose gel showing DNA fragments produced by gradient PCR amplification of the *gag* gene from 500 ng ACH-2 DNA. GOPF and GOPR were used as primers. Lanes one and fifteen contain a 1 Kb plus DNA ladder (Invitrogen). The expected 1.7 Kb fragment is labelled. $MgCl_2$ concentrations of either 2 mM or 3 mM and annealing temperatures of 58 – 58.3 – 58.9 – 59.7 – 60.9 – 62.3 – 64 – 65.4 – 66.5 – 67.3 – 67.8 - 68°C were used as indicated. Sterile distilled water was used in place of template DNA as negative control.

Optimisation of Gag amplification

It is known that that the HIV-1 DNA load decreases during long-term HAART [33][34]. Since PCR amplification of the *gag* gene from a HIV-1-infected patient sample was shown to be unsuccessful, we speculated that that our PCR strategy might be insufficiently sensitive to amplify *gag* from a HAART-treated HIV-1-infected patient with reduced proviral load. The sensitivity of the PCR was therefore determined by amplifying various copy numbers of the HIV-1 vector pNL4-3 using GOPF and GOPR as primers (table 1). The expected 1.7 Kb fragment was visible for 1 x 10^6 and 1 x 10^5 copies of the vector (figure 3). However, there was no fragment visible for copy numbers below 1 x 10^5 (figure 3). These results suggest our PCR strategy might indeed be insufficiently sensitive to amplify *gag* from a HAART-treated HIV-1-infected patient with reduced proviral load.

The PCR conditions were therefore optimised by varying the annealing temperature and the $MgCl_2$ concentration. A gradient PCR with various annealing temperatures and two different $MgCl_2$ concentrations was performed using 10^4 copies of the HIV-1 vector pNL4-3 to assess sensitivity and ACH-2 DNA to assess specificity. For pNL4-3, expected 1.7 Kb fragments were visible on the gel (figure 4A). The strongest signal for the expected 1.7 fragment was produced at an annealing temperature of 64°C and a $MgCl_2$ concentration of 2mM (figure 4A). For ACH-2, expected 1.7 Kb fragments were also visible (figure 4B). There appeared to be a negative correlation between the formation of additional fragments and annealing temperature but not between the formation of additional fragments and $MgCl_2$ concentration (figure 4B). Together, these results show that PCR amplification of the *gag* gene was most sensitive and specific at an annealing temperature of 64°C and a $MgCl_2$ concentration of 2mM.

Gag amplification in HIV-1 positive patient samples after optimisation

After optimisation of the PCR conditions, amplification of the *gag* gene was attempted using DNA from HAART-treated clade B HIV-1-infected patients 2, 3, 4, 5 and 6 (P2, P3, P4, P5 and P6) and GOPF and GOPR as primers (table 1). There was no expected 1.7 Kb fragment visible for the patient samples (figure 5). Moreover, there were additional fragments visible for the patient samples and for the positive control (figure 5). Thus, PCR amplification of the *gag* gene was still unsuccessful using DNA from HAART-treated clade B HIV-1-infected patients despite optimisation of the PCR.

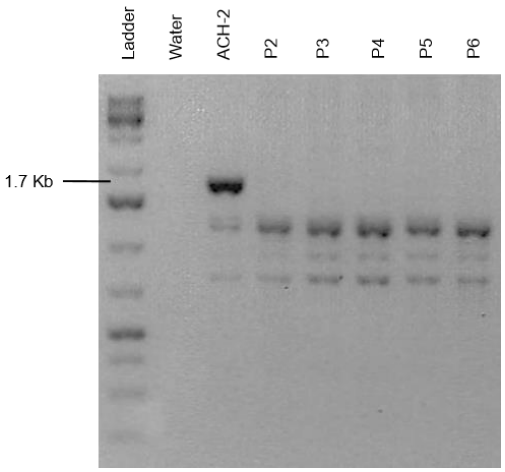

Figure 5. Ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification of the *gag* gene in HAART-treated clade B HIV-1-infected patient samples using GOPF and GOPR as primers. The amount of DNA used as template was 300 ng. Lane one contains a 1 Kb plus DNA ladder (Invitrogen). The expected 1.7 Kb fragment is labelled. The HIV-1 positive cell line ACH-2 was included as positive control and sterile distilled water was used in place of template DNA as negative control. P2-P6 = HAART-treated clade B HIV-1-infected patients 2-6.

Copy number HIV-1 in HIV-1 positive patient samples

Despite optimisation of the PCR strategy, amplification of the *gag* gene in HAART-treated clade B HIV-1-infected patient samples was still unsuccessful. We hypothesised that this might result from low levels of HIV-1 DNA in the patient samples. To test this hypothesis, the HIV-1 copy number in patients P2, P3, P4, P5 and P6 was determined using an established duplex-qPCR for HIV-1 DNA and for the single copy cellular *CCR5* gene. As expected, all samples were positive for *CCR5* (figure 6A). In addition, DNA from P2 and P6 appeared to be positive for HIV-1 (figure 6A). However, the qPCR signal for these patients lay outside of the standard curve, therefore yielding apparent levels of less than one copy per reaction making the results for P2 and P6 unreliable. DNA from P3, P4 and P5 was negative for HIV-1.

As a robust HIV-1 copy number could not be measured for patient samples P2-P6, we screened samples from three additional HAART-treated clade B HIV-1-infected patients (P7-P9) by duplex qPCR. As well as being positive for *CCR5*, all three patient samples were also positive for HIV-1 (figure 6B), yielding copy numbers from 1 to 100 or more per reaction.

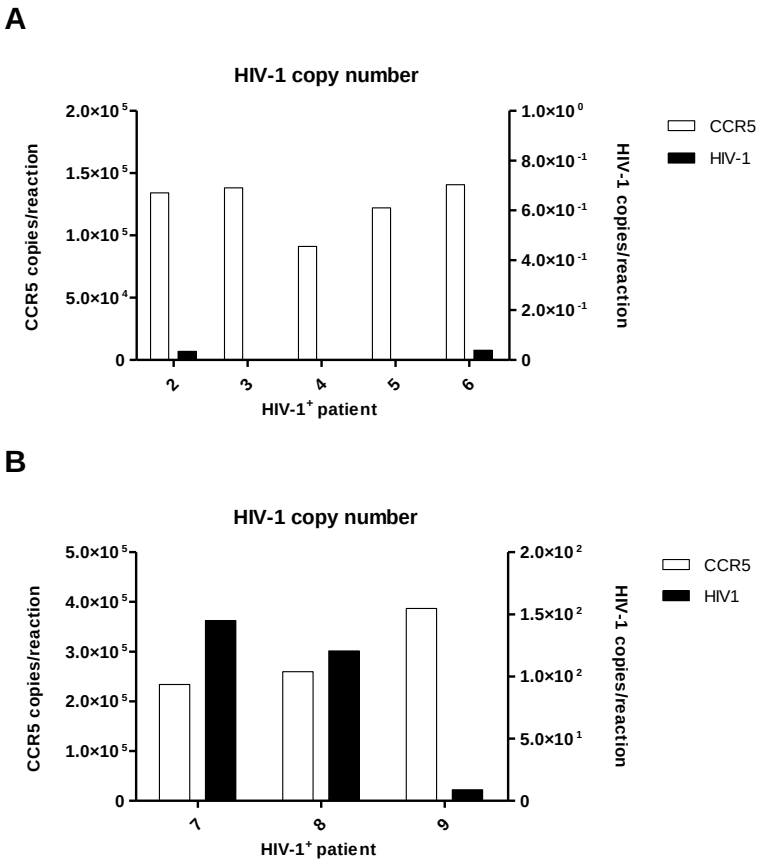

Figure 6. HIV-1 copy numbers in HAART-treated clade B HIV-1-infected patient samples. The HIV-1 copy number in the HIV-1-infected patient samples was determined using duplex-qPCR for *CCR5* and HIV-1. (A) HIV-1 copy numbers in patients 2, 3, 4, 5 and 6. (B) HIV-1 copy numbers in patients 7, 8 and 9. CCR5 was used as a reference gene. Results represent the means of a single experiment performed in triplicate.

Gag amplification in HIV-1 positive patient samples

We then tested our PCR strategy on the patient samples shown to contain detectable levels of HIV-1 DNA. PCR amplification of the *gag* gene was performed on DNA from patients P7, P8 and P9 using GOPF/GOPR, 793F/2280R, 793F/1444R, 1621F/2280R and 1190F/2018R primer pairs (table 1). Although primer pair GOPF/GOPR yielded the expected 1.7 Kb fragment for the three patient samples, the signal was at least seven times weaker than that for the positive control (figure 7). Similarly, primer pair 793F/2280R gave the predicted 1.5 Kb fragment for all three samples, but the signal was at least fourteen times weaker than that for the positive control (figure 7). For primer pair 793F/1444R, the signal for the expected 652 bp fragment for the three patient samples was about five times weaker than that for the positive control (figure 7). Primer pair 1621F/2280R also yielded the expected 660 bp fragment for the three patient samples, but the signal was at least five times weaker than the signal for the positive control (figure 7). For primer pair 1190F/2018R, the predicted 829 bp fragment was not visible (figure 7). In addition, multiple additional fragments were observed with all five PCR primer pairs in both the positive control and the patient

samples (figure 7). PCR amplification of the *gag* gene from the patient samples for all primer pairs was therefore considered to be insufficient for sequencing in terms of both sensitivity and specificity.

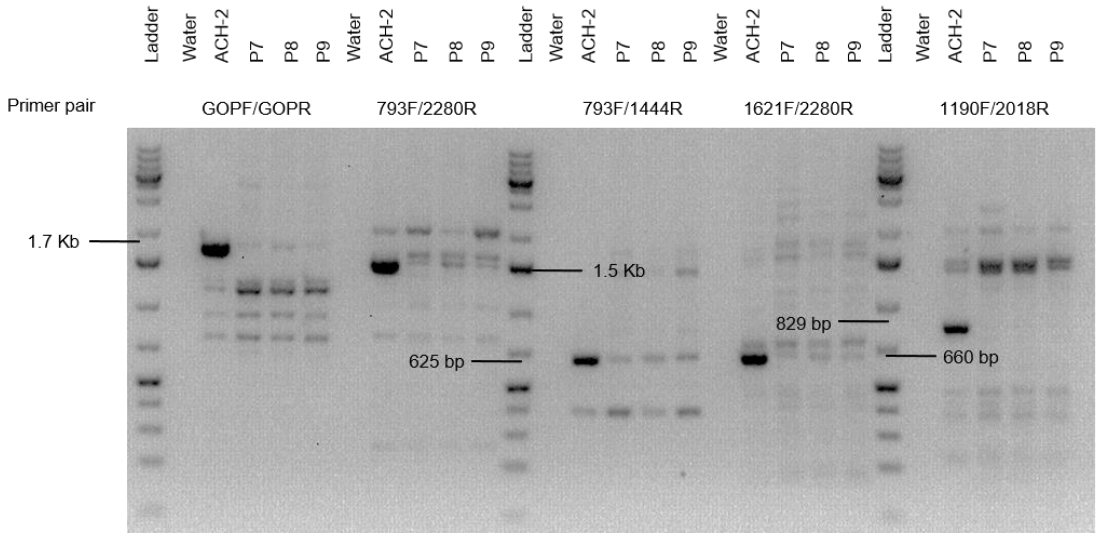

Figure 7. Ethidium bromide stained agarose gel showing DNA fragments produced by PCR amplification of the *gag* gene in HAART-treated clade B HIV-1-infected patients 7, 8 and 9. The expected product size for each primer pair is labelled. Lanes one, twelve and twenty-three contain a 1 Kb plus DNA ladder (Invitrogen). The HIV-1 positive cell line ACH-2 was included as positive control and sterile distilled water was used in place of template DNA as negative control. P7-P9 = HAART-treated clade B HIV-1-infected patients 7-9.

Gag amplification in HIV-1 positive patient samples by nested PCR

As amplification of the *gag* gene in from the patients was still insufficient for sequencing, we aimed to improve sensitivity and specificity of the PCR using a nested approach. The nested PCR was performed on DNA from patients P7 and P9. Primer pair GOPF/GOPR was used for the outer PCR but did not yield a visible 1.7 Kb fragment for P7 and P9 (data not shown). However, subjecting the PCR products of P7 and P9 to a second round amplification using primer pairs 793F/2280R, 793F/1444R and 1190F/280R resulted in bands of 1.5 Kb, 652 bp and 1 Kb, respectively, as expected (Figure 8). Although multiple additional fragments were also visible, their signals were weaker than those of the specific fragments. This nested PCR amplification of the *gag* gene therefore provided products suitable for sequencing of the patient samples.

Gag sequencing in HIV-1 positive patient samples

Next, the successfully amplified PCR products from patients 7 and 9 (P7 and P9) were Sanger sequenced using primers 793F, 1190F, GSP1, 1444R and 2280R and aligned with the HXB2 sequence upon which consensus peptides are based. The amino acid sequence from ACH-2 showed 98.9% identity with that of HXB2 (table 3 and figure S2). The amino acid sequences from P7 and P9 were 92.3% and 93.2% homologous to HXB2, respectively. In addition, amino acid substitutions were present in the SL9 CTL-epitope in both P7 and P9 (figure S2). These amino acid substitutions were in agreement with the amino acid sequences of the naturally occurring SLYNTIAVL (6I8V) and SLFNTIAVL (3F6I8V) variants (figure S1). Moreover, P9 had multiple Gag sequences, suggesting the presence of multiple proviral sequences (figure S2). Thus, the Gag sequences derived from HAART-treated clade B HIV-1-infected patients 7 and 9 were shown to be significantly divergent from the consensus HXB2 sequence.

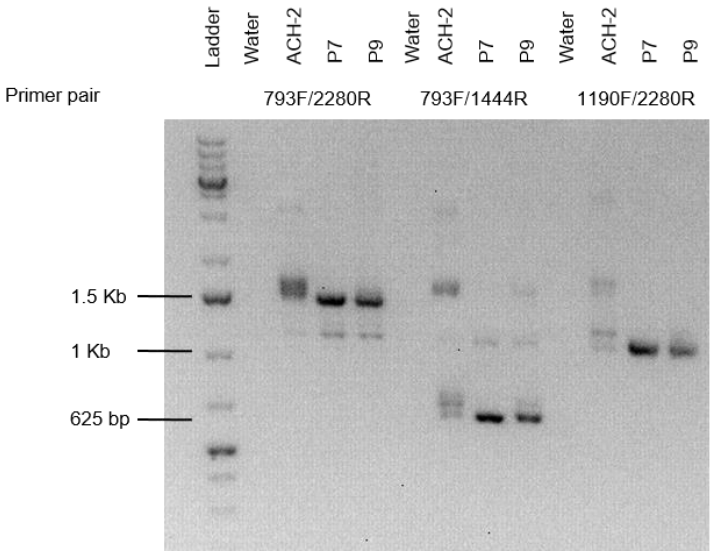

Figure 8. Ethidium bromide stained agarose gel showing DNA fragments produced by nested PCR amplification of the *gag* gene in HAART-treated clade B HIV-1-infected patients 7 and 9. The expected product sizes for primer pairs 793F/2280R, 793F/1444R and 1190F/2280R (1.5 Kb, 652 bp and 1 Kb respectively) are labelled. The amount of DNA used in the outer PCR as template was 300 ng. Lane one contains a 1 Kb plus DNA ladder (Invitrogen). The HIV-1 positive cell line ACH-2 was included as positive control and sterile distilled water was used in place of template DNA as negative control. P7 and P9 = HAART-treated clade B HIV-1-infected patients 7 and 9.

Table 3. Sanger sequencing results for the *gag* gene from HAART-treated clade B HIV-1-infected patients.

Sample	No. of AA* different from reference	AA different from reference (%)	AA comparable to reference (%)
ACH-2	5	1.1	98.9
P7	35	7.7	92.3
P9	31	6.8	93.2

* AA = amino acid

Gag amplification in different HIV-1 subtypes

To evaluate the breadth of coverage of our nested PCR strategy, amplification of the *gag* gene was performed for PBMCs infected with clade A, B, C, D, F or G HIV-1 using primer pair GOPF/GOPR for the outer PCR and 793F/2280R for the inner PCR. PBMCs infected with each of the isolates yielded the expected 1.7 Kb fragment after the first PCR reaction, the outer PCR (data not shown). In addition, DNA from the infected PBMCs gave the expected 1.5 Kb fragment after the second PCR reaction, the inner PCR (figure 9). DNA from uninfected PBMCs did not yield the expected fragments with either PCR, as expected. The nested PCR strategy for *gag* gene amplification therefore appeared to be applicable to PBMCs infected with a wide range of HIV-1 clades.

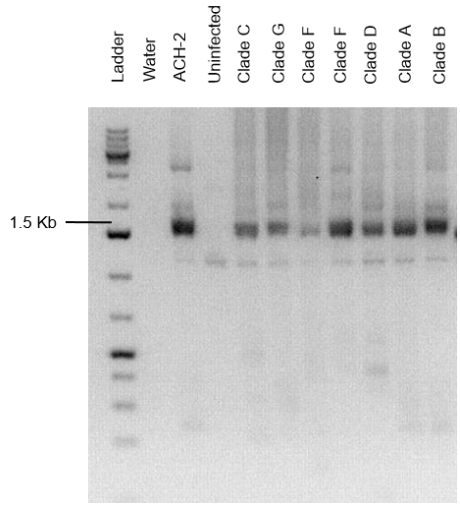

Figure 9. Ethidium bromide stained agarose gel showing DNA fragments produced by nested PCR amplification of the *gag* gene from uninfected PBMCs and PBMCs infected with clade A, B, C, D, F or G HIV-1. 793F and 2280R were used as primers for this inner PCR. The amount of DNA used as template in the outer PCR was 300 ng. Lane one contains a 1 Kb plus DNA ladder (Invitrogen). The expected 1.5 Kb fragment is labelled. The HIV-1 positive cell line ACH-2 was included as positive control and sterile distilled water was used in place of template DNA as negative control.

Discussion

The latent HIV-1 reservoir in HIV-1-infected individuals on HAART is the major barrier to finding a cure for HIV-1 infection [35]. Recent studies suggest that these latent HIV-1 reservoirs can be eliminated by HIV-1-specific CTLs using an HIV-1-specific CTL-based vaccine [30][36] However, mutations in the latent HIV-1 reservoir form a major obstacle to this approach [37]. Vaccines based on CTLs stimulated with autologous HIV-1 peptides can potentially overcome this obstacle. A recent study showed that the number of responders and the magnitude of the responses increased when CTLs were specific for autologous peptides compared to when CTLs were specific for consensus peptides [25]. Moreover, another study showed that naive $CD8^+$ T cells primed by autologous HIV-1 presenting DCs more effectively kill $CD4^+$ T cells infected with autologous HIV-1 in HIV-1-infected individuals on HAART than did memory $CD8^+$ T cells [30]. It is therefore hypothesised that DC-primed HIV-1-specific naive $CD8^+$ T cells are more effective when primed with autologous Gag peptides than when primed with consensus peptides. To address this hypothesis, the aim of our study was to assess the variation of HIV-1 *gag* sequences in PBMCs obtained from long-term HAART-treated HIV-1-infected individuals. We demonstrated using a newly established method for the sequencing of the *gag* gene, that Gag sequences from HAART-treated clade B HIV-1-infected individuals differ considerably from consensus.

In this study, we were able to amplify and sequence the *gag* gene in long-term HAART-treated clade B HIV-1-infected patient samples. We focused on sequencing of the *gag* gene because the HIV-1 Gag protein is preferentially targeted by HIV-1-specific CTLs during acute and chronic infection [19]. The PCR and sequencing strategy established in our study was shown to be successful for HIV-1 subtypes A, B, C, D, F and G, although this was not demonstrated for other *gag* amplification and sequencing strategies [38][39][40][41][42]. Current methods used to generate *gag* sequences are mainly focused on RNA [30][43][44][45][46][47][48]. In addition, most methods are not focused on B clade HIV-1-infected patients undergoing HAART [44][46][47][48]. We aimed to sequence DNA rather than RNA from HIV-1-infected patients undergoing HAART because the latent reservoir does not produce sufficient amounts of virus during HAART but rather persists in a DNA form as an integrated provirus [11]. There are methods available that use DNA to generate *gag* sequences [32][38][39][40][41][42]. However, these methods are

also not focused on B clade HIV-1-infected patients undergoing HAART [32][38][39][41] or do not provide sequence data for the entire *gag* gene [39]. Ultimately, we were able to sequence 90% of the *gag* gene using our newly established amplification and sequencing strategy. We aimed to sequence the complete gene because this would help identify the locations of mutations important for CTL recognition and activity. This seems particularly of interest since a recent study suggested that CTL-based HIV-1 vaccines should target epitopes in areas where CTL escape mutations are associated with substantial viral fitness costs [49].

Our present study shows that Gag sequences from HAART-treated clade B HIV-1-infected individuals indeed differ from the consensus. It is known that a single amino acid substitution can substantially reduce or eliminate susceptibility to CTLs [22]. Our finding together with this recent evidence supports the hypothesis that CTLs specific for autologous peptides would provide a more effective response than those specific for consensus peptides. Amino acid differences were observed throughout the entire Gag, including the immunodominant HLA-A*0201-restricted, HIV-1 p17 gag77–85 epitope (SLYNTVATL; SL9). The SL9 variants observed were SLYNT**I**A**V**L and SL**F**NT**I**A**V**L. Recent evidence showed that SL9 variants can be recognised as well as or better than the consensus sequence, but only when CTLs are stimulated with sequences matching the variants [50], supporting the use of autologous peptides for stimulation. Another study showed that CTLs are able to recognise SL9 variants, but that the efficiency of recognition was variant and donor dependent [51]. This finding indicates that vaccines based on CTLs specific for autologous gag peptides might induce a stronger T cell response than CTLs specific for consensus gag peptides in some but not all HIV-1-infected patients. In addition, our sequencing results suggest that one of the HAART-treated clade B HIV-1-infected patient samples contained multiple proviruses, suggesting that multiple peptides covering the same Gag location would be needed to eliminate all proviruses. Taken together, these findings support the idea that CTLs stimulated with autologous peptides would be more effective than those stimulated with consensus peptides in certain HIV-1-infected individuals.

A novel method for sequencing the *gag* gene in PBMCs obtained from HAART-treated HIV-1-infected individuals was established in our study. However, our conclusions were based on results obtained using very limited numbers of HIV-1-infected individuals and therefore unsuitable for statistical analysis. Furthermore, the sequencing performed in our study was performed in PBMCs from HAART-treated clade B HIV-1-infected individuals. Although we were able to amplify *gag* in PBMCs infected with clades A, B C, D, F and G of HIV-1 *in vitro*, the PCR amplification strategy has not yet been tested with PBMCs from patients infected with these different HIV-1 subtypes. Neither has the strategy been tested with samples from acutely infected patients or untreated patients. It would therefore be useful to test our newly established sequencing strategy in a larger cohort of diverse HIV-1-infected patients.

In conclusion, we demonstrated using a newly established method for the sequencing of the *gag* gene, that Gag sequences from HAART-treated clade B HIV-1-infected individuals differ considerably from consensus. This sequencing approach would facilitate the development of immunotherapeutic strategies involving autologous Gag peptides, assuming that DC-primed HIV-1-specific naive $CD8^+$ T cells would be more effective when primed with autologous Gag peptides than when primed with consensus peptides.

Acknowledgments

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Supplementary files

Supplementary Methods

Peptide stimulation and intracellular cytokine staining

Multiparametric intracellular cytokine staining (ICS) was tested upon activation of $CD8^+$ T cells by the CEF peptide pool (a pool consisting of 23 MHC class I-restricted viral peptides from human CMV, EBV and influenza virus) and upon activation of $CD4^+$ and $CD8^+$ T cells by Staphylococcal enterotoxin B (SEB) as described previously [52]. In brief, PBMCs ($1x10^7$ cells/ml) from three healthy adults were left unstimulated (negative control) or were stimulated with 2 µg/mL CEF (Mabtech AB, Nacka Strand, Sweden) or 4µg/mL SEB (Sigma-Aldrich, St. Louis, USA) in the presence 4 µg/ml anti-CD107a (BV605) (BioLegend, London, United Kingdom) to detect degranulation, 1µg/ml co-stimulatory antibodies anti-CD28 and anti-CD49d and 1x protein transport inhibitor cocktail (all eBioscience, Waltham, MA, USA). PBMCs were then cultured in a humidified incubator with 5% CO_2 at 37°C overnight. After incubation, PBMCs, together with a compensation control containing 50% live and 50% dead cells, were washed three times with cold PBS and stained with Fixable Viability Dye-eFluor 780 (eBioscience) at a final concentration of 1:1000 for 30 min at 4°C in the dark. After incubation, cells were washed twice with cold flow cytometry staining buffer (eBioscience) and fixed with 1x IC fixation buffer (eBioscience) for 30 min at 4°C in the dark. After incubation, cells were permeabilised with 1x IC permeabilisation buffer (eBioscience) and incubated for 20 min at RT in the dark. Thereafter, cells were washed once with 1x IC permeabilisation buffer and incubated in a pretitrated optimal concentration of Human FC Receptor Binding Inhibitor Purified (eBioscience) for 20 min on ice. Subsequently, cells were stained with pretitrated optimal concentrations of anti-CD3-Alexa-Fluor 700, anti-CD4-FITC, anti-CD8a-PerCP-eFluor 710, anti-IFNγ-PE-Cy7, anti-IL-2-PE, anti-TNFα-eFluor 450 and anti-IL-17-APC (all eBioscience) for 45 min at RT in the dark. Thereafter, stained samples were washed once with 1x IC permeabilisation buffer and twice with flow cytometry staining buffer. After washing, stained samples were resuspended in flow cytometry staining buffer and stored at 4°C in the dark until analysis. Stained samples were acquired using an LSR-II cytometer with FACSDiva Software (BD Bioscience). Single stained and negative Ultracomp compensation beads (eBioscience) were acquired for each experiment before sample acquisition and used to calculate the compensation matrix. All FACS analyses were performed using FlowJo® software (Treestar, Inc; OR) using the following gating strategy: lymphocytes; live; $CD3^+$; $CD4^+$ or $CD8^+$. The $CD4^+$ and $CD8^+$ populations were then analysed separately for the expression of each of the five stimulation markers (CD107a, IFNγ, IL-2, TNFα, IL-17).

Supplementary Results

Intracellular cytokine staining

The ICS was intended to be used to evaluate the response in patient PBMC to consensus and autologous peptide stimulation. As a test of principle, PBMCs from three healthy donors were stimulated with CEF or SEB. The CEF peptide pool is known to activate CMV, EBV and influenza-specific $CD8^+$ T cells and SEB generically activates $CD8^+$ and $CD4^+$ T cells. Responding cells were to be identified by their expression of intracellular cytokines (IFNγ, IL-2, TNFα and/or IL-17) and surface expression of the cytotoxicity marker CD107a [53]. Representative data from donor one are shown in figures S3 and S4. As expected, stimulation with the CEF peptide pool resulted in an increased number of $CD8^+$ cells expressing IFNγ, TNFα and CD107a (and, to a lesser extent, IL-2) but not IL-17. $CD4^+$ cells were not stimulated by this pool of CD8-epitopes. In contrast, exposure to SEB resulted in high levels of stimulation of both

CD4⁺ and CD8⁺ cells.

Supplementary Figures

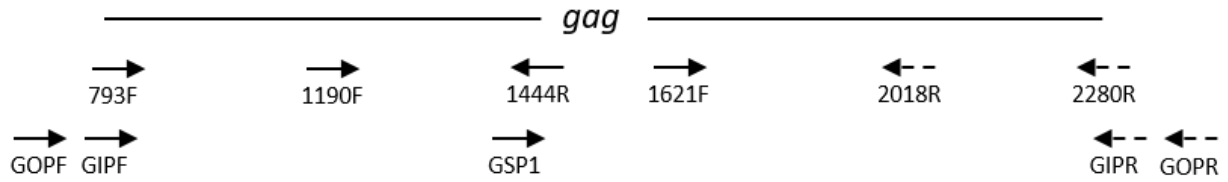
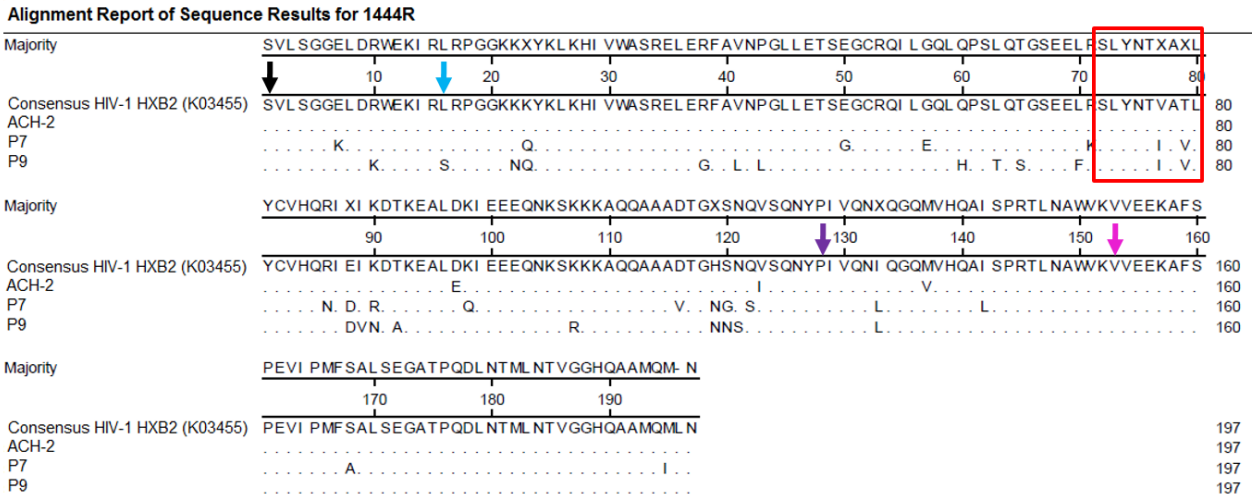


Figure S1. PCR and sequencing primer positions on the *gag* gene. Solid arrows indicate forward primers and dashed arrows indicate reverse primers.

A



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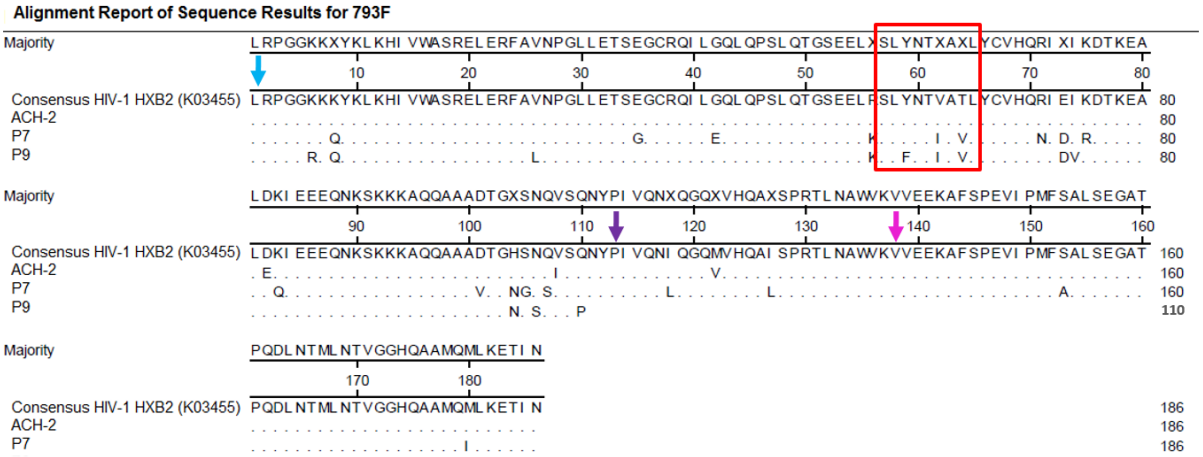
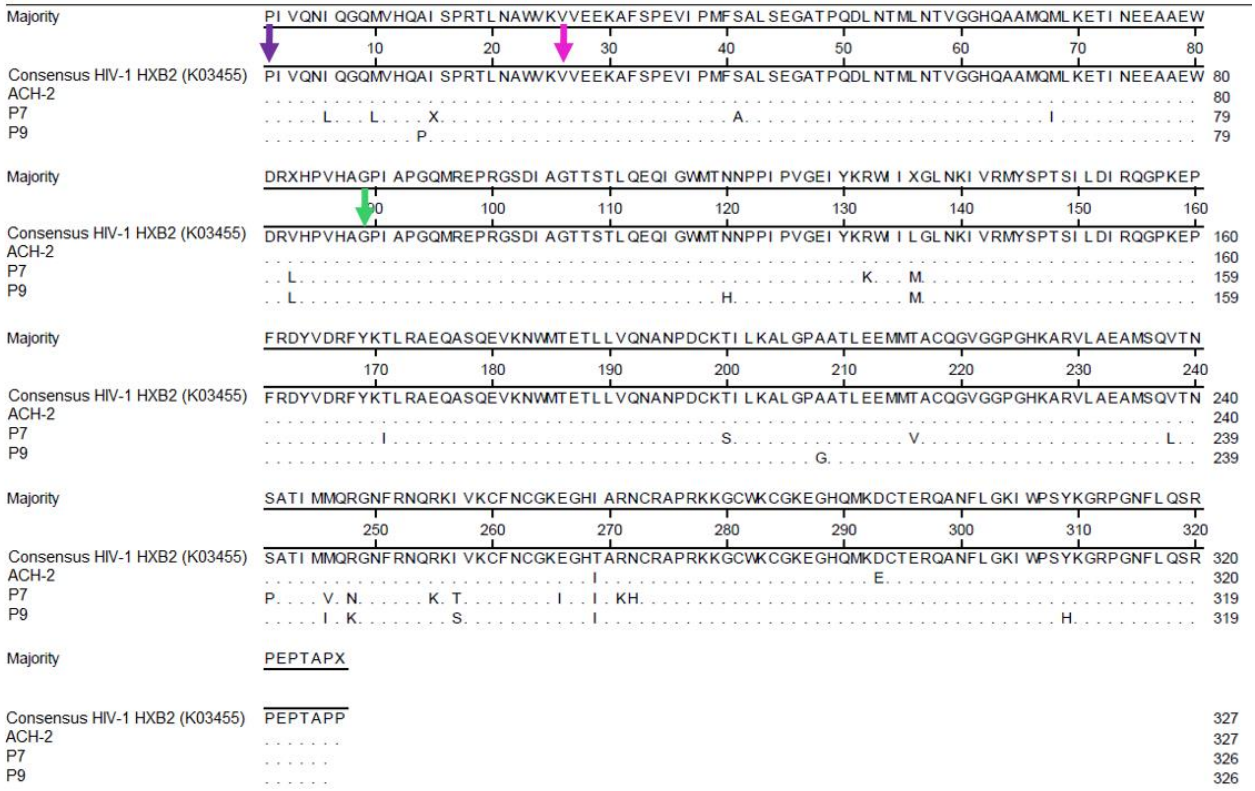


C

Alignment Report of Sequence Results for 2280R



D

Alignment Report of Sequence Results for 1190F

E

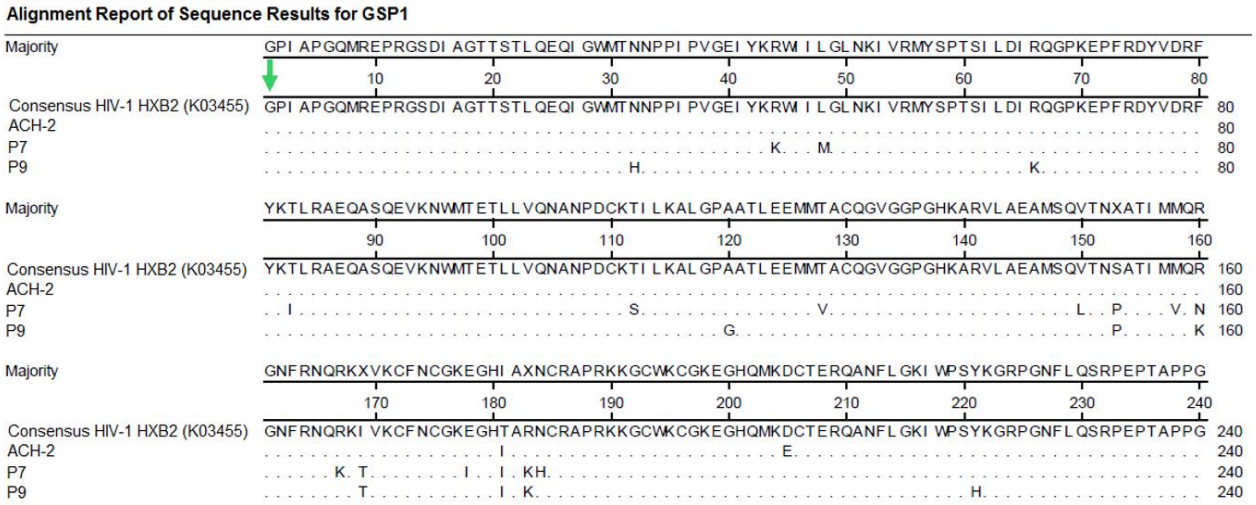


Figure S2. Alignment reports of Gag sequences from HAART-treated clade B HIV-1-infected patients 7 and 9 derived by Sanger sequencing. Analysis was performed using SeqMan Pro and MegAlign software using HXB2 as a reference sequence. Alignments are shown for sequences generated using the following primers: (A) 1444R; (B) 793F; (C) 2280R; (D) 1190F; (E) GSP1. The start of the sequence for each primer is indicated by arrows; black arrow: 1444R, blue arrow: 793F, purple arrow: 2280R, pink arrow: 1190F, green arrow: GSP1. The SL9 epitope is marked in red. P7 and P9: HAART-treated clade B HIV-1-infected patient 7 and 9.

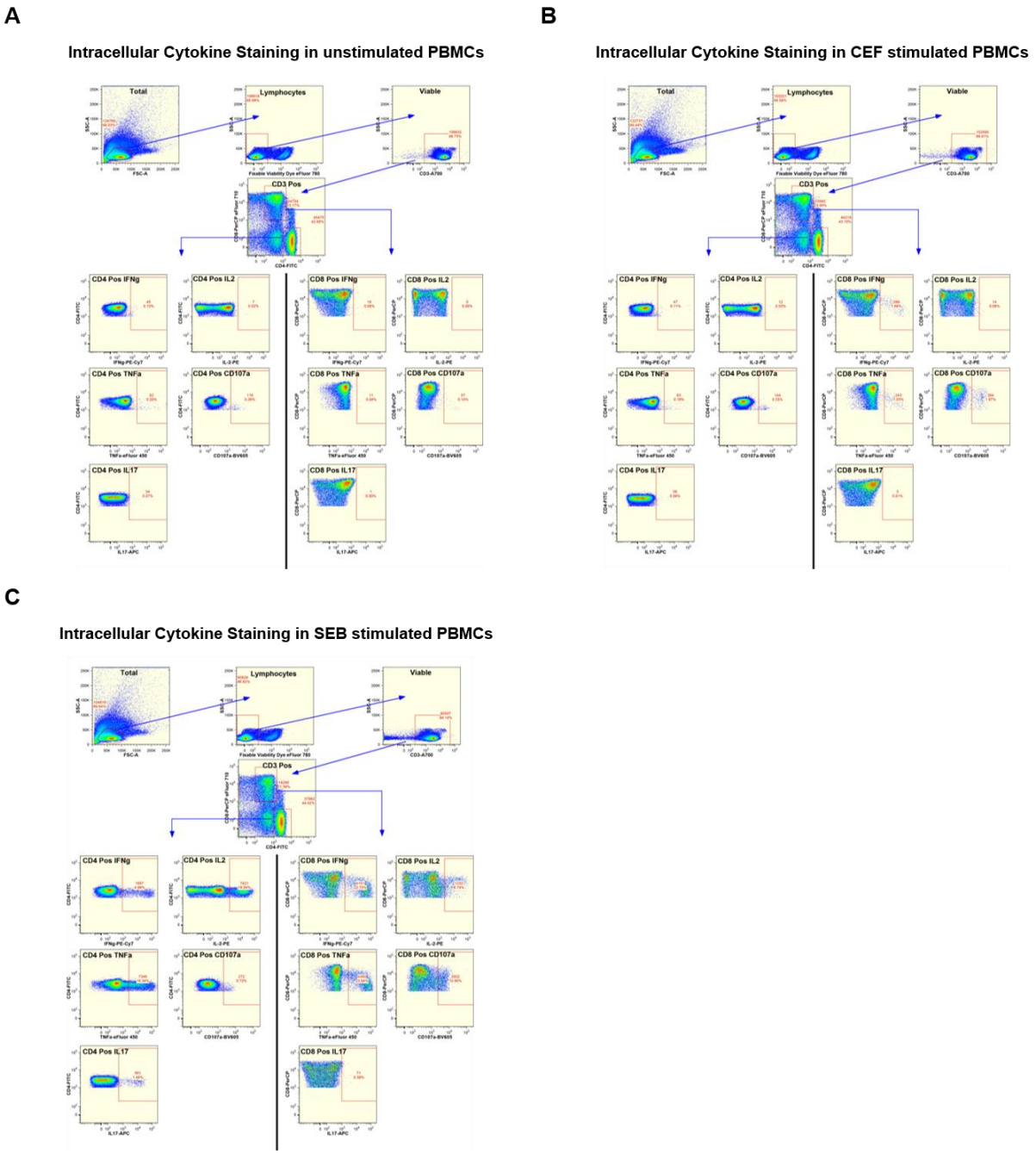


Figure S3. Multiparametric intracellular cytokine staining in PBMCs from a healthy donor after stimulation with CEF or SEB. ICS was performed without stimulation (A) or after stimulation of PBMCs from a healthy donor with CEF (B) or with SEB (C). FACS analyses were performed using FlowJo® software (Treestar, Inc; OR) using the following gating strategy; lymphocytes; live; CD3+; CD4+ or CD8+. The CD4+ and CD8+ populations were then analysed separately for the expression of each of the five stimulation markers (CD107a, IFNγ, IL-2, TNFα, IL-17). CEF = peptide pool consisting of 23 MHC class I-restricted viral peptides from human CMV, EBV and influenza virus. SEB = Staphylococcal enterotoxin B.

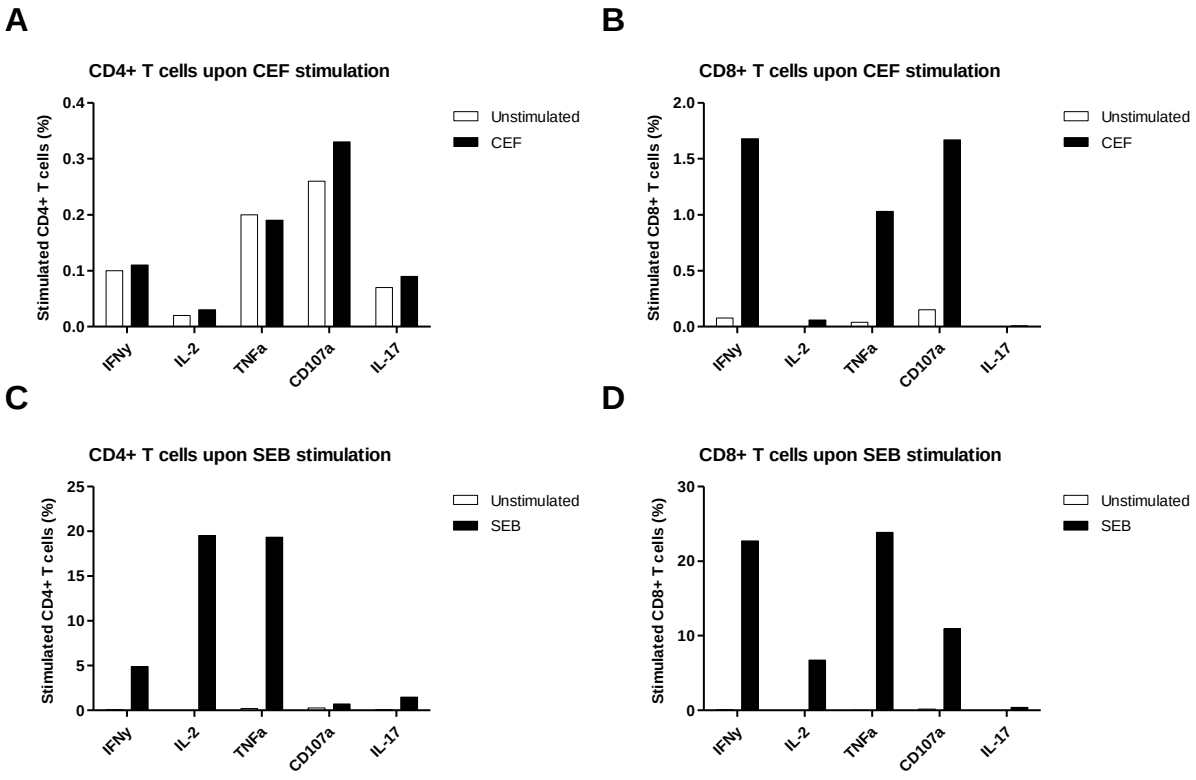


Figure S4. Graphical representation of the FACS data shown in figure S3. Percentages of $CD4^+$ and $CD8^+$ T cells positive for IFNγ, IL-2, TNFα CD107a or IL-17 without stimulation or after stimulation with CEF or SEB. Percentages of $CD4^+$ or $CD8^+$ T cells positive for the different markers after stimulation with CEF or SEB compared with unstimulated cells: $CD4^+$ (A) and $CD8^+$ (B) cells after CEF stimulation; $CD4^+$ (C) and $CD8^+$ (D) cells after SEB stimulation. CEF = peptide pool consisting of 23 MHC class I-restricted viral peptides from human CMV, EBV and influenza virus. SEB = Staphylococcal enterotoxin B.