Deep sequencing of the HIV-1 polymerase gene for characterisation of cytotoxic T-lymphocyte epitopes during early and chronic disease stages

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Research

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Abstract

Background

Despite multiple attempts, there is still no effective HIV-1 vaccine available. The HIV-1 polymerase (pol) gene is highly conserved and encodes cytotoxic T-lymphocyte (CTL) epitopes. In this study, deep sequencing was employed for characterisation of HIV-1 Pol CTL epitopes in mostly paired samples obtained during early and chronic stages of infection. Deep sequencing data was then compared to Sanger sequencing data only in samples obtained at baseline.

Results

Fifty-two participants were enrolled in the study. Their median age was 28 years (interquartile range: 24–32 years) and the majority of participants (92.3%) were female. Illumina minority variant analysis identified a significantly higher number of CTL epitopes (n = 65) compared to epitopes (n = 8) identified through Sanger sequencing. Most of the identified epitopes mapped to reverse transcriptase (RT) and integrase (IN) regardless of the method of sequencing. There was a significantly higher proportion of minority variant epitopes in RT (n = 39, 60.0%) compared to IN (n = 17, 26.2%) and PR (n = 9, 13.8%), p = 0.002 and < 0.0001, respectively. However, no significant difference was observed between the proportion of minority variant epitopes in IN versus PR, p = 0.06. Some epitopes were detected in either early or chronic HIV-1 infection whereas others were detected in both stages. Different distribution patterns of minority variant epitopes were observed in sample pairs; with some increasing or decreasing over time, while others remained constant. Some of the identified epitopes have not been previously reported for HIV-1 subtype C. There were also variants that could not be mapped to reported CTL epitopes in the Los Alamos HIV database.

Conclusion

Deep sequencing revealed many Pol CTL epitopes, including some not previously reported for HIV-1 subtype C. The findings of this study support the inclusion of RT and IN epitopes in HIV-1 vaccine candidates as these proteins harbour many CTL epitopes. Variants that were not mapped within CTL epitopes could represent new epitopes.

Introduction

The HIV/AIDS pandemic has been a global crisis for four decades [1–2]. At the end of 2020, there were 37.6 million people living with HIV globally, 1.5 million new HIV-1 infections, and 690 000 AIDS-related deaths [3]. South Africa has 7.9 million people living with HIV-1 (PLWH), making it the country with the highest number of infections in the world [4–5]. This highlights the need to better understand HIV-1 natural immune responses in order to bolster the efforts of developing effective HIV-1 vaccines.

During early HIV-1 infection, effective cytotoxic T-lymphocyte (CTL) immune responses play an important role in the control of viraemia, by contributing to suppression of the viral load (VL) to a set-point [6]. CTL responses target and kill virus-infected cells, via recognition of viral peptide epitopes and releasing cytokines and cytotoxic granules that facilitate cell killing [7–9]. Viral peptide epitopes are presented on the surface of infected cells by the human leukocyte antigen type I (HLA I) trans-membrane proteins encoded by HLA-A, B and C alleles [10–12]. The targeted epitopes undergo immune selection pressure, which leads to the emergence of viral escape mutations [13–14]. This results in evasion of the immune system, loss of immune control and ultimately progression to AIDS [7, 10]. The majority of epitopes that play a role in the control of viraemia have been extensively studied and reported for HIV-1 Gag [15–16]. However, some studies show that HIV-1 Pol also harbours important CTL epitopes that contribute to the control of HIV-1 viraemia [17–19], hence pol is included in some HIV-1 vaccine candidates [20–25]. There are limited data on the characterisation of HIV-1 Pol CTL...
epitopes in early and chronic HIV-1 disease stages. The aim of this study was to characterise and assess the evolution of CTL epitopes encoded by HIV-1 pol during early and chronic stages of HIV-1 infection, using deep sequencing methods.

Materials And Methods

Study population

This was a retrospective study that used stored plasma samples that were collected from individuals in the Tshwane district of South Africa. Participants enrolled in this study had a confirmed diagnosis of early or chronic HIV-1 infection and were antiretroviral therapy (ART) naïve. They were identified in a study that screened for early HIV-1 infection in individuals who had a negative rapid test at point-of-care facilities, and diagnosis was confirmed through HIV-1 VL, antibody (enzyme-linked immunoassay), p24 antigen, Western blot and avidity testing [26]. Most of the participants were followed up, and thus samples at two time-points were available (at baseline and follow-up) for analysis.

Nucleic acid extraction and amplification of HIV-1 pol

Total nucleic acids were manually extracted from plasma samples using the QIAamp UltraSens Virus kit (Qiagen, Hilden, Germany). Samples with a VL > 1000 copies/ml were extracted from a plasma input volume of 500 µl, which was adjusted to 1 ml using phosphate-buffered saline (PBS). Samples with a VL ≤ 1000 copies/ml were extracted directly from 1 ml plasma input volume to increase nucleic acid yield. Extraction was performed according to the manufacturer’s instructions except for the first centrifugation step, which was optimized to 800 relative centrifugal force (RCF) instead of the recommended 1200 RCF. This modification facilitated a more efficient dissolution of the pellet. Nucleic acids were eluted in a volume of 60 µl and all the eluates were stored at -70 ºC immediately after extraction.

The complete HIV-1 pol gene was amplified in all samples using an in-house nested PCR method, employing the SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Invitrogen, Carlsbad, California, USA). Each PCR reaction was performed in a 50 µl reaction, which included 5 µl of extracted RNA / DNA template, 2X reaction mix containing 2.4 mM magnesium sulphate (MgSO4), 0.4 mM of each deoxynucleotide triphosphate (dNTPs), 10 mM sense primer, 10 mM anti-sense primer, 5 units (U) of enzyme mix for (first round) or 1U of Platinum Taq polymerase (for second round), and nuclease-free water. The first-round amplicon was used as template for the second-round amplification (www.lifetechnologies.com) (Supplementary Table 1). One or two extracted HIV-1-negative controls were included in each experiment to assess potential contamination.

The following sets of primers were used for amplification of the complete pol gene: SM-F1 (outer forward) 5’-GCG GCT ACA TTA GAA GAA ATG ATG-3’ (HXB2 1807–1830) and SM-R1 (outer reverse) 5’-GCC AAG TAT TGT AGA GAT CCT ACC T-3’ (HXB2 5462–5488), SM-F3 (inner forward) 5’-AGA TTG TTA AAT GTT TCA ACT GTG G-3’ (HXB2 1952–1976) and SM-R3 (inner reverse) 5’-CTC CTG TAT GCA AAC CCC AAT A-3’ (HXB2 5245–5266). The nested primers amplified a fragment size of 3268 bp.

Sanger sequencing and sequence analysis

Sanger sequencing was performed on PCR amplicons, but only for baseline samples. Sequencing was performed commercially in six overlapping regions covering the entire pol ORF (Inqaba Biotechnical Industries, Tshwane, South Africa) (Supplementary Table 1). Sequences were edited in CLC Main Workbench 21 software (Qiagen, Hilden, Germany) and consensus sequences were generated, viewed in BioEdit 7.2.5 (https://bioedit.software.informer.com/download/) and aligned using the online version of the MAFFT program (https://mafft.cbrc.jp/alignment/server/). The HIV-1 HXB2 reference sequence (K03455.1) was used for nucleotide numbering. Phylogenetic analysis was performed on the multiple alignment and the ratio of nonsynonymous to synonymous (dN-dS) mutations within the HIV-1 Pol region was computed using MEGA software (https://www.megasoftware.net/). A dN-dS ratio ≥ 5 was used to identify codons under high
selection pressure and these sites were subsequently mapped to CTL epitopes documented in the Los Alamos HIV database [27].

### Illumina sequencing and analysis of consensus sequences

Sequencing of PCR amplicons was performed at Inqaba Biotechnical Industries (Pretoria, South Africa), a commercial NGS service provider. Briefly paired-end libraries (2 x 300 bp) were prepared using the NEBNext® Ultra™ II DNA library prep kit for Illumina® (New England Biolabs, USA) and sequencing was performed on an Illumina MiSeq instrument (Illumina, USA).

Some samples (n = 12) that were viewed to have poor quality scores were sent for repeat sequencing to the National Institute for Communicable Diseases (NICD), Sequencing Core Facility, Johannesburg, South Africa. Multiplexed paired-end libraries (2 x 300 bp) were prepared using the Nextera™ XT DNA Library Sample Preparation kit (Illumina Inc., California, United States) according to the manufacturer's instructions and sequencing was carried out on an Illumina MiSeq instrument (Illumina Inc., California, United States).

Illumina sequencing data was analysed in the PASeq program ([https://paseq.org/](https://paseq.org/)) using the feature that excludes APOBEC-induced hypermutations, and consensus sequences were generated. The quality of the Illumina reads was assessed in PASeq and CLC Genomics Workbench 21 (Qiagen, Hilden, Germany). PASeq-generated Illumina consensus sequences were viewed in BioEdit 7.2.5 software and aligned to HIV-1 reference sequences obtained from the GenBank database, in the MAFFT program available online. Sanger and Illumina consensus sequences obtained from baseline samples were compared through phylogenetic analysis in MEGA software and were observed to correctly cluster together (Supplementary Fig. 1).

### Analysis of Illumina sequencing reads for minority variants

Deep sequencing reads were mapped to an HIV-1 subtype C reference (AY162225.1) in CLC Genomics. Minority variants were analysed using a low-frequency variant detection tool in CLC Genomics, setting the cut-off for significance at 1% and the minimum frequency for variants at 5%. Minority variants were assessed in all participant samples and only nonsynonymous variants were mapped to HIV-1 CTL epitopes in the Los Alamos HIV database, to identify the epitope they fall within. Thus, in this study, minority variant epitopes were defined as epitopes existing at proportions above 5% but less than 20%. The Los Alamos HIV database was also used to predict human leukocyte antigen (HLA) allotypes that may be involved in presenting the identified epitopes to CTLs. Evolution within Pol CTL epitopes was assessed by comparing the proportion of minority variants between baseline and follow-up samples, and in early and chronic HIV-1 disease stages.

### Data analysis

Descriptive statistics was used to present median values and interquartile range (IQR) for age, HIV-1 VL and sequencing depth. Fisher's exact test was used to assess if there was an association between the distribution of mutations and HIV-1 disease stage for epitopes identified through Sanger sequencing. Two sample t-test was used for comparing the proportions of minority variant epitopes among the different regions of the Pol protein. A p-value of ≤ 0.05 was considered statistically significant. All statistical tests were performed on the STATA 16.0 software package (StataCorp LP, College Station, TX, USA). The statistical analyses of dN-dS ratios were computed on the MEGA programme using the HyPhy test for selection. Due to the small sample size, codons with dN-dS ratios of ≥ 5 were considered for assessment of epitopes under immune selection pressure. GraphPad Prism v9.1.2 (GraphPad Software, San Diego, California, USA) was used to prepare graphs for the frequently recognised minority variant epitopes among the different regions of the Pol protein, and also for showing the dynamics of these epitopes in sample pairs obtained during early and chronic HIV-1 infection stages.

### Results

#### Demographics
This study enrolled 52 participants with HIV-1 infection, 34 of whom had paired plasma samples. Their median age was 28 years (IQR: 24–32 years), and majority were females (92.3%). There were 15 participants (28.9%) with early HIV-1 infection and 37 (71.1%) with chronic HIV-1 infection. The median HIV-1 VL at baseline was $2.8 \times 10^4$ copies/mL (IQR: $8.6 \times 10^3 - 9.1 \times 10^4$ copies/mL). The median interval between baseline and follow-up sampling was 4 weeks (IQR: 3 – 8 weeks) (Table 1).
| Sample ID | Sex | Age (years) | HIV Stage | HIV VL | Total reads (x10^6) | HIV reads (x10^5) | Sequencing depth (x) | FU interval (weeks) | Total reads (x) | HIV reads (x) | Sequencing depth (x) |
|-----------|-----|-------------|-----------|--------|---------------------|------------------|---------------------|-------------------|-----------------|--------------|---------------------|
| 261       | M   | 33          | E         | 8.4    | 415 560            | 198 157          | 17408               | No FU             |                 |              |                     |
| 2504      | F   | 24          | E         | 3.7    | 396 640            | 288 841          | 25452               | 2                 | 483 620        | 308 253      | 27268               |
| 3469      | F   | 20          | E         | 3.3    | 79 948             | 38 927           | 3139                | 9                 | 181 956        | 104 874      | 9169                |
| 5041      | M   | 23          | E         | 2.2    | 146 352            | 108 572          | 10023               | No FU             |                 |              |                     |
| 6512      | F   | 23          | E         | 1.7    | 155 222            | 114 138          | 10269               | 2                 | 246 184        | 181 098      | 16380               |
| 6727      | F   | 28          | E         | 4.8    | 91 218             | 60 365           | 5230                | 2                 | 202 824        | 127 955      | 11522               |
| 6638      | F   | 28          | E         | 1.9    | 167 878            | 108 348          | 9913                | 6                 | 309 802        | 222 628      | 20221               |
| 6582      | F   | 24          | E         | 6.2    | 311 276            | 201 897          | 17355               | 6                 | 857 888        | 602 248      | 49753               |
| 9498      | F   | 34          | E         | 5.0    | 145 514            | 100 637          | 8871                | No FU             |                 |              |                     |
| 9049      | F   | 20          | E         | 1.6    | 363 168            | 226 752          | 19829               | 3                 | 1 724 070      | 1 406 644    | 65497               |

ID – identity, M = male; F = female; HIV = human immunodeficiency virus; E = early HIV infection; C = chronic HIV infection; VL = viral load; x = times; FU = follow-up
| ID  | Gender | Age | Stage | Baseline sequencing | Follow-up sequencing |
|-----|--------|-----|-------|---------------------|---------------------|
| 8575 | F      | 27  | E     | \(9.3 \times 10^4\) | 355 040 238 087 21126 | 8 |
| 8047 | M      | 31  | E     | \(1.2 \times 10^6\) | 386 426 308 249 28180 | 2 |
| 7084 | F      | 28  | E     | \(3.3 \times 10^8\) | 199 022 113 257 9843 | 4 |
| 6743 | F      | 26  | E     | \(2.7 \times 10^4\) | 87 982 43 575 3928 | 7 |
| 6737 | F      | 24  | E     | \(2.2 \times 10^3\) | 270 528 192 684 16793 | No FU |
| 639  | F      | 30  | C     | \(6.5 \times 10^3\) | 851 104 651 917 60785 | 7 |
| 641  | F      | 30  | C     | \(7.0 \times 10^4\) | 1 334 058 969 199 45128 | 4 |
| 843  | F      | 21  | C     | \(2.9 \times 10^4\) | 328 480 278 706 16615 | 5 |
| 921  | F      | 44  | C     | \(9.7 \times 10^3\) | 919 452 599 019 49637 | No FU |
| 1121 | F      | 27  | C     | \(8.0 \times 10^4\) | 126 868 90 889 8203 | 2 |
| 1213 | F      | 36  | C     | \(3.2 \times 10^4\) | 279 080 228 046 14505 | No FU |
| 1475 | F      | 32  | C     | \(4.4 \times 10^4\) | 1 165 614 772 938 35990 | 9 |

ID – identity, M = male; F = female; HIV = human immunodeficiency virus; E = early HIV infection; C = chronic HIV infection; VL = viral load; x = times; FU = follow-up
| ID  | Baseline sequencing | Follow-up sequencing |
|-----|---------------------|----------------------|
| 2340 | F 22 C | 1.4 x 10^4 | 3 144 374 | 2 260 428 | 119694 | No FU |
| 2678 | F 30 C | 2.2 x 10^5 | 406 036 | 291 734 | 16510 | 4 | 1 300 644 | 1 060 811 | 49394 |
| 2696 | F 18 C | 3.9 x 10^2 | 2 426 068 | 1 624 274 | 137760 | No FU |
| 3253 | F 28 C | 8.9 x 10^4 | 1 342 710 | 893 218 | 41590 | 3 | 2 060 282 | 1 337 210 | 115839 |
| 3387 | F 37 C | 7.9 x 10^4 | 1 769 858 | 1 107 226 | 95026 | No FU |
| 3474 | F 21 C | 1.6 x 10^4 | 241 256 | 164 637 | 14848 | 12 | 289 220 | 194 533 | 17234 |
| 9986 | F 28 C | 9.7 x 10^4 | 187 066 | 116 633 | 9830 | 2 | 90 100 | 57 543 | 4772 |
| 3606 | F 24 C | 1.5 x 10^4 | 558 718 | 186 290 | 19453 | No FU |
| 3869 | F 32 C | 2.1 x 10^5 | 1 413 006 | 905 554 | 42165 | 4 | 1 678 940 | 1 181 291 | 98302 |
| 3880 | F 30 C | 7.5 x 10^3 | 1 569 698 | 1 138 834 | 101974 | 3 | 2 339 288 | 1 600 920 | 132141 |
| 3910 | F 33 C | 2.8 x 10^4 | 929 580 | 715 365 | 33309 | 3 | 2 858 950 | 1 977 216 | 157648 |
| 3912 | F 27 C | 3.2 x 10^4 | 167 708 | 120 889 | 10826 | 8 | 364 208 | 270 552 | 23731 |

**ID** – identity, **M** = male; **F** = female; **HIV** = human immunodeficiency virus; **E** = early HIV infection; **C** = chronic HIV infection; **VL** = viral load; **x** = times; **FU** = follow-up
| ID  | Gender | Age | Disease Status | Baseline sequencing | Follow-up sequencing |
|-----|--------|-----|---------------|---------------------|---------------------|
|     |        |     |               | E  F  C | x  | E  F  C | x  |                |                    |
| 3920| F      | 20  | C             | 6.6 10^4 | 558 718 | 186 290 | 16933 | 8     | 741 498 | 533 342 | 44809   |
| 3935| F      | 33  | C             | 2.4 10^5 | 1 733 492 | 1 367 261 | 63663 | 8     | 3 256 | 998 1 870 | 279 | 130685 |
| 4351| F      | 35  | C             | 2.6 10^3 | 1 091 330 | 855 414 | 39830 | 3     | 114 350 | 71 708 | 5819   |
| 4198| F      | 19  | C             | 1.7 10^3 | 1 523 160 | 925 214 | 77596 | No FU |        |        |        |
| 5054| F      | 26  | C             | 2.7 10^4 | 392 770 | 294 322 | 26607 | 2     | 323 594 | 241 595 | 21969 |
| 6380| F      | 25  | C             | 1.1 10^4 | 354 870 | 254 132 | 23050 | 4     | 229 160 | 156 127 | 13011 |
| 6565| F      | 28  | C             | 5.6 10^3 | 1 827 778 | 1 280 993 | 76473 | 3     | 121 716 | 95 006 | 8145   |
| 6671| F      | 35  | C             | 1.4 10^4 | 935 030 | 676 975 | 40433 | No FU |        |        |        |
| 6649| F      | 32  | C             | 2.1 10^4 | 1 163 568 | 755 545 | 65939 | 2     | 1 482 096 | 998 909 | 83275 |
| 6640| F      | 37  | C             | 3.0 10^3 | 376 388 | 248 289 | 21017 | 5     | 1 471 394 | 1 041 570 | 93224 |
| 6596| F      | 31  | C             | 3.8 10^3 | 1 318 526 | 1 082 538 | 50406 | 8     | 1 346 566 | 910 739 | 78503 |
| 6509| M      | 36  | C             | 1.0 10^5 | 655 472 | 520 480 | 23577 | No FU |        |        |        |

ID – identity, M = male; F = female; HIV = human immunodeficiency virus; E = early HIV infection; C = chronic HIV infection; VL = viral load; x = times; FU = follow-up
| ID   | Gender | Age | Stage of HIV | Baseline sequencing | Follow-up sequencing | FU |
|------|--------|-----|--------------|---------------------|----------------------|----|
| 9915 | F      | 30  | C            | $1.4 \times 10^4$ | 489/752, 372/610     | 21830 | No FU |
| 9895 | F      | 31  | C            | $4.8 \times 10^3$ | 458/108, 328/446     | 18433 | No FU |
| 9854 | F      | 20  | C            | $1.2 \times 10^4$ | 393/220, 336/159     | 20018 | No FU |
| 8828 | F      | 27  | C            | $4.1 \times 10^4$ | 1/331, 985/767       | 45900 | 14/94, 59/609, 5106 |
| 7959 | F      | 40  | C            | $1.4 \times 10^5$ | 538/128, 453/795     | 23053 | No FU |
| 6990 | F      | 26  | C            | $1.7 \times 10^4$ | 143/560, 101/490     | 9056  | 6/179, 136/949, 12630 |

ID – identity, M = male; F = female; HIV = human immunodeficiency virus; E = early HIV infection; C = chronic HIV infection; VL = viral load; x = times; FU = follow-up

### Amplification of HIV-1 pol and phylogenetic analysis

The complete HIV-1 *pol* gene was successfully amplified in all project samples using the in-house nested PCR method. Phylogenetic analysis of Illumina consensus sequences showed that most study sequences clustered with HIV-1 subtype C strains. All baseline and follow-up sequences for each participant correctly clustered together (Fig. 1), showing accurate amplification and sequencing of viral genes from the same participant.

### Pol CTL epitopes based on Sanger consensus sequences

Eight CTL epitopes, with amino acid residues under high selection pressure, were identified. The majority of the identified epitopes were located within RT. The distribution of escape mutations was comparable between early and chronic HIV-1 disease stages (Table 2). However, these escape mutations were identified mostly in participants with chronic HIV-1 infection (Supplementary Table 2). One or more escape mutations were observed in each epitope. Two other codons were identified to be under high selection pressure but could not be mapped to the reported CTL epitopes in the Los Alamos HIV database (Table 2).
Table 2
Nonsynonymous mutations in Pol CTL epitopes assessed from Sanger consensus sequences

| pol protein | Epitope position | Wild type CTL epitope | Escape CTL mutation | Early HIV samples: n = 15 (%) | Chronic HIV samples: n = 34 (%) | P-value |
|-------------|------------------|-----------------------|---------------------|-----------------------------|--------------------------------|---------|
| PR | PR (11–20) | VTIKIGGQLK | I15V | 73,3 | 59,4 | 0,345 |
| PR | PR (30–38) | DTVLEDMLN | M36I/L | 98 | 79 | 0,702 |
| RT | RT (33–41) | ALVEICTEM | V35T/K/M | 100 | 100 | N/A |
| RT | RT (202–210) | IEELRQHLL | I202V | 10 | 17,7 | 1,000 |
| RT | RT (269–277) | QIYAGIKVK | A272P/G/S* | 80 | 55 | 0,345 |
| Not within epitope RT 329 | | | | | | |
| | | | I329V/L | 20 | 5,8 | 0,160 |
| IN | Not within epitope IN 206 | | | | | |
| | | | T206S | 20 | 15 | 0,687 |
| IN | IN (218–227) | TKIQRVYY | K219N/Q | 10 | 17,6 | 1,000 |
| IN | IN (278–288) | DDCVSRQED | S283D/G | 100 | 95 | 1,000 |

*Codon had significant dN/dS ratio (p-value = 0.001). CTL = cytotoxic T-lymphocyte; N/A = not applicable; PR = protease; RT = reverse transcriptase; IN = integrase; Pol = polymerase.

Pol CTL epitopes based on analysis of minority variants

The median sequencing depth (for baseline and follow-up) was 23052X (IQR: 13011–49753X) (Table 1). Minority variant analysis identified 65 frequently targeted Pol CTL epitopes. There was a significantly higher proportion of epitopes in RT (n = 39, 60.0%) compared to IN (n = 17, 26.2%) and PR (n = 9, 13.8%), p = 0.002 and < 0.0001, respectively. However, no significant difference was observed between the proportion of minority epitopes in IN versus PR, p = 0.06. The majority of these epitopes were identified in participants with chronic HIV-1 infection compared to those with early HIV-1 infection. Some epitopes were only identified in either early or chronic HIV-1 infection, whereas others were identified in both stages of disease (Fig. 2). Different distribution patterns of minority variant epitopes were observed. Some variant epitopes increased or decreased between baseline and follow-up, while others remained constant between these two time-points (Fig. 3, Supplementary Table 3). There were minority variants (2 in early and 26 in chronic HIV-1 infection) that could not be mapped to the reported CTL epitopes in the Los Alamos HIV database (Supplementary Table 3).

Pol CTL epitopes and predicted HLA class I presentation

HLA class I allotypes that possibly present the identified epitopes were predicted from the Los Alamos HIV database. Some participants recognised more epitopes than others. This was observed for participants 8047 and 6743 who each recognised seven epitopes during early HIV-1 infection. The most commonly targeted epitope during early HIV-1 infection was SAAVKAACW (IN 123–131), located in IN, and possibly presented by HLA-B*58:01 [27]. The same trend was noticed in chronic HIV-1 infection where the most frequently targeted epitope, MASDFNLPPPV (IN 22–31), was also located in IN and possibly presented by HLA-A*02 [27]. Some of the frequently targeted epitopes identified in this study have never been reported for HIV-1 subtype C. HLA class I allotypes that possibly present the identified epitopes in this study have been reported previously in the South African population [11–12, 28–32]. Some epitopes were predicted to be recognised by multiple HLA class I allotypes. There were also frequently recognised epitopes that seemed to have similar HLA class I restriction such as ALQDSGLEV (RT 485–494) and RAMASDFNL (IN 20–28) in early HIV-1 infection (Table 3).
| Epitope position | Wild type and variant epitope sequences | Subtype identified for epitope | Predicted HLA | Participants |
|------------------|----------------------------------------|-----------------------------|---------------|--------------|
| PR (68–76)       | GKKAIGTVL E...R...M... | Subtype B and C | **B*15:03; B*13:02; C*02** | 3469, 8047, 6743 |
| RT (139–149)     | TPGIRQYNNVL ........I....F | Subtype C | **B*81:01; B*81** | 6737, 261, 9049 |
| RT (243–251)     | PIQLPEKDS ..........E....N | Subtype C | Not reported | 6737, 6743 |
| RT (268–277)     | SQIYPGIKVR ......S......N | Subtype C | **A*74:01** | 6743, 6638 |
| RT (485–493)     | ALQDSGLEV .....N...... | Subtype B | **A*02:01; A*02** | 8047, 6743 |
| RT (516–525)     | ELVNQIEQL .....K......V...K... | Not reported | **A*02** | 9049, 8047, 6743 |
| IN (20–28)       | RAMASDFNFL K...........V... | Not reported | **A*02:01; A*02** | 6582, 3469, 6743 |
| IN (123–131)     | SAAVKAACW ...T.......M......S... | Subtype C | **B*58:01** | 7084, 9049, 8047, 6743 |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen
### Early HIV infection

| Epitope position | Wild type and variant epitope sequences | Subtype identified for epitope | Predicted HLA | Participants |
|------------------|----------------------------------------|-------------------------------|---------------|-------------|
| IN (135–146)     | IQEFGIPYNPQ .H.........................| Subtype C B*15:03             | B*15:03       | 2504, 8047  |
|                  |                                        |                               |               |             |
| IN (213–220)     | LQKQITKI .................................| Subtype B B*52:01             | B*52:01       | 9049, 8047, 8575 |
|                  |                                        |                               |               |             |
| IN (263–271)     | RKAKIIKDY .................................| Subtype C B*15:03             | B*15:03       | 9049, 8047, 8575 |
|                  |                                        |                               |               |             |

### Chronic HIV infection

| Epitope position | Wild type and variant epitope sequences | Subtype identified for epitope | Predicted HLA | Participants |
|------------------|----------------------------------------|-------------------------------|---------------|-------------|
| PR (11–20)       | VTIKIGGQLK .................................| Subtype B and C A*03:01; A*11:01 | A*03:01; A*11:01 | 6990, 641, 3253, 3910, 3912, 3920, |
|                  |                                        |                               |               |             |
| PR (30–38)       | DTVLEEMNL .................................| Subtype A and B A*68:02; A*28; A*68 | A*68:02; A*28; A*68 | 3253, 6565, 9986 |
|                  |                                        |                               |               |             |
| PR (34–42)       | EEINLPGKW .................................| Subtype A, C and D A*11; A*68; B*08 | A*11; A*68; B*08 | 843, 3880 |
|                  |                                        |                               |               |             |
| PR (42–50)       | WKPKMIGGI .................................| Subtype B Cw3                  | Cw3           | 6649, 6990, 1121, 1475, 3253 |
|                  |                                        |                               |               |             |
| PR (56–66)       | VRQYDQIPIEI F............................| Subtype B B*13                | B*13          | 6649, 3253, 3920, 3935, 6565 |
|                  |                                        |                               |               |             |
| PR (68–76)       | GKKAIGTVL .................................| Subtype B and C B*15; B*15:03; B*13:02; C*02 | B*15; B*15:03; B*13:02; C*02 | 3253, 3910, 3869, 5054, 9986 |
|                  |                                        |                               |               |             |
| PR (80–90)       | TPVNIIRNML .................................| Subtype C B*81:01; B*81        | B*81:01; B*81 | 1475, 3253, 3910, 3920, 5054, 6596 |
|                  |                                        |                               |               |             |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen.
| Early HIV infection |  |
|--------------------|---|
| **PR (91–99)**     | **TQIGCTLNF** |
| **Subtype B and C** | **B*15:01; B*15:03** |
|                    | **639, 1475, 3253, 9986** |
| **PR (99)-RT (8)** | **FPISPIETVP** |
| **Subtype B**      | **B*54:01** |
|                    | **6649, 641, 843, 1121, 1475, 3253, 3935, 2678** |
| **RT (57–66)**     | **NTPVFAIKKK** |
| **Subtype B and C** | **A*11; A*68; B*08** |
|                    | **6649, 3253** |
| **RT (73–82)**     | **KLVDRELK** |
| **Subtype A, B, C and D** | **A*03:01; A*34; A*29; B*18; B*80** |
|                    | **641, 3910, 3869, 6565** |
| **RT (105–113)**   | **SVTVLDVGD** |
| **Subtype B**      | **Not reported** |
|                    | **843, 3920, 3935** |
| **RT (113–120)**   | **DAYFSVPL** |
| **Subtype B**      | **A*24; B*51:01; B*51** |
|                    | **1475, 3253** |
| **RT (118–127)**   | **VLPDEGFRKY** |
| **Subtype C**      | **B*35:01; B*35:02; B*42:01** |
|                    | **1121, 8828** |
| **RT (136–144)**   | **NNETPGIRY** |
| **Subtype C**      | **B*18:01; B*18** |
|                    | **6649, 1475, 3869, 3920** |
| **RT (149–159)**   | **LPQGWKGSPIAI** |
| **Subtype C**      | **B*39:10; B*42:01** |
|                    | **3253, 3910, 9986** |
| **RT (159–167)**   | **IFQSSMTKIL** |
| **Subtype A, B and C** | **Not reported** |
|                    | **843, 5054** |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30].
Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen
| RT (171–181) | FRAQNPEIVY  | Not reported | Not reported | 6649, 843, 1475, 3253, 3474, 3869, 3920, 3935, 5054 |
|--------------|-------------|--------------|--------------|-------------------------------------------------|
| RT (181–189) | YQYMDDLYV   | Subtype A, B, C and D | A*02:01; A*02 | 641, 6565                                        |
| RT (192–202) | DLEIGQHRTKI | Subtype B    | A*03         | 6649, 6990, 1121, 3920, 3935, 6596, 8828          |
| RT (203–212) | EELREHLLKW  | Subtype C    | B*44:03      | 6990, 1121, 3253, 3474, 3869, 3920, 3935, 6565, 6509, 7959 |
| RT (233–241) | ELHPDRWTv   | Subtype B and C | Not reported | 641, 1121, 3912, 3935, 6565                        |
| RT (240–248) | TVQPIVLPE   | Subtype B    | Not reported | 6649, 3474                                        |
| RT (304–312) | AENREILKE   | Subtype B    | Not reported | 843, 3253, 3910                                   |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen.
| RT (317–327) | VYYDPSKDLIA | Subtype C | Not reported | 6990, 1121, 1475, 3253, 3910, 3935 |
| RT (329–339) | IQKQGQGWY | Subtype B | B*39:01 | 5054, 6565, 3387, 6509 |
| RT (340–352) | QIYQEPKNLTKG | Subtype B | A*11; A*11:01 | 641, 843, 1475, 3474, 3910, 6380 |
| RT (356–366) | KMRTAHTNDVK | Subtype B | A*03:01; A*03 | 6649, 6990, 641, 1121, 1475, 3253, 3920, 5054, 9986 |
| RT (367–375) |QLTEAVHKI | Subtype C | Not reported | 3880, 6565, 8828 |
| RT (375–383) | IAMESIVIW | Subtype B and C | B*53:01; B*35:08; B*57:02; B*57:03; B*58:01; C*12 | 6990, 639, 3912, 3935 |
| RT (379–388) | SIVIWGKTPK | Subtype B | A*11:01 | 3880, 6990, 843, 1121, 3474, 3920, 3935, 9986 |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen.
| RT       | Sequence                | Subtype | HLA Allele | References          |
|----------|-------------------------|---------|------------|---------------------|
| RT (397–406) | TWETWWTEYW               | Subtype B | B*44 | 6640, 1121, 3912, 3920 |
|          | G................       |         |            |                     |
| RT (416–425) | FVNTPPLVKL     | Subtype C | B*07 | 641, 3253, 3935, 7959 |
|          | I................       |         |            |                     |
|          | T...........         |         |            |                     |
| RT (432–441) | EPIAGAETFY       | Subtype C | B*35:01 | 641, 843, 3910, 3869, 5054, 6509, 7959 |
|          | L................       |         |            |                     |
|          | M................       |         |            |                     |
|          | V................       |         |            |                     |
|          | Y.................     |         |            |                     |
| RT (448–457) | RETKIGKAGY     | Subtype B | A*29 | 843, 1121, 3253, 3869, 3920, 3935, 9986 |
|          | R................       |         |            |                     |
|          | V................       |         |            |                     |
|          | R................       |         |            |                     |
| RT (461–469) | KGRQKVTL         | Subtype B | B*08:01 | 1121, 1475, 3910, 3920, 3935, 9986, 6509, 7959, 9854 |
|          | R.....................  |         |            |                     |
|          | M.............       |         |            |                     |
|          | I..................... |         |            |                     |
|          | A..................... |         |            |                     |
| RT (468–476) | SLETTNQK       | Subtype C | A*74:01 | 6640, 6990, 1475, 6509, 7959 |
|          | R.....................  |         |            |                     |
| RT (477–486) | TELQAIQLAL     | Subtype C | B*18:01 | 641, 843, 3910 |
|          | R.....................  |         |            |                     |
| RT (491–501) | SEVNIVTDSQY   | Subtype C | B*44:03 | 3880, 6649, 6565 |
|          | V..................... |         |            |                     |
|          | M..................... |         |            |                     |
| RT (509–518) | QPDKSESELV   | Subtype A, B, C and D | B*07 | 1121, 3869 |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen.
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| RT (519–527) | NQIEQLIKK | Subtype B | Not reported | 6640, 6649, 843, 3869, 5054, 8828, 9986 |
| RT (526–534) | IKKEKIYLA | Subtype B | Not reported | 6649, 641, 3920, 3935, 9986 |
| RT (550–559) | KLVSQGIRKV | Subtype A, B, C and D | A*02:01 | 639, 1121, 3869, 9986 |
| IN (22–31) | MASDFNLPPIV | Not reported | A*02 | 6649, 641, 843, 1121, 1475, 3910, 3869, 3920, 3935, 4351, 5054, 6565 |
| IN (33–43) | AKEIVASCDKC | Not reported | Not reported | 6649, 6990, 3253, 3920, 2678 |
| IN (68–76) | LEGKIILVA | Subtype B | B*40:06 | 6990, 639, 3253 |
| IN (78–86) | HVASGYIEA | Subtype B | B*54:01 | 1121, 3935, 6565 |
| IN (101–105) | ILKLAGRWPVK | Subtype C | A*03:01 | 1121, 1475, 3474, 3869, 5054, 2678 |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen.
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| IN (114–123) | HTDNSNFTS | Subtype B | Cw*05 | 1121, 3869 |
|---------------|------------|-----------|-------|------------|
|               |            |           |       |            |
| IN (123–132)  | SAAVKAACWW | Subtype C | B*58:01 | 3880, 641, 843, 3935, 8828 |
|               |            |           |       |            |
| IN (135–143)  | IQFEFGIPYNQ | Subtype C | B*15:03 | 1475, 6565 |
|               |            |           |       |            |
| IN (164–172)  | QVRDQAELH | Subtype C | A*02:05 | 3869, 3920 |
|               |            |           |       |            |
| IN (185–194)  | FKRKGIGGY | Subtype A, B and C | B*15:03; B*27:05; C*01 | 3920, 3935 |
|               |            |           |       |            |
| IN (206–213)  | TDIOKTKE | Subtype B | B*40:02 | 3935, 6380, 6565 |
|               |            |           |       |            |
| IN (213–220)  | LQKQITKI | Subtype B | B*52:01 | 843, 1475 |
|               |            |           |       |            |
| IN (241–250)  | LLWKGEGAVV | Not reported | A*02:01 | 6640, 641 |
|               |            |           |       |            |
| IN (259–268)  | VPRRKVKK | Subtype B and C | B*08:01 | 843, 3253, 3910, 3912, 5054 |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen
Early HIV infection

| IN (278–288) | DDCVAGRQDED | Not reported | Not reported | 3253, 3910, 3935, 8828 |

| S | D. | N |

HLA alleles in boldface are those that have been identified (reported) in South Africa or southern Africa [11, 28–30]. Alleles underlined are those that were reported to be protective [11, 28–29, 49, 50]. PR = protease; RT = reverse transcriptase; IN = integrase; HLA = human leukocyte antigen

Discussion

To our knowledge, this is the first study to assess the evolution of HIV-1 Pol CTL epitopes in samples obtained during early and chronic disease stages in a setting where HIV-1 subtype C is predominant. We identified epitopes within all three regions of Pol, unlike previous studies that have only characterised epitopes within RT and PR [16, 29, 33]. This highlights the advantage of using a PCR method that amplifies the complete pol gene. Baseline and follow-up sequences for each participant correctly clustered together, indicating accurate amplification and sequencing of viral genes belonging to the same participant. It was expected that the majority of sequences would cluster with HIV-1 subtype C as this is the most common subtype in the southern African region [34–35]. A small number of non-subtype C strains were detected, which could have been introduced through migration, and this has also been reported in previous South African studies [36–37].

Many epitopes were identified through the analysis of minority variants obtained by deep sequencing compared to majority variants obtained by Sanger sequencing. This shows the advantage of using deep sequencing for characterisation of Pol CTL epitopes as this method can detect variants that exist below 20% of the total virus population, which are not readily detected by Sanger sequencing [38–39]. Some identified minority variant epitopes have never been reported for HIV-1 subtype C and were possibly missed by previous studies that employed Sanger sequencing. The use of Sanger sequencing in most studies has most likely underestimated the presence of CTL epitopes in Pol. Other studies have also shown increased detection of CTL epitopes when using next-generation sequencing (NGS) methods [40–41]. This shows an advantage in the use of NGS for characterising HIV-1 CTL epitopes in future research studies.

In this study, CTL epitopes were identified in all regions of Pol, but mostly in RT and IN. Some of these epitopes such as GKKAI GTVL (PR 68–76) and IAMESIVIW (RT 375–383) (Table 3) have been previously reported to induce CTL responses [42–43]. These data indicate that all three regions of Pol are immunogenic and may play a role in the control of viraemia and the establishment of a viral set-point during early HIV-1 infection, in agreement with previous studies that assessed CTL responses against the HIV-1 proteome [42–44]. In a study that assessed CTL responses during acute HIV-1 infection, Kim et al showed that the larger proportion of targeted epitopes were within Pol as compared to other regions of the HIV-1 proteome, and some of these epitopes stimulated strong CTL responses [42]. Ojwach et al reported that CTL responses towards epitopes in RT and IN during acute and chronic HIV-1 infection induced mutations that decreased viral replicative fitness, indicating that RT and IN harbour crucial CTL epitopes [45]. Fewer epitopes were identified in PR especially during early HIV-1 infection and this could indicate that this protein harbours more subdominant CTL epitopes that are mostly recognised later during the course of infection [46]. Thus, PR might have a limited role in control of viremia during early HIV-1 infection. CTL epitopes encoded by pol have also been reported to play a role in the control of viremia in long-term non-progressors (LTNPs) and elite controllers (ECs) [47–48].
HLA class I allotypes play an important role in the control of HIV-1 viraemia [29]. Several HLA class I alleles that encode allotypes predicted to present epitopes identified in this study are known to be present in the South African population, including HLA-B*58:01 and HLA-A*02 that present SAAVKACW (IN 123–131) and MASDFNLPPIV (IN 22–31), respectively [28, 30, 49]. Past gene association studies showed that alleles within the HLA-B group correlate with greater viral control than alleles in the HLA-A and HLA-C groups [28–29, 50]. Payne et al found that HLA-B*58:01 was associated with slower HIV-1 disease progression and was protective against HIV-1 infection [51], which could explain why some participants such as 8047 and 6743 recognised more epitopes, as they also recognised an epitope reported to be presented by HLA-B*58:01.

A hierarchy in epitope presentation may be an explanation for recognition of more epitopes during chronic HIV-1 infection. Immunodominant epitopes could be preferentially presented earlier during the course of infection, followed by presentation of subdominant epitopes later [52]. Immunodominant epitopes are often located in variable domains whereas subdominant epitopes usually map to more conserved regions of the HIV-1 proteome [18, 53]. The HIV-1 pol gene is more conserved than gag and env [54] and therefore harbours more subdominant epitopes [18]. Examples of subdominant responses being more effective in controlling viraemia have been shown in past studies [55–56].

The evolution of Pol CTL epitopes was monitored by assessing minority variants in sample pairs. Minority variant epitopes that increased in proportion from baseline to follow-up may have replaced the wild-type epitopes over time and facilitated immune escape [57]. Fitness cost is the likely explanation for minority variant epitopes that decreased in proportion over time, and these may have been out-competed by new variants [57]. The proportion of some minority variant epitopes remained constant between the two time-points, and these could also represent variants that do not replicate efficiently and are hence maintained at lower levels within the HIV-1 quasispecies [40, 57]. Some variant sites were located outside of reported epitopes (Supplementary Table 3), and these may represent new unreported epitopes or could highlight important adjacent sites that may affect epitope processing and presentation [13, 58]. Viral evolution was also assessed by comparing sequences from early and chronic HIV-1 stages. Data from this study showed that evolution within CTL epitopes began quite early following infection, as epitopes with escape mutations were detected in participants who had early HIV-1 infection [59]. Many epitopes with escape mutations were detected in participants in the chronic HIV-1 stage, indicating that viral evolution of HIV-1 results from immune selection pressure that occurs throughout the course of infection and diverse viral populations evolve that constantly evade immune responses [57, 60].

All three expressed proteins (PR, RT and IN) of Pol harbour CTL epitopes and this highlights the importance of including pol in the design of a vaccine candidate [2, 20]. Some researchers have included RT and IN in the design of HIV-1 vaccines [22–23, 53]; this is supported by the findings of our study as the majority of epitopes mapped to these two proteins. Conserved epitopes have been shown to provide cross-clade protection against HIV-1 infection [2, 18]. Some of the conserved Pol epitopes such as IETVEPKV (RT 5–12) and SVPLEANF (RT 117–126) (Table 3), that were previously observed in a study that looked at using a vaccine candidate with conserved immunogens were also identified in our study [18]. The immune responses directed towards epitopes in conserved regions are usually subdominant but may provide protection against multiple HIV-1 subtypes [17, 53]. Many studies that included Pol epitopes in vaccine candidates showed stimulation of effective CTL responses [18, 56, 61–62]. Ahmed et al, showed control of HIV-1 replication in vitro through stimulation of Pol CTL responses by a vaccine that contained subdominant epitopes [56].

Limitations of this study include the small sample size and fewer participants with early HIV-1 infection. The duration of infection in participants with chronic HIV-1 infection was not known. HIV-1 VL was not measured in the participants at follow-up, therefore no correlation between VL and the number of minority variant epitopes detected could be made. Functional CTL responses against identified epitopes were not determined and genomic DNA samples were unavailable to conduct HLA class I genotyping. Longitudinal samples were only obtained at two time-points for most of the participants at a median interval of 4 weeks and this might not be sufficient for assessing viral evolution.

Conclusions
Deep sequencing revealed many HIV-1 Pol CTL epitopes, including some which have never been reported for HIV-1 subtype C. Immune selection pressure on Pol CTL epitopes was observed during early HIV-1 infection highlighting the possibility that these epitopes might contribute to the control of viraemia and the establishment of a VL set-point. Variable patterns of distribution of epitopes in sample pairs reflect the ongoing generation of escape mutants capable of evading the immune responses throughout the course of infection. The findings of this study support the inclusion of RT and IN in potential HIV-1 vaccine candidates, as these regions harbour the majority of CTL epitopes. Variants that could not be mapped to known epitopes in the Los Alamos HIV database might represent new unreported epitopes. Future research, using deep sequencing, is needed for better characterisation of Pol epitopes as this protein contains highly conserved epitopes that have the potential to provide HIV-1 cross-clade protection.

**Abbreviations**

HIV-1
Human immunodeficiency virus type 1
PLWH
People living with HIV
CTL
Cytotoxic T-lymphocyte
VL
Viral load
HLA I
Human leukocyte antigen type 1
ART
Antiretroviral therapy
RCF
Replicative centrifugal force
RT-PCR
Reverse transcriptase polymerase chain reaction
RNA
Ribonucleic acid
DNA
Deoxyribonucleic acid
dNTPs
Deoxynucleotide triphosphate
ORF
Open reading frame
NGS
Next generation sequencing
IQR
Interquartile range
Gag
group-specific antigen
Pol
Polymerase
Env
Envelope
PR
Declarations

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Author contributions

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Investigation: PN, SHM.

Supervision: SHM, CTT.

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Data analysis: PN, SHM, CTT, TCQ, SL, AR, AI.

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Writing – review & editing: PN, SHM, CTT, TCQ, SL, AR, AI.

Competing interest

The authors declared no competing interest.

Ethics approval and consent to participate
Ethics approval was obtained for this study from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, South Africa: Reference number 282/2020. Written consent was obtained from all participants in the parent study (Ethics reference: 295/2015) [26].

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this manuscript and its supplementary information files.

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Figures
Figure 1

Neighbour-joining phylogenetic tree of all samples including sample pairs. Group O strain of HIV-1 was used for rooting. Only bootstrap values above 70% are shown. Baseline and follow-up (fu) sequences of the same participant correctly clustered.
Figure 2

Frequently recognized Pol CTL epitopes in early and chronic HIV infection. These are epitopes that were recognized by more than one participant. (A) Epitopes within the protease (PR) region. (B) Epitopes within the reverse transcriptase (RT) region. (C) Epitopes within the integrase (IN) region. Many epitopes were located within RT and IN as compared to PR, and most were found in chronic HIV infection. CTL = cytotoxic T-lymphocytes.
Figure 3

Dynamics of minority variants in sample pairs during (A) early HIV infection and (B) chronic HIV infection. (i) Some variants either increased in proportion between baseline and follow-up or remained at / above the detection limit of 5% (black solid line). (ii) Other variants decreased in proportion over time and were detected below 5% at follow-up. (iii) There were variants that increased in proportion over time to become majority variants and were thus maintained above 20% (black dotted line). (iv) Some minority variants that were detected at follow-up existed as majority variants at baseline. The median interval between baseline and follow-up sampling was 4 weeks (IQR: 3 – 8 weeks).

Supplementary Files

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- SupplementaryFigure1.pdf
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