Possible clearance of transfusion-acquired *nef*/LTR-deleted attenuated HIV-1 infection by an elite controller with CCR5 Δ32 heterozygous and HLA-B57 genotype

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Abstract

Background: Subject C135 is one of the members of the Sydney Blood Bank Cohort, infected in 1981 through transfusion with attenuated *nef*/3′ long terminal repeat (LTR)-deleted HIV-1, and has maintained undetectable plasma viral load and steady CD4 cell count, in the absence of therapy. Uniquely, C135 combines five factors separately associated with control of viraemia: *nef*/LTR-deleted HIV-1, HLA-B57, HLA-DR13, heterozygous CCR5 Δ32 genotype and vigorous p24-stimulated peripheral blood mononuclear cell (PBMC) proliferation. Therefore, we studied in detail viral burden and immunological responses in this individual.

Methods: PBMC and gut and lymph node biopsy samples were analysed for proviral HIV-1 DNA by real-time and nested PCRs, and *nef*/LTR alleles by nested PCR. HIV-specific antibodies were studied by Western blotting, and CD4+ and CD8+ T lymphocyte responses were measured by proliferation and cytokine production *in vitro*.

Results: PBMC samples from 1996, but not since, showed amplification of *nef* alleles with gross deletions. Infectious HIV-1 was never recovered. Proviral HIV-1 DNA was not detected in recent PBMC or gut or lymph node biopsy samples. C135 has a consistently weak antibody response and a substantial CD4+ T cell proliferative response to a previously described HLA-DR13-restricted epitope of HIV-1 p24 *in vitro*, which augmented a CD8+ T cell response to an immunodominant HLA-B57-restricted epitope of p24, while his T cells show reduced levels of CCR5.

Conclusions: Subject C135's early PCR and weak antibody results are consistent with limited infection with a poorly replicating *nef*/LTR-deleted strain of HIV-1. With his HLA-B57-restricted gag-specific CD8 and helper HLA-DR13-restricted CD4 T cell proliferative responses, C135 appears to have cleared his HIV-1 infection 37 years after transfusion.

Keywords: HIV-1, CD4, CCR5, Nef

Introduction

Possible 'correlates of immune protection' in HIV-1 infection have been widely reviewed [1–4]. However, from tens of millions of individuals living with HIV globally, there is only one documented case of apparent clearance of HIV-1, the Berlin Patient, who underwent allogeneic bone marrow transplantation from a CCR5 Δ32 homozygous donor [5,6].

Earlier, after approximately 10 years of the epidemic, we originally observed a small group of individuals living with HIV-1, the Sydney Blood Bank Cohort (SBBC), who remained asymptomatic [7]. These subjects were subsequently found to be infected with a *nef*/long terminal repeat (LTR)-deleted strain of HIV-1 [8], and attenuation of the transmitted viral strain appeared to be the common factor in this asymptomatic cohort. This is consistent with data from experimental infection with *nef*-deleted simian immunodeficiency virus (SIV) [9], but overall, *nef* deletion is not a common finding in cohorts of long-term non-progressors (LTNPs) [10–12]. Alternatively, LTNP cohorts exhibit over-representation of CCR5 Δ32 heterozygous individuals [13–15], implicating reduced viral co-receptor expression in limiting viraemia. In the case of the Berlin Patient, it is plausible that ablative therapy and transplantation with progenitors that lacked expression of CCR5 were the main reason for the dramatic reduction of viral burden, although it cannot be ruled out that a low-grade graft versus host immune response could also have played a role [5], since other studies found that such protocols reduce HIV DNA in peripheral blood mononuclear cells (PBMCs) to below

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detectable levels, but ultimately recipients' HIV plasma viraemia can rebound in the absence of antiretroviral therapy (ART) from an unknown reservoir [16,17].

However, there is clear evidence for immune control of viral burden, as indicated by the prevalence of HLA-B27 and HLA-B57 genotypes amongst LTNP [18]. Up to half of LTNPs with undetectable plasma viral load, in the absence of ART, referred to as Elite Controllers, are HLA-B57 [19–21], consistent with the consensus that HIV-specific CD8+ cytotoxic T lymphocytes (CTLs), particularly Gag-specific CTL, contribute to control of viraemia [3]. Another select group of LTNP subjects includes those with unusually strong CD4 lymphoproliferative responses to p24 [22,23], which may also exert anti-p24 cytotoxicity [24,25]. Furthermore, one report suggested that individuals with HLA-*DRB1*13* genotype have higher CD4 lymphoproliferative responses to p24 and associated control of viraemia [26], while HLA-*DRB1*13* was over-represented in elite controllers [27]. HLA-*DRB1*1303* and HLA-*DRB1*1302* were also found to be associated with lower plasma viral loads in two other studies [28,29].

We have investigated immunological and virological parameters in one particular individual, subject C135, who was transfused with the SBBC strain of *nef*/LTR-deleted attenuated HIV-1, as detailed previously [30], and maintained a steady CD4 T cell count, unlike some other subjects in this cohort [31,32]. Subject C135 is also CCR5 Δ32 heterozygous [31], has HLA-B57 and HLA-*DRB1*13:02:01* alleles [23], and a vigorous PBMC proliferative response to HIV-1 p24 [23,33]. Therefore, in this individual, five well-defined factors associated with LTNP are combined. We now show that C135 has a potent CD4+ T cell response to an HLA-DR13-restricted epitope in HIV-1 p24 *in vitro*, which in turn appears to assist CD8+ T cells to proliferate in response to HLA-B57-restricted epitopes of p24. These responses are associated with complete control over replication of HIV-1 *in vivo* for over 30 years, in the absence of ART, to the point where cells latently infected with HIV-1 DNA can no longer be detected in PBMC, or in biopsy samples of either lymph node or gut-associated lymphoid tissue.

Methods

Subjects

Subject C135, a 72-year-old male, is a member of the SBBC who was transfused in 1981 with a unit of erythrocytes from donor D36, later shown to be infected with *nef*/3' LTR-deleted virus [30,31]. Other SBBC transfusion recipients from this donor were also LTNPs [7,8,31,32], consistent with attenuation of the transmitted viral strain. C135 was identified in 1996 by tracing of recipients of transfusions from donor D36 and remains completely asymptomatic after a total of 37 years. Detailed serological findings for this subject are described in the results section. C135 is CCR5 Δ32 heterozygous [31] and HLA A1,33; B50,57; DR7,13 [23]. The HLA-DRB1 alleles, by high-resolution typing, are 07:01:01 and 13:02:01.

Healthy adult controls were recruited from university and hospital staff. The study was approved by the Australian Red Cross Blood Service–New South Wales Institutional Ethics Committee, and written informed consent was obtained.

HIV-1 Western blots

Prospectively collected longitudinal serum samples from C135 were examined for antibodies to HIV-1 using the BioRad New LAV Blot I (BioRad, Marnes La Coquette, France), according to the manufacturer's instructions.

Amplification of HIV-1 proviral DNA

Proviral DNA was amplified from patient PBMC DNA using an ultrasensitive PCR method based on a biphasic booster amplification protocol and nef5' and LTR3' primers, and the *nef*/LTR product was cloned and sequenced, as previously described [30,34].

In addition, five other assays for HIV-1 proviral DNA have been used, including (1) the diagnostic Roche Amplicor Monitor HIV DNA PCR, with a reported sensitivity of 5 copies per 10,000 PBMCs (Roche Diagnostics, Basel, Switzerland); (2) a published PCR assay for HIV-1 *gag* DNA with an estimated sensitivity of 10 copies per 1,000,000 cells [35]; (3) a published sensitive real-time PCR for HIV-1 DNA using *gag* primers, capable of detecting <10 copies per 300,000 cells [36,37]; (4) a published real-time PCR for HIV-1 DNA using *pol* primers, capable of detecting <3 copies per 100,000 cells [38,39] [40]; and (5) nested PCR for HIV-1 *gag* [41] with a sensitivity <<10 copies per 300,000 cells [42].

DNA for assays 3–5 was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany).

The single-copy assay was used to quantify plasma viral load as previously described [43].

Altogether, these assays were performed in four independent laboratories.

Lymphocyte immunophenotyping and cell sorting

Lymphocyte immunophenotyping of fresh whole-blood samples from C135 between 2009 and 2014 was performed as previously described [44,45].

Memory CD45RO+ CD4+ cells for HIV DNA analysis were purified from PBMC using a FACSAria (BD Biosciences, San Jose, CA, USA), as previously described [37,39]. Similarly, CD4+ T cells were also purified from gut and lymph node biopsy samples (see further) by cell sorting.

Lymphocyte function assays

From 1996 to 2006, PBMCs from C135 were assayed for proliferation in response to HIV-1 p24 in a 6-day ^{3}H-thymidine uptake assay, as previously described [33]. PBMCs were also labelled with either carboxyfluorescein diacetate succinimidyl ester (CFSE) or Cell Trace Violet (Thermo Fisher Scientific, Waltham, MA USA), and cultured in the presence or absence of recombinant HIV-1 p24, or with pools of overlapping peptides or individual peptides from the sequence of HIV-1 Gag, as previously described [23,46,47]. Proliferation was optimised by supplementation at day 5 of culture with low doses of exogenous interleukin (IL)-15 (0.5 ng/mL; R&D Systems, Minneapolis, MN, USA) and IL-21 (0.1 ng/mL; BioSource International, Camarillo, CA, USA), followed by a further 5 days of culture. This culture system has been extensively validated to provide an optimal ratio of antigen-specific proliferation to background proliferation [48]. At the end of the culture, PBMCs were stained with CD3-PerCP-Cy5.5 or -Pacific Blue, CD4-Alexa Fluor 700 and CD8-APC-Cy7, CD25-APC and CD71-PE (BD Biosciences, San Jose, CA, USA) and analysed on a four-laser LSR II (BD Biosciences), as previously described [48]. All proliferation experiments contained negative control cultures in the absence of added HIV-1 Gag antigenic peptides and positive control cultures containing phytohaemagglutinin (PHA), respectively, and were performed on at least three occasions.

Intracellular cytokine (ICC) assays, in response to HIV-1 Gag peptides, were performed using fresh sodium heparin-anticoagulated

whole blood, as previously described [36,49], with a validated cut-off for positive results of 0.08% (mean from HIV uninfected healthy adult controls ($n=20$) plus 3× SD) [50]. All ICC experiments included negative control cultures and positive control cultures containing Staphylococcal enterotoxin B (SEB), respectively, and were performed on at least two occasions.

Lymph node and gut biopsies

Ultrasound-guided fine needle biopsy (FNB) was performed in 2014 under local anaesthesia, as previously described [51], using a 25-gauge needle inserted into a single lymph node in the inguinal chain. Cells were transferred into RPMI/10% FCS, pelleted by centrifugation and resuspended, and CD4 T cells were accurately counted using immunophenotyping in Trucount™ tubes (BD Biosciences), as previously described [51]. The remaining cells were cell sorted for CD4+ T cells for HIV DNA analysis.

C135 underwent routine endoscopy and colonoscopy screening in 2009 and provided 10 pinch biopsy samples taken from each of three sites: left colon, right colon and terminal ileum. No abnormalities were detected during the procedure. Single-cell suspensions were prepared from these biopsy samples, as previously described [44,45]. CD45+ CD4+ lymphocytes and CD45– epithelial cell adhesion molecule (EpCAM)+ epithelial cells were counted using Trucount™ tubes. Remaining cells were cell sorted for CD4+ T cells for HIV DNA analysis.

CCR5 expression on memory CD4+ and CD8+ T lymphocytes

Fresh whole-blood samples from C135 and healthy adult controls, respectively, were stained exactly in parallel within 1 hour of venepuncture using an optimised indirect immunofluorescence method for CCR5, as previously described [52,53]. Briefly, 100 µl of whole blood was incubated with 1 µg of purified anti-CCR5 monoclonal antibody (mAb), clone 2D7 (BD Biosciences) for 1 hour on ice, washed twice with PBS, incubated with 1/50 dilution of PE-labelled affinity purified Fab'2 goat anti-mouse IgG (H+L chain) (Jackson ImmunoResearch, West Grove, PA), washed once, incubated with 1/10 dilution of normal mouse serum for 10 minutes at RT, then incubated for a further 15 minutes at RT with directly conjugated CD3, CD4, CD8 and CD45RO-ECD (Beckman Coulter, Hialeah, FL) mAb. Red cells were lysed with Optilyse C (Beckman Coulter), washed once with PBS, fixed with 0.5% paraformaldehyde in PBS and analysed on the LSR II flow cytometer, as described earlier.

Statistical analysis

Results are expressed as medians and interquartile ranges, analysed using Prism versions 6 and 7 (GraphPad, La Jolla, CA, USA).

Results

HIV serology and CD4 T cell counts

C135 presented in 1996 (Figure 1a), 15 years after infection, as HIV antibody positive by routine diagnostic enzyme immunoassay, but with an indeterminate Western blot (WB) pattern [30], as shown in Figure 1b, according to standard Australian interpretative guidelines [54]. An indeterminate WB serology was maintained for all available samples, with weak p18, p24 and gp160 bands between 1996 and 2007. These results would be interpreted as HIV positive, according to Centers for Disease Control and Prevention guidelines [55], and also in combination with positive nucleic acid testing for HIV-1 on at least two occasions (see further), by revised Australian criteria [56].

Later WB results from five samples between 2011 and 2017 have been weakly positive for gp160, with all other bands negative. In contrast, the other SBBC members had full WB reactivity [7,30]. The WB pattern for C135 is uncommon and can be seen in some primary HIV subjects early during seroconversion [54].

CD4 T cell counts for C135 over the 21 years between 1996 and 2017 remained steady (median: 505 cells/µl), as did the CD4:CD8 ratio (median: 1.03), as shown in Figure 1c.

Activated CD38+ HLA-DR+ cells were 0.6%, 1.9% and 1.2% of CD8 T cells on three occasions, respectively, between 2009 and 2014 [compared to a median of 1.5% (interquartile range: 1.0%–2.0%) for 54 healthy adult controls in a contemporaneous study [49]]. Therefore, C135's PBMCs have shown no change in CD8 activation levels since he had a median of 2.0% from nine samples between 1996 and 1998, in our earlier report in 1999 [57].

Thus, C135 differs from other LTNP subjects with *nef*-deleted HIV-1, whose CD4 cell counts have eventually declined [58], including all but two other members of the SBBC [31].

HIV-1 *nef*/LTR DNA PCR assays and sequencing

Proviral HIV-1 DNA was originally detected in PBMC from C135 in 1996, and the number of copies was estimated to be 2–5 copies per 100,000 PBMCs [30]. Longitudinal PBMC samples from C135 were screened for the presence of the *nef*/LTR region of proviral HIV-1 using a boosted PCR (Table 1), as previously described [30,34]. Four samples from 1996 and 1997 were positive; however, all subsequent samples tested were negative by the boosted PCR (Table 1).

Amplification of samples from 1996 to 1997 with primers nef5' and LTR3' gave 660, 664-, 664- and 660-bp products, respectively, on the occasions for which a positive signal was obtained (Table 1), compared with an 882-bp product for HIV-1_{NL4-3}. Cloning and sequencing of the *nef*/LTR amplimer (25.09.96) showed deletions of 1, 72, 4 and 11 bp from the *nef*-alone region relative to HIV-1_{NL4-3}, and 139 bp from the *nef*/LTR overlap. This 139-bp deletion includes the deletion common to all SBBC strains [30,34], equivalent to nucleotides 9281–9435 of HIV-1_{NL4-3}, and includes the 5' nuclear factor (NF)-κB binding sequence. Other features common to the SBBC strain of viruses include a GA-rich sequence

Table 1. PCR amplification of proviral DNA encoding the *nef*/LTR region

Sample date	*nef*/LTR PCR result	Length of *nef*/LTR bp
13.02.96	–	na
11.03.96	–	na
09.05.96	+	660
25.09.96	+	664
18.11.96	+	664
24.03.97	+	660
14.07.97	–	na
04.11.98	–	na
19.10.99	–	na
28.02.00	–	na
23.05.01	–	na
08.05.02	–	na
05.06.04	–	na

na: not applicable.

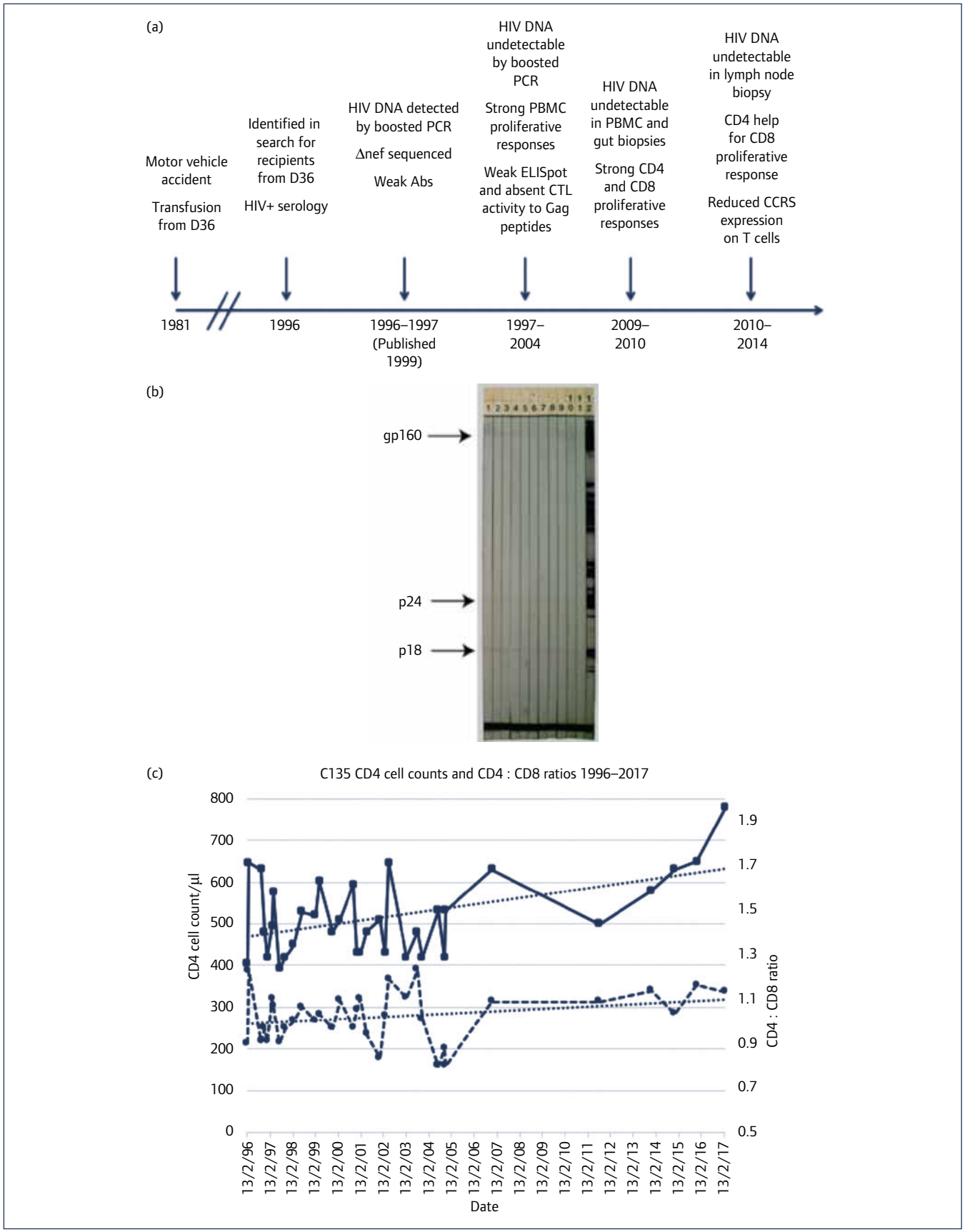


Figure 1. Timeline of studies, HIV-1 serology and CD4 T cell counts for subject C135. (a) Timeline of transfusion, testing, sampling and studies for C135. See text for details. (b) Western blot analysis of longitudinal serum samples from C135, consistently showing faint bands at p18, p24 and gp160, for the following dates: 25/9/96 (lane 1), 14/4/97 (lane 2), 14/7/98 (lane 3), 23/8/99 (lane 4), 17/10/00 (lane 5), 23/8/01 (lane 6), 1/10/02 (lane 7), 23/9/03 (lane 8), 4/11/04 (lane 9), 29/6/07 (lane 10), kit negative control (lane 11), and kit positive control (lane 12). Note that the bands on the original blots are faint to the naked eye but easily visible. The level of brightness of the original digital photographic image has been reduced by 25% to make bands easier to visualise for this report, but makes the bands on the positive control (lane 12) appear less distinct than those on the original blots. (c) Longitudinal CD4 T cell counts (solid line) and CD4 : CD8 ratios (dashed line) for C135 from 1996 to 2017. Trend lines (linear regression) are shown for each data series. CTL: cytotoxic T lymphocyte; ELISpot: enzyme-linked absorbent immunospot; PBMC: peripheral blood mononuclear cell

Table 2. PCR results for C135 samples

Sample	Date	PCR	Amount of sample	Result	Limit of detection	Method reference
PBMC	2002–2004	*gag* DNA PCR	5×10^6 cells	Negative	<10 copies/10^6 cells	[35]
Whole blood	2006–2007	Roche Qualitative Monitor v1.5	0.5 mL whole blood	Negative	5 copies/10,000 cells	Roche product insert
Gut biopsy CD4 T cells	August 2009	*gag* DNA PCR	156,000 cells	Negative	<10 copies/300,000 cells	[36,37]
PBMC	March 2010	*gag* DNA PCR	83,000	Negative	<10 copies/300,000 cells	[36,37]
Memory CD4 T cells from PBMC	March 2010	*gag* DNA PCR	2×10^6 cells	Negative	<10 copies/300,000 cells	[36,37]
PBMC	September 2014	*pol* DNA PCR	6.23×10^6 cells	Negative	<3 copies/100,000 cells	[40]
Lymph node CD4 T cells	September 2014	nested *gag* PCR	76,000 cells	Negative	<<10 copies/300,000 cells	[42]
Plasma	April 2014	*gag* RNA PCR	14 ml	Negative	<0.3 copy/mL	[43]

PBMC: peripheral blood mononuclear cell.

block at a position equivalent to HIV-1_{NL4-3} 9258–9270 bp, together with duplicated NF-κB and Sp1 sequences, which differ in arrangement between cohort viruses [8,30,34]. The sequence of C135 includes an additional Sp1 and a partial NF-κB binding sequence [30].

The C135 sequence encodes a truncated Nef open reading frame of 19 amino acids due to the introduction of a termination codon (TAA) brought into phase by the deletion of a single nucleotide equivalent to nucleotide 8842 of HIV-1_{NL4-3} [30]. However, translation beginning at a second ATG codon (at nucleotides 8822–8824 with respect to HIV-1_{NL4-3}), in a different reading frame, would encode a protein containing 96 amino acids of Nef, although, of the conserved blocks, only a complete block B would be retained.

HIV DNA PCR assays of peripheral blood mononuclear cells and purified CD4 T cells

Table 2 summarises PCR results from C135 from 2002 onwards, from samples including PBMC, whole blood, and purified CD4 T cells from PBMC, gut and lymph node biopsies.

HIV DNA PCR testing of fresh whole blood, using samples from 2006 and 2007, were negative by the qualitative diagnostic Roche Amplicor HIV-1 DNA test.

Negative results were also obtained for fresh PBMC samples by three other assays. The first PCR assay for HIV-1 *gag* DNA in PBMC had an estimated sensitivity of 10 copies per 1,000,000 cells [35]. This assay readily detected HIV DNA in PBMC from other members of the SBBC (NK Saksena, unpublished data).

The second assay, a real-time PCR for HIV-1 DNA in PBMC using Gag primers, capable of detecting <10 copies per 300,000 cells [36,37], was also negative. This assay was also negative on 2×10^6 highly purified memory CD45RO+ CD4 T cells, but this assay readily detected HIV DNA in PBMC and cell-sorted CD4 T cells from other HIV-positive subjects [36,37].

The third assay was a real-time PCR for HIV-1 DNA using *pol* primers, capable of detecting <3 copies per 100,000 cells [38,39]. A total of 53 replicates were assayed using this assay, from an equivalent of 6.23×10^6 PBMCs, and all replicates were negative.

Furthermore, plasma samples have always remained negative by routine diagnostic HIV-1 plasma viral load assays [31,33] and were also negative in 2014 using the single-copy assay [43]. The latest diagnostic HIV viral load result from 2017 was <20 copies/mL.

Importantly, HIV-1 has never been isolated from PBMC samples from C135, despite numerous attempts [30,59], using coculture techniques that resulted in successful isolation of viral strains from other members of the SBBC [7,8,59].

Lymph node and gut biopsies

Lymph node cells were obtained from C135 by ultrasound-guided FNB of an inguinal lymph node in 2014. The number of CD4 T cells recovered was 148,043 cells, which was towards the low end of the observed normal range for inguinal lymph node FNBs from 10 healthy adult controls (73,701–1,666,592), as previously described [51].

HIV DNA in cell-sorted FNB CD4 T cells was negative using a nested *gag* PCR [41], recently optimised for assays of antigen-specific CD4 T cells [42].

CD4+ T lymphocytes were also isolated from gut biopsy samples in 2009. The number of CD4 T cells in pooled single-cell suspensions derived from biopsy samples from terminal ileum, left colon and right colon for C135 was 28,415 CD4 T lymphocytes/10^6 EpCAM+ epithelial cells, similar to the median for results for these three sites, from 21 HIV-uninfected adults of 26,381 CD4 T lymphocytes/10^6 EpCAM+ epithelial cells, as previously described [45].

HIV DNA was negative in the 156,000 cell-sorted C135 gut biopsy CD4 T cells (Table 2) using real-time PCR [37].

Lymphocyte function assays

PBMC from C135 consistently showed a strong p24-specific proliferative response from 1996 to 2006 (Figure 2a), as previously described [23,33,60]. We now confirm that this response was due to proliferation of CD4+ T lymphocytes (Figure 2b), which is characteristic of LTNP [22,61]. Furthermore, when responses to individual HIV-1 Gag peptides were studied previously [23], the PBMC proliferative response was found to be directed to the 15-mer WMTNNPPIPVGEIYK, which we now also confirm as a CD4 proliferative response (Figure 2b). This sequence contains an epitope previously reported to be presented on HLA-*DRB1*1301* [26], and C135, by high-resolution typing, has the HLA-DRB1 alleles 07:01:01 and 13:02:01.

Moreover, PBMC also showed a CD8 T-lymphocyte proliferative response to the pool of 123 HIV-1 Gag overlapping peptides (see Figure 2b, middle histogram, bottom left quadrant). Such a CD8 proliferative response is characteristic of HLA-B57 LTNP [62]. We attempted to map CD8+ T-lymphocyte responses to

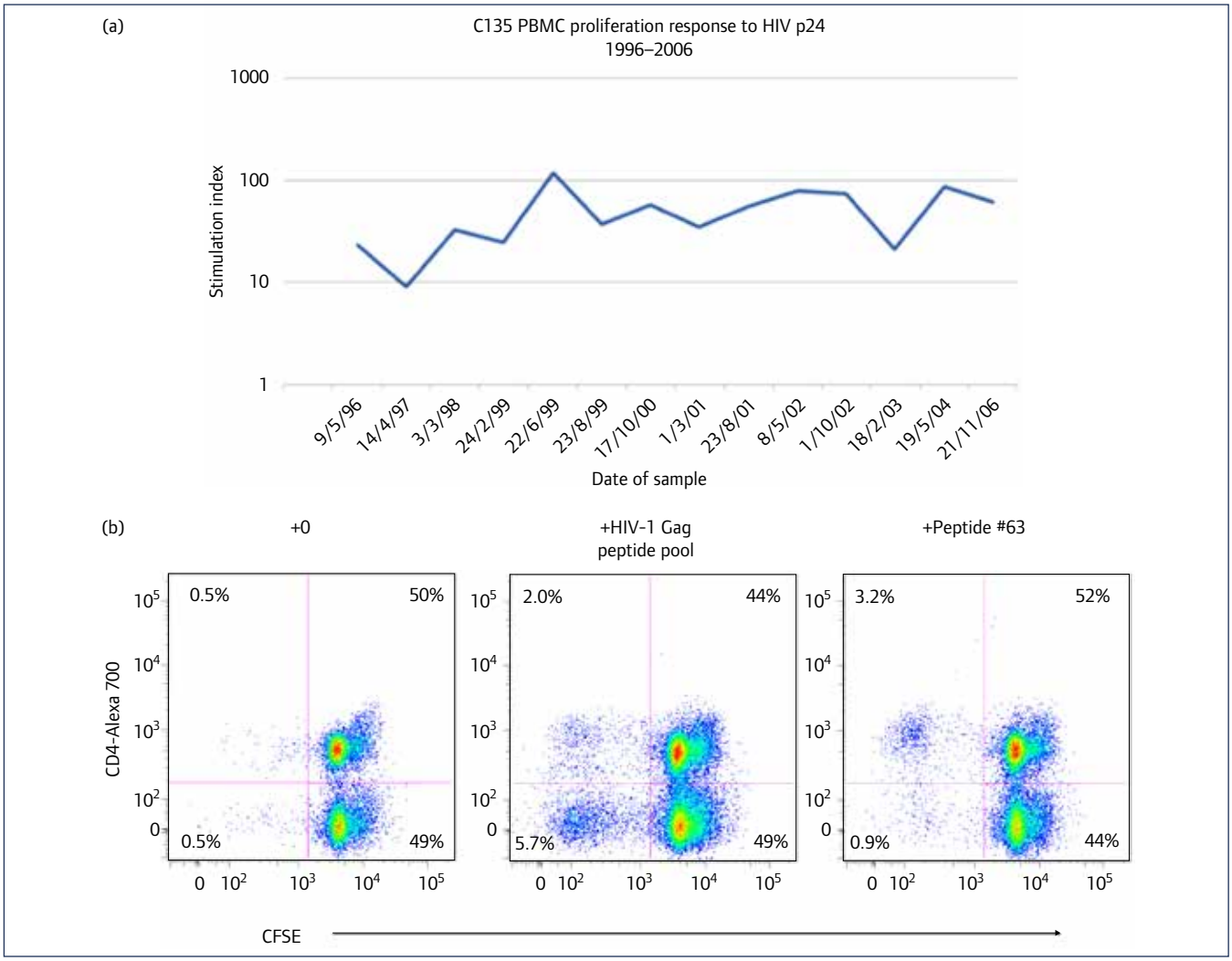


Figure 2. CD4 and CD8 T cell responses to HIV-1 Gag antigens for subject C135. (a) Stimulation indices for C135's PBMC proliferation in response to HIV p24, for longitudinal samples from 1996 to 2006, using the ^{3}H-thymidine uptake assay. (b) Flow cytometric analysis showing proliferating CD4+ T lymphocytes in CFSE-labelled PBMC from C135, in response to culture with HIV-1 Gag peptide pool (middle histogram) or with peptide #63, WMTNNPPIPVGEIYK (right histogram), compared with culture in the absence of antigen (left histogram). (c) Flow cytometric analysis showing proliferation of CD8+ T lymphocyte in CFSE-labelled PBMC from C135, in response to culture with the CD8 Gag peptide #60 (AGTTSTLQEQIGWMT) in the presence (right histogram) and absence (left histogram) of the CD4 peptide #63 (WMTNNPPIPVGEIYK). (d) Flow cytometric analysis showing proliferation of CD8+ T lymphocyte in CFSE-labelled PBMC from C135, in response to culture with the CD8 Gag peptides #40 (KVVEEKAFSPEVIPM) and #41 (EKAFSPEVIPMFSAL) in the presence (right histogram) and absence (left histogram) of the CD4 peptide #63 (WMTNNPPIPVGEIYK). CFSE: carboxyfluorescein diacetate succinimidyl ester; PBMC: peripheral blood mononuclear cell

individual peptides, but only minimal CD8+ responses were found in the absence of a concurrent CD4 response (see left histograms, Figures 2c,d), even culturing optimally with the addition of low doses of exogenous IL-15 and IL-21 [48]. However, a clear CD8 proliferative response was observed when PBMC were incubated with the 15-mer peptide containing the well-characterised HLA-B57 p24 epitope, TSTLQEQIGW [63,64], in the presence of the CD4 response to the CD4-specific p24 peptide, WMTNNPPIPVGEIYK (Figure 2c). Another well-described HLA-B57 epitope, KAFSPEVIPMF [63], also gave a very weak CD8 proliferative response, again only in the presence of the CD4 antigenic peptide WMTNNPPIPVGEIYK (Figure 2d).

Previous experiments with PBMC from C135 have shown weak interferon (IFN)-γ responses and absent CTL responses immediately *ex vivo* [23,33,65]. Using the ICC assay, we found a small, but distinct, CD4 IFN-γ+ response to HIV-1 Gag peptides (Figure 3), which represented around 0.1% of CD4+ T cells in fresh whole blood. Even though this is a relatively low level of antigen-specific CD4+ T cells, it still reaches our validated cut-off for positive ICC results (0.08%), determined for HIV-uninfected controls as part of a vaccine trial [50]. The population of IFN-γ+ CD4+ T cells clearly also made IL-2 and were CD127 positive (IL-7Rα+), but were CTLA-4 negative (Figure 3), consistent with their proliferative phenotype.

Cell surface CCR5 expression

C135 is CCR5 Δ32 heterozygous [31], and we examined expression of CCR5 on T cells in fresh whole blood, as shown compared with T cells from healthy adult controls, stained and analysed in parallel (Figure 4). The results show that C135 has a demonstrably lower level of CCR5 expression on his CD4+ T cells (Figure 4a), but not a dramatically lower percentage of CCR5+ CD4+ T cells. The difference is even clearer when CD8+ T cells are examined (Figure 4b), again with a lower expression level seen as a lower fluorescence intensity, but not as a lower CCR5+ percentage.

It should be noted that PBMC from C135 were able to be infected with laboratory strains of HIV-1 *in vitro*, although this required depletion of inhibitory CD8+ lymphocytes [23].

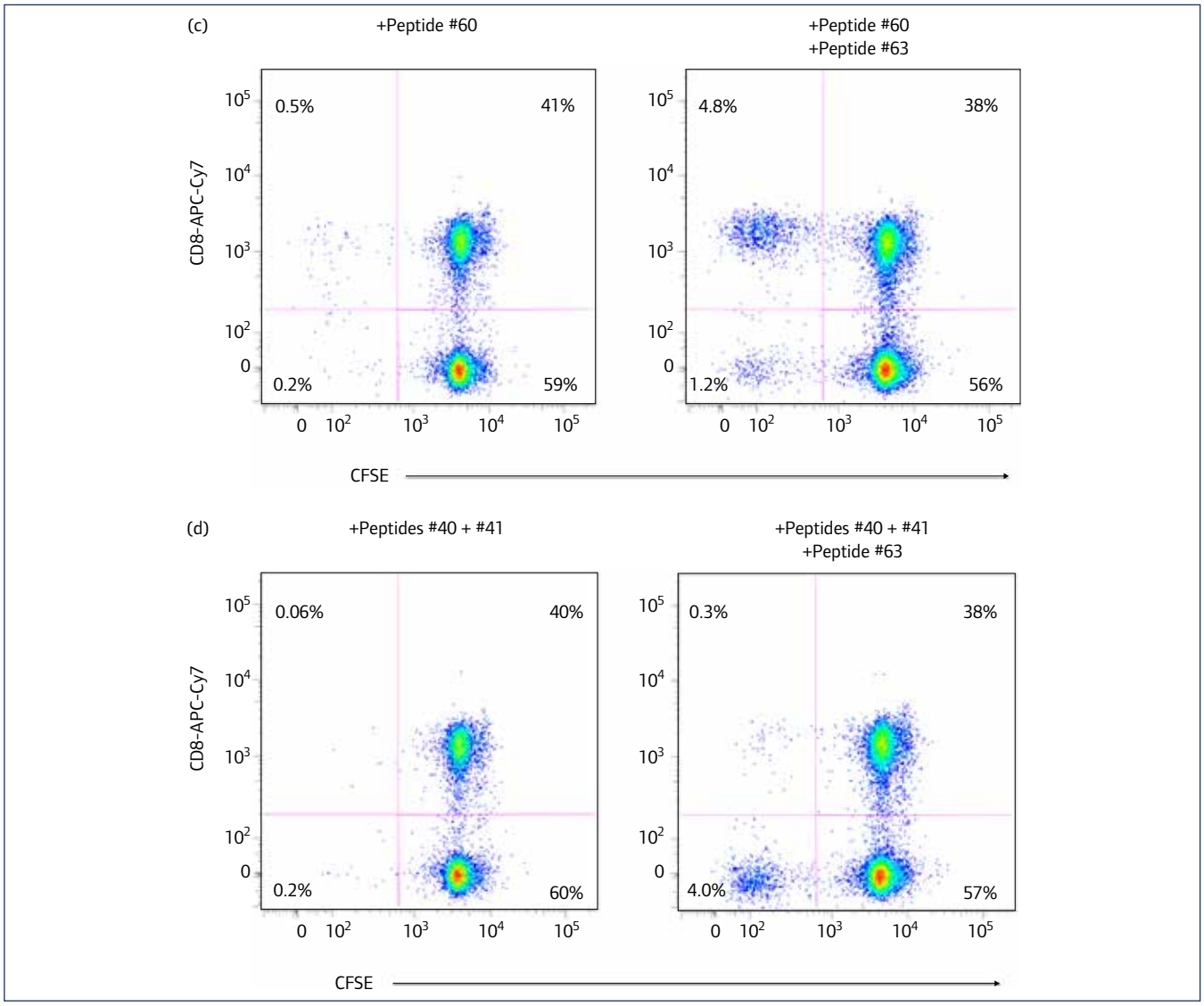


Figure 2. continued.

Discussion

Subject C135 is distinct from other members within the highly unusual SBBC, infected with the same *nef*/LTR-deleted HIV-1, and compared with other elite controllers. For over 37 years, C135 has maintained undetectable plasma viral load, an uncommon indeterminate WB pattern, and steady CD4 cell count and CD4 : CD8 ratio within the normal range, without CD8 activation.

What really sets C135 apart as unique is having had detectable HIV DNA in PBMC samples from around 15 years after infection, but subsequent negative results on a range of assays of HIV DNA in PBMC, purified memory CD4 T cells and lymph node and gut biopsy samples. In contrast, two of the other SBBC recipients, C49 and C64, with consistently undetectable plasma viral loads without ART, have a readily detectable reservoir of HIV-1 DNA in PBMC, and C64 has had virus isolated on one occasion from PBMC [31,34,59] (and NK Saksena, unpublished data). A leukapheresis sample has not been available from C135 but could possibly be tested in future, more exhaustive studies of viral outgrowth assays of replication-competent HIV-1.

One study of PBMC from patients on ART reported that two out of 30 were negative for HIV DNA, but all patients had HIV DNA detected in rectal CD4 cells [66]. Three early studies of elite controllers reported only one negative HIV DNA PCR result out of a total 25 elite controllers [11,20,21], but analysis of tissue samples was not reported. One large study of 46 controllers found only one subject negative using a sensitive assay for HIV RNA in plasma, and this subject was positive for proviral DNA in PBMC [67]. Another study of four highly selected elite controllers, with weak indeterminate HIV WBs similar to C135, has also found very low quantitative levels of viral burden, but with at least one positive assay of HIV DNA from PBMC, or from biopsy samples of colon or ileum, as well as one instance of viral outgrowth in high input coculture assay [68]. In these latter four cases, proliferative CD8 responses to HIV-infected autologous CD4 T cells and granzyme B-associated cytotoxicity were a feature, but CD4 proliferative responses were not reported. Only two of these cases were HLA-B57 and one had some evidence of a *nef* deletion but still had replication-competent viral outgrowth [68]. Furthermore, another study of elite controllers reported significantly elevated activation of circulating CD8 T cells [69]. Altogether, subject C135 differs from other elite controllers in the literature.

The combination of the antibody results and the T-lymphocyte HIV Gag peptide-specific proliferation and cytokine responses, which are consistent with this subject's HLA typing, and together with

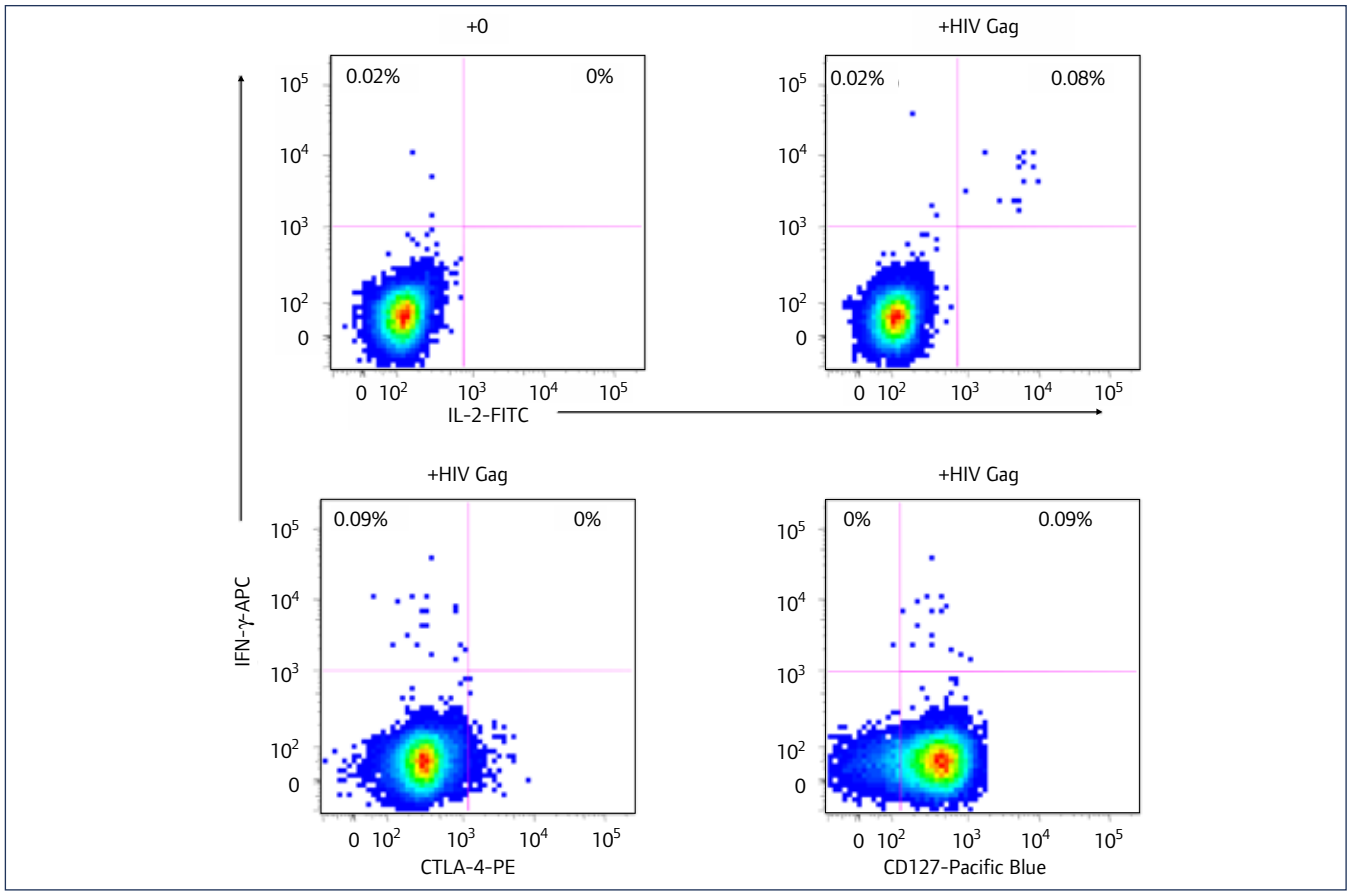
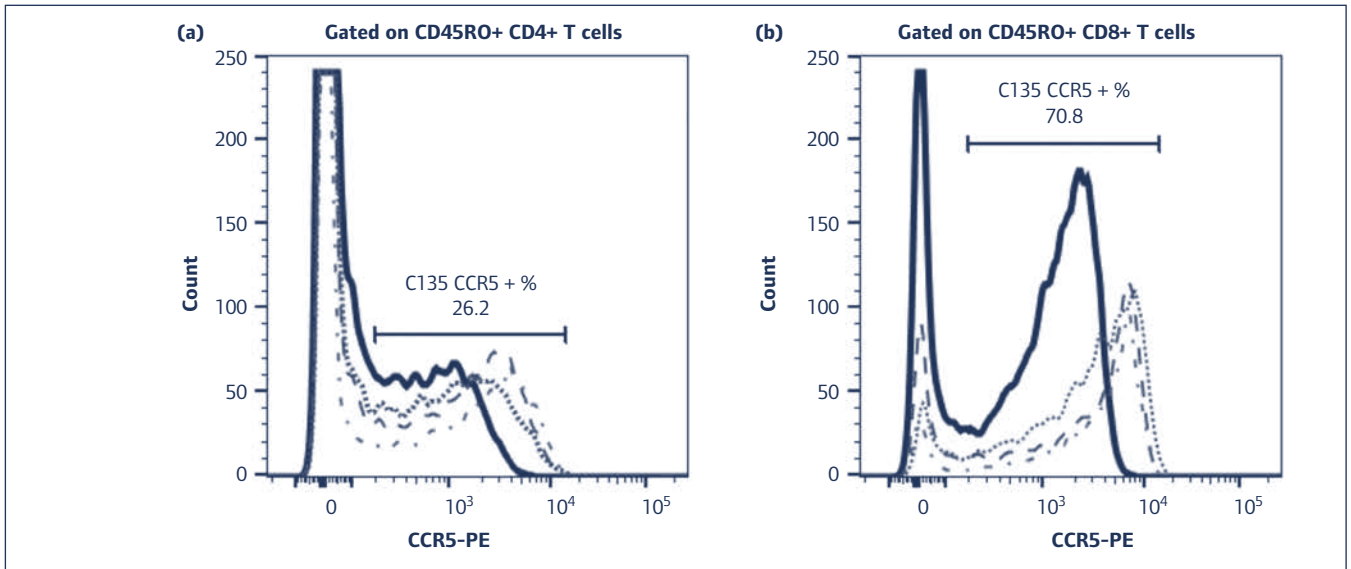


Figure 3. Intracellular cytokine assays. Antigen-specific CD4+ T cells by intracellular cytokine assay for subject C135. Intracellular flow cytometric analysis of the CD4+ T cell cytokine response in fresh whole blood from C135, cultured with a pool of overlapping HIV-1 Gag peptides, showing the production of IFN-γ and IL-2, the lack of production of the inhibitory receptor CTLA-4, in combination with the expression of the IL-7Rα chain, CD127, a marker of long-term memory T cells. Background production of IFN-γ and IL-2 in the absence of HIV-1 Gag peptides is shown in the upper left histogram. IFN: interferon; IL: interleukin



Subject	CCR5+ % of CD45RO+ CD4	CD4 CCR5+ geometric mean		CCR5+ % of CD45RO+ CD8	CD8 CCR5+ geometric mean
C135	26.2	724		70.8	1366
C009	49.1	1490		86.7	3337
C005	39.8	1897		77.0	3048
C001	56.7	1188		88.3	2856

Figure 4. Relatively low level of CCR5 expression on CD4 and CD8 T lymphocytes from subject C135. (a) Flow cytometric histogram of CCR5 expression on CD4+ T lymphocytes from C135 (solid black line) and three healthy adult controls (dashed histograms), respectively. (b) Flow cytometric histogram of CCR5 expression on CD8+ T lymphocytes from C135 (solid black line) and three healthy adult controls (dashed histograms), respectively

the early PCR results, convincingly argues that C135 has been weakly infected with a poorly replicating HIV-1 strain. C135 has no other risk factors, and it is believed that he has not been exposed to HIV-1 except in the case of the transfusion in 1981 with an HIV-infected unit from the SBBC donor D36 [31]. Furthermore, consistent longitudinal results argue strongly against any mistaken identity or laboratory error in a single sample. Additionally, the sequencing data from the early PCR positive samples from C135 yielded a sequence distinct from, but still closely related to, sequences obtained from other SBBC cohort samples, further arguing against simple contamination or mistaken samples.

We and others have found that primary HIV-1 infection in subjects who undergo treatment initiation very early can demonstrate a weakening of their HIV-specific antibody levels [70–73]. More recently, it has been reported that HIV-positive patients who underwent allogeneic stem cell transplantation and subsequently exhibited undetectable HIV DNA showed declining titres of HIV-1 antibodies [17,74]. Similarly, the Berlin Patient has exhibited a decline in HIV-specific titres [6], and while it was interpreted that such antibody titre declines may have been due to lack of antigen, in these allogeneic stem cell transplant cases, this was possibly confounded by the conditioning regimens. Altogether, in these cases, and in elite controllers [67,68], low antibody levels correlated with unusually low levels of viral burden.

The limited development of the anti-HIV-1 antibody response in C135 and the very low HIV DNA proviral load suggest that HIV-1 replication was restricted very early after infection, consistent with previous observations of low viral load in HLA-B57 individuals during acute infection [64]. Also, the limited antibody response to HIV envelope proteins suggests that neutralising antibodies played a minor role, if any, in limiting infection. In fact, plasma samples from C135 showed little or no activity when tested for neutralisation of a range of clade B isolates [59].

C135 has a CD4 cell count and a CD4 : CD8 ratio in the normal range, and within those CD4+ T cells, along with other SBBC members, has a high proportion of circulating memory phenotype cells [57]. We tested purified memory CD4 T cells from C135's PBMC and obtained a negative PCR result. We also tested CD4+ T cells from lymphoid tissue and gut biopsy samples from C135, which were negative. Using the same methods, for samples from other subjects living with HIV, we have previously readily measured HIV DNA in memory CD4 T cells from PBMCs [36,37,39,41], gut biopsy samples [44,45] and lymph node fine needle biopsy samples [51].

Another reservoir that may harbour infected cells over such a long period could be central nervous system (CNS), but sampling of this compartment is problematic, and we have evidence that even mild symptomatic CNS involvement is reflected in HIV DNA levels in circulating CD4 T cells [40]. The transfusion donor, D36, developed HIV-associated dementia after 18 years of being infected with the same strain of *nef*/LTR-deleted HIV-1 [34]. However, donor D36 had a detectable plasma viral load and declining CD4 cell count [31] and elevated CD8 T cell activation, in contrast with C135 [57]. The undetectable viraemia, steady CD4 cell count and lack of activation of CD8 T cells, over more than three decades, argues against a smouldering infection in a non-lymphoid reservoir in C135.

C135 has a CCR5 Δ32 heterozygous genotype and reduced cell surface expression of CCR5, which is known to be associated with LTNP status [13,15]. Furthermore, the *nef*/LTR-deleted strain of HIV-1 with which he was transfused has clearly been shown to be attenuated *in vivo* [8]. Nef is believed to reduce cell surface expression of HLA class I molecules, and its lack may have helped the Gag-specific CD8 CTL in C135 to rapidly identify infected cells and limit viral burden early in infection [64,75]. One report has described that, amongst rhesus macaques immunised with *nef*-deleted SIV, those macaques with major histocompatibility complex class I alleles previously known to be associated with control of SIV infection exhibited better subsequent control of acute infection with heterologous strains of SIV [76], suggesting synergy between *nef* deletion and CD8 responses, analogous to subject C135.

The present studies of PBMC proliferation demonstrate that CD4 T cells from C135 may provide help for potent immunological HLA-B57-restricted CD8 T cell control of HIV-1 infection. C135 had responses to two of the best-studied HLA-B57 CD8+ CTL epitopes in p24. The proliferative CD4 response to the sequence WMTNNPPIPVGEIYK adjacent to helix 7 has previously been reported to be associated with viral control in HLA-*DRB1*1301* individuals [26]. While much effort has been made to delineate protective CD8 T cell responses, proliferative Gag-specific CD4 T cell responses are even more highly associated with LTNP status [22,61]. Previously we found that PBMCs from C135 have shown weak IFN-γ responses and absent CTL responses immediately *ex vivo* [23,33,65]. In contrast with the IFN-γ and CTL results, we had previously found that PBMCs from C135 exhibited strong *in vitro* CD8 antiviral suppressive activity [23], which has also been reported for other HIV-positive LTNP and elite controllers [23,77–79]. Furthermore, PBMCs from C135 protected reconstituted severe combined immunodeficiency mice from superinfection with HIV-1, in an adoptive transfer model [80].

C135's CD4 response augmented his CD8 response to HLA-B57-restricted HIV-1 Gag peptides. The first CD8 epitope, TSTLQEQIGW, has been described to mutate relatively readily, but with a fitness cost [81]. The other small C135 CD8 response is to the epitope, KAFSPEVIPMF, which is located in the highly conserved helix 2 of the N-terminal domain of p24, and mutation of a proline in this helix rendered HIV-1 non-infectious [82]. We, and others, have described LTNP subjects with very large populations of proliferative CD4+ CTL that also targeted a nearly identical epitope in helix 2 [24,83]. These results are similar to a previous study where IL-2 produced by CD4 T cells stimulated with an HIV-1 Nef peptide dramatically enhanced a CD8 response to a different Nef peptide [84], but the CD4 enhancing activity was lost after resolution of acute infection. For subject C135, the CD4 proliferative activity has been maintained for over 30 years, as also reported for memory CD4 T cell responses to vaccinia virus [85].

While it would be desirable to design vaccines to stimulate appropriate targeting of conserved residues in helices 2, 6 and 7 of HIV p24 [86], it is difficult to conceive how non-HLA-B57, -B27 or -DR13 subjects could be vaccinated against exactly the same epitopes. Yet helices 2, 6 and 7 and the cyclophilin binding loop may represent a potential therapeutic target for a small molecule [87]. However, a therapeutic approach to block Nef function is completely unclear at present, even though *nef* deletion appears to be the best described attenuation for either HIV-1 or SIV *in vivo*, and inhibition of Nef may be critical to T cell control of the HIV-1 reservoir [88].

We interpret the results for C135 as a probable case of clearance of HIV-1 infection, resulting from a combination of plausible immune, host genetic and viral attenuation mechanisms. However, with only a single individual subject, caution is still required yet provides some hope that a similar combination of favourable pressures can be more generally applied to HIV-1 infection with a similar outcome.

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Authors' contributions

JZ, WBD, MC, CMLM, PHC, KS, BW, SP, PRG, NS, NKS and AK designed experiments, and JZ, WBD, MC, CMLM, PC, KS, BW, KMcB, WH-N, BH, MB and YX performed experiments. MD recruited and performed endoscopy. SR provided clinical care for subject C135 and identified the clinical possibility of viral clearance. AK, KK, DAC, JSS and JL supervised the recruitment and management of LTNP cohorts. JZ, WBD, MC, CMLM, PHC, KK, SP and AK analysed the data. JZ, WBD, MC, CMLM and AK vouch for the data and analysis and wrote the paper.

Conflict of interest

The authors do not disclose any financial conflicts of interest.

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