Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression

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An HIV-1-seropositive volunteer was infused with an expanded autologous cytotoxic T lymphocyte (CTL) clone directed against the HIV-1 nef protein. This clone was adoptively transferred to determine whether supplementing CTL activity could reduce viral load or improve clinical course. Unexpectedly, infusion was followed by a decline in circulating $CD4^+$ T cells and a rise in viral load. Some of the HIV isolates obtained from the plasma or $CD4^+$ cells of the patient were lacking the nef epitope. These results suggest that active CTL selection of viral variants could contribute to the pathogenesis of AIDS and that clinical progression can occur despite high levels of circulating HIV-1-specific CTLs.

The identification of the components of the immune system that prevent the initiation and propagation of HIV-1 infection and the development of AIDS will foster the development of prophylactic and therapeutic strategies for populations at risk. In general, induction of major histocompatibility complex-(MHC)-restricted cytotoxic T lymphocyte (CTL) responses is thought to confer protection both against dissemination of infection and development of disease in different human disorders and animal models of viral diseases[1-11]. In acute infection with HIV and simian immunodeficiency virus (SIV), reduction in viral load is associated with the generation of antigen-specific CTLs[12,13]. On the other hand, persistence of CTLs in at least one viral model system has been associated with immunopathological effects[14] and may contribute to abnormalities observed in other clinical disorders[15].

A high frequency of HIV-1-specific CTLs has been found in HIV-1-seropositive individuals and appears to wane with disease progression[16-27]. It is unclear if the loss of such responses is causally related to the development of AIDS or merely reflects the pervasive depression of immune responsiveness associated with disease progression.

In this study, the question posed was whether supplementing HIV-1-specific CTLs in an HIV-1 seropositive patient (1) would be safe and have salutary effects by suppressing viral load and would promote a more favourable clinical course; (2) would have no objective clinical, immunological or virological effects; or (3) would yield unfavourable consequences.

Characterization of an HIV nef-specific clone

A CTL clone with specificity against a conserved region of nef was selected for study because this viral protein is expressed early and in high concentration in infected cells. The nef CTL clone 4N225 was derived by limiting dilution from an HIV-1-seropositive individual and was found to recognize a 10-residue peptide (^{73}QVPLRPMTYK^{82}) optimally in association with HLA-A3.1 (Fig. 1*a*). The clone was able to lyse target cells sensitized with truncations of this peptide at higher concentrations. To identify the critical residues that could affect recognition by this CTL clone, a series of peptides with alanine substitutions of residues with side chains within the epitope were used to pulse target cells. Changes at position 77 (R→A) or position 80 (T→A) resulted in a 10^4–10^5 increase in the concentration of peptide required to sensitize targets for lysis; changes at position 76 (L→A) and 79 (M→A) resulted in a 10- to 100-fold increase in the concentrations of peptide required, whereas substitutions at positions 73–75, 78, 81, 82, had no effect on lytic responses. CTL recognition was also impaired by an R→G change at position 77, whereas a T→S change at position 80 restored the ability of this peptide to sensitize target cells for lysis indicating that the polarity of the residue could be a determinant for recognition. Single amino acid changes at most positions were tolerated, although these results suggest that some substitutions of key residues outside of the anchoring positions could result in complete loss of recognition by HLA-A3.1-restricted nef-specific CTLs.

To determine whether the nef-specific CTLs could recognize the native nef product during acute HIV-1 infection, HLA-A3 matched and mismatched target cells were infected with HIV-1 and used as target cells for the CTL assay. Only the HLA-A3 matched, HIV-1-infected target cells were lysed (Fig. 1*b*). The clone 4N225 was examined next for its ability to inhibit replica-

tion of HIV-1 isolates from the donor patient (Fig. 1*c*). Freshly sorted patient CD8⁺ cells, or the 4N225 cloned cells, but not autologous cells from a control CD8⁺ clone without HIV-1 specific cytolytic activity, were able to inhibit viral replication within the patient's CD4⁺ cells in a dose-dependent manner. Since the 4N225 clone could recognize a conserved CTL epitope, lyse HIV-1-infected target cells, and inhibit replication of the patient's autologous virus, it was deemed a suitable candidate for use in the adoptive transfer studies.

Adoptive transfer of the autologous nef-specific clone

Graded numbers of CTLs were infused during two time periods. A limited course of interleukin-2 (IL-2) (seven doses during three days) was administered only after the first series of infused CTLs. Circulating CD8⁺ counts were transiently increased following all infusions (Fig. 2*a*). No sustained increase of nef-specific CTL activity was found in the circulating population (Fig. 2*b*). This may be due to the fact that the patient had a high baseline nef-specific CTL response and that adoptively transferred activated T cell lines (for example, tumour-infiltrating lymphocytes) tend to localize within lymphoid tissues, liver, and lungs[28]. The transient increases in CD8⁺ counts could have been caused by displacement of the endogenous CD8⁺ populations by the infused CTL clone from the lymphoid tissues. CD8⁺ cell counts returned to baseline values after about 30 days. In contrast, the transient increase of CD4⁺ cells, especially after the first CTL infusion, was followed by marked declines in CD4⁺ counts (Fig. 2*c*). Although one cannot be certain that this decline was causally related to CTL infusion, accelerated loss of CD4⁺ cells was observed, particularly following the second series of cell transfers. Despite the patient's decline in CD4⁺ counts below 50 cells per cm^3, and clinical deterioration as evidenced by weight loss and development of cryptosporidiosis, marked CTL activity of fresh peripheral blood mononuclear cells (PBMC) against nef and other HIV-proteins was preserved (Fig. 2*d*).

Changes in HIV viral load after CTL infusion

We tried to determine whether CD4⁺ cell loss was associated with a rise in proviral load by quantitative polymerase chain reaction (PCR). Total proviral DNA (integrated and unintegrated) appeared stable during the periods at the time of infusion of cells and IL-2, and immediately after IL-2 administration; however, a marked (fourfold) rise in proviral DNA was observed within eight months of the first infusion, and further increases were found after the second infusion (Fig. 3*a*). These increases in proviral load paralleled the declines in CD4⁺ cell counts. Similarly, p24 antigen values were stable for 20 months before the first CTL infusion, and increased after the initiation of CTL therapy (Fig. 3*b*).

Selection of HIV variants

Specimens were obtained to determine whether a rise in viral load was accompanied by the selection of viral variants, especially within the CTL epitope. Based on the earlier results showing a tolerance for a single-residue substitution at most positions within the nef epitope and a previously published study suggesting that changes could be observed within CTL epitopes over time[29], we anticipated that sequence variants might be present within the epitope of this patient, given his documented persistent CTL activity to this epitope over many years. Examination of viral sequences of several specimens obtained before CTL infusion showed the presence of two major forms of the nef CTL sequence in the patient's circulation (Table 1*a*,*b*). One sequence was prototypic of the published sequences[30] ([73]QVPLRPMTYK[82]), whereas the major variant contained a V→I change at position 74. No deletions were observed within the nef epitope in the 379 viral clones examined from the pretreatment plasma samples (Table 1*a*, line 1). On the basis of the previous alanine substitution studies, the V→I change was not expected to affect CTL recognition. Indeed, using a peptide with a V→I substitution at position 74, no differences were seen in sensitization of targets for lysis (data not shown). In contrast, sequences of viral clones obtained both from the plasma (Table 1*a*, lines 2 and 3, and Table 1*b*, nef D) and cultured CD4⁺ cells (Table 1*a*, line 4, and Table 1*b*, Bl and nbl samples) from the patient at times after CTL infusion showed the presence of a variety of viral sequences

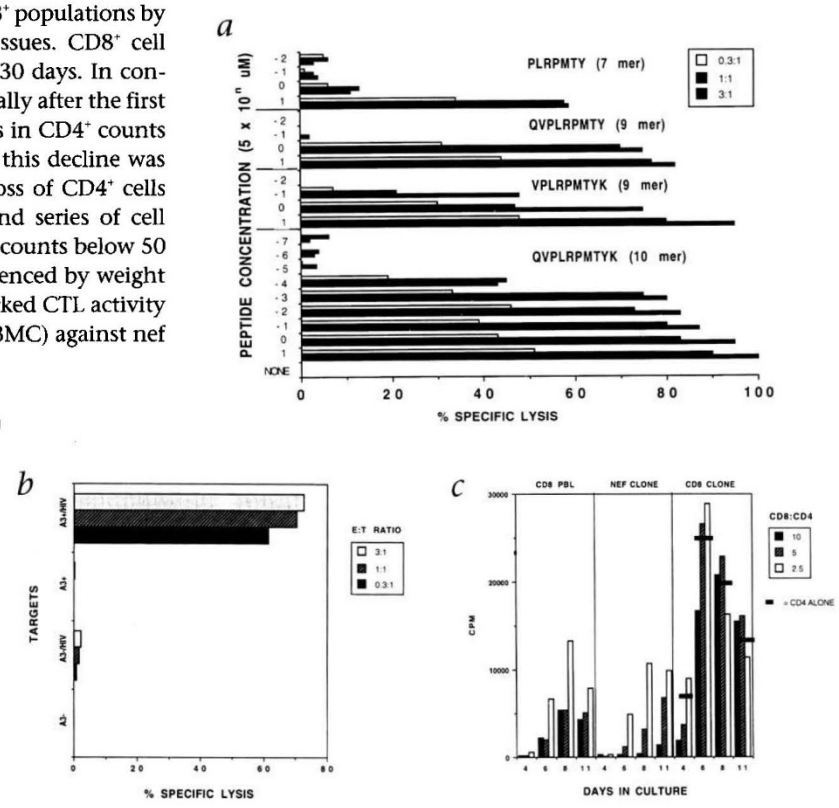

Fig. 1 Specificity and anti-HIV properties of nef-specific clone. *a*, Mapping of the fine specificity of nef-specific clone. Per cent specific lysis was determined against target cells pulsed with indicated concentrations of peptide in a 4-hour ^{51}Cr release assay. *b*, Nef-specific clone lysis of HIV-infected targeted cells. HLA-A3 matched and mismatched LCLs infected with HIV_{LAI} were used as target cells as described in the Methods section. *c*, Inhibition of HIV replication of autologous virus nef-specific clone. Freshly sorted autologous CD8⁺ cells, the nef specific clone, and a CD8⁺ autologous clone without lytic activity against HIV, were added in the indicated ratios to sorted and activated CD4⁺ cells as described in the Methods section. RT activity was determined in culture supernatants on indicated days. Results of RT values of CD4⁺ cells cultured in the absence of CD8⁺ cells is shown in the bars of the last panel.

with deletions in the nef CTL epitope (Table 1*b*). These changes were both in-frame deletions and some nef defective forms (frameshift with stop codons). In the last samples analysed, derived from cultures of $CD4^+$ cells from the patient six months after the last CTL infusion, up to 20% of the isolates had nef CTL epitope deletions. Of these isolates, about 35% contained in-frame deletions. Approximately 78% had large in-frame deletions of greater than 40 residues or frameshifts with stop codons that might preclude functional protein expression. Many of the deletions started at amino acid 74 near the amino terminus of the epitope, suggesting that this site may be prone to errors in reverse transcription and that their selection is facilitated by the population of nef-specific CTLs. In total, we estimate that 22% of the sequences with deletions would encode a functional nef gene product.

Discussion

In this study, clinical progression and CD4 decline occurred despite the presence of CTLs to nef and other HIV proteins. By supplementing the extremely large number of CTLs to a particular epitope, we demonstrate that viral variants with deletions in the CTL epitope can be selected in humans. However, it is unlikely that CTL selection of the variant isolates with deletions alone was responsible for the progressive nature of this patient's disease. The majority of isolates still contained the wild type nef sequence and cytolytic activity to other proteins remained intact throughout the course of this patient's $CD4^+$ decline. We have no evidence that viruses with these particular nef deletions are more or less cytopathic *in vitro* or *in vivo*, although biological changes have been demonstrated with changes in expression or segments of nef and envelope proteins. In the former[31], deletions resulted in a less pathogenic strain of virus. Other possibilities that may account for the increased viraemia and the patient's clinical progression following the adoptive transfer of CTLs include (1) induction and secretion of virus-enhancing cytokines (for example, tumour necrosis factor and γ-interferon), which are produced by this CTL clone when stimulated with specific antigen; (2) release of free virions from infected cells lysed by CTLs and dissemination to other tissue compartments; and (3) inappropriate dosing of CTLs that favoured untoward effects.

Adoptive transfer of human cytomegalovirus (HCMV)-specific CTL clones has yielded encouraging results in preventing HCMV disease in individuals undergoing bone marrow transplantation[32]. Under those circumstances, increasing the frequency of CTLs appears to limit the re-emergence of localized reactivation of HCMV. Because the viral load should be much less in HCMV patients, as compared with individuals chronically infected with HIV-1, the chances for selection of HCMV variants would be reduced.

The fact that CTLs can select for viral escape mutant was first demonstrated *in vivo* with lymphocytic choriomeningitis virus (LCMV) in mice[14]. Similar selective processes were believed to have occurred in HIV-infected persons where amino acid substitutions were observed within a gag-specific CTL epitope over time, and also resulted in loss of lytic activity against the altered sequence[29]. Others have shown that target cells pulsed with some synthetic peptides, containing residue changes that mimic natural variant isolates, are not recognized by CTL directed against the same prototypic CTL epitopes[33,34]. In a recent report, a high mutation rate within anchoring residues of CTL epitopes in nef was believed to account for the failure of HIV-1-seropositive individuals bearing the appropriate haplotypes to generate CTLs to these sites[35]. These results, however, differ from a report that sequence changes were not observed over time within a dominant CTL gag epitope in SIV-infected monkeys who developed an AIDS-like disorder[36]. These disparate observa-

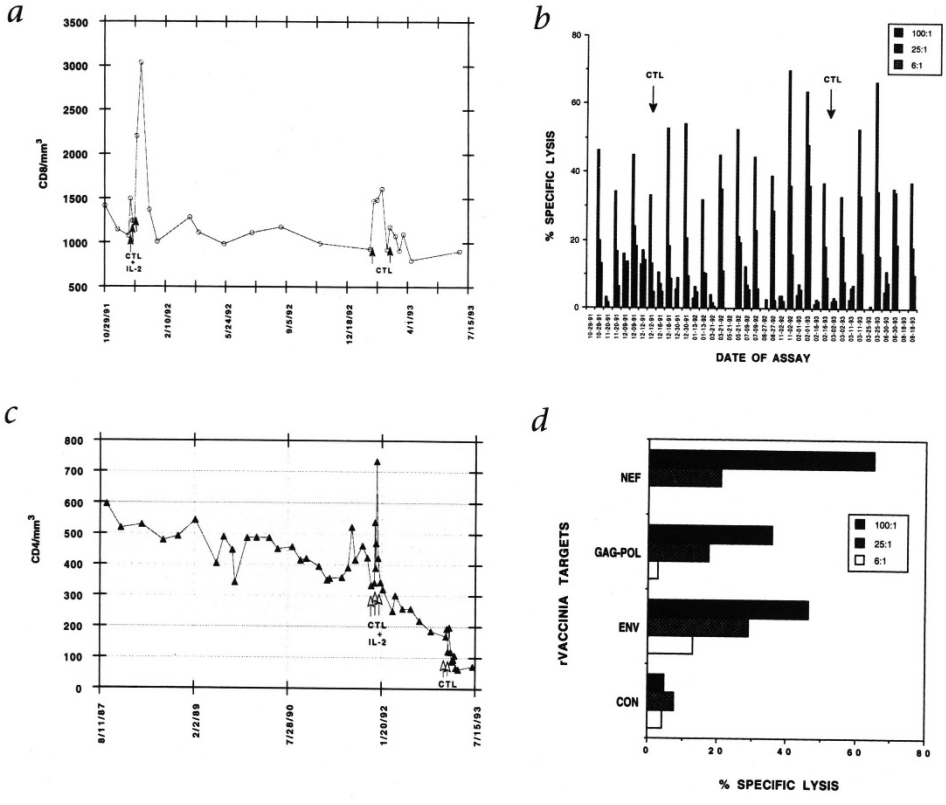

Fig. 2 Peripheral cell lymphocyte phenotypes and CTL activity before and after transfer of nef-specific CTL clone. *a*, Peripheral $CD8^+$ cell count over time *b*, HLA-A3-restricted nef-specific CTL response over time. PBMC were separated on the days indicated and added to the HMY-A3 transfected, ^{51}Cr-labelled target cells. The paired results for each assay date indicates per cent specific lysis of unpulsed target cells and per cent specific lysis of target cells pulsed with peptide QVPLRPMTYK, respectively. *c*, Peripheral CD4+ cell count over time. *d*, HIV-1-specific CTL responses of PBMC from patient at completion of trial (8/93). An autologous LCL was infected with recombinant vaccinia viruses expressing nef, envelope (IIIB), or gag-pol proteins and used as targets in CTL assay as described[18,21]. Clones derived from PBMC cultures were found to be MHC-restricted.

tions might reflect subtle differences in mutational rates and in selection pressures directed at particular sequences of the CTL epitopes within the respective viruses. Alternatively, new structural or functional advantages (or disadvantages) for the mutated gag proteins could have been conferred to the emerging mutant virions.

In the case of the patient under study, his baseline pool of viruses before CTL infusion appeared to contain two major forms of the A3-restricted epitope: either V or I at position 74. The patient's PBMC and the infused CTL clone had identical lytic responses (both in sensitivity and magnitude) against target cells pulsed with peptides composed of either version of the CTL epitope. Although this patient had documented CTL activity for at least five years against this epitope, it could be argued that this variant virus (V to I) emerged either independently or as a consequence of CTL immune selective pressure. From the results obtained utilizing target cells pulsed with peptides containing alanine substitutions within the A3-restricted nef epitope, it appears that only changes at the single residue 77 (R) would result uniformly in a viral variant with a substantial selective advantage over the patient's prevailing strains, because of the virtual absence of CTL reactivity against those peptide-sensitized target cells. However, the observation that infusion of the nef-specific CTL selected different variants that deleted large segments of the CTL epitope, clearly demonstrates that active immune selection is occurring *in vivo*. Given the rapid turnover of the viral population *in vivo*, selection might occur quickly after infusion of activated CTL. Selection may not be occurring uniformly throughout the circulation and lymphoid system. More likely, the CTLs distribute to separate, heterogeneous compartments (for example, within individual lymph nodes) where partial viral containment and selection of different variants may occur independently at the various sites. It is unclear how long the adoptively transferred CTL persist in these tissues. If these variants continue to accumulate over time, it would support the notion that CTLs directed to other epitopes within other HIV-1 proteins (Fig. 2*d*) either are inefficient in containing new variants as they are established (presuming that those other epitopes were unchanged) or are inaccessible to the tissue compartments at a time when selection is occurring.

It is unclear whether selection of variants will pose a problem in devising therapies utilizing CTL to contain HIV-1 infection. The results do raise concern for strategies that are directed to single viral sequences and the selective pressures that could be applied by the cellular immune system. On the other hand, such an approach might be useful for directed selection of viral variants with changes in regulatory or structural proteins that are known to yield less pathogenic viruses. This would be of utility only in therapeutic circumstances where replication of the

Table 1 Selection of variant viral sequences by nef-CTL clone

***a* Frequency of variant viral sequences over time**

	Wild type	*nef* clone type Epitope ^{74}V→I	Epitope deletion
Pre-treatment (plasma) 31 May 90 & 5 Jun. 91	200/379	179/379	0/379
Post-infusion 1 (plasma) 27 Oct. 92	8/10	n.d.	2/10
During infusion 2 (plasma) 8 Feb. 93	50/68	17/68	1/68
Post-infusion 2 (supernatant of CD4$^+$ cells cultured with PBMC) 14 Sept. 93	394/497	n.d.	103/497

n.d. wild type specimens not sequenced to determine the number of ^{74}V→I variants.

***b* nef deletions observed after CTL infusion**

		73 ⟷ 82					
pre-treatment							
wild type	QEDEEVGFPV	RPQVPLRPMT	YKGAIDLSHF	LKEKGGLEGL	VYSQKRQDIL	DLWVYHTQGY	FPDWQNYTPM
74 V→I (in epitope)	----------	---I------	----------	----------	----------	----------	----------
63 D→E	--E-------	----------	----------	----------	----------	----------	----------
90 F→L	----------	----------	---------L	----------	----------	----------	----------
post-treatment							
nef D (in frame)	----------	----		----------	----------	---	
B1-28-1214 (in frame)	----------	----			----	----------	----------
B1-30-1214 (in frame)	----------					----------	----------
B1-31-1214 (in frame)	----------					------	----------
B1-26-1214	---				KISL	ICGSTTHKAT	SLIGRTTHQ*
B1-25-1214	----------	----				SVGLPHTRL	LP*
B1-27-1214	----------	-----				VGLPHTRL	LP*
nbl-14-1214 (in frame)	---D				----------	----------	----------
nbl-15-1214 (in frame)	--A						----------
nbl-18-1214	----------	----		*			
nbl-20-1214	----------	----		KGGTGRA	SLLPEKTRYP	*	
nbl-17-1214	------				TPRKDKISL	ICGSTTHKAT	SLIGRTTHQ*
nbl-21-1214	-------*						
nbl-19-1214	--------					TTHKAT	SLIGRTTHQ*
nbl-16-1214	----------	--				TTHKAT	SLIGRTTHQ*

Pretreatment sequences were derived from RNA samples from plasma on 31 May 90 and 5 Jun. 91; post-treatment nef D sequence was derived from two different RNA samples from plasma on 27 Oct. 92 and 8 Feb. 93; all remaining sequences (B1 and nbl series were obtained from supernatants of CD4$^+$ cells cocultured with PBMC from samples of 14 Sept. 93. *indicates the presence of a stop codon.

parental virus could be contained or eliminated by other immunological, cellular, or chemotherapeutic interventions.

The observation that this patient continued to maintain potent CTL activity against a number of HIV-1 proteins casts doubt on an exclusive role of CTL in preventing clinical progression to AIDS. Furthermore, it raises concern that high quantities of CTLs, especially directed to a single epitope in the setting of a large viral load, may contribute to the immunopathogenesis of AIDS, similar to that seen in chronic LCMV infection in mice[14]. However, it is possible that in the absence of this HIV-1-specific CTL activity, this patient's clinical deterioration might have been more accelerated. Further insights into the effects of adoptive cellular therapy with HIV-1-specific CTL to different epitopes and protein specificities will be garnered from prospective clinical trials. Populations of polyclonally activated and expanded $CD8^+$ populations containing HIV-1-specific CTL have been achieved[37], and it remains to be seen whether such therapies will be advantageous.

Methods

Nef-specific clone. $CD8^+$ peripheral blood mononuclear cells from a patient with HIV-1-specific CTLs to nef[21] were cloned by limiting dilution in RPMI-1640 (M.A. Bioproducts) and 10% FCS in the presence of 50,000 irradiated heterologous feeder cells, PHA (1 μg ml^{-1}, Burroughs-Wellcome) and IL-2 (10% volume, Pharmacia, Inc., Silver Spring, Maryland, and 10 U ml^{-1}, Cetus, Emeryville, California), as described[18]. Clones were screened for CTL activity against lymphoblastoid cell line (LCL) infected with recombinant vaccinia virus-expressing nef, and positive cultures were expanded in the presence of IL-2. Clone 4N225 was derived from a culture cloned at 1 cell per well. The T cell receptor of this clone was determined to be: Vα 25.1, Jα45, Vβ12.6a, Jβ 1.3, Cβ1 (S.K., L.S. Johnson, U. Utz, unpublished work). Heterologous irradiated feeder cells from HIV-1 seronegative donors and 1:50,000 dilution of OKT3 ascites fluid (kindly provided by R. Mittler, Bristol-Myers, Wallingford, Connecticut) were added to cultures once every two to three months for clonal expansion.

CTL assays and target cells. The HMY2.CIR lymphoblastoid cell line transfected with the gene coding for HLA-A3.1 and labelled with ^{51}Cr (Amersham) was pulsed with synthetic peptides from nef and used as target cells. CTL clones were added at different E:T ratios, incubated for 4.5 hours at 37 °C in a 5% CO_2 incubator, and supernatant fluids were harvested using a Skatron system to determine percentage of specific lysis as described[18].

LCL cells derived from HLA-typed $HLA\text{-}A3^+$ and $HLA\text{-}A3^-$ volunteers were screened for CD4 expression and for their ability to be infected with $HIV\text{-}1_{LAI}$ as determined by reverse transcriptase production in cell-free culture supernatants. LCL (1×10^6 cells) from one HLA-A3-matched (A3, Aw34, B14, B51, Cw3, Cw8) and one HLA-A3-mismatched donor (A2, A29, B14, Bw57, Cw7, Cw8) were infected at a multiplicity of infection of 0.1 ($HIV\text{-}1_{LAI}$) and HIV-1-infected cells were harvested for use in the CTL assay before significant cytolysis was noted in both sets of cultures (typically 2–3 days post infection).

HIV replication assays. For assay of HIV production, freshly separated PBMC from the patient were sorted into $CD4^+$ and $CD8^-$ fractions by FACS. Reanalysis of sorted cells showed 98–99% purity of the respective populations. $CD4^+$ cells were placed in 24-well Costar plates at 10^6 cells ml^{-1} in the presence of 100 U μl^{-1} IL-2 and 1:1,000 dilution of anti-CD2 ($T11_{2+3}$, kindly provided by E. Reinherz) for 18–24 hours. Cells were harvested, washed, and replated at 50,000 cells per well in 100 ml of RPMI + 10% FCS + 100 U ml^{-1} IL-2 in a 96-well Costar plate. Sorted $CD8^+$ autologous cells (kept at 4 °C overnight in RPMI-1640 + 10% FCS) or cloned T cells were added to the $CD4^+$ cells at indicated ratios. Control cultures included $CD8^+$ or $CD4^+$ cells cultured alone. Culture supernatant (100 μl) was harvested every 2–3 days, and samples were kept frozen at –70 °C for assay for reverse transcriptase and/or p24 antigen production.

RT was assayed by the incorporation of $[^{32}P]$-TTP on a poly(A) template as described[38]. 24 HIV-1 antigen was assayed using a commercial kit (Du Pont) following the manufacturer's directions.

Lymphocyte cell surface markers. PBMC were stained with monoclonal antibodies (OKT-3, OKT-4, OKT-8; Ortho Diagnostics, Raritan, New Jersey) and enumerated by single-colour flow cytometry (Epics Profile I, Coulter, Hialeah, Florida). Absolute lymphocyte count was calculated by a Coulter Counter determination of the number of white blood cells (WBCs) and simultaneous differential staining of the specimens. The number of cells of a particular phenotype was derived by

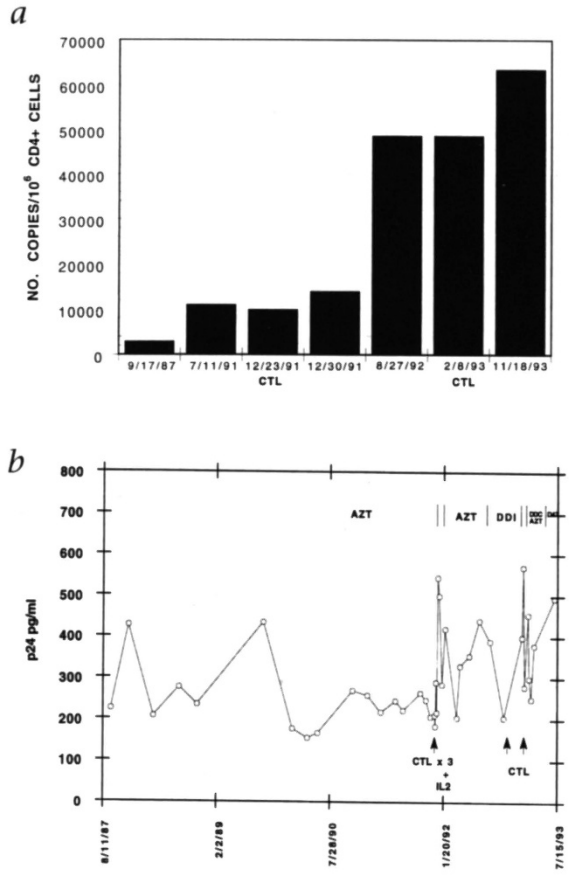

Fig. 3 HIV-1 viral load and p24 serum values over time. *a*, PCR amplification of HIV-1 DNA in sorted $CD4^+$ T cell populations isolated from sequential peripheral blood cell samples before and after CTL transfer. The percentage of $CD4^+$ cells containing HIV-1 DNA was 0.3% in 9/87, 1% in 12/91 (time of the first CTL transfer), increased to 5% in 8/92, remained unchanged (5%) in 2/93 (time of second CTL transfer), and increased to 6.5% in 11/93 after second CTL transfer. All the samples were analysed in the same PCR reaction. PCR amplification and semiquantitative analysis were performed as described[39]. Results are expressed as number of copies per 10^6 cells. *b*, p24 values in serum over time. Serum samples were taken at times indicated and stored frozen at –70 °C in a repository. p24 values were determined using a Coulter kit according to the manufacturer's directions.

multiplying the absolute lymphocyte count by the percentage of cells stained with the appropriate monoclonal antibody.

Expansion of CTL clone for adoptive transfer studies. CTLs cultured in upright T175 cm^2 flasks (Nunc) were expanded until a total of approximately 3×10^9 cells was achieved. Cells were cryopreserved with FCS at 2×10^7 cells ml^{-1} in Nunc vials. With initiation of the clinical study, cells were thawed rapidly in a 37 °C water bath and then diluted 10-fold with cold AIM (Gibco) supplemented with 5% heat inactivated FCS (Sigma), 2 mM glutamine (Whittaker), and Fungizone (Gibco) slowly over about 5 minutes in 50-ml conical tubes (Falcon). Cells were centrifuged at 400*g* for 10 minutes, and resuspended in media supplemented with 7,200 IU ml^{-1} IL-2 (Cetus). For the first series of infusions (December 1991), cells first were placed into T175 cm^2 flasks and supplemented with allogeneic feeders at a ratio of 2:1 allogeneic feeders. For the second series of infusions (March 1993), cells were placed into polyolefin tissue culture bags (Fenwal) supplemented with allogeneic feeder cells at ratios of >2:1 CTL and stimulated with OKT-3 (10 ng ml^{-1}, Ortho). Cultures were monitored two or three times per week using an automated WBC counter (Coulter S Plus 5 Cell Counter, Coulter Electronics) and manual WBC counts with measurement of trypan blue (Gibco) viability. Cells were maintained between $1.0–3.0 \times 10^6$ cells ml^{-1}. Cells were restimulated with allogeneic feeders and OKT-3 every 3–4 weeks. Allogeneic cells were obtained from volunteer donors, who were required to pass all American Association of Blood Banks and Food and Drug Administration requirements for blood donation. Two methods of leukocyte resources were used. First, buffy coats were prepared by centrifugation of whole blood that was collected into quadruple packs with CPDA-1 anticoagulant (4R6402, Fenwal, Baxter Healthcare) at 4,600*g* in a Sorval centrifuge for 5 minutes. The bags were placed into a plasma expressor, and the buffy coat was collected into one of the empty transfer packs. Second, donors underwent lymphocytopheresis on a CS-3000 (Fenwal) blood cell separator according to the following parameters: a whole-blood flow rate set to 50–60 ml min^{-1}, acid citrate dextrose anticoagulant ratio of 1:11, 373*g* (1,600 r.p.m.), interface detector setting 150 and pumpbacks programmed to occur at 3.5-minute intervals. Cells collected in this manner were resuspended and centrifuged in a Sorval RC3C at 20 °C, 277*g* for 10 minutes. Platelet-rich plasma was removed, and the leukocyte concentrate was resuspended in normal saline. Mononuclear cells were isolated by using automated Ficoll-Hypaque density gradient sedimentation using the Fenwal CS-3000 blood cell separator. Briefly, cells were underlayed with Ficoll-Hypaque in the collection chamber and washed in the Grandulo chamber. Feeders were irradiated with 2,500 cGy in a gamma cell irradiator before use in culture. On the day of infusion, the CTLs were harvested and washed with normal saline (McGaw) using the Fenwal CS-3000 blood cell separator. Cells were resuspended in normal saline supplemented with 2.0% human serum albumin (Miles) and filtered (4C2100, blood component recipient set, Fenwal) before infusion. Cells were not infused earlier than two weeks after the addition of irradiated feeder cells. Samples of cells were examined for lack of transformation by testing growth on several occasions in the absence of IL-2 *in vitro* and in SCID mice *in vivo*.

Clinical study. After obtaining informed consent, 28×10^9 cloned cells were infused in the patient in December 1991 in three escalating doses during a two-week period. IL-2 (1.7×10^6 unit; Cetus) every eight hours for seven doses was administrated intravenously after the last of the first series of CTL infusions. The patient received 12×10^9 CTL (January 27, 1993) and 13×10^9 CTL (2 Mar. 1993) for the second course of therapy. No IL-2 was administered with these cell infusions.

Amplification and quantitation of HIV-1 DNA in $CD4^+$ cells. Sorting of $CD4^+$ T-cell populations was performed as previously described[39]. For DNA PCR, sorted $CD4^+$ T cells (grade of purity 97–98%) were lysed and 10-fold serial dilutions were obtained by mixing different amounts of cell lysates corresponding to 2×10^3, 2×10^2, and 2×10^0 $CD4^+$ T cells with Jurkat (leukaemia T-cell line) cell lysates (corresponding to 5×10^4 cells) in a total volume of 50 µl. DNA was amplified using a primer pair specific for the gag (SK145/101) gene segment, and amplified products were hybridized to the specific probe (SK102 for gag) that had been end-labelled using [^{32}P]ATP. Products of hybridization were analysed by electrophoresis in 10% polyacrylamide gels and visualized by autoradiography. As a positive control, 10-fold serial dilutions of cell lysates from 8E5, a chronically infected T-cell clone containing one proviral copy per cell were used[38]; as a negative control lysis buffer (LB) and cell lysates from 5×10^4 Jurkat cells were used. In order to perform semiquantitative analysis of HIV-1 DNA copies, the intensity of the radioactive signal for each sample was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, California) and compared with those obtained in 10-fold serial dilutions of cell lysates from 8E5. A simple regression curve was fitted for the 10-fold dilutions (10^3, 10^2, 10^1, 10^0) of the standard (8E5) cell lysates, and the equation obtained was used to determine the amount of target sequence in the patient sample. Results are expressed as number of copies/10^6 $CD4^+$ cells.

p24 quantitation. Serum samples were taken at times indicated and stored frozen at –70 °C in a repository. p24 values were determined using a Coulter kit according to the manufacturer's directions.

Extraction of RNA from plasma sera and virus stocks and DNA sequencing. In order to determine variability in the region of nef containing the CTL epitope, sequences of the nef gene encompassing amino acid residues approximately 58 to 81 were determined. Each sample for nucleic acid extraction was mixed with an equal volume of 5 M guanidine thiocyanate, 0.5% *N*-laurylsarcosine, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate, pH 7.0. The solution was made 0.2 M with sodium acetate, 100 µg of tRNA was added and extracted with an equal volume of water-saturated phenol containing 10% chloroform. Phases were separated by centrifugation and the nucleic acid in the aqueous phase was precipitated with isopropanol (50% final). The nef region of interest was amplified by using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, Connecticut). The primer used in the reverse transcriptase reaction was the oligomer 5′–CAU CAU CAU CAU TGGTGTGTAGTTCTGCCAATC–3′, where the nucleotide 13 represents nucleotide pair number 383 of the nef gene consensus, and nucleotide 33 represents number 363 (ref. 29). The second primer used in the PCR mix addition was 5′–CUA CUA CUA CUA TTGTGCCTGGCTAGAAGCA–3′, where nucleotide 13 represents nucleotide pair number 160 and 31 represents number 178. The PCR reaction conditions were an initial 2 minutes at 94 °C, followed by 35 cycles of 1.5 minutes at 94 °C, 1.5 minutes at 55 °C, and 1.5 minutes at 72 °C, followed by an 8.5-minute incubation at 72 °C. The PCR products were cloned by reacting with uracil DNA glycosylase while annealing to the pAMP vector plasmid of the Clone AMP System (Life Technologies, Inc., Gaithersburg, Maryland). Competent DH5α were transformed with the annealed vector. Plasmid DNA was purified by using the Magic MiniPrep DNA Purification System (Promega). Inserts were verified by *Bam*HI-*Eco*RI digestion followed by electrophoresis in 1.5% agarose gels. Positive clones were sequenced using the SP6 Promoter Primer (Life Technologies) by a modification of the USB Sequenase Version 2.0 Protocol using 35-Sequetide (NEN-Du Pont) and separated by electrophoresis in 6% sequencing gels at 70 W for approximately two hours. The gels were dried and exposed to X-ray film overnight at –70 °C.

Nucleic acid hybridization analysis of nef clone populations. Transformants containing molecularly cloned nef sequences from the indicated times were streaked directly onto nitrocellulose filters on ampicillin-containing plates, in triplicate. The plates were incubated overnight at 37 °C, and the filters were removed and processed as described[40] except that the filters were air-dried and exposed to ultraviolet light using the Stratalinker (StrataGene Cloning Systems, LaJolla, California). Differential hybridization was performed using an oligomeric probe representing the CTL epitope: 'probe epitope' 5'–CAGGTACCTTTAAGACCAATGACT–3' (nef gene consensus nucleotide 218 to 241) and an oligomeric probe representing a distant portion of the nef gene: 'probe nef' 5'–ACCACACACAAGGCTACT–3' (nef gene consensus nucleotide 344 to 361). Both oligomers were labelled in a reaction using 30 pmol oligomer, 20 μCi [^{32}P]dATP, in the presence of polynucleotide kinase in 70 mM TrisHCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT. The labelled oligomers were purified using Quik-Spin (Boeringer Mannheim) columns.

Hybridization reactions with labelled 'probe nef' were carried out at 50 °C overnight in 6× SSC, 5× Denhardt's, 0.1% SDS, 25 mM sodium phosphate, 1.25 mM sodium pyrophosphate. Filters were washed three times in 6× SSC, 0.1% SDS at 50 °C for 2 minutes per wash. Hybridization and wash conditions for reactions with "probe epitope" were the same except that 60 °C was used. The filters were exposed to film overnight, and colonies were discriminated by their high signal, gray signal, or absence of signal using the probe epitope, while having a strong signal for probe nef.

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