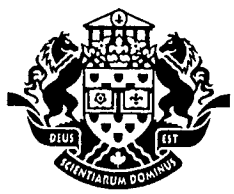

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MODULATION OF CD8 ANTIVIRAL FACTOR BY HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

BY

GREG BEAUDOIN

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Abstract

CD8+ T cells secrete CD8 Antiviral Factor (CAF), an unidentified soluble factor that inhibits replication of HIV-1 by suppression of viral transcription. To date, the effect of Highly Active Antiretroviral Treatment (HAART) on CAF production in HIV+ individuals is inconclusive. Therefore, we have examined CAF production by CD8+ T cells of HIV-positive patients on various treatment regimens, using a transcription-based assay. We found that HAART does not significantly modulate CAF compared to untreated controls, but NNRTI treatment may increase CAF in some patients, as this group demonstrated a higher proportion of effective suppressors of viral gene expression. These trends were not seen at the level of viral replication, which shows that determination of CAF at the level of virus replication does not mirror results found using a transcription-based assay. These results suggest that immunological changes induced by NNRTI-based therapy may affect the ability of CD8+ T cells to produce CAF.

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General Introduction

Human Immunodeficency Virus (HIV) is the causative agent of the Acquired Immune Defiency Syndrome (AIDS). Disease progression typically begins with a symptomatic acute infection followed by a long period of clinical latency, during which symptoms are minimal. Clinical latency is characterized by a generally low viral load, high levels of HIV-specific CD8 T cells, and slowly declining number of CD4 T cells. At the point where a patient succumbs to AIDS, viral load increases in conjunction with a dramatic drop in CD4 T cell numbers, and HIV-positive individuals become highly susceptible to opportunistic infections due to complete immune dysregulation.

CD8 T cells play several important roles in controlling HIV-1 infection (reviewed in [1]). HIV-specific cytotoxic CD8 T cells (CTL) target infected cells and induce apoptosis through granzyme B/perforin and/or Fas-FasL pathways. Furthermore, both nonspecific and HIV-specific CD8 T cells release soluble factors which inhibit viral replication without killing the infected cell. These CD8 T cell-derived soluble factors include the β-chemokines MIP-1α, MIP-1β and RANTES, which inhibit viral entry at the cell surface by blocking viral coreceptors, and the CD8 T cell Antiviral Factor (CAF), which blocks transcription of HIV-1 mRNA.

The Identity of CAF

CD8 T cells were first shown to suppress HIV-replication by Walker *et al.* in 1986 [2], yet the protein factor(s) involved in CAF activity remains unknown to this day. It was thought that CAF was identified in 1995 when it was discovered that CD8-derived β-

chemokines inhibit viral replication by an unknown mechanism [3]. Soon afterward, several groups simultaneously showed that chemokine receptors (ie. CCR5 and CXCR4) serve as HIV coreceptors required for cell entry [4-9] and that soluble chemokines were capable of blocking viral entry [5, 10]. However, by this time it had been established that CAF suppressed viral replication at the level of transcription [11-16], not cell entry, and it has since been shown that CAF activity, particularly the inhibition of LTR-mediated gene expression [17], is not mediated by chemokines [17-20]. CAF has also been found not to affect reverse transcription or provirus integration [21].

At present, the identity of CAF remains unknown. It could consist of one or more protein factors, and thus far is known not to include previously identified antiviral cytokines [19, 22] (reviewed in [23]). Possible CAF candidates have been investigated and eliminated, such as granzymes A and B [24], and IL-16 [25], which is known to suppress LTR activation [26, 27]. Extensive studies of CD8 T cells supernatants from HIV-positive individuals showed that CAF secretion could be inhibited by monensin and brefeldin-A, that its secretion pattern differed from that of various known cytokines. Furthermore, this group demonstrated two separate CAF activities: one which was >50kDa and heparin binding, and one that was <10 kDa and did not bind heparin [28]. The former was later discovered to be a modified form of bovine antithrombin III, and CD8 T cells were shown to mediate the modifications necessary for its antiviral action [29]. In CD8 T cell lines derived from HIV-positive children, CAF was found to have a molecular weight between 3 and 10 kDa [30]. CAF is not contained within cytotoxic granules of CD8 T cells [31], and is most likely secreted in the same fashion as cytokines [28].

Recently, new CAF candidates have been proposed, each derived from CD8 T cells and demonstrating antiviral activity. Natural Killer Cell Enhancing Factors (NKEF)-A and -B were found to be secreted by CD8 T cells from both HIV-positive and -negative individuals, and plasma levels of these proteins were higher in Long Term Non-Progressors (LTNP) (individuals who remain asymptomatic after more than 10 years of being HIV-positive and demonstrate consistently low viral loads) than in other groups. NKEF-A/B are part of the peroxiredoxin family of proteins; other proteins of this type have antiviral properties mediated through downregulation of the NF-κB pathway [32]. Geiben-Lynn *et al.* proposed that although NKEF-A/B are indeed novel CD8 T cell -derived antiviral proteins, they are not actually CAF because these proteins were also isolated from HIV-negative individuals. However, as it has been well established that HIV-negative individuals also have CAF activity [13, 33-36], the possibility remains that NKEF-A/B are in fact a component of CAF. α-defensins 1, 2 and 3, secreted from a small sub-population of CD8 T cells, were found to have β-chemokine-independent antiviral activity; they were then proposed to be the major non-chemokine component of CAF [37]. These proteins have sequence similarities to retrocyclin, a human homologue to a monkey minidefensin; retrocyclin has been shown to have potent antiviral activity, acting at a stage before viral reverse transcription [38]. It has since been shown that α-defensins do in fact have antiviral activity but have no effect on viral gene expression; furthermore, these proteins were not detectable in CD8 T cells by others [39, 40]. Furthermore, Zhang *et al.*, who conducted the initial α-defensin study [37], have recently

retracted their findings [41]. Therefore, α-defensins are not likely to be the major component of CAF.

Gene expression analysis was recently performed on CD8 T cells from a set of twins, one of which was HIV-positive and had higher CAF, and the other HIV-negative. Forty-nine genes were identified as being upregulated in the twin with higher CAF. Other HIV-positive individuals exhibiting high CAF were screened for elevated levels of these genes, and three genes were found to be expressed at significantly higher levels in these subjects: NKG7, a transmembrane protein associated with granules in NK cells and CTL; ICT1, a differentiation marker in a colon carcinoma cell line; and a hypothetical protein, FLJ13949 [42]. The significance of these genes in CAF remains to be determined.

Mechanism of CAF Activity

CAF blocks transcription of HIV-1 mRNA by inhibiting activation of the Long Terminal Repeat (LTR), the viral promoter [11-16]. The LTR is transactivated by the viral protein Tat, as well as by several transcription factors such as NFκB and Sp1 (reviewed in [43] and [44]). CAF activity is not restricted to HIV-1 infection; it also inhibits LTR activity of HIV-2, SIV [2, 16], FIV [45], HTLV-1 and RSV [12]. Suppression of LTR activation by CAF is not HLA-restricted, and is mediated by soluble factors [11]. Although not completely dependent on cell-cell contact, HLA compatibility or presentation of viral peptides to the T Cell Receptor (TCR), it appears in some cases that CAF is enhanced if CD8 T cells are cocultured with autologous HIV-infected CD4 T cells [46, 47]. This

lends credibility to the notion that there exists more than one type of CAF activity, as will be discussed below.

It is not clear whether CAF enters the target cell and mediates its antiviral effects intracellularly, or whether CAF interacts with surface proteins on the target cell, and induces a metabolic pathway that leads to production of factors involved in the direct inhibition of LTR activity. When cell-free supernatants containing CAF are treated with protease inhibitors such as leupeptin, they lose most of their CAF activity. This suggests that CAF activity involves either a proteolytic event, or possibly proteins that interact with protease inhibitors [48]. This is in agreement with the previously mentioned CD8 T cell-mediated modification of bovine anti-thrombin III into an antiviral factor [29]. Other evidence further suggests the involvement of a metabolic pathway induced by CAF. In infected target cells, STAT1 activation and IRF-1 induction were found to be necessary downstream events of CAF activity [49]. These observations could initially be construed as IFN-γ activity because IFN-γ, which suppresses LTR activation, is produced by STAT-1 activation and subsequent IRF-1 induction [50, 51]. However, in this system IFN-γ was not responsible for CAF activity, as shown by control experiments using neutralizing antibodies to IFN-γ. It is still possible that CAF activity involves a similar pathway to that initiated by IFN-γ; this cytokine has been shown to upregulate the HLA Class II transcriptional factor (CIITA), which apart from activating transcription of HLA Class II genes also blocks the function of HIV-1 Tat [52], a mechanism similar to that of CAF. CAF may also be protein-kinase dependent, and its inhibitory activity can be blocked by

a protein synthesis inhibitor [14]. Later findings demonstrated that CAF from HIV-positive, but not HIV-negative, individuals was inhibited by cyclohexamide [28].

At the level of the LTR, the NFAT-1 binding site is essential for suppression of Tat-mediated gene expression by CAF [53], whereas the NF-κB binding site is required for the suppression of PMA/ionomycin-induced gene expression by CAF [12]. Knuchel *et al.* also implicated NF-κB in CAF (derived from nonhuman primates)-mediated inhibition of SIV LTR activation [14]. In addition, the AP-1 site appears to be involved in CAF activity, as its binding is increased in CAF treated cells [54]; this site overlaps the COUP-1 site, a negative regulator of HIV in T cells [55-57].

Immune Control of CAF Production and Function

IL-2 increases CAF activity in CD8 T cells from LTNP and some progressors, whereas IL-4 and IL-10 can decrease this activity. The latter effect can be prevented or reversed with the addition of IL-2, demonstrating the importance of this cytokine in CAF induction [58]. IL-2 is also required to induce anti-SIV activity in human CD8 T cells [59]. Stimulation with antibodies to CD28 increases CAF activity, as does co-culture with macrophages; blocking of the interaction between CD28 and its macrophage receptor, B7, abrogates CAF production [60]. Following CD28 stimulation, IL-2 and IL-2 receptor are both upregulated, and if IL-2 is prevented from binding its receptor, CAF increases are not observed even after CD28 stimulation [60]. Therefore, CD28 signaling from macrophages enhances CAF activity by rendering CD8 T cells more responsive to

autocrine IL-2. Coculture with mature dendritic cells (DC) has also been implicated in CAF induction; this effect was mediated by DC-derived IL-15 [61].

While it has been well established that CAF strongly inhibits replication of T-tropic viruses, there remains controversy as to what effect CAF has on macrophage infection, an important component of HIV transmission and latency. CD8 T cells have been shown to suppress HIV replication in primary macrophages [19, 34, 62] and in the macrophage cell line U1 [19]. However, another study showed that CD8 T cell-derived soluble factors from asymptomatic HIV+ individuals upregulate replication of syncytia-inducing (SI) and non-syncytia-inducing (NSI) HIV strains in human macrophages [63], as well as viral replication and gene transcription in monocytic cell lines [64]. It is possible in the latter cases that CAF activity was masked by other CD8 T cell-derived factors that strongly upregulate M-tropic virus replication. Indeed, it was later shown that upregulation of LTR activity in monocytic cells is pertussis-toxin sensitive (implicating a G protein coupled receptor), and is associated with increases in TNF-α; antibodies to this cytokine abrogated the increase in gene transcription. Because CD8 T cell-mediated LTR suppresion in CD4 T cells is insensitive to pertussis toxin, it appears that separate CD8 T cell activities are used to modulate HIV transcription in different cell types [65]. This has been further substantiated by Maslove *et al.*, who demonstrated that NF-κB and NFAT-1 binding is enhanced during the upregulation of LTR activation in monocytes by CD8 T cell-derived soluble factors, contrasting the decreased binding observed in CAF-treated T cells [54]. This study also showed that CD8 T cell soluble factors increase binding at the AP-1 site within the LTR, which overlaps the COUP-1 site; COUP-1 is a

positive regulator of HIV in monocytes [66, 67] and brain cells [66], and is a negative regulator in T cells [55-57].

Little is known about which viral characteristics render HIV and other lentiviruses susceptible to CAF. SIV-1 mutants with defective *nef* genes are more susceptible to human CAF than their wild-type counterparts [59]. In another study, a CD8 T cell clone's ability to suppress an NSI HIV strain but not an SI strain was found to be due to differences in the envelope gene between the two viral genotypes [68].

CAF Production by CD8 T Cell Sub-populations

CD8+CD28+ T cells have been shown to more strongly suppress M-tropic HIV than CD8+CD28- [69]. Landay *et al.* have shown that activated CD8 T cells (CD8+HLA-DR+) and CD8 T cells expressing CD28 are associated with a stronger CAF response [70]. Similarly, in a comparison between CD8 T cell subsets of HIV-positive and HIV-negative individuals, it was determined that CD8 T cells expressing an activated phenotype (CD38+ and/or HLA-DR+) had higher CAF activity in HIV-positive individuals than in the HIV-negative group [71]. However, when analyzing various panels of CD8 T cell lines that each produce varying levels of CAF, there does not appear to be a correlation between an activated phenotype and CAF activity [72, 73]. This suggests that activation state might not be the only determinants of CD8 T cells antiviral activity. Bucci *et al.* have identified a particular subset of CD8 among cats that produced the majority of CAF activity. CD8 T cells expressing low levels of the CD8 β-chain (CD8α+βlo) were found to produce the majority of CAF, and to expand preferentially in

asymptomatic FIV+ cats [74]. It is not yet known whether this phenomenon exists in humans as well.

The Multifactorial Nature of CAF

CAF activity may be comprised of more than one protein component, and thus would not necessarily result from a single protein's activity. This is suggested by numerous conflicting reports on the identity of CAF itself (see above), whether CAF is virus specific (i.e. only found in HIV-positive individuals), whether CAF induction is HLA-restricted and therefore requiring cell-cell contact, and whether CD8 T cells can inhibit HIV replication in ways other than suppression of the LTR and secretion of β-chemokines. Primary CD8 T cells and CD8 T cell lines have been shown to possess either contact-mediated, HLA-restricted antiviral activity (usually in the context of viral epitopes) or soluble antiviral activity which is not virus specific, or both. Tsubota *et al.* showed that cytotoxic CD8 T cells can suppress HIV in a contact-dependent, HLA-restricted fashion [75]. This effect was also seen in CD8 T cells from HIV-negative individuals [13]. Van Baalen *et al.* have suggested that CTL-derived CAF (from HIV-specific CD8 T cells) is HLA-restricted and HIV-antigen-restricted. In their system, CTL lines specific for a particular viral antigen (Rev) were able to effectively suppress viral replication through both cytotoxic and non-cytotoxic actions; however, at a later time point, a viral escape mutant developed with a mutation in the Rev gene, and this mutant was able to effectively replicate, indicating that both types of inhibition previously observed had been abrogated [76]. In another study however, CAF from LTNP and HIV-negative individuals was not dependent on cell-cell contact and was not HLA-restricted

[33]. Feline CAF also appears to be a soluble factor whose production is not HLA-restricted [45]. Rhesus macaques may have two types of CAF activity; one which is produced by unstimulated CD8 T cells and requires cell-cell contact and HLA-restriction, and another more potent activity which is produced by activated CD8 T cells without cell-cell contact or HLA-restriction [77]. This study also showed that TCR-stimulation with a viral gag peptide induced CAF activity in macaques [77]. An HIV-specific CD8 clone was shown to have soluble CAF activity as well as a stronger, contact-mediated HLA-restricted suppressive activity [78]. Similarly, Yang *et al.* created an HIV-specific clone whose CAF production was contact dependent and HLA-restricted; after CAF was secreted however, its antiviral effects were not HLA-restricted and required no cell-cell contact [79]. Mackewicz *et al.* showed that HLA-compatibility enhances CD8 antiviral activity but is not completely essential [47]. Also, thymic peptides enhance the CAF response, presumably triggering CD8 in an antigen-specific way similar to that induced by cell-cell contact [80].

Contrary to earlier beliefs that exposure to HIV was a prerequisite for CAF activity to be present within an individual [11, 81], CAF activity has been repeatedly demonstrated in HIV-negative individuals [13, 33-36]. Wang *et al.* have demonstrated that allo-immunization of HIV-negative women with their partner's unmatched leukocytes elicits an upregulation of CAF, as well as protective chemokines [69, 82, 83]. In another study, two types of CAF activity were observed: one which was present in HIV-positive and HIV-negative individuals and was lost upon disease progression in HIV-positive individuals, and another that was only found in HIV-positive individuals and was

maintained throughout disease progression [84]. Geiben-Lynn *et al.* demonstrated that cyclohexamide could inhibit CAF activity from HIV-negative but not HIV-positive individuals, suggesting either a different suppressive activity for each or that CAF components are preformed within HIV-positive individuals [28].

Some cell types other than CD8 have been shown to possess non-CAF antiviral activities, such as CD4 T cells [85-87], NK cells [88] and monocytes [85], indicating that within the immune system there are multiple types of HIV-suppressive activities. It is therefore possible that CD8 cells produce more than one type of antiviral activity.

Relationship Between CAF and HIV-infection/AIDS

In the context of HIV infection, data is inconclusive as to the effects of infection on CAF activity. Blackbourn *et al.* showed that CAF activity in lymphoid tissue-derived CD8 T cells negatively correlated with viral load in the lymphoid tissues [89], but no such correlation was observed in peripheral blood [89, 90]. In another study, a negative correlation was found between blood viral load and CAF in baboons [91]. Others have shown that before HAART, CAF inversely correlates with viral load, but that after treatment the two are positively correlated [92]. Past studies have shown either a positive [93-95] or a negative [96] correlation between CAF and CD4 counts in untreated HIV-positive individuals. Furthermore, improved clinical status, as defined by the 1995 Centers for Disease Control and Prevention class groups, was shown not to be a determinant of CAF levels [97]. This is corroborated by Neeltje *et al.*, who showed that there was no correlation between CD4 T cell counts and CAF [34]. In some cases, higher

levels of CAF and β-chemokines before HAART were predictive of better virus suppression after treatment [98].

CAF activity is not limited to humans, as CAF activity has been detected in chimpanzees, baboons, rhesus macaques, african green monkeys and cats [45, 91, 99-102]. Non-human primates and monkeys with high CAF usually show resistance to HIV-1/2 and SIV infection, or to virus-induced disease progression [99, 100], and FIV-positive asymptomatic cats typically have high levels of CAF [45, 103]. As with humans, CAF activity in animals acts at the level of transcription [14, 91, 104]. Notably, chimpanzees are naturally resistant to HIV-induced disease, and high CAF activity in both HIV-positive and HIV-negative chimpanzees appears to be a critical factor in this resistance. When CD8 T cells are removed from Peripheral Blood Mononuclear Cell (PBMC) cultures, chimpanzee CD4 T cells are readily infected by HIV-1 [99]. African green monkeys are also highly resistant to SIV-induced disease progression. CD8 T cells make up a very high proportion (~80%) of their total PBMC, and these cells have very high CAF activity against SIV, as well as against HIV during *in vitro* infection of human CD4 T cells [100]. CD8 T cells have also been shown to mediate prevention of superinfection in baboons and cats [105, 106], either by preventing the initial integration of the second virus, or preventing its spread.

Although CAF mediates potent antiviral activity in *ex vivo* systems, there is only some evidence that it suppresses or prevents viral replication *in vivo*. In rhesus macaques dually infected with CXCR4- and CCR5-specific SHIV, the CCR5-specific virus

dominated (as expected), until CD8 T cells were removed from the monkeys, at which point CXCR4-specific virus emerged [107]. This indicates that CD8 T cells play a role in suppression of CXCR4-specific viruses *in vivo*. In human infection, only two groups are known to regularly demonstrate a correlation between disease resistance and high CAF activity: LTNP, [33, 95, 108, 109], and Exposed-Uninfected (EU) individuals, who are frequently exposed to potential infection, through regular i.v. drug use or continued sexual contact with an HIV-positive partner, yet do not seroconvert [85, 87, 110-112]. In both groups, CD4 T cells *in vitro* are more readily infected by HIV when CD8 T cells are removed from culture, compared to whole PBMC. These findings are mirrored in non-human primates and monkeys as well. For example, HIV-1 exposed uninfected rhesus macaques have higher CAF levels, as well as β-chemokine levels, than unexposed controls [113].

It seems as though HIV exposure isn't required for CAF activity to be present, but in general, HIV-positive individuals, LTNP and individuals exposed to the virus without seroconverting tend to more frequently demonstrate higher levels of CAF. It's not clear whether, in the case of EU individuals, CAF activity is high as a result of exposure to HIV, or whether CAF activity was already present before exposure. Rhesus macaques demonstrate increased CAF and β-chemokine levels after immunization with live attenuated SIV [77], and cats immunized with FIV also showed increases in CAF activity [45]. However, in another study macaques co-immunized with HIV and SIV were still infected at a later time point by SHIV, despite their having significantly increased CAF

and β-chemokine levels after immunization [114]. This indicates that in some cases, upregulation of CAF after exposure to HIV might not be sufficient to block infection.

Antiretroviral Therapy and CAF

Treatment of HIV infection has progressed to a point where patients are typically able to live longer, more productive lives than in the past. Treatment originally consisted of nucleotide analogues called Nucleotide Reverse Transcriptase Inhibitors (NRTI). These are inserted into the viral genome during reverse transcription, and subsequently block the progression of reverse transcription because normal nucleotides cannot be linked to NRTIs. More effective drugs were later introduced; these included Protease Inhibitors (PI), which block maturation of viral particles by inhibiting polyprotein processing by the viral protease, and Non-Nucleotide Reverse Transcriptase Inhibitors (NNRTI), chemical inhibitors of the viral reverse transcriptase enzyme. Therapy regimens with a single class or type of drug resulted in high emergence rates of drug-resistant viruses. Combination therapy, known as Highly Active Anti-Retroviral Therapy (HAART), is more likely to suppress emergence of drug-resistant mutants (reviewed in [115] and [116]). HAART typically consists of at least one PI or one NNRTI, in combination with two different NRTI.

The effects of HAART on CAF activity within HIV+ individuals have recently been studied, and conflicting results have arisen. In one longitudinal study, CAF was shown to decrease significantly in 76% of patients on HAART, while it increased or was maintained in untreated patients [117]. Similarly, CAF from HIV-positive individuals

undergoing HAART was found to decrease to levels associated with HIV-negative individuals in another study [92]. Conversely, Chun *et al.* showed that patients who commenced HAART shortly after acute infection had significantly higher β-chemokine-independent CAF than LTNP, untreated patients and patients who commenced treatment during chronic infection [118]. Similarly, Kottilil *et al.* showed higher CAF in HAART-treated patients compared with untreated controls [88]. Results from these studies were based on replication-based assays to determine CAF, and most do not control for confounding factors that might inhibit steps of the infection cycle other than that of gene transcription. Our goal was to determine the effects of HAART on CAF-mediated inhibiton of the LTR, using an assay that determines CAF activity by the ability of cell-free supernatants from PHA-stimulated CD8 T cells to suppress LTR-driven gene expression. Furthermore, in light of the lack of available data on the modulation of CAF production by HIV-infection, we sought to determine which infection-associated events might mediate CAF production.

Statement of Objectives

1. To determine whether CAF activity in HIV-positive individuals on HAART differs from that of untreated HIV-positive individuals and HIV-negative individuals.
2. To determine whether viral load, CD4 T cell counts, co-culture with HIV-infected CD4 T cells, and HIV-infection of CD8 T cells, have a modulatory effect on CAF production.

Abbreviations

CTL: Cytotoxic T Lymphocyte
FasL: Fas Ligand
FIV: Feline Immunodeficiency Virus
HIV: Human Immunodefiency Virus
HTLV-1: Human T Cell Leukemia Virus
IRF-1: Interferon Regulatory Factor
MIP-1α/β: Macrophage Inflammatory Protein
NK: Natural Killer
PHA: Phytohemagglutinin
PMA: Phorbol Myristate Acetate
RANTES: Regulated on Activation Normal T Cell Expressed and Secreted
RSV: Rous Sarcoma Virus
SDF-1α: Stromal Derived Factor
SHIV: Simian/Human Immunodefiency Virus
SIV: Simian Immunodefiency Virus
STAT1: Signal Transducer and Activator of Transcription 1

MATERIALS AND METHODS

Cell culture

Jurkat T cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 200 U/ml penicillin and 200 μg/ml Streptomycin, at 37°C and 5% CO_2. Primary CD8 T cells were cultured in the same conditions as Jurkat cells, except media was supplemented with 20% fetal calf serum and 20 U/ml IL-2, unless otherwise indicated. HTLV-1-transformed CD8 T cell lines were cultured as with primary cells, except media was supplemented with 100 U/ml IL-2.

Patient selection

HIV+ patients already involved in a separate study with the Ottawa Hospital Infectious Diseases Unit consented to providing 20-30 ml of whole blood for this study. Age-matched patients were divided into three groups depending on their treatment regimen: PI, patients on protease inhibitor-containing therapy (n=13); NNRTI, patients on non-nucleoside reverse transcriptase inhibitor-containing therapy (n=13); and UN, patients that had been off treatment > 6 months, or that were drug-naïve (n=12). In both treated groups, selection criteria were predetermined in order to ensure effective antiviral therapy in the majority of subjects. These criteria included an undetectable viral load (VL) (<50 copies/ml) and a CD4 count of >200 cells/mm^3 blood, with the following exceptions: one PI patient (A2) had a CD4 count of 97 cells/ml, one PI patient (A12) had a viral load of 204 copies/ml, and one PI patient (A4) had a VL of 14,672 copies/ml and a CD4 count of 90 cells/ml. T cell counts and viral load data were assessed between 0-3 months prior to

CAF analysis (see APPENDIX B for viral load and CD4 T cell counts upon blood sample acquisition). No such selection criteria were included for the untreated group. A control group of HIV-seronegative individuals (not age-matched) was also included (n=13).

Peripheral Blood Mononuclear Cell (PBMC) isolation and CD8 T cell isolation

PBMC were separated from whole blood by Ficoll-Paque^{TM} PLUS gradient separation, washed twice with PBS and cultured at $1x10^6$ cells/ml. CD8 T cells were isolated from cultured PBMC using Magnetic Affinity Cell Sorting (MACS) CD8 Microbeads and MS^+/RS^+ columns, according to the manufacturer's instructions. Briefly, cells were washed twice with MACS buffer (5% bovine serum albumin, 2mM EDTA in PBS) and incubated for 20 minutes at 4°C with CD8-specific microbeads. Cells were passed through the magnetic separation column, washed, eluted with MACS buffer, and washed twice with PBS. Cells were cultured at $1x10^6$ cells/ml and activated with 5 μg/ml PHA.

Isolation of CAF-containing cell-free supernatants from primary CD8 T cells

Freshly isolated and PHA-activated CD8 T cells were cultured for three days, washed twice with PBS and resuspended in the original culture volume of fresh media in the absence of PHA. In some cases, cell numbers on day 3 were significantly lower than the original culture concentration; these cells were resuspended at half the original culture volume in order to bring the concentration to approximately $1x10^6$ cells/ml. Cells were cultured for 3 days, and then pelleted. Supernatants were removed and heat-inactivated (56°C for 60 minutes), then stored at -80°C. Cells were resuspended in PBS and counted

in replicate using the Trypan Blue dye exclusion method, until reproducibility of cell numbers was achieved.

DEAE-DEXTRAN transient transfection of Jurkat T cells

Jurkat T cells were grown to confluence, resuspended at $1x10^6$ cells/ml in fresh media for 18 hours, and then pelleted and resuspended in an equal volume of fresh media for 2-3 hours. Cells were washed twice in serum-free RPMI 1640, and resuspended at $6x10^6$ cells/ml in transfection buffer containing 250 μg/ml DEAE-Dextran, 50mM Tris pH 7.3, in serum-free RPMI. 1 μg/ml pLTRCAT, with LTR sequence of BRU strain to position +77 directing CAT [119], and 0.5 μg/ml pSVtat [120] were added. Cells were incubated in a 37°C water bath with 50RPM shaking, for 45 minutes. Transfected cells were pelleted, washed twice with serum-free RPMI 1640, and cultured at $3.75x10^6$ cells/ml. Each transfection was performed on a maximum of $30x10^6$ cells. If more were needed for a particular assay, separate transfections were performed, and transfected cells were pooled and redistributed before treatment with CD8 T cell supernatants. (Note: optimization of this method for reproducibility is described in APPENDIX A)

Determination of CAF activity

In order to control for variable cell density at the end of the six day culture period, cell-free CD8 T cell supernatants were adjusted with standard media so that the amount of supernatant originating from $0.5x10^6$ cells would equal 1ml (this figure was $0.8x10^6$ for determination of CD8 T cell line-derived CAF). Thus, CAF activity was measured as the amount of suppressive activity per $0.5x10^6$ cells. Freshly transfected Jurkat T cells were

aliquoted in 12-well tissue culture plates ($1.875x10^6$ cells/well), and 1ml CAF-containing cell-free CD8 T cell supernatants were added to each well. Cells were cultured for 24 hours, then activated with 25ng/ml PMA and 2μM ionomycin. After 18 hours of activation, cells were washed twice with PBS and resuspended in 0.5 M Tris. Cells were lysed using 3 freeze-thaw cycles (freeze: ethanol-dry ice bath, 5 minutes; thaw: 37°C water bath, 5 minutes) with complete vortexing after each thawing cycle. Lysates were cleared of cell debris by centrifuging at 14000 xg for 15 minutes. Cleared lysates were removed and protein concentrations were determined using Bio-Rad Protein Assay reagent and a BSA standard curve (0-10 μg/ml). For CAF determination, 10 μg of total lysate protein were loaded into a CAT ELISA plate (Roche), and volumes were adjusted to normalize total protein concentration. CAT concentrations were determined following the ELISA kit instructions. CAF activity for each CD8 supernatant was determined as % suppression of CAT expression, relative to a media-treated control, using the following formula: $CAF=100x([CAT]_{media}-[CAT]_{CD8})/ [CAT]_{media}$. Negative CAF values were expressed as 0% suppression.

Determination of IFN-γ and IL-16 concentration

IFN-γ and IL-16 concentrations in cell-free supernatants were determined using ELISA kits according to the manufacturer's instructions.

HIV-1 infection of PBMC

PBMC from healthy donors were cultured at $2x10^6$ cells/ml and activated with 2.5 μg/ml PHA for 3 hours. After 2 hours activation, 2 μg/ml polybrene was added. Cells were

washed twice with PBS, resuspended at $1x10^6$ cells/ml, and inoculated with 300 $TCID_{50}$ HIV-1 IIIB, or mock-infected with an equal volume of media. After 3 days, CD8 T cells were isolated as above, and cultured for 3 days at $1x10^6$ cells/ml. Cell-free supernatants were isolated and tested for CAF activity, as above.

HIV-1 infection of Jurkat T cells

Jurkat T cells were infected as with PBMC, except activation was done with 25 μg/ml PMA. After 5 days, Jurkats were resuspended at $5x10^6$ cells/ml in fresh media, and aliquoted in a 96-well round-bottom tissue-culture plate ($0.75x10^6$ cells/well), for determination of HIV-1 suppressive activity.

Determination of HIV-1 replication suppression

Cell-free CD8 supernatants, with volumes normalized as above, were added to HIV-infected Jurkat T cells, and cells were cultured for 24 hours. Jurkat supernatants were then measured for p24 concentrations, using a p24 Antigen Capture Assay Kit. HIV-1 suppressive activity was determined with the same method as CAF determination, as percent suppression of viral replication relative to a media-treated control.

Activation marker expression on primary CD8 T cells after PHA stimulation

CD8 T cells from the PI, NNRTI and HIV-negative group were isolated as described above, and cultured as in the CAF determination assay. Cells were analyzed for CD28, CD38, HLA-DR and apoptosis levels by flow cytometry on days 0, 3 and 6.

Flow cytometry

CD8 cells were washed twice with PBS and once with Flow Binding Buffer (FBB; 15.4 mM NaN_3, 5 μg/ml BSA in PBS), then resuspended in 100 μl FBB and incubated with appropriate FITC-conjugated antibodies (100 μl/ml) or Annexin V-FITC (100 μl/ml) and propidium iodide (50 μl/ml) for 20 minutes in the dark at room temperature. Surface antigen expression/apoptosis was then determined using a Coulter EPICS Altra flow cytometer (Coulter) and data was analyzed using Expo32 software (Coulter). FITC-conjugated antibodies: anti-human CD28, anti-human CD38, anti-human HLA-DR. Apoptosis determination was performed using Annexin-FITC Apoptosis Detection Kit I, which included propidium iodide.

HTLV-1 transformation of CD8 T cells

CD8 T cells isolated from PBMC of healthy donors were cocultured with an equal amount of freshly irradiated (10000 rad, 27 minutes) HTLV-l-producing MT2 cells. Freshly irradiated MT2 cells were added every 3-4 days for 2 weeks. Transformed CD8 cells were separated from MT2 cells as described above for isolation of CD8 T cells from PBMC. Separation was performed 3 times in succession. Transformed CD8 T cells were set up in a limiting dilution assay in a 96-well plate, and allowed to expand. Cells were later transferred to larger culture dishes until a sufficient number of cells were obtained. Cells were characterized for CD4 and CD8 expression by flow cytometry. (Transformation work was done by Naveed Gulzar in Dr. Karen Copeland's lab)

HIV-1 infection of CD8 T cell lines

CD8 T cell lines were counted and resuspended at $2x10^6$ cells/ml, and activated with 2.5 μg/ml PHA for 3 hours. Cells were then washed twice and inoculated with 300 $TCID_{50}$ HIV-1 IIIB, or mock-infected with an equal volume of media. HIV- and mock-infected cells were cultured for 7 days and media was supplemented with 100 U/ml IL-2 every 2 days. On day 7, cells were washed and cultured in fresh media for 3 days. CD8 T cell line supernatants were collected on day 10, heat inactivated, stored at -80°C, and then later assessed for CAF activity as described above. Expression levels of CD28, CD38 and HLA-DR were determined on days 0, 7 and 10 by flow cytometry, as described above.

Statistical Analysis

Significance of CAF differences between groups was determined using the Student's t-test. Correlations between CAF activity and various parameters were determined using the Spearman Rank Order Correlation test. Calculations were performed using SigmaStat 3.0 software.

PRODUCT SOURCES

Fetal Calf Serum: Wisent
IL-2: AIDS Research and Reference Reagent Program of the NIH
PHA: Sigma
PMA: Sigma
DEAE-Dextran: Sigma
Ionomycin: Sigma
Polybrene: Sigma
RPMI 1640: Gibco
Streptomycin: Gibco
Penicillin: Gibco
Ficoll-Paque™ PLUS: Pharmacia Fine Chemicals
MACS CD8 microbeads and magnetic columns: Miltenyi Biotec
Bio-Rad Protein Assay reagent: Bio-Rad
p24 Antigen Capture Assay Kit: AIDS Vaccine Program
Anti-human CD28, anti-human CD38, anti-human HLA-DR: Pharmingen
Annexin-FITC Apoptosis Detection Kit I: Pharmingen
CAT ELISA kit: Roche
IFN-γ ELISA kit: BD Biosciences
IL-16 ELISA kit: Biosource International
SigmaStat 3.0 software: SPSS Inc.

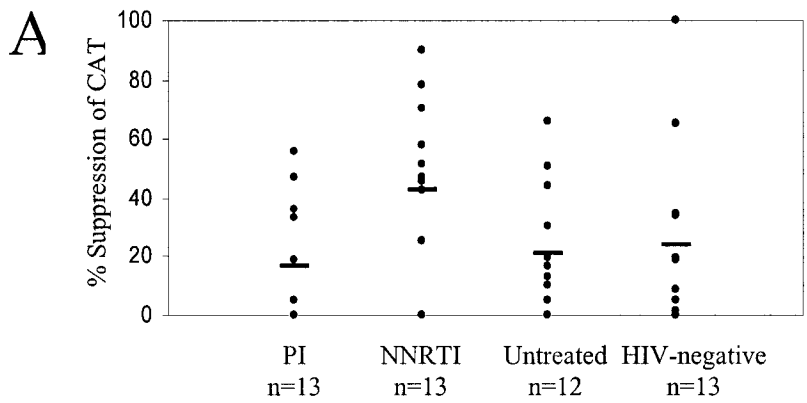
RESULTS

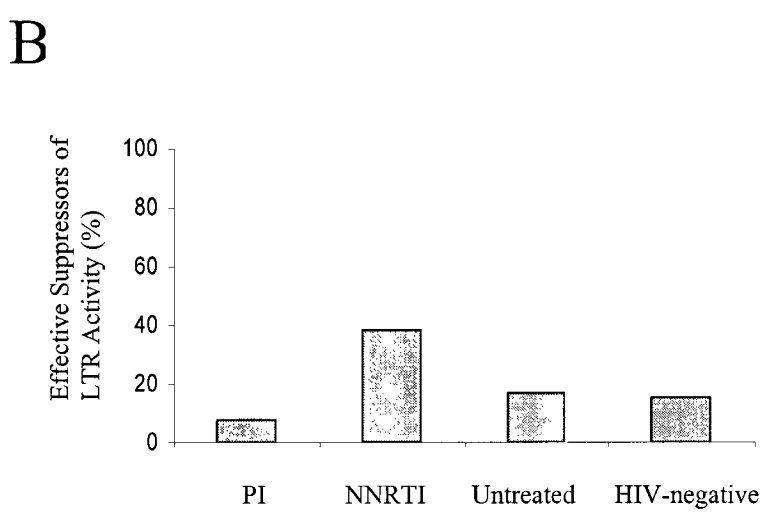
HAART has no effect on CAF-mediated inhibition of LTR-driven gene expression.

In order to determine whether antiretroviral treatment increases or decreases CAF, cell-free supernatants of PHA-activated CD8 T cells of patients on HAART, untreated patients and healthy controls were assessed for their relative abilities to suppress LTR-driven gene expression. Jurkat T cells were transiently transfected with a CAT reporter gene under the direction of the HIV LTR, as well as a Tat transactivating protein vector, which activates the LTR. Cells were then treated with cell-free supernatants from PHA-activated CD8 T cells from all groups. Transfected cells were also activated with PMA and ionomycin to further enhance baseline reporter gene production. CAF activity was measured as percent CAT suppression compared to a media-treated control. No significant differences in mean CAT suppression were observed between any of the groups (Figure 1A). Protease inhibitor-treated patients (PI) suppressed an average of 19.7% of CAT expression (range: 0-55.6%); non-nucleoside reverse transcriptase inhibitor-treated patients (NNRTI) suppressed an average of 39.2% (range: 0-90.2%); untreated patients (UN) suppressed an average of 21.2% (range: 0-65.6%); HIV-negative controls (NEG) suppressed an average of 25.8% (range: 0-100%). Results were also interpreted in order to reflect only individuals who effectively suppressed the LTR (>50% suppression), similar to interpretations by others [121]. We found that a higher proportion of NNRTI (38.5%) effectively suppressed LTR activity, compared with the other groups (PI: 7.7%; UN: 16.7%; NEG: 15.4%) (Figure 1B). No association was observed between CAF and the type of protease inhibitor or of non-nucleoside reverse transcriptase inhibitor prescribed to the patient (APPENDIX B). IFN-γ and IL-16

Figure 1. Suppression of CAT expression by cell-free CD8 T cell supernatants.
CD8 T cell supernatants were added to Jurkat T cells transiently transfected with pLTR-CAT and svTat. After 24h Jurkats were stimulated with PMA/ionomycin, and lysed 18h later. Cell lysates were evaluated for CAT concentration by ELISA. % suppression is calculated relative to a control sample treated with media alone. **A)** Suppressive activity of whole groups of patients. Black bars indicate mean % suppression for each group (PI, NNRTI, Uninfected: n=13; Untreated: n=12). **B)** Percentage of each group that efficiently suppressed LTR activity (i.e. >50% suppression). PI: patients on protease inhibitor treatment; NNRTI: patients on non-nucleoside reverse transcriptase inhibitor treatment.

Figure 1


A
% Suppression of CAT
100
80
60
40
20
0
PI
n=13
NNRTI
n=13
Untreated
n=12
HIV-negative
n=13


B
Effective Suppressors of LTR Activity (%)
100
80
60
40
20
0
PI
NNRTI
Untreated
HIV-negative

suppress LTR activation, and concentrations of these cytokines were determined in cell supernatants by ELISA, in order to determine whether LTR-suppression observed in this system could be attributed to higher levels of one or both of these cytokines. After cytokine concentrations were determined in CD8 T cell supernatants, these values were adjusted according to the volume of supernatant used in each transfection sample, to more accurately reflect the actual concentration of each cytokine in the transfection system. No correlation between CAF and IFN-γ levels was observed in any of the groups. A positive correlation was observed between CAF and IL-16 levels in the PI group but not the others ($p<0.05$, $r=0.638$) (APPENDIX B).

CAF-mediated LTR suppression does not correlate with CD4 T cells numbers, and inversely correlates with viral load in untreated HIV-positive individuals

Spearman Rank Order Correlation tests were performed in order to determine whether CD4 T cell levels and viral load were determinants for CAF activity in our system. Viral load negatively correlated with CAF in the untreated group ($p=0.0018$, $r=-0.776$). Within the PI group, a positive correlation was observed between CAF and relative CD4 T cell percentages ($p=0.018$, $r=0.639$). No correlation was found between CAF and CD4 T cell counts, CD8 T cell counts and relative CD8 T cell percentages in any of the three HIV-positive groups (APPENDIX B).

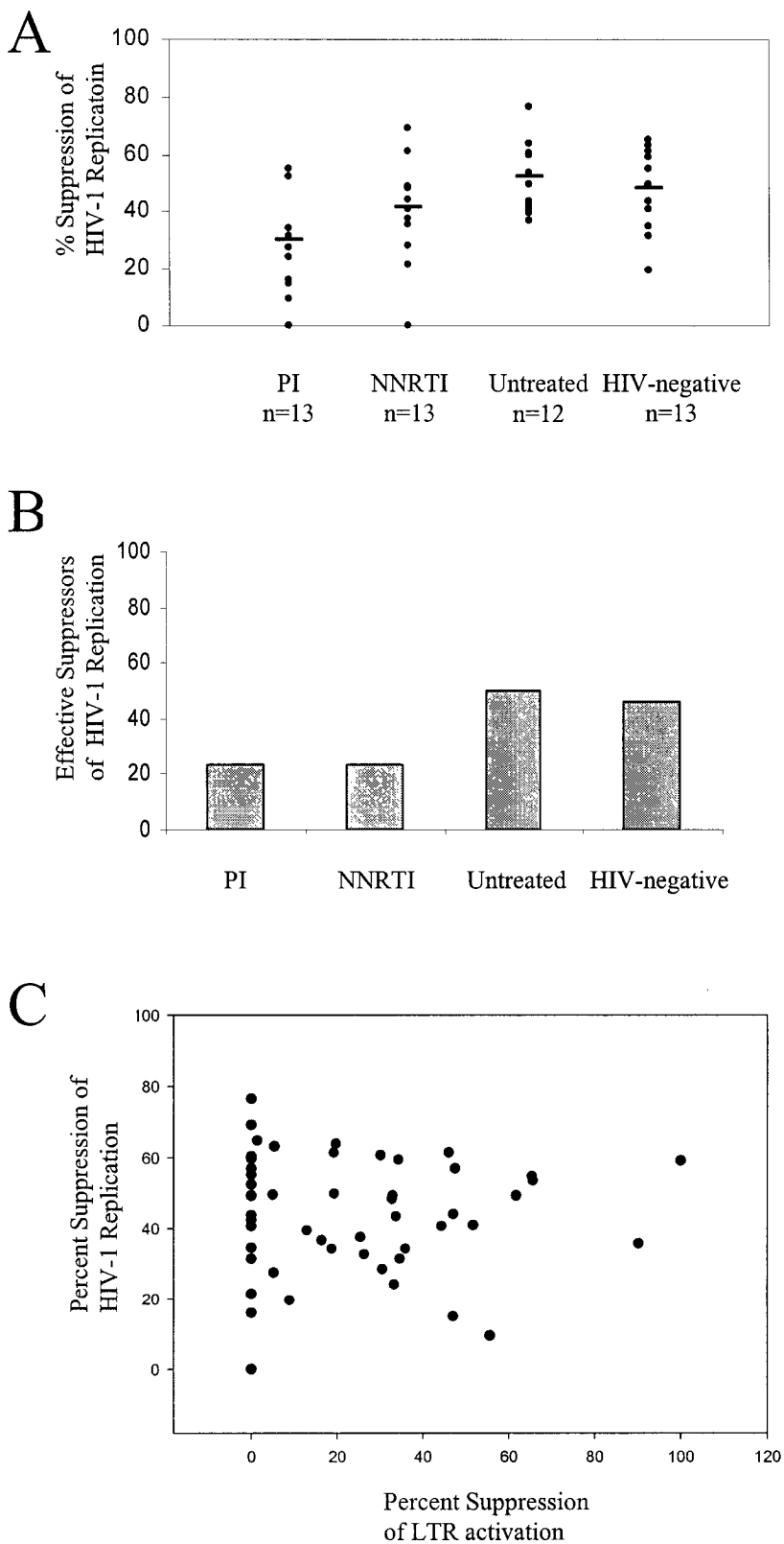
CAF-mediated LTR-suppression does not correlate with CD8 T cell-mediated inhibiton of HIV-1 IIIB replication during acute infection

CAF activity inhibits HIV replication at the level of transcription, but it is not clear whether CD8-mediated inhibition of viral replication during acute infection assays accurately reflects suppression of the LTR by CAF. We sought to determine whether levels of suppression observed in our transient transfection system would be mirrored in an acute infection assay. Jurkat T cells were infected with HIV-IIIB, a β-chemokine-insensitive strain, and treated with the same cell-free CD8 T cell supernatants as in the transfection assay, or with media as a control. After 24 hours, viral replication was measured by supernatant p24 concentrations (Figure 2A). We found that the PI group suppressed an average of 30.2% of HIV replication (range: 0-59.5%), NNRTI suppressed an average of 41.8% (range: 0-69.2%), UN suppressed an average of 52.3% (range: 36.7-76.6%), and NEG suppressed an average of 48.4% (range: 19.6-64.8%). The PI group inhibited significantly less viral replication than the untreated group ($p<0.0008$), and the NNRTI group demonstrated suppression levels lower than the untreated group; this difference did not reach significance ($p=0.052$). When analyzing the proportions of each group that effectively suppressed viral replication (>50% suppression), we found that 23.1% of PI and NNRTI, 50% of UN and 46.2% of NEG demonstrated effective suppression (Figure 2B). No correlations were noted between suppression of viral replication and CD4 T cell counts, relative CD4 T cell percentages, CD8 T cell counts or relative CD8 T cell percentages (APPENDIX B). Furthermore, there was no correlation

Figure 2. Suppression of HIV-1 replication by cell-free CD8 T cell supernatants; comparison of transcription-based and replication-based inhition assays

CD8 T cell supernatants were added to HIV-infected Jurkat T cells, and p24 concentrations in Jurkat supernatants were determined after 24 hours by ELISA. % suppression is calculated relative to a control sample treated with media alone. **A)** Suppressive activity of whole groups of patients. Black bars indicate mean % suppression for each group (PI, NNRTI, Uninfected: n=13; Untreated: n=12). **B)** Percentage of each group that efficiently suppressed viral replication (i.e. >50% suppression). **C)** Comparison of suppressive activity against LTR and HIV for all subjects. PI: patients on protease inhibitor treatment; NNRTI: patients on non-nucleoside reverse transcriptase inhibitor treatment.

Figure 2


A
% Suppression of HIV-1 Replicatoin
100
80
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0
PI n=13
NNRTI n=13
Untreated n=12
HIV-negative n=13
B
Effective Suppressors of HIV-1 Replication
100
80
60
40
20
0
PI
NNRTI
Untreated
HIV-negative
C
Percent Suppression of HIV-1 Replication
100
80
60
40
20
0
0
20
40
60
80
100
120
Percent Suppression of LTR activation

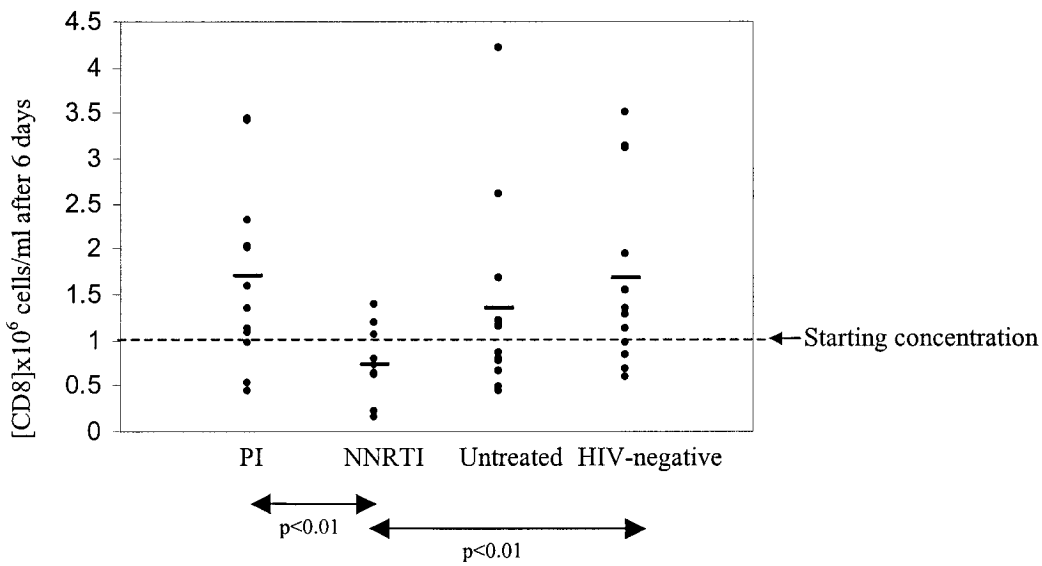
between levels of CAF and levels of suppression of HIV replication for each individual (Figure 2C).

CD8 T cells from patients on NNRTI-therapy respond differently to PHA activation compared to cells from patients on PI-therapy

We observed that after 3 days of PHA-stimulation followed by 3 days without PHA, CD8 T cells from the NNRTI group tended to be less numerous than those from the other groups. All cells were cultured at a density of $1x10^6$ cells/ml on day 0, and at day 6, the average density of NNRTI cells was $0.73x10^6$ cells/ml, compared to $1.68x10^6$ cells/ml in the PI group, $1.33x10^6$ cells/ml in the UN group and $1.67x10^6$ in the HIV-negative group (Figure 3). The differences between PI and NNRTI and between HIV-negative and NNRTI were significant ($p<0.01$). It was unclear whether the cells were dying in culture, or whether they were slow to proliferate after PHA stimulation. In a small cohort of patients and HIV-negative donors (PI, NNRTI: n=4; untreated: n=7), flow cytometric analysis was performed to determine expression levels of activation markers (CD28, CD38 and HLA-DR) *ex vivo* (day 0), after PHA stimulation (day 3) and after recovery from PHA stimulation (day 6). Apoptosis levels were also assessed by flow cytometry, by analysis of Annexin-V binding in the absence of propidium-iodide staining. Untreated HIV-positive patients could not be studied due to low availability of blood samples from these individuals. No differences in apoptosis levels were observed between any of the groups at any time point. Activation receptor expression kinetics were assessed on days 0 (*ex vivo*, pre-activation), 3 (post activation) and 6 (activation recovery) (APPENDIX C). Mean expression levels are shown in Table 1. PI and NNRTI expressed nearly equal levels of CD28 on day 0, although the range of expression was much smaller in NNRTI.

Figure 3. Concentration of CD8 T cells after PHA stimulation.
CD8 T cells were isolated from PBMC, cultured at $1x10^6$ cells/ml and activated with PHA for 3 days. Cells were then washed with PBS and cultured another 3 days without PHA. Concentration was determined on day 6 by Trypan Blue exclusion method. Black bars indicate mean concentration for each group (PI, NNRTI, Uninfected: n=13; Untreated: n=12). PI: patients on protease inhibitor treatment; NNRTI: patients on non-nucleoside reverse transcriptase inhibitor treatment.

Figure 3


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
[CD8]x10^6 cells/ml after 6 days
Starting concentration
PI
NNRTI
Untreated
HIV-negative
p<0.01
p<0.01

HIV-negative (NEG) could be divided into two groups: NEG CD28hi, who showed 89.1±1.1% CD28 expression on day 0 (n=2), and NEG CD28lo, who showed 27.9±7.7% CD28 expression on day 0 (n=5). On day 3, CD28 levels increased in all groups. By day 6, CD28 levels continued to increase in PI and NNRTI, were unchanged in NEG CD28hi and decreased slightly in NEG CD28lo (Figure 4A). CD38 expression on day 0 was similar between PI, NNRTI and NEG CD28hi, and was lower in NEG CD28lo. As expected after PHA activation, CD38 expression increased dramatically in all groups by day 3. CD38 expression continued to increase by day 6, after PHA had been absent for 3 days (Figure 4B). HLA-DR expression was the most varied among the four groups. On day 0, PI and NNRTI were similar in their HLA-DR expression levels. NEG CD28hi HLA-DR expression on day 0 was significantly lower than NNRTI ($p<0.05$). NEG CD28lo HLA-DR expression on day 0 was significantly lower than PI ($p<0.001$), NNRTI ($p<0.05$) and NEG CD28hi ($p<0.05$). In all cases, HLA-DR expression increased after 3 days of PHA stimulation. PI, NNRTI and NEG CD28hi expressed similar levels of HLA-DR, while NEG CD28lo HLA-DR expression was significantly lower than PI ($p<0.0001$), NNRTI ($p<0.001$) and NEG CD28hi ($p<0.001$). By day 6, HLA-DR expression had returned to the same level (or lower) as day 0 expression in NNRTI and NEG CD28hi, but not in PI or NEG CD28lo (Figure 4C). Significant differences in expression were observed between PI and NNRTI ($p<0.05$), PI and NEG CD28hi ($p<0.001$), PI and NEG CD28lo ($p<0.0001$), NNRTI and NEG CD28hi ($p<0.01$), and NNRTI and NEG CD28lo ($p<0.05$).

Figure 4. Expression of activation markers on activated CD8 T cells
CD8 T cells were isolated from PBMC, cultured at $1x10^6$ cells/ml and activated with PHA for 3 days. Cells were then washed with PBS and cultured another 3 days without PHA. Activation marker expression was determined by flow cytometry. **A)** CD28 expression; **B)** CD38 expression; **C)** HLA-DR expression. PI: patients on protease inhibitor treatment; NNRTI: patients on non-nucleoside reverse transcriptase inhibitor treatment; NEG CD28hi: HIV-negative donors who expressed >90% CD8+CD28+ on day 0; NEG CD28lo: HIV-negative donors who expressed <15% CD8+CD28+ on day 0.

Figure 4

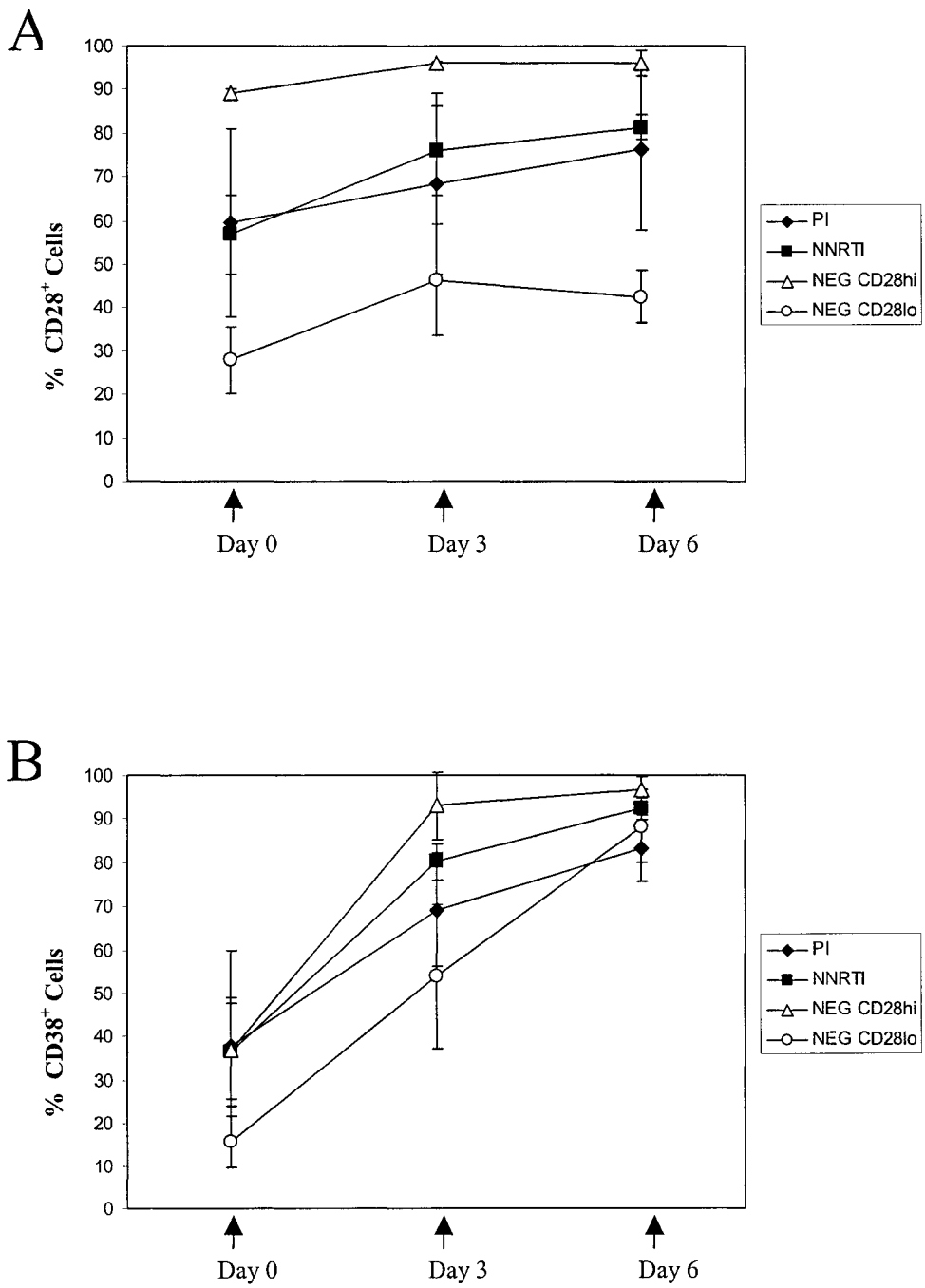

A
100
90
80
70
60
50
40
30
20
10
0
% CD28+ Cells
Day 0
Day 3
Day 6
PI
NNRTI
NEG CD28hi
NEG CD28lo
B
100
90
80
70
60
50
40
30
20
10
0
% CD38+ Cells
Day 0
Day 3
Day 6
PI
NNRTI
NEG CD28hi
NEG CD28lo

Figure 4

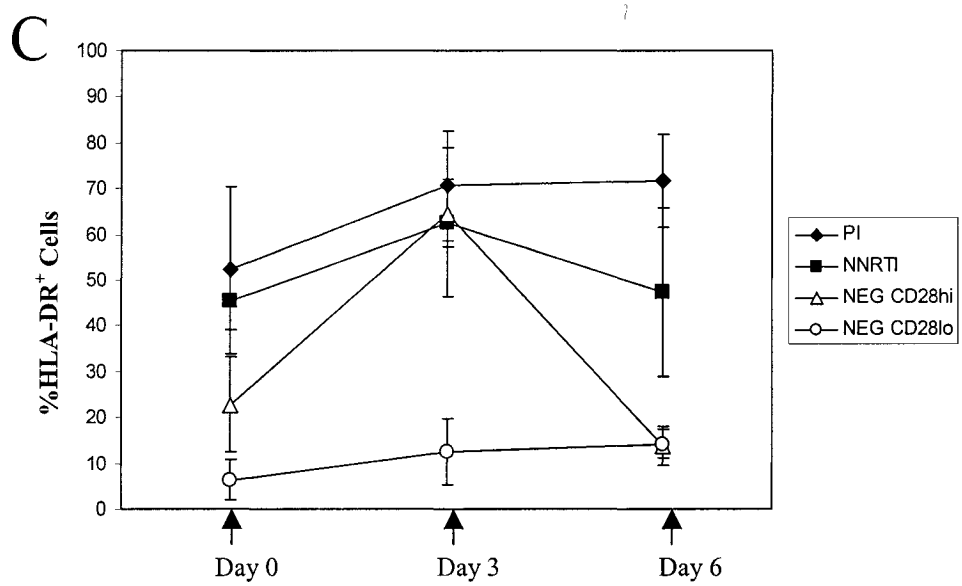

C
100
90
80
70
60
50
40
30
20
10
0
%HLA-DR+ Cells
Day 0
Day 3
Day 6
PI
NNRTI
NEG CD28hi
NEG CD28lo

Table 1. Activation receptor expression kinetics

	Day	PI	NNRTI	NEG CD28hi	NEG CD28lo
CD28	**0**	59.4±21.5	56.8±9.0	89.1±1.1	27.9±7.7
	3	68.5±20.8	76.0±10.2	96.2±0.3	46.4±12.9
	6	76.2±18.4	81.4±2.8	96.0±3.0	42.5±6.1
CD38	**0**	37.8±22.1	36.4±12.5	36.7±11.0	15.7±6.0
	3	69.2±13.1	80.2±4.1	93.0±7.7	53.9±16.6
	6	83.1±7.6	92.4±2.5	96.7±3.0	88.3±8.4
HLA-DR	**0**	52.2±18.2	45.5±6.4	22.8±10.4	6.3±4.4
	3	70.6±12.1	62.6±16.2	64.6±7.4	12.5±7.1
	6	71.6±10.2	47.4±18.3	13.8±4.3	14.3±3.0

CD8 T cells from patients treated with protease inhibitors (PI), non-nucleoside reverse transcriptase inhibitors (NNRTI), and HIV-negative controls, were activated with PHA for 3 days, then washed and cultured without PHA for 3 days. Surface expression of CD28, CD38 and HLA-DR were determined on day 0 (pre-PHA), day 3, and day 6.

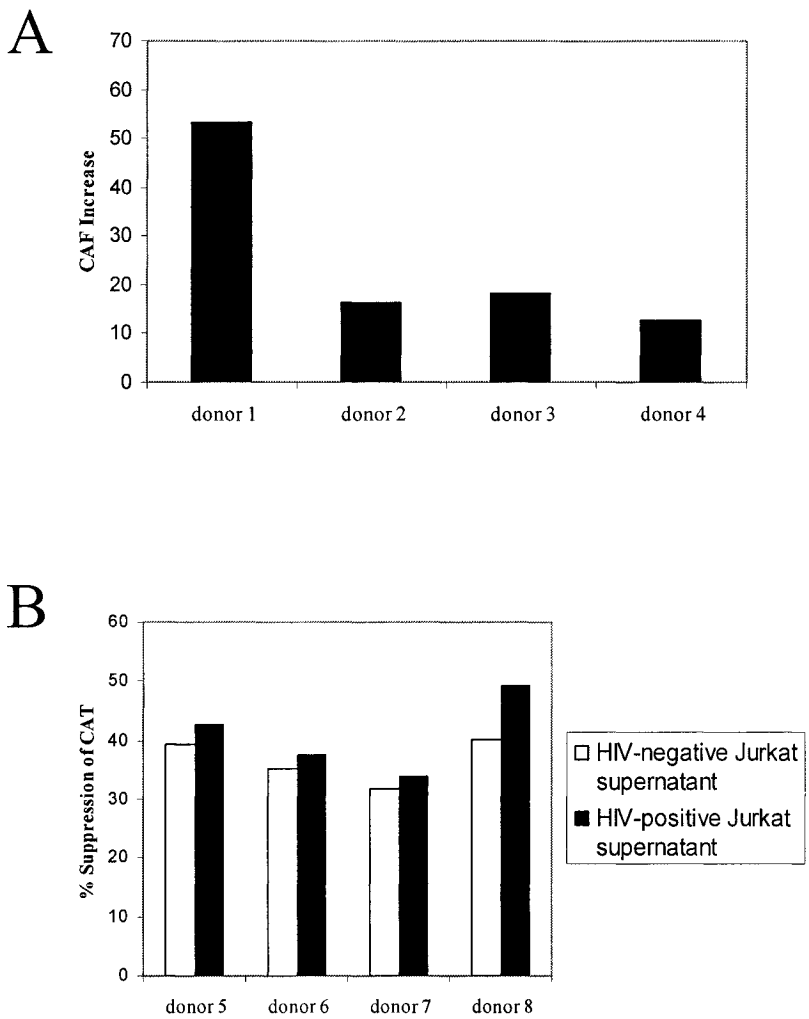
CD8 T cells cocultured with HIV-infected CD4 T cells demonstrate higher CAF activity than CD8 T cells cocultured with mock-infected CD4 T cells

It is not clear which immune parameters are necessary for induction of CAF in CD8 T cells. We sought to determine whether HIV-infected CD4 T cells could induce CD8 T cells to produce CAF. Whole PBMC from 4 HIV-negative donors were inoculated with HIV-1 IIIB or mock-infected with media, and cultured for 3 days. While a small proportion of CD8 T cells might be infected in this system, the major target of HIV-IIIB remains CD4 T cells. After 3 days, CD8 T cells were isolated and cultured 3 more days. Cell free supernatants were heat-inactivated, then assessed for CAF activity. Coculture with HIV-infected CD4 T cells increased CAF activity in all four cases, compared to mock-infected controls (Figure 5A, Table 2). In one case, the increase was dramatic (mock: 0% suppression; HIV-positive: 53.2% suppression). The other three cases showed more moderate increases of 16.3%, 18.2% and 12.6% suppression of LTR

Figure 5. CAF modulation by HIV-infected CD4 T cell coculture and HIV-infected CD4 T cell-derived soluble factors

A) PBMC from healthy donors were infected with HIV-1 IIIB, or mock-infected, and cultured for 3 days. CD8 T cells were then isolated from PBMC and cultured for 3 days. Cell-free supernatants were collected, heat inactivated and tested for CAF activity, as in Figure 1. **B)** Jurkat T cells were infected with HIV-1 IIIB, or mock-infected, and cell-free supernatant was collected after 5 days. Primary CD8 T cells were activated and treated with either mock- or HIV-infected Jurkat cell-free supernatant for 3 days, then washed and cultured for 3 days. Cell-free CD8 T cell supernatant was assessed for CAF activity, as in Figure 1.

Figure 5


A
70
60
50
40
30
20
10
0
CAF Increase
donor 1
donor 2
donor 3
donor 4
B
60
50
40
30
20
10
0
% Suppression of CAT
donor 5
donor 6
donor 7
donor 8
HIV-negative Jurkat supernatant
HIV-positive Jurkat supernatant

activity. No correlation was observed between CAF activity and concentrations of IFN-γ or IL-16 (data not shown).

Table 2. CAF activity after coculture with HIV infected CD4 T cells

	Donor 1		Donor 2		Donor 3		Donor 4	
	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+
CAF (%)	0	53.2	0	16.3	38.0	56.2	66.9	79.5
Δ CAF	53.2		16.3		18.2		12.6	

PBMC from HIV-negative donors were infected with HIV-1 IIIB (HIV+) or mock-infected (HIV-), and cultured 3 days. CD8 T cells were then isolated using magnetic affinity beads, and were cultured 3 days. Cell-free supernatants were harvested from CD8 T cells and assessed for CAF activity. ΔCAF was calculated by subtracting HIV- CAF from HIV+ CAF.

Soluble factors secreted by HIV-infected CD4 T cells do not induce CAF in CD8 T cells

Having shown that coculture with HIV-infected CD4 T cells can induce CAF production in CD8 T cells, we next investigated whether this effect was due to soluble factors secreted by CD4 T cells, and whether cell-cell contact was required for the observed CAF increases. We acutely infected isolated CD4 T cells from two HIV-negative individuals, heat-inactivated the resulting supernatants, and cultured isolated CD8 T cells from a different donor with these supernatants. CD8 T cells were cultured in CD4 T cell conditioned media for 3 days, then washed and cultured in fresh unconditioned media for 3 days. CAF activity in cell-free supernatants was then assessed as described above. Preliminary data was inconclusive, as it appeared that in some cases HIV-infected CD4 T cell supernatants increased CAF, while in others CAF was decreased (APPENDIX D). In order to eliminate several unwanted variables from this system, Jurkat T cells were used as the infection target. Jurkats were infected with HIV-1 IIIB or mock-infected, and the

resulting supernatants were tested for CAF induction capabilities against CD8 T cells from four different HIV-negative donors. In each case, a slight increase in CAF was observed in CD8 T cells treated with HIV-infected CD4 T cell supernatant, but this increase was not significant (Figure 5B). No correlation was observed between CAF activity and concentrations of IFN-γ or IL-16 (data not shown).

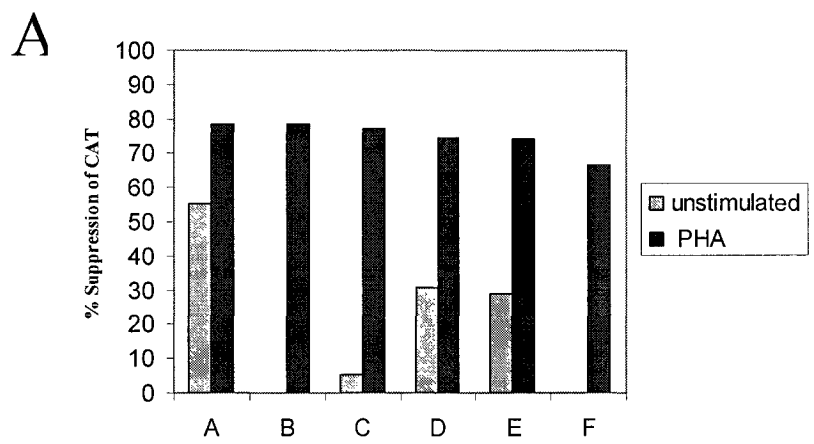
HIV-infection of CD8 T cell lines increases LTR suppressive activity

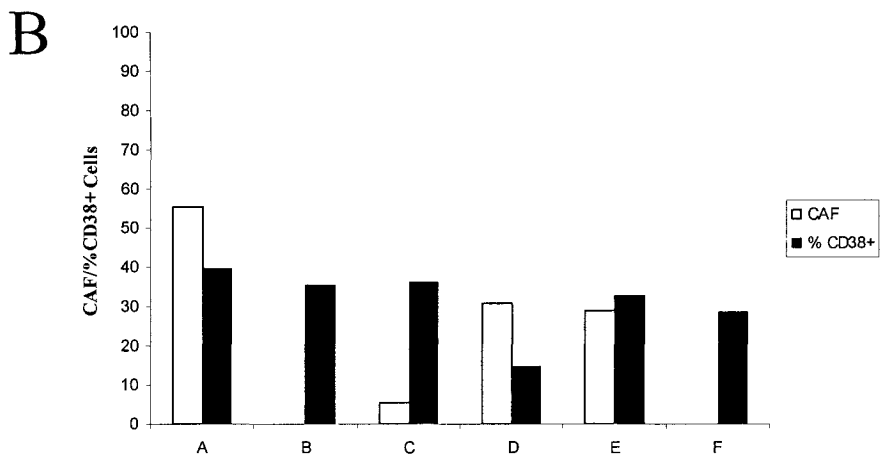
HTLV-1-transformed CD8 cell lines (originating from HIV-negative donors) were found to possess CAF activity. Some required PHA activation, while others did not (Figure 6A). Expression analysis of activation markers showed that these cell lines all expressed very low levels of CD28, medium levels of CD38, and high levels of HLA-DR (APPENDIX D). The only variation observed between the cell lines was CD38 expression, and no correlation was found between spontaneous CAF activity and levels of CD38 (Figure 6B). We chose three of these cell lines and found they were readily infected with HIV-1 IIIB (Figure 6C), despite the fact that they do not express CD4, the main HIV receptor (data not shown).

CD8 T cells can be infected by HIV-1, and the effects of infection on CD8 T cell function are not well characterized. We sought to determine what effect HIV infection would have on CAF production. Two CD8 T cell lines were HIV- and mock-infected, cultured for 7 days with periodic replenishment of IL-2, and then resuspended in fresh media. After 3 days, cell-free supernatants were assessed for CAF activity, as described above. LTR suppressive activity was increased by HIV-infection in both cell lines (Figure 6D).

Figure 6. Infection of CD8 T cell lines and CAF determination
HTLV-1-transformed CD8 T cell lines (designated A-F) were assessed for CAF activity and permissibility to HIV-infection. **A)** Cell lines were assessed for CAF activity with and without PHA-stimulation. CAF was determined as in Figure 1. **B)** CD38 expression was analyzed for each cell line and compared with spontaneous CAF activity. **C)** Cell lines were infected with HIV-1 IIIB, and p24 levels in the supernatant were determined at days 7, 10 and 14. **D)** HIV- and mock- infected cell lines were cultured for 7 days with periodic IL-2 replenishing, then resuspended in fresh media for 3 more days. On day 10, cell-free supernatants were assessed for CAF activity, as described in Figure 1.

Figure 6


A
% Suppression of CAT
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
F
unstimulated
PHA


B
CAF/%CD38+ Cells
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
F
CAF
% CD38+

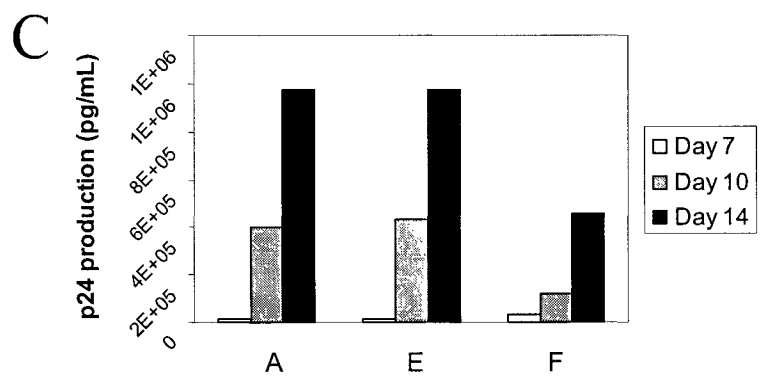
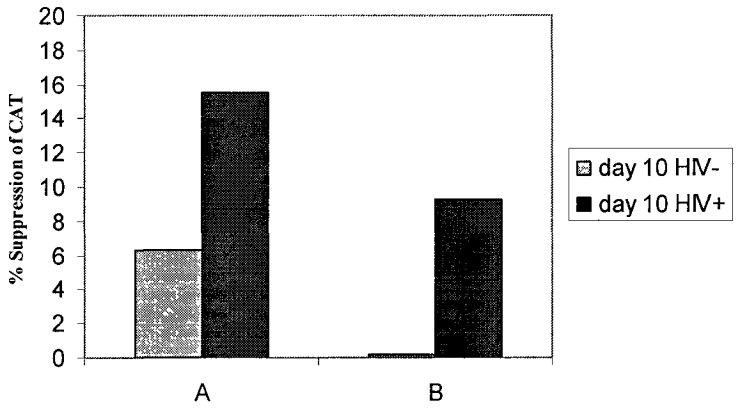

C
p24 production (pg/mL)
1E+06
1E+06
8E+05
6E+05
4E+05
2E+05
0
A
E
F
Day 7
Day 10
Day 14

Figure 6

D


20
18
16
14
12
10
8
6
4
2
0
% Suppression of CAT
A
B
day 10 HIV-
day 10 HIV+

In cell line A, HIV-infected cells suppressed 15.5% of gene expression whereas mock-infected cells suppressed less than half of that amount (6.3%). In cell line B, HIV-infected cells suppressed 9.3% of gene expression, and mock-infected demonstrated no suppression at all. In both cases, increases in CAF were mirrored by increases in IFN-γ and IL-16 concentrations (Table 3).

Table 3. CD8 T cell line-derived LTR-suppressive activity, IFN-γ concentrations and IL-16 concentrations

Cell Line Supernatant	% LTR Suppression	[IFN-γ], pg/ml[a]	[IL-16], pg/ml[a]
A: Mock-infected	6.3	55.1	69.0
A: HIV-infected	15.5	96.4	242.0
B: Mock-infected	0	26.1	16.2
B: HIV-infected	9.3	47.5	66.2

% suppression of LTR-driven gene expression is shown for two CD8 T cell lines, each either mock- and HIV-infected, as well as relative concentrations of IFN-γ and IL-16. [a]Values were initially determined for cytokine concentrations in neat cell-free supernatants, and were adjusted to reflect the input volume of supernatant relative to the entire volume of the transfection system.

DISCUSSION

The effects of antiretroviral therapy on CAF production are not clear. In a cross-sectional analysis of 26 patients undergoing HAART (13 on protease inhibitor therapy (PI), 13 on non-nucleoside reverse transcriptase inhibitor therapy (NNRTI)), we have shown that there is no significant difference in CAF-mediated suppression of the LTR compared with untreated controls (UN) and HIV-negative individuals (NEG), when analysis of the entire patient population was performed. CAF in the NNRTI group was slightly, but not significantly ($p<0.1$) higher than PI and UN. The lack of significance seen here was due to a high degree of variability within each group; a larger sample size would probably allow these differences to become more apparent. Analysis of the proportions of individuals in each group that effectively suppressed LTR activity (>50% suppression, described in [121]) showed that 41.7% of NNRTI demonstrated effective suppression, compared to 16.7% or less in the other three groups (Figure 1B). Furthermore, the actual suppression values for all effective suppressors tend to be much higher in the NNRTI group compared to PI and UN. The cause of the trend towards elevated CAF in NNRTI is not clear, because little is known about the comparative effects of PI and NNRTI-based therapy on CD8 T cell function. Smith *et al.* showed that CD8 T cells from NNRTI-treated patients expressed higher levels of CD38 and HLA-DR than PI-treated patients [122]. An activated phenotype, involving both CD38 and/or HLA-DR expression, has been associated with higher CAF activity [70, 71]. Therefore, this might explain why more NNRTI in our system had higher levels of CAF. However, others have found that there is no difference in CD8 T cell activation in either type of treatment [123, 124], and that when patients switch from PI- to NNRTI-based therapy, there is no change in CD8 T

cell activation [125]. Therefore, we cannot be certain at this time that heightened activation states were responsible for CAF variations in our system.

It is most likely that the LTR suppression observed within our system is the result of true CAF activity (defined as CD8-derived soluble factors which inhibit viral replication by inhibiting LTR activation), and not confounding non-CAF factors. Protein expression, and by association gene expression, was controlled for by determining the amount of reporter gene detected in a set amount of total protein. β-chemokines were not controlled for because these chemokines do not interfere with LTR-mediated gene expression [17]. Statistical analysis was performed to determine whether the levels of inhibition observed were due to IL-16 or IFN-γ-mediated suppression of the LTR, both of which have been shown to suppress LTR activation [26, 126]. In only one group (PI) was a correlation observed between upregulation of IL-16 and increased CAF activity. However, it is not likely that the observed increases in IL-16 contributed to LTR-suppression. The supernatant sample with the highest amount of IL-16 (410.3 pg/ml of transfection media) also had the highest CAF (55.6%), whereas another supernatant sample with slightly less IL-16 (383.9 pg/ml of transfection media) showed no CAF activity, indicating that the maximal levels of IL-16 detected within this system were insufficient to inhibit LTR activity. Also, Mackewicz *et al.* found that the minimum concentration of IL-16 required to suppress HIV replication was 1 μg/ml [25], approximately $2x10^3$-fold higher than levels seen in our system. Therefore, our results are reflective of true CAF activity.

Others have shown CAF activity to decrease after antiretroviral therapy. For example, one study showed that patients undergoing mono- or dual-therapy maintained consistently high CAF for 52 weeks, but that CAF activity abruptly disappeared as soon as these patients commenced HAART [92]. In this study, mono- or dual-therapy consisted of an NRTI, a PI, or both. Stranford *et al.* showed that CAF decreased significantly from pre-treatment levels in 76% of patients undergoing HAART, and that over the same time period, untreated controls either maintained CAF activity or an increase was observed [117]. Contrary to these findings, others have shown that patients undergoing HAART had higher CAF than untreated controls [88]. Similarly, Chun *et al.* showed that patients who commenced HAART shortly after primary infection had much higher CAF than patients who commenced HAART during chronic infection, as well as LTNP and untreated controls [118]. This is reflective of findings by Oxenius *et al*, who found that HIV-specific CD8 T cell function was preserved in patients who commenced HAART during primary infection or concurrent to seroconversion; in patients where HAART-commencement was delayed, HIV-specific CD8 T cell function was lost [127].

Two of the above-mentioned CAF studies used acute infection assays to determine CAF activity [92, 117]. Specifically, acutely infected CD4 T cells were cocultured with CD8 T cells, and CAF was determined as suppression of p24 production. The viral strain used in these studies, HIV-1_{SF33}, is a T-tropic strain, meaning that it preferentially infects CD4 T cells using CXCR4 as a coreceptor. Therefore, β-chemokines were not controlled for in this study, as they would have minimal inhibitory effects against that particular viral strain. However, replication of HIV-1_{SF33} could still theoretically be suppressed by IL-16

and IFN-γ, neither of which was controlled for. Accounting for IL-16 is important, as serum levels of this cytokine have been shown to increase dramatically (1213%) in patients undergoing HAART, compared to untreated controls [128]. In addition, there was no evidence in either study that the viral inhibition observed was mediated by soluble factors. Because all experiments were performed in a CD4-CD8 T cell coculture system, unknown cell-cell-contact-mediated methods of inhibition could possibly have been contributing to the antiviral effects observed. Furthermore, in the above-mentioned studies where CAF was found to be increased in patients on HAART, an endogenous infection system was used to detect CAF [88, 118]. In those cases, CD8 T cells were assessed for their ability to suppress replication of endogenous HIV in autologous CD4 T cells. These viral strains were sensitive to β-chemokine inhibition, and in both cases CAF activity was shown to be β-chemokine independent. Also, although coculture systems were used, data was corroborated with either a transwell culture system (where CD8 and CD4 T cells are separated by a semi-permeable membrane) [118] or by treatment of CD4 T cells with cell-free CD8 T cell supernatant [88]. These results show that the effects being observed were mediated by CD8 T cell-derived soluble factors independently of β-chemokines. However, controls for IL-16 and IFN-γ were not included.

None of the four studies mentioned above examined CAF-mediated suppression of the LTR [88, 92, 117, 118]. CAF has been shown repeatedly to inhibit LTR activation at the level of transcription [11-16], and in one study was specifically shown not to interfere with reverse transcription or provirus integration [21]. It is entirely possible that CD8 T

cells possess antiviral activity acting on stages other than gene transcription or viral entry. This is reflected by the multitude of contrasting CAF data already published. Some studies show CAF to be HLA-restricted [13, 75], while others have shown it to act independently of HLA-restriction [33, 45]. Also, some have shown more than one type of CAF activity within the same individual [28, 77, 84]. These studies do not demonstrate that the observed antiviral activity is being mediated at the level of transcription. Thus, it is possible that CD8 T cells secrete antiviral factors other than CAF, IL-16 and IFN-γ (which block viral gene transcription) and β-chemokines (which block viral entry), and that these unknown factors act at stages other than gene expression. Our studies have controlled for all known CD8 T cell-derived antiviral factors other than CAF, and furthermore data analysis is based on LTR-driven gene transcription, and so is more indicative of true CAF activity than some other studies.

We have demonstrated that within untreated HIV-positive individuals, there is an inverse correlation between viral load and CD8 T cell-mediated LTR suppression. This is in agreement with a previous study of baboon-derived CD8 T cell-mediated suppression of viral replication, performed by Locher *et al* [91]. This contradicts other findings, where no correlation was seen between blood viral load and CAF [89, 90]. We have also shown that within each group studied, there is no correlation between CD4 T cell counts and CD8 T cell-mediated LTR suppression, in agreement with findings by Neeltje *et al* [34]. Others have shown either a positive [93-95] or a negative [96] correlation between CD4 T cell counts and CD8 T cell-mediated suppression of viral replication in infection

assays. Our results, derived from a transcription-based system, are more likely to reflect true CAF activity.

We investigated whether the data collected in our LTR suppression system would be mirrored in an acute infection assay. Using HIV-1 IIIB-infected CD4 T cells treated with the same cell-free supernatants as in the transcription-based assay, we showed a different pattern of inhibition of viral replication, compared to that of LTR suppression. While absolute numbers of suppression cannot be compared between the two assays, trends over the population studied can. In the transcription inhibition assay, we observed no significant difference in overall suppression over the whole study group, but that a higher proportion of NNRTI effectively suppressed the LTR. The acute infection assay showed that untreated HIV-positive individuals had significantly higher suppressive capabilities than PI ($p<0.0008$) and slightly higher suppressive capabilities than NNRTI ($p=0.052$). Also, both UN and NEG demonstrated a higher proportion of effective suppressors than PI and NNRTI. These results reflect findings by others [92, 117], who also showed lower CD8 T cell-mediated suppression of viral replication in individuals on HAART. In our system, no correlation was observed between LTR suppression and HIV suppression (Figure 2C). We found no correlation between viral suppression and IL-16 or IFN-γ. Also, β-chemokines were not controlled for because the strain used for infection, HIV-1 IIIB, is β-chemokine insensitive. SDF1-α, the CXCR4 ligand, was not controlled for because it has been shown to not be produced by CD8 T cells [18]. Taken together, these findings indicate that CD8 T cell-mediated suppression of viral replication *in vitro* could occur independently of chemokines and known LTR suppressors (IL-16, IFN-γ, CAF),

and that these levels of suppression do not always reflect CAF-mediated LTR suppression. The majority of CAF studies in the past have used either acute infection or endogenous infection assays to determine CD8 T cell antiviral activity. In particular, this was the case in previous studies performed to determine the association between HAART and CAF [88, 92, 117, 118]. Any studies using acute or endogenous infection assays make the presumption that all antiviral activity being observed is occurring at the level of transcription. However, it cannot be ruled out that unknown CD8 T cell-derived factors other than CAF are involved. We have shown that CD8 T cell-mediated suppression of viral replication is not necessarily reflective of CAF-mediated suppression of LTR activation.

In our system designed to assess CAF derived from CD8 T cell supernatants from HIV-positive individuals, we observed that CD8 T cells from patients receiving NNRTI-based therapy tended to be less numerous than CD8 T cells from all other groups at the end of the culture period. Statistical analysis revealed that this difference in cell numbers was significant ($p<0.01$) when compared to PI-treated individuals, which is of interest because NNRTI are a newer form of treatment and their immunomodulatory effects have not been completely characterized yet. Analyzing PHA-activated CD8 T cells from a small cohort of patients on HAART and HIV-negative individuals (selection criteria was identical to CAF studies; untreated HIV-positive individuals were not tested due to lack of blood samples for additional work), we found that abnormal apoptosis was not the reason for the observed difference. Activation receptor expression analysis was also

performed to determine if any phenotypic differences between the groups might explain why NNRTI CD8 T cells were responding differently to culture conditions. We found that within the HIV-negative group, 2/7 expressed very high levels of CD28 *ex vivo* (NEG CD28hi), and 5/7 expressed much lower levels of CD28 (NEG CD28lo). In all groups tested, we found that CD38 expression was upregulated after PHA-stimulation, and either increased further or was maintained after PHA was removed. HLA-DR expression increased in all groups after PHA-stimulation, and in two of the groups (NNRTI and NEG CD28hi), HLA-DR expression returned to baseline levels (or lower) three days after PHA was removed. Within the other two groups (PI and NEG CD28lo), HLA-DR expression did not return to baseline at the end of the culture period. Of interest to us was the difference between PI and NNRTI. At baseline, both expressed similar levels of HLA-DR, and both increased expression similarly after PHA activation. Once PHA was removed, NNRTI expression of HLA-DR returned to baseline levels (47.4±18.3%) while PI expression remained as high as it was immediately after PHA activation (71.6±10.2%). It is not clear how this differential response to PHA activation would contribute to the differences in viability we observed. Commencement of HAART has been repeatedly associated with a decline in CD8 T cell activation [129-132], but similar to findings by others [123, 131], we have found that CD38 and HLA-DR expression is generally higher in patients on HAART compared with HIV-negative controls. It is possible that persistent viral challenge (even below the range of detection) in the bloodstream might maintain elevated levels of CD8 T cell activation. This activated state could render CD8 T cells more sensitive to PHA stimulation *in vitro*; if over activated, these cells might undergo Activation Induced Cell Death (AICD).

Although excessive apoptosis was not detected in our system, it is possible that death occurred between days 0 and 3 (after PHA stimulation and before cells were analyzed a second time for apoptosis levels), or between day 3 and day 6. However, this does not account for the observation that only NNRTI CD8 T cells were less numerous than PI. Both groups expressed similar levels of HLA-DR on day 0 and 3, yet differed on day 6. If CD8 T cells are indeed undergoing AICD in our system, it is possible that PI CD8 T cells are resisting AICD due to direct pro-survival effects conferred to them by PI treatment. It has been established that PI treatment has anti-apoptotic effects [133], possibly because the HIV protease (the target of PI) is similar to human caspases, which are involved in apoptosis. Furthermore, PI have been shown to prolong cell survival by blocking entry into the cell cycle [134]. Therefore, overactivated CD8 T cells from PI might resist AICD *in vitro*, and would persist over the entire culture period. This could explain the elevated levels of activated viable PI CD8 T cells that we observed in conjunction with the decrease in NNRTI CD8 T cells. This model is contrary to findings by others, who have demonstrated that CD8 T cells from patients on PI or on NNRTI therapy respond identically to PHA activation [124].

To date it is not clear which immune parameters are involved in CAF production, most notably in the context of HIV infection itself. We have shown that coculture with autologous HIV infected CD4 cells enhances CAF production in CD8 T cells from HIV-negative individuals. This suggests that, although CD8 T cells are capable of producing CAF without exposure to HIV or to HIV-infected cells, contact-mediated signaling from infected cells can increase CAF activity. Others have shown results similar to ours in

HIV-positive individuals, measuring CAF activity as the suppression of viral replication within the actual coculture [47, 78]. Our data shows that HIV-induced CAF increases are also observed in HIV-negative individuals, and that this suppression is observed at the level of gene transcription. It is not yet known whether the CAF increases shown here are HLA-restricted, and whether viral antigen presentation was involved in the CAF increases. These factors have been implicated in CAF activity previously [13, 75], but it is not yet known whether they are prerequisites for CAF activity, or rather enhancing factors. We have shown here that soluble factors secreted by HIV infected CD4 T cells do not increase CAF activity in HIV-negative individuals. Similarly, Yang *et al.* showed that in a CD8 T cell line, CAF production was HLA-restricted and cell-cell contact dependent, but that its subsequent antiviral actions were mediated by soluble factors and did not require HLA-restriction or cell-cell contact [79]. Therefore, it appears that contact-mediated signaling events between CD4 and CD8 T cells are required for the increases in CAF seen in our coculture system.

We developed a group of HTLV-1-transformed CD8 T cell lines, and some of these cell lines spontaneously produced CAF without external activation. All cells were in a permanently activated state due to their ubiquitous expression of HLA-DR, and further activation with PHA was able to significantly increase CAF production in all cell lines tested (Figure 6A). We found no correlation between spontaneous CAF production and activation levels, as indicated by the variability seen in CD38 expression in each cell line (Figure 6B). This is in agreement with other findings, where cell line-derived CAF was not dependent on cellular activation phenotypes [72, 73]. CAF derived from primary

CD8 T cells appears to be dependent on an activated phenotype [70, 71]. This contrast indicates that factors other than cellular activation are involved in the modulation of CAF production.

In addition to CD4 T cells, HIV infects CD8 T cells, yet it is not known whether infection of CD8 T cells modulates CAF production. We found that HIV-infected CD8 T cell lines showed increased suppressive activity towards LTR-driven gene expression. IFN-γ and IL-16 concentrations were determined, and a correlation between CAF increases and levels of both cytokines was observed. While IL-16 levels were far below those known to suppress viral replication *in vitro*, IFN-γ levels were high enough to possibly be responsible for the LTR suppression observed. While characterizing these cell lines, we found that depleting one cell line's supernatant of IFN-γ reduced LTR-suppressive activity from 58.1% to 41.7% (Appendix E). In this case, the relative concentration of IFN-γ in the transfection supernatant was 77.7 pg/ml, indicating that IFN-γ at that concentration was capable of mediating 16.4% suppression. In the cell line infection system, IFN-γ levels (Table 3) were similar to that seen in the depletion experiments, indicating that the increases in suppression we observed were likely due to increased levels of IFN-γ. Neutralizing antibody studies would indicate whether this cytokine was entirely responsible for the observed LTR suppression.

Conclusion

We have found that there is no significant increase or decrease in CAF activity in HIV-positive individuals being treated by HAART. Patients treated on NNRTI-based therapy

tended to have more potent CAF activity, but overall there was substantial variability within each group. A negative correlation between CAF and viral load was found in untreated HIV-positive individuals, and we found no correlation between CD4 T cell counts and CAF activity. We found that using replication-based assays to determine CAF activity does not necessarily reflect the ability of CD8 T cells to suppress LTR activation, demonstrating that future studies by others should include transcriptional suppression assays to produce more accurate data. We also corroborated previous findings that CAF activity can be induced by HIV infected CD4 T cells, and that cell-cell contact is required for CAF induction but not for its subsequent antiviral activity. Finally, we showed that HIV-infection of CAF-producing CD8 T cell lines increases their anti-viral capabilities, and that this action is most likely mediated by increases in IFN-γ and not in CAF. There is substantial uncertainty remaining as to the identity of CAF, its mechanism of action, and what factors mediate its production and function. Our results will help uncover the mechanisms by which CAF is produced, which in turn could lead to potential therapy alternatives, if a method to trigger CAF *in vivo* is found.

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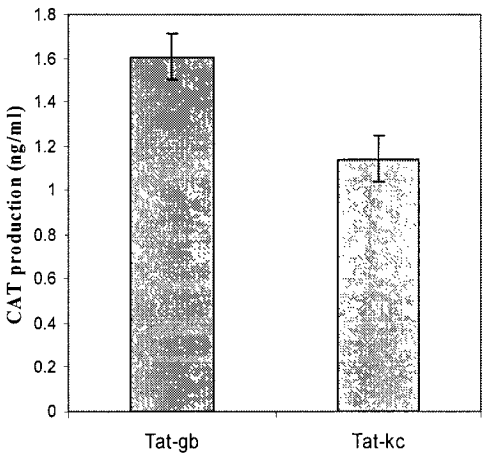
APPENDIX A. Optimization of LTR-CAT/Tat transfection for reproducibility



Two separate transfections were carried out, each with the same LTR-CAT construct and different Tat constructs (Tat-gb and Tat-kc). Transfected Jurkat cells were distributed into four separate wells and treated as described in Materials and Methods. CAT expression was determined for each sample, and average results are shown here.

APPENDIX B. Patient clinical information, suppression levels, cytokine secretion

i) PI

	Rx	CD4 cells/μl	%CD4	VL	CAF[b]	α-HIV[c]	IFN-γ[d] pg/ml	IL-16[d] pg/ml
A1	RTV/SQV	746	22.8	<[e]	0	55.2	4.86	3.4
A2	KAL	97	14.5	<	0	0	20.50	22.0
A3	RTV/SQV	315	21.2	<	0	31.5	1.00	23.0
A4	LPV	90	8.3	14672	5.2	27.6	10.00	81.5
A5	SQV/RTV	623	31.0	<	35.9	34.3	0.06	257.4
A6	NFV/SQV	644	26.6	<	33.3	24.2	<	316.6
A7	RTV/SQV	722	55.1	<	55.6	9.5	<	410.3
A8	IDV	1020	40.4	<	47.0	15.0	2.30	203.9
A10	FTV/RTV	536	25.1	<	0	16.0	0.23	141.9
A11	FTV	548	30.5	74	0	52.4	2.82	232.2
A12	ATZ	929	52.0	<	18.7	34.3	<	248.6
A13	FTV/RTV	319	21.1	<	26.3	32.8	<	126.6
A14	FTV/RTV	415	41.9	<	34.3	59.5	1.14	802.2

ii) NNRTI

	Rx	CD4 cells/μl	%CD4	VL	CAF	α-HIV	IFN-γ pg/ml	IL-16 pg/ml
B3	NVP	791	37.8	<	0	21.5	11.00	328.0
B5	EFV	667	33.1	<	0	0	10.00	143.0
B7	EFV	655	25.7	<	25.4	37.6	3.98	72.0
B8	NVP	492	24.9	<	0	69.2	<	47.5
B10	EFV	673	29.2	<	47.1	44.1	0.14	109.2
B11	EFV	475	17.8	<	46.0	61.4	0.02	184.0
B12	EFV	1202	43.8	<	51.7	41.0	<	247.1
B13	EFV	679	26.6	<	90.2	35.8	0.97	314.5
B14	EFV	1402	47.1	<	70.2	28.5	<	385.2
B15	EFV	886	37.9	<	78.4	48.4	<	192.4
B16	EFV	396	18.0	<	42.7	49.3	<	192.4
B17	EFV	829	50.6	<	58.1	49.3	0.34	58.1
B18	EFV	745	31.3	<	0	57.0	<	383.9

iii) Untreated

	CD4 cells/μl	%CD4	VL	CAF	α-HIV	IFN-γ pg/ml	IL-16 pg/ml
C1	361	20.2	97816	5.0	49.6	1.50	53.0
C2	8	1.4	153772	0	76.6	6.50	11.0
C3	640	40.5	6842	65.6	53.6	0.99	40.5
C4	339	29.6	1174	44.3	40.7	<	109.0
C5	307	21.2	5617	19.7	63.9	<	268.2
C6	410	29.9	218	30.1	60.7	<	229.8
C7	460	45.1	45456	16.4	36.7	<	186.8
C8	60	9.8	79402	12.9	39.5	<	57.3
C9	136	15.5	115233	9.8	60.4	<	230.8
C10	506	21.0	7413	0	43.8	0.70	375.7
C11	624	39.0	57060	0	59.8	<	229.2
C12	192	22.3	238115	50.9	42.5	<	129.6

iv) HIV-negative

	CAF	α-HIV	IFN-γ pg/ml	IL-16 pg/ml
D1	1.4	64.8	9.49	320.0
D2	19.3	49.9	9.49	215.0
D3	0	40.7	17.50	115.5
D4	34.6	31.5	25.50	43.5
D5	8.9	19.6	<	68.2
D6	0	49.3	<	113.1
D7	5.4	63.2	<	246.8
D8	19.2	61.4	<	140.9
D9	0	34.6	7.94	264.6
D10	33.7	43.5	10.16	316.8
D11	100	59.2	36.40	211.1
D12	65.4	54.8	1.20	232.6
D13	47.5	57.0	11.17	375.1

a) RTV, Ritonavir; SQV, Saquinavir; KAL, Kaletra (Lopinavir/Ritonavir); LPV, Lopinavir; NFV, Nelfinavir; IDV, Indinavir; ATZ, Atazanavir; NVP, Nevirapine; EFV, Efavirenz b) CAF: % suppression of LTR activation. c) α-HIV: % suppression of HIV replication. d) concentrations are adjusted to reflect the relative concentration of each cytokine in transfection supernatant. e) "<" signifies samples which were below the level of detection

APPENDIX C. Activation marker expression in PHA activated patient samples

i) PI

	%CD28$^+$			%CD38$^+$			%HLA-DR$^+$			% apoptosis		
	0	**3**	**6**	**0**	**3**	**6**	**0**	**3**	**6**	**0**	**3**	**6**
A15	81.7	89.0	91.3	66.8	87.8	91.3	72.7	88.0	83.3	3.2	0.8	18.3
A17	55.9	58.3	72.8	32.0	58.0	73.0	53.1	70.0	72.0	5.0	3.2	2.0
A18	68.5	82.4	89.1	38.9	68.1	83.3	54.5	62.6	58.3	1.2	3.7	1.6
A19	31.4	44.3	51.6	13.6	62.7	84.8	28.3	61.9	72.7	1.1	30.	0.6

ii) NNRTI

	%CD28$^+$			%CD38$^+$			%HLA-DR$^+$			% apoptosis		
	0	**3**	**6**	**0**	**3**	**6**	**0**	**3**	**6**	**0**	**3**	**6**
B10	54.8	69.4	82.2	42.1	75.4	92.5	52.5	65.0	74.3	1.1	4.9	1.7
B12	54.0	85.9	84.9	34.5	85.4	91.6	44.7	62.6	34.5	0.4	4.5	3.6
B13	69.7	83.4	80.3	49.2	79.8	95.6	47.6	41.7	42.9	1.5	2.1	3.1
B14	48.9	65.3	78.3	20.0	80.4	89.7	37.2	81.3	37.9	0.3	8.6	0.8

iii) HIV-negative

	%CD28$^+$			%CD38$^+$			%HLA-DR$^+$			% apoptosis		
	0	**3**	**6**	**0**	**3**	**6**	**0**	**3**	**6**	**0**	**3**	**6**
D6	90.3	96.0	95.8	28.9	87.6	94.6	15.5	59.3	10.8	0.4	2.0	0.3
D7	88.7	96.4	96.2	44.5	98.5	98.8	30.2	69.8	16.9	16.8	3.9	0.9
D9	26.9	24.7	37.4	20.9	26.9	88.1	5.0	15.1	10.6	0.2	4.1	1.1
D10	40.8	48.9	37.3	9.6	56.2	73.9	10.7	10.3	13.8	0.3	6.6	1.3
D11	27.8	46.1	51.4	14.3	52.8	92.0	1.5	6.3	14.9	0.1	0.9	0.7
D12	20.6	55.8	40.5	22.8	70.4	94.4	11.1	23.6	18.9	0.5	0.4	0.5
D13	23.6	56.4	46.1	10.7	63.3	93.3	3.1	7.3	13.3	0.4	0.4	0.4

APPENDIX D. CAF in CD8 T cells treated with cell-free supernatant from autologous HIV-infected CD4 T cells

CD4 donor	Da		Ty		Da		Ty	
CD8 donor	Dw		Dw		Al		Al	
HIV +/-	+	-	+	-	+	-	+	-
CAF	46.2	41.1	41.7	39.3	10.4	53.6	56.1	0

APPENDIX E. CD8 T cell line characterization

i) IFN-γ depletion of cell line XE3, and CAF data before and after depletion

	$[IFN\text{-}\gamma]_{pg/ml}$	CAF
Undepleted	332	58.1
Depleted	8	41.7

ii) CAF and activation marker expression

	CAF	%$CD28^+$	%$CD38^+$	%$HLA\text{-}DR^+$
A	55.4	1.0	42.0	99.8
B	0	2.1	35.3	99.8
C	5.4	3.1	36.1	99.6
D	30.9	0.7	14.8	99.6
E	29.0	1.7	32.6	99.8
F	0	1.8	28.5	99.7