Published in final edited form as:

Cell Immunol. 2011 ; 266(2): 154–164. doi:10.1016/j.cellimm.2010.09.011.

Secretion of MIP-1 β and MIP-1 α by CD8⁺ T-lymphocytes Correlates with HIV-1 Inhibition Independent of Coreceptor Usage

Kevin O. Saunders^{a,b}, Cavin Ward-Caviness^c, Robert J. Schutte^{a,f}, Stephanie A. Freel^{a,d}, R. Glenn Overman^{a,d}, Nathan M. Thielman^f, Coleen K. Cunningham^f, Thomas B. Kepler^{a,c}, and Georgia D. Tomaras^{a,b,c,d,g,*}

Kevin O. Saunders: Kevin.saunders@duke.edu; Cavin Ward-Caviness: Cavin.ward-caviness@duke.edu; Robert J. Schutte: Robert.schutte@duke.edu; Stephanie A. Freel: Stephanie.freel@duke.edu; R. Glenn Overman: Glenn.overman@duke.edu; Nathan M. Thielman: n.thielman@duke.edu; Coleen K. Cunningham: Coleen.cunningham@duke.edu; Thomas B. Kepler: keple003@mc.duke.edu

^aDuke Human Vaccine Institute, Duke University Medical Center, Durham, North Carolina, USA, 27710

^bDepartment of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina, USA, 27710

^cDepartment of Bioinformatics and Biostatistics, Duke University Medical Center, Durham, North Carolina, USA, 27710

^dDepartment of Surgery, Duke University Medical Center, Durham, North Carolina, USA, 27710

^fDepartment of Medicine, Duke University Medical Center, Durham, North Carolina, USA, 27710

^gDepartment of Immunology, Duke University Medical Center, Durham, North Carolina, USA, 27710

Abstract

CD8⁺ T-lymphocytes can utilize noncytolytic mechanisms to suppress HIV-1 replication through the secretion of soluble factors. The secretion of MIP-1 β , MIP-1 α , IP-10, MIG, IL-1 α , and interferon gamma correlated most strongly with soluble noncytolytic suppression ($p < 0.0001$). Since the noncytolytic response is impaired by histone hyperacetylation, we examined the ability of histone hyperacetylation to alter the expression of immune-related genes. MIP-1 α and IP-10 were also among the genes that were down-regulated by histone hyperacetylation. We define a multifactorial cytokine profile of CD8⁺T lymphocytes capable of mediating noncytolytic suppression of CXCR4-tropic HIV-1 replication.

Keywords

CD8⁺ T-lymphocyte; Noncytolytic Suppression; Cytokines; Chemokine; HIV-1

© 2010 Elsevier Inc. All rights reserved.

*Corresponding author. Mailing address: Rm 4079 MSRBII, 106 Research Drive, Duke University Medical Center, Durham, NC 27710, Telephone: +1 (919) 681-5598 Fax: +1 (919) 684-5230 gdt@duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Early *in vitro* studies determined that CD8⁺ T-lymphocytes can suppress human immunodeficiency virus type 1 (HIV-1) infection [1]. The importance of the CD8⁺ T-lymphocyte response to HIV-1 infection is highlighted by the appearance of HIV-1-specific CD8⁺ T-lymphocytes concurrent with the initial decline in HIV-1 viral load [2; 3], and the appearance of escape mutations within the first fifty days of infection [4]. Included in the initial CD8⁺ T-lymphocyte response during the decline in viral load is a subset of cells that suppress HIV-1 replication without lysis of infected cells [5]. *In vivo* studies using simian immunodeficiency virus infection of rhesus macaques, have shown the reduction in viremia is also mediated by CD8⁺ T-lymphocytes without the lysis of SIV-infected cells [6; 7; 8]; thus demonstrating the noncytolytic CD8⁺ T-lymphocyte response as an important component for the control of viremia that should be further characterized.

The noncytolytic CD8⁺ T-lymphocyte response includes the inhibition of HIV-1 transcription through unknown mediators [9; 10; 11]. The initial work of Walker and colleagues, which was later confirmed by several other groups, demonstrated that CD8⁺ T-lymphocytes could inhibit HIV-1 replication when grown in direct contact with infected cells, or across a semi-permeable membrane [12; 13; 14]. This result suggests the secretion of soluble antiviral factors as a mechanism for inhibition of HIV-1 replication. Although cell contact is not necessary for suppression in all cases, allowing cell contact generally increases the magnitude of suppression [12; 15]. Indeed, CD8⁺ T-lymphocytes that potentially suppress HIV-1 replication when in direct contact with infected targets may display only negligible suppression of HIV-1 replication across semi-permeable membranes [12]. The increased suppression of HIV-1 replication is not due to target cell lysis, as the number of CD4⁺ T-lymphocytes does not change and these CD8⁺ T-lymphocytes demonstrated minimal cytolytic activity in chromium release assays [15; 16]. Thus, potent contact-mediated and soluble noncytolytic suppression of HIV-1 replication are distinct responses.

The factor(s) involved in soluble noncytolytic suppression have been difficult to identify. Fractionation of supernatants from CD8⁺ T-lymphocytes demonstrated that several fractions exhibit moderate suppressive activity, and that combining these fractions produces suppression equal to unfractionated supernatant [17]. Moreover, prothymosin- α secretion by CD8⁺ T cells can inhibit HIV replication in macrophages but not T-lymphocytes [18]. Taken together, it is likely that noncytolytic suppression is mediated by multiple factors. For CCR5-tropic viruses, it is clear that the soluble secretion of the natural ligands, β -chemokines, from CD8⁺ T cells [19] and CD4⁺ T cells [20; 21] can mediate virus inhibition. For CXCR4- tropic viruses that are not sensitive to β -chemokine mediated inhibition; the natural ligand, stromal derived factor 1 (SDF-1), was examined and not found to be responsible for suppression [22]. However, we have found that MIP-1 β along with CD107 on CD8⁺ T cells was associated with CD8⁺ T-lymphocytes capable of mediating virus inhibition of both CXCR4 and CCR5 viruses [23], suggesting that this β -chemokine may possess more direct or indirect inhibitory functions than previously known. Identification of the molecules secreted by noncytolytic suppressive CD8⁺ T-lymphocytes may define the factors responsible for mediating virus inhibition as well as define a cytokine signature of noncytolytic suppression.

We previously demonstrated that the production of a subset of the CD8⁺ T cell derived factors associated with noncytolytic suppression is regulated by histone acetylation [24]. Exposure of CD8⁺ T-lymphocytes to an inhibitor of histone deacetylation led to diminished contact-mediated and soluble noncytolytic suppression of HIV-1. Histone acetylation is generally associated with the activation of transcription [25]. Therefore, it was proposed that negative regulators of CD8⁺ T cell antiviral factors were transcribed after inhibition of

histone deacetylation, which resulted in the decrease in suppressive capacity [24]. The ability of histone acetylation to decrease the expression of genes involved in the immune response to HIV-1, could aid in the identification of factors associated with noncytolytic suppression. Thus, the genes involved in the immune response that are regulated by histone deacetylation in CD8⁺ T-lymphocytes should be defined.

It is estimated that less than 1% of HIV-1 infected individuals maintain low levels of viremia and are asymptomatic in the absence of anti-retroviral therapy [26; 27]. Virus controllers (VCs) are a distinct subset of asymptomatic anti-retroviral naïve HIV-1 infected individuals with normal CD4 counts and viral loads less than 5000 for at least one year [23]. It is unclear what types of immune responses result in sustained control of HIV-1 viremia in these individuals. Multiple cohorts containing asymptomatic HIV-infected subjects have reported overrepresentation of the controlling HLA allele B*57 [28; 29], which is responsible for a robust cytotoxic CD8⁺ T-lymphocyte response [reviewed in 30]. The virus controllers in this study display potent virus inhibition, which can include both the cytotoxic and noncytolytic mechanisms of suppression of HIV-1 replication [23]. Contact-mediated and soluble noncytolytic suppression have been observed with CD8⁺ T-lymphocytes from untreated asymptomatic HIV-1 infected individuals [31; 32], and could contribute to the virus inhibition exerted by our cohort of virus controllers. Thus, the relative contribution of the soluble noncytolytic antiviral response in this cohort of virus controllers at both the individual donor and cohort-level warrants further study.

In this cohort of virus controllers, all subjects demonstrated the ability to inhibit HIV-1 replication in a CD8⁺ virus inhibition assay [23], but only one of these subjects also had strong soluble suppression. We further probed the soluble activity from primary CD8⁺ T-lymphocytes and from a CD8⁺ T-lymphocyte cell line to determine if particular cytokine concentrations correlated with the amount of suppression of HIV-1 replication. We found that VC CD8⁺ T-lymphocytes exhibiting soluble noncytolytic suppression secreted higher levels of MIP-1 α , MIP-1 β , IP-10, and MIG than primary CD8⁺ T-lymphocytes without this type of response. Additionally, the expression in CD8⁺ T-lymphocytes of 17 genes involved in the host immune response was regulated by histone deacetylation; furthermore, 7 of these genes were more highly transcribed by CD8⁺ T lymphocytes with soluble noncytolytic suppression. We have defined a soluble cytokine profile that significantly correlates with the noncytolytic response. This profile along with the cellular markers of CD8⁺ T cell responses (MIP-1 β and CD107) [23] may enable better characterization and measurement of protective CD8⁺ T cell responses.

2. Materials and Methods

2.1. Study Participants

Virus controllers were defined as HIV-1-infected individuals with CD4⁺ T cell counts greater than 385 cells/ μ L and viral loads less than 5000 copies/mL. Virus controllers were recruited from the Duke Adult and Pediatric Infectious Disease Clinics. Informed consent was obtained from all study participants according to a Duke University IRB approved protocol. Seronegative PBMCs were isolated from blood donors from the Red Cross.

2.2 Cell culture

PBMCs from seronegative donors or virus controllers were activated for 3 days with anti-CD3 and anti-CD28 in RPMI (20% FBS, 100 U/mL Penicillin, 100 μ g/mL Streptomycin, 20 U/mL IL-2). To enrich for CD4⁺ cells, the CD8⁺ cells in PBMCs were labeled using CD8 MicroBeads (Miltenyi Biotechnology) and depleted with an autoMACS Cell Separator (Miltenyi Biotechnology). CD8⁺ cells were isolated using the CD8⁺ T Cell Isolation kit and autoMACS Cell Separator (Miltenyi Biotechnology). CD8-depleted PBMC contained less

than 3.5% CD8⁺ cells, and isolated CD8⁺ cells contained less than 2.2% CD4⁺ cells as determined by Flow Cytometry (FACS Caliber). The HVS-transformed CD8⁺ T-lymphocyte cell line from an asymptomatic HIV-1 infected subject, JR-HVS [33], was cultured in AIM-V (20 % FBS, 40 U/mL IL-2). TZM-bl cells were cultured in DMEM (10% FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin).

2.3. Valproic Acid Treatment of CD8⁺ T-lymphocytes

CD8⁺ T-lymphocytes were cultured in AIM-V supplemented with 2.0 mM valproic acid (Calbiochem) or PBS as a vehicle control for 16 h at 1.8×10^6 cells/mL. After 16 hours of pretreatment, a subset of the CD8⁺ T-lymphocytes was washed with PBS, resuspended in complete growth media, and used as effector cells in direct contact CD8⁺ suppression assays. Total RNA was extracted from the remaining treated cells to be analyzed by Real-time Reverse transcriptase-PCR Array (SABiosciences).

2.4. Direct Contact CD8⁺ T-lymphocyte Suppression Assay

Pooled seronegative CD4⁺-enriched cells were infected with a single cycle CXCR4-tropic HIV-1_{NL4-3} pseudotyped luciferase reporter virus for 2 h in a 50 mL conical tube [34; 35]. This pseudotyped virus contains a modified NL4-3 genome that encodes luciferase at the *nef* locus, and is limited to a single round of infection by frameshift mutated *env*[35]. HIV-1 infected CD4⁺ cells were plated with CD8⁺ T cells at a 0.5:1 effector to target (E:T) ratio or cultured alone. The cells were lysed with Luciferase Cell Culture Lysis reagent (Promega) and one freeze-thaw cycle 72 hours post infection. Luciferase expression was quantified with the Luciferase Assay System (Promega) on a Centro LB luminometer (Berthold) operating Microwin Software. The level of HIV replication in the CD8⁺ T cell co-cultures was calculated as the log₁₀ reduction in replication compared to infected CD4⁺ T cells as follows: $\log_{10}([\text{luciferase in the CD8}^+ \text{ T cell co-culture}] / [\text{luciferase expression in the replication control}])$.

2.5. Soluble CD8⁺ T-lymphocyte Suppression Assay

TZM-bl cells were trypsinized and washed once with complete growth media. 1.6×10^4 TZM-bl cells were plated in the lower chamber of a 96-well 0.4 µM Pore Transwell system (Corning) in complete AIM-V. The CXCR4-tropic HIV-1_{IIIIB} was used to infect the TZM-bl cells at 0.5 multiplicities of infection. CD8⁺ T-lymphocytes were plated in the upper chamber of the Transwell system at various effector to target ratios at the time of infection of the target cells. 48 hours post-infection LTR-driven luciferase expression was measured using the BriteLite Plus system (Perkin Elmer). The percent suppression of HIV-1 replication was calculated as follows: $[1 - (\text{luciferase expression by TZM-bl cells in the presence of CD8}^+ \text{ T cells}) / (\text{luciferase expression in the absence of CD8}^+ \text{ T cells})] \times 100\%$.

2.6. Real-time Reverse-Transcriptase PCR Array

Total RNA was extracted from valproic acid and PBS-treated CD8⁺ T-lymphocytes using QIAshredders (Qiagen) and the RNeasy Plus kit (Qiagen) and following the manufacturer's protocol. To determine RNA quality, 100 ng of RNA was fractionated by agarose gel electrophoresis followed by visualization of ribosomal RNA with SYBR Safe (Invitrogen) and ultraviolet light. RNA was purified of contaminating genomic DNA and reverse transcribed with the RT² First Strand cDNA Kit (SABiosciences). cDNA was analyzed by Human Common Cytokine and Human Chemokine and Receptor PCR arrays using RT² SYBR® Green / ROX® qPCR Master Mix (SABiosciences) per the manufacturer's conditions on a 7500 Real-time PCR machine (Applied Biosystem). Changes in gene expression in valproic acid and PBS-treated CD8⁺ T cells was calculated using the $\Delta\Delta C_t$ analysis of threshold cycles by the SABioscience PCR Array Data Analysis Web

Portal [36]. For this analysis gene expression was normalized to GAPDH, β -actin, $\beta 2$ microglobulin, Ribosomal protein L13a, and Hypoxanthine phosphoribosyltransferase 1.

2.7. Cytokine and Chemokine Luminex Analysis

Cell culture supernatant was collected prior to luciferase measurement at the conclusion of soluble suppression assays. Triton X-100 was added to the supernatant to a final concentration of 1% to inactivate HIV-1 virions. The supernatants were diluted 2.5 fold in AIM-V and stored at -80°C until the luminex analysis was performed. Seventy-six individual human cytokines and chemokines were examined using Milliplex MAP Human Cytokine/Chemokine Panels I, II, and III per the manufacturer's protocol on a Bio-Rad Luminex machine. Infected target cells alone were considered the baseline and are designated as 0:1 E:T ratio. Spearman correlations were performed with Prism 5.0a (Graphpad). Hill functions were calculated for percent suppression and protein concentration with the R statistical software package using the following formula: $y = 100 \times x^r / (x^r + c^r)$, where y = percent suppression, x = protein concentration, and $r = 1$.

3. Results

3.1. Soluble CD8⁺ T-lymphocyte noncytolytic suppression of HIV-1 replication is rare among Virus Controllers

Soluble CD8⁺ T-lymphocyte noncytolytic suppression has been assessed in asymptomatic HIV-1 positive patients at different stages of infection [37; 38], however its presence in this cohort of virus controllers (VC) has yet to be examined. Therefore, we examined the ability of CD8⁺ T-lymphocytes from 19 virus controllers to inhibit HIV-1 replication when separated from target cells by a semi-permeable membrane. These virus controllers have been shown previously to inhibit virus replication in virus inhibition assays [23]. The HVS-transformed cell line, JR-HVS with documented noncytolytic suppressive capacity, was used as a positive control for noncytolytic suppression [11; 33; 34]. CD8⁺ T-lymphocytes from seronegative donors were used as negative controls for soluble noncytolytic suppression. Ten of the 19 (53%) VC CD8⁺ T-lymphocytes showed no soluble noncytolytic suppression of HIV-1 replication (Fig. 1). Eight (42%) other VC CD8⁺ T-lymphocytes possessed levels of suppression that were similar to seronegative CD8⁺ T-lymphocytes. VC18 CD8⁺ T-lymphocytes was the only virus controller that demonstrated significantly better soluble noncytolytic suppression than seronegative CD8⁺ T-lymphocytes (Student's t -test; $p < 0.01$), although these cells did not suppress as well as JR-HVS CD8⁺ T-lymphocytes. Thus, VC18 and JR-HVS were classified as soluble suppressors (SS) and the seronegatives and remaining VCs were referred to as non-soluble suppressors (NSS). Overall, these findings suggest that potent soluble CD8⁺ T-lymphocyte noncytolytic suppression is quite rare or that very close proximity of cells that cannot be achieved with the transwell culture system is required.

3.2. CD8⁺ T-lymphocytes with soluble noncytolytic suppression secrete high concentrations of multiple cytokines

Cytokine secretion from CD8⁺ T-lymphocytes with soluble noncytolytic suppression has not been fully characterized. Thus, we used a multiplex bead-based assay to analyze 76 different cytokines potentially secreted by CD8⁺ T-lymphocytes during soluble noncytolytic suppression of HIV-1 replication. The 76 cytokines consisted of interleukins (ILs), colony stimulating factors (CSFs), interferons (IFNs), chemokines, and proinflammatory cytokines. The concentration of cytokines was compared between SS and NSS-CD8⁺ T-lymphocytes to identify cytokines that may distinguish soluble noncytolytic suppressive activity. In particular, we focused on cytokines that trended upward as the E:T ratio increased, and were expressed at least 3-fold higher by SS CD8⁺ T-lymphocytes than the NSS-CD8⁺ T-

lymphocytes from NEG1 and VC3 at a 1:1 E:T ratio. Among the interleukins, colony stimulating factors, interferons, and proinflammatory cytokines analyzed, JR-HVS and VC18 CD8⁺ T-lymphocytes secreted higher levels of IFN- γ and IL-1 α than virus controller and seronegative CD8⁺ T-lymphocytes that lack soluble noncytolytic suppression (Fig. 2). Specifically, JR-HVS secreted 31 and 38-fold higher amounts of IFN- γ and IL-1 α , respectively, compared to NSS VC3 at a 1:1 E:T ratio. VC18 secreted 6 and 24-fold more of these same cytokines than VC3, respectively, at a 1:1 E:T ratio. Analysis of the chemokine responses revealed 5 proteins that were secreted highly by SS-CD8⁺ T-lymphocytes (Fig. 2). Among these chemokines were the β -chemokines, MIP-1 α and MIP-1 β . Compared to VC3, JR-HVS and VC18 secreted at least 7.5-fold more of these β -chemokines. Additionally, the α -chemokines I-TAC, MIG, and IP-10 were secreted highly by SS-CD8⁺ T-lymphocytes (Fig. 2). At a 1:1 E:T ratio, the concentration of these chemokines were at least 60-fold greater in JR-HVS and VC18 than VC3 cultures. In total, we found that 7 of the 76 (9.2%) cytokines were secreted at least 3-fold higher by SS-CD8⁺ T-lymphocytes than NSS-CD8⁺ T-lymphocytes.

3.3. The cytokine profiles of JR-HVS and VC18 CD8⁺ T-lymphocytes differ in magnitude and composition

Since VC18 and JR-HVS CD8⁺ T-lymphocytes possess different magnitudes of soluble noncytolytic suppression (Fig. 1), we hypothesized that there should be differences in the magnitude and/or identity of the cytokines comprising their responses. Indeed, JR-HVS secreted more IFN- γ , IL-1 α , MIP-1 α , and MIP-1 β than VC18 (Fig. 2). I-TAC, MIG, and IP-10 were also secreted higher by JR-HVS than VC18 CD8⁺ T-lymphocytes. Additionally, we found that IL-13, TNF- α , GM-CSF, and I-309 were secreted highly only in suppression cultures containing JR-HVS CD8⁺ T-lymphocytes (Fig. 3A). Thus, both the magnitude and composition of the soluble noncytolytic suppressive response differed between JR-HVS and VC18 CD8⁺ T-lymphocytes.

3.4. Other cytokines that are not components of the noncytolytic suppressive cytokine profile

Numerous cytokines have been reported to inhibit HIV-1 replication, and some of which have been proposed to have a role in noncytolytic suppression. We analyzed the presence of these antiviral cytokines in transwell cultures containing SS and NSS-CD8⁺ T-lymphocytes. IFN- α , IL-4, IL-10, and IL-16 have been documented as anti-HIV factors [39; 40; 41; 42], however we observed low, indistinguishable levels of these cytokines in our assays across different CD8⁺ T-lymphocytes at various E:T ratios (Fig. 3B). Furthermore, we did not observe distinguishable concentrations of the anti-HIV factors MDC [43], TARC [44], or SDF-1 [45], between SS-CD8⁺ T-lymphocytes and NSS-CD8⁺ T-lymphocytes.

3.5. MIP-1 β and MIP-1 α strongly correlate with the magnitude of soluble noncytolytic suppression of CXCR4- tropic virus

To further identify cytokines that distinguish soluble noncytolytic suppressive CD8⁺ T-lymphocytes, we correlated the concentration of each cytokine with the percent suppression in the CD8⁺ T-lymphocyte transwell culture. We hypothesized that if the cytokine was an important indicator of soluble suppression then the cytokine should correlate with soluble suppression by VC, JR-HVS, and seronegative CD8⁺ T-lymphocytes. Therefore, we combined the protein concentrations and percent suppression into one large data set for analysis. Among all 76 cytokines, MIP-1 β most strongly correlated with soluble noncytolytic suppression by all of the different CD8⁺ T-lymphocytes (Table 1, Spearman $r = 0.891$, $p < 0.0001$). The concentration of MIP-1 β possessed a nonlinear relationship with percent suppression (Fig. 4) suggesting that a certain threshold must be reached for the most potent suppression to occur. MIP-1 α , IP-10, and MIG were also strongly correlated with

soluble noncytolytic suppression (Spearman $r = 0.867, 0.827, \text{ and } 0.801$ respectively, $p < 0.0001$ for all). Concentrations of IL-1 α and IFN- γ correlated strongly with soluble noncytolytic suppression, although not as well as MIG (Spearman $r = 0.799, p < 0.0001$ for both). In concordance with MIP-1 β , these cytokines possessed a nonlinear relationship with percent suppression also (Fig. 4). These cytokines strongly correlated with soluble noncytolytic suppression whether suppression by JR-HVS or VC18 CD8 $^{+}$ T-lymphocytes was analyzed individually or combined together (Table 1). These cytokines were also identified as cytokines secreted higher by both SS-CD8 $^{+}$ T-lymphocytes than NSS-CD8 $^{+}$ T-lymphocytes (Fig. 2). Except for I-TAC, the cytokines with Spearman correlation coefficients below 0.798 were not secreted highly by both SS-CD8 $^{+}$ T-lymphocytes. I-TAC was expressed highly by SS-CD8 $^{+}$ T-lymphocytes, but its concentration varied in cultures with relatively low levels of soluble suppression, which decreased its correlation coefficient (Fig. 2). In total, the concentrations of MIP-1 β , MIP-1 α , IP-10 and MIG, as well as IL-1 α and IFN- γ were indicative of all levels of soluble noncytolytic suppression and distinguish SS and NSS-CD8 $^{+}$ T-lymphocytes.

3.6. Histone hyperacetylation decreases noncytolytic suppression and alters the expression of cytokine and cytokine receptors

We previously reported that inhibiting histone deacetylation decreases the ability of CD8 $^{+}$ T-lymphocytes to suppress HIV-1 infection [24]. We sought to define the changes in expression of genes with immune function that may account for the attenuation of CD8 $^{+}$ T-lymphocyte noncytolytic suppression. SS-CD8 $^{+}$ T-lymphocytes were incubated in a histone deacetylase inhibitor, valproic acid (VPA), and subsequently used as effector cells in direct contact CD8 $^{+}$ T-lymphocyte suppression assays. HIV-1 replication was reduced by 1.25 and 1 log in the presence of untreated JR-HVS and VC18 CD8 $^{+}$ T-lymphocytes (Fig. 5A). After the inhibition of histone deacetylation, suppression of HIV-1 replication by JR-HVS and VC18 CD8 $^{+}$ T-lymphocytes was significantly reduced (Student's t -test; $p < 0.05$ and $p < 0.01$ respectively). RNA was isolated from a portion of the CD8 $^{+}$ T-lymphocytes that were used as effectors in the suppression assays to examine changes in gene expression. The fold change in expression of 164 genes, involved in the host immune response, after inhibition of histone deacetylation was determined for JR-HVS and VC18 CD8 $^{+}$ T-lymphocytes. A 3-fold change in expression cut-off was determined based on five times the standard error of the mean fold change for β -actin. In concordance with the function of histone deacetylases as transcription repressors [reviewed in 46], gene expression increased more frequently than decreased after inhibition of histone deacetylation (Fig. 5B). In JR-HVS CD8 $^{+}$ T-lymphocytes, 15 genes were down-regulated and 23 genes were up-regulated after inhibition of histone deacetylation (Fig. 5B). In VC18 CD8 $^{+}$ T-lymphocytes, inhibition of histone deacetylation led to the down-regulation of 4 genes and up-regulation of 54 genes (Fig. 5B). BMP4, IL-1F6, and IL-2 were up-regulated in both cell types (Table 2). Since inhibition of histone deacetylation decreased suppression, we hypothesized that the molecules important for suppression were decreasing in expression. Therefore, we chose to focus on genes whose expression was down-regulated after treatment. MIP-1 α and Lymphotoxin β (LTB) were the only genes down-regulated in both types of CD8 $^{+}$ T-lymphocytes (Fig. 5C and 5D). M-CSF and Lymphotoxin α (also termed TNF- β ; LTA) were down-regulated in both CD8 $^{+}$ T cell types, although these genes were only greater than 3-fold down-regulated in VC18 CD8 $^{+}$ T-lymphocytes. Six of the cytokines identified in our protein expression analysis were also down-regulated in JR-HVS CD8 $^{+}$ T-lymphocytes after inhibition of histone deacetylation (Table 3). MIP-1 α and IP-10 correlated highly with soluble noncytolytic suppression and were down-regulated in SS-CD8 $^{+}$ T-lymphocytes by histone hyperacetylation (Table 3). I-TAC was also secreted by both SS-CD8 $^{+}$ T-lymphocytes and down-regulated by histone hyperacetylation in JR-HVS cells (Fig. 2 and 5C). GM-CSF, IL-13, and I-309 were down-regulated by histone hyperacetylation in JR-HVS CD8 $^{+}$ T-lymphocytes (Fig. 5C), and

secreted uniquely by JR-HVS CD8⁺ T-lymphocytes during noncytolytic suppression (Fig. 3A). Histone hyperacetylation downregulated LTB, but its secretion could not be assessed since it is a transmembrane protein. Taken together, histone deacetylation regulates the expression of a subset of genes whose secretion directly correlates with soluble noncytolytic suppression.

3.7. MIP-1 α is regulated by histone deacetylation and expressed highly in CD8⁺ T-lymphocytes with soluble noncytolytic suppression

The identification of genes down-regulated by histone hyperacetylation provided us with a set of candidate genes that may be indicative of a SS-CD8⁺ T-lymphocyte phenotype. To identify genes that may be important markers of SS-CD8⁺ T-lymphocytes, we examined the mRNA transcript levels of each of the candidate genes in untreated CD8⁺ T-lymphocytes for those genes more highly expressed in SS-CD8⁺ T-lymphocytes. MIP-1 α was expressed significantly higher in JR-HVS and VC18 CD8⁺ T-lymphocytes compared to seronegative CD8⁺ T cells (Student's *t*-test, *p* = 0.0003 and 0.0437 respectively; Fig. 6A). M-CSF, LTA, and I-309 were also expressed higher in SS-CD8⁺ T-lymphocytes compared to seronegative cells, with the increase being significant only for JR-HVS CD8⁺ T-lymphocytes (*t*-test, *p* = 0.0002, 0.0165, and 0.0062 respectively). Additionally, MCP-1 and BCA-1 were expressed higher in VC18 and significantly higher in JR-HVS CD8⁺ T-lymphocytes than NSS-CD8⁺ T-lymphocytes (*t*-test *p* = 0.015 and 0.033 respectively). Although IL-3 was expressed higher by SS-CD8⁺ T-lymphocytes, the increase was not significant.

Similar to the protein analysis, JR-HVS and VC18 CD8⁺ T-lymphocytes expressed different magnitudes of the identified genes (Fig. 6). Although VC18 expressed higher levels than seronegative CD8⁺ T-lymphocytes for 7 of the 17 genes identified, these levels were lower than JR-HVS CD8⁺ T-lymphocytes for each of the genes. In addition to expressing higher levels of the above 7 genes, JR-HVS CD8⁺ T-lymphocytes expressed significantly high levels of IL-9, GM-CSF, IL-13, and IL-8 (Fig. 6B). This difference further demonstrates the heterogeneity between cells exhibiting noncytolytic suppression. Lastly, not all of the genes that were down-regulated in CD8⁺ T-lymphocytes were expressed higher in SS-CD8⁺ T-lymphocytes than NSS-CD8⁺ T-lymphocytes. The mRNA transcripts of the remaining 6 genes were not increased in SS-CD8⁺ T-lymphocytes (Fig. 6C).

4. Discussion

A comprehensive description of a suppressive CD8⁺ T-lymphocyte response may aid in determining the type of CD8⁺ T-lymphocyte response that should be elicited by therapeutics or a protective HIV-1 vaccine. We detail here a comprehensive analysis of cytokine expression, using two approaches, from primary CD8⁺ T lymphocytes and JR-HVS CD8⁺ T-lymphocytes capable of noncytolytic HIV-1 inhibition. We analyzed the mRNA levels of 164 genes and the secretion of 76 different proteins to compile a profile of cytokines expressed by CD8⁺ T-lymphocytes exhibiting soluble noncytolytic antiviral activity. These two approaches identified 19 different cytokines that may be involved in noncytolytic suppression (Table 3). Multiple genes were identified in both approaches, underscoring the multifactorial nature of this response (Table 3). IP-10 protein concentrations directly correlated with soluble noncytolytic suppression, but mRNA levels were shown to be similar among seronegative, VC18, and JR-HVS CD8⁺ T-lymphocytes. Quantification of IP-10 mRNA is difficult since it has a high turnover rate and requires stabilization by other factors [47], which may account for why the mRNA levels were not different among the different types of CD8⁺ T-lymphocytes. Thus in understanding the noncytolytic response, protein analyses should be done to ensure that important genes are not omitted.

Additionally, we analyzed soluble noncytolytic suppression in a panel of virus controllers and found this facet of the response was rare in our cohort of virus controllers. This finding supports a previous claim that soluble noncytolytic suppression in particular does not correlate with high CD4 counts or with clinical status [32]. However, these individuals do exhibit contact mediated suppression indicating that they suppress HIV-1 replication through alternative mechanisms [23]. This observation is similar to those made with other types of asymptomatic HIV-infected individuals [48]. VC18 was the only virus controller that significantly suppressed HIV-1 through soluble mediators. VC18 has been infected for more than 4 years and maintains a CD4 count of approximately 1550 CD4⁺ cells/ μ L. Apart from one viral blip of 2000 copies/mL, the viral load of VC18 remains close to 150 copies/mL with 1 undetectable reading within the last year. VC18 is also positive for HLA-B*27, an HLA-B allele associated with slower progression to AIDS [49]. However, Saez-Cirion *et al.* have shown that in at least one instance HLA-B*27 positive HIV controllers lack soluble noncytolytic suppression despite potent contact-mediated virus inhibition [48].

We extended a previous finding that epigenetics regulates the ability of CD8⁺ T-lymphocytes to suppress HIV-1 replication by analyzing the changes in gene expression due to histone hyperacetylation. This study identified genes with immune functions regulated by histone deacetylation in CD8⁺ T-lymphocytes. Specifically, LTB and MIP-1 α were strongly regulated by histone deacetylation in both JR-HVS and VC18 CD8⁺ T-lymphocytes. However, many other factors were not similarly regulated in these cells. As JR-HVS CD8⁺ T cells are highly activated transformed cells that express high levels of cytokines, epigenetics may regulate gene expression differently in these cells. Furthermore, epigenetic regulation of noncytolytic suppression may be due to changes in multiple genes involved in noncytolytic suppression that vary between CD8⁺ T-lymphocytes. This observation may explain why purification of a single factor that blocks HIV-1 transcription has proven difficult. Epigenetics only regulates a portion of the suppressive response, as noncytolytic suppression is not completely blocked by an inhibitor of histone deacetylases. Therefore, this approach provided a way to focus on a subset of genes that may be important, but is not an exhaustive screen.

Our profile includes several genes that have been studied previously in the context of the CD8⁺ T-lymphocyte noncytolytic suppression. Among these genes were MIP-1 α , MIP-1 β , IL-13, I-309, IP-10, and GM-CSF [17; 19; 50; 51; 52; 53]. I-309 and IP-10 mRNA levels in CD8⁺ T cells from an HIV-1 infected subject have been previously correlated with contact-mediated noncytolytic suppression [53]. Our study corroborates this finding for soluble noncytolytic suppression with protein and mRNA analysis from multiple subjects, and suggests that these cytokines could be markers of a noncytolytic suppressive CD8⁺ T-lymphocyte response. Although our study did not address the function of the strongly correlated cytokines, several lines of investigation have shown MIP-1 β to be particularly important in HIV-1 pathogenesis. The β -chemokines are most noted for their inhibitory effects on HIV-1 entry [19], but there is some evidence that they may also inhibit HIV-1 replication after entry, consistent with noncytolytic suppression [54]. In a previous study, MIP-1 β , in addition to CD107, was identified as a marker strongly associated with virus inhibition by virus controller and HIV-1 vaccinee CD8⁺ T cells [23]. Recent work indicates that MIP-1 β positive CD8⁺ T-lymphocytes exert immune pressure early in HIV-1 infection (Ferrari G, personal communication). Also, Cocchi *et al.* has reported that the ability of CD8⁺ T-lymphocytes to secrete high levels of MIP-1 β and MIP-1 α correlates with an asymptomatic HIV-1 infection [55].

IL-13, I-309, and GM-CSF are expressed in CD8⁺ T-lymphocytes with noncytolytic suppression [17; 52], however their role in suppression is still debated. Notably, IL-13 inhibits HIV-1 replication in monocytes without disrupting integration or maturation of the

virus [56]. Therefore, the mechanism of IL-13 is similar to the mechanism proposed for noncytolytic suppression. GM-CSF has also been shown to inhibit HIV-1 replication in monocytes [57], suggesting it could be important for suppression of HIV-1 replication in CD4⁺ T-lymphocytes. IL-8 is also intriguing as it has been shown to inhibit both HIV-1 entry [58] and transcription [59]. I-309 [51], and MCP-1 [60] all inhibit HIV-1 entry, but effects on HIV-1 gene expression have yet to be determined. Future studies should aim to distinguish whether our identified genes are solely markers of a suppressive phenotype, or the suppressive mediators of the noncytolytic response.

Within the suppressive profile are cytokines that are induced by or elicit similar signaling pathways. The interferon gamma response genes, IP-10 and MIG, as well as IFN- γ itself were found to directly correlate with soluble noncytolytic suppression. Additionally, the IFN- γ response gene I-TAC was secreted highly by SS-CD8⁺ T-lymphocytes. IP-10, MIG, and I-TAC are ligands for CXCR3 [61], thus demonstrating redundancy in the cytokine profile of SS-CD8⁺ T-lymphocytes. GM-CSF and IL-3 are regulated by histone deacetylation and were differentially expressed by SS and NS-CD8⁺ T-lymphocytes. The receptors for these cytokines belong to the GM-CSF receptor family, and are heterodimers containing the same signaling common β chain [62; 63]. Interestingly, signaling of the common β chain and IFN- γ receptor leads to activation of STAT1 [64; 65], which has been shown to be required for noncytolytic suppression of HIV-1 [66]. This result may explain why neutralization of only one or a few cytokines has not impacted noncytolytic suppression since the cytokines in this response may have redundant signaling. Thus, future investigation could address the inhibition of signaling molecules upstream of STAT1 activation to elucidate the mechanisms of noncytolytic suppression.

Acknowledgments

We thank Michael Archibald for technical assistance with suppression assays, and Thaddeus Gurley and Dr. M. Anthony Moody for flow cytometry support. We also express our gratitude to Dr. John Schmitz of the UNC-CH CFAR Immunology Core for performing HLA typing. We thank the Duke CFAR Clinical Core Investigators: Drs. Sunita Patil, Jason Stout, Gary Cox, David Holland, Charles Hicks, Suzanna Naggie, Mehri McKeller, Deverick Anderson, Ann Mosher, Vivian Chu, Steve Taylor and Ross McKinney and the patients and staff of the Duke Adult and Pediatric Infectious Disease Clinics. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: T2M-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. This work was supported by grants RO1-AI-052779 (G.D.T.), F31-AI-078715 (K.O.S.), Molecular Virology and Clinical Cores of the Duke University Center for AIDS Research P30-AI-64518 awarded by the National Institute of Allergy and Infectious Disease.

References

1. Walker CM, Moody DJ, Stites DP, Levy JA. CD8⁺ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science*. 1986; 234:1563–6. [PubMed: 2431484]
2. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol*. 1994; 68:4650–5. [PubMed: 8207839]
3. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8⁺ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*. 1994; 68:6103–10. [PubMed: 8057491]
4. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV, Keele BF, Learn GH, Turnbull EL, Salazar MG, Weinhold KJ, Moore S, Letvin N, Haynes BF, Cohen MS, Hraber P, Bhattacharya T, Borrow P, Perelson AS, Hahn BH, Shaw GM, Korber BT, McMichael AJ. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med*. 2009; 206:1253–72. [PubMed: 19487423]
5. Levy JA, Mackewicz CE, Barker E. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8⁺ T cells. *Immunol Today*. 1996; 17:217–24. [PubMed: 8991383]

6. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, Irwin CE, Safrit JT, Mittler J, Weinberger L, Kostrikis LG, Zhang L, Perelson AS, Ho DD. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med*. 1999; 189:991–8. [PubMed: 10075982]
7. Klatt NR, Shudo E, Ortiz AM, Engram JC, Paiardini M, Lawson B, Miller MD, Else J, Pandrea I, Estes JD, Apetrei C, Schmitz JE, Ribeiro RM, Perelson AS, Silvestri G. CD8+ lymphocytes control viral replication in SIVmac239-infected rhesus macaques without decreasing the lifespan of productively infected cells. *PLoS Pathog*. 6:e1000747. [PubMed: 20126441]
8. Wong JK, Strain MC, Porrata R, Reay E, Sankaran-Walters S, Ignacio CC, Russell T, Pillai SK, Looney DJ, Dandekar S. In vivo CD8+ T-cell suppression of siv viremia is not mediated by CTL clearance of productively infected cells. *PLoS Pathog*. 2010; 6:e1000748. [PubMed: 20126442]
9. Chen CH, Weinhold KJ, Bartlett JA, Bolognesi DP, Greenberg ML. CD8+ T lymphocyte-mediated inhibition of HIV-1 long terminal repeat transcription: a novel antiviral mechanism. *AIDS Res Hum Retroviruses*. 1993; 9:1079–86. [PubMed: 8312050]
10. Mackewicz CE, Blackbourn DJ, Levy JA. CD8+ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription. *Proc Natl Acad Sci U S A*. 1995; 92:2308–12. [PubMed: 7534418]
11. Tomaras GD, Lacey SF, McDanal CB, Ferrari G, Weinhold KJ, Greenberg ML. CD8+ T cell-mediated suppressive activity inhibits HIV-1 after virus entry with kinetics indicating effects on virus gene expression. *Proc Natl Acad Sci U S A*. 2000; 97:3503–8. [PubMed: 10725407]
12. Walker CM, Levy JA. A diffusible lymphokine produced by CD8+ T lymphocytes suppresses HIV replication. *Immunology*. 1989; 66:628–30. [PubMed: 2497065]
13. Tomaras GD, Greenberg ML. CD8+ T cell mediated noncytolytic inhibition of human immunodeficiency virus type I. *Front Biosci*. 2001; 6:D575–98. [PubMed: 11229882]
14. Brinchmann JE, Gaudernack G, Vartdal F. CD8+ T cells inhibit HIV replication in naturally infected CD4+ T cells. Evidence for a soluble inhibitor. *J Immunol*. 1990; 144:2961–6. [PubMed: 1969879]
15. Walker CM, Erickson AL, Hsueh FC, Levy JA. Inhibition of human immunodeficiency virus replication in acutely infected CD4+ cells by CD8+ cells involves a noncytotoxic mechanism. *J Virol*. 1991; 65:5921–7. [PubMed: 1920621]
16. Wiviott LD, Walker CM, Levy JA. CD8+ lymphocytes suppress HIV production by autologous CD4+ cells without eliminating the infected cells from culture. *Cell Immunol*. 1990; 128:628–34. [PubMed: 1972661]
17. Geiben-Lynn R, Kursar M, Brown NV, Kerr EL, Luster AD, Walker BD. Noncytolytic inhibition of X4 virus by bulk CD8(+) cells from human immunodeficiency virus type 1 (HIV-1)-infected persons and HIV-1-specific cytotoxic T lymphocytes is not mediated by beta-chemokines. *J Virol*. 2001; 75:8306–16. [PubMed: 11483776]
18. Mosioan A, Teixeira A, High AA, Christian RE, Hunt DF, Shabanowitz J, Liu X, Klotman M. Novel function of prothymosin alpha as a potent inhibitor of human immunodeficiency virus type 1 gene expression in primary macrophages. *J Virol*. 2006; 80:9200–6. [PubMed: 16940531]
19. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science*. 1995; 270:1811–5. [PubMed: 8525373]
20. Kaur G, Tuen M, Virland D, Cohen S, Mehra NK, Munz C, Abdelwahab S, Garzino-Demo A, Hioe CE. Antigen stimulation induces HIV envelope gp120-specific CD4(+) T cells to secrete CCR5 ligands and suppress HIV infection. *Virology*. 2007; 369:214–25. [PubMed: 17765942]
21. Saha K, Bentsman G, Chess L, Volsky DJ. Endogenous production of beta-chemokines by CD4+, but not CD8+, T-cell clones correlates with the clinical state of human immunodeficiency virus type 1 (HIV-1)-infected individuals and may be responsible for blocking infection with non-syncytium-inducing HIV-1 in vitro. *J Virol*. 1998; 72:876–81. [PubMed: 9420304]
22. Lacey SF, McDanal CB, Horuk R, Greenberg ML. The CXC chemokine stromal cell-derived factor 1 is not responsible for CD8+ T cell suppression of syncytia-inducing strains of HIV-1. *Proc Natl Acad Sci U S A*. 1997; 94:9842–7. [PubMed: 9275213]

23. Freel SA, Lamoreaux L, Chattopadhyay PK, Saunders K, Zarkowsky D, Overman RG, Ochsenbauer C, Edmonds TG, Kappes JC, Cunningham CK, Denny TN, Weinhold KJ, Ferrari G, Haynes BF, Koup RA, Graham BS, Roederer M, Tomaras GD. Phenotypic and functional profile of HIV-inhibitory CD8 T cells elicited by natural infection and heterologous prime/boost vaccination. *J Virol*. 2010; 84:4998–5006. [PubMed: 20200250]
24. Saunders KO, Freel SA, Overman RG, Cunningham CK, Tomaras GD. Epigenetic regulation of CD8(+)T-lymphocyte mediated suppression of HIV-1 replication. *Virology*. 2010
25. Hebbes TR, Thorne AW, Crane-Robinson C. A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J*. 1988; 7:1395–402. [PubMed: 3409869]
26. Grabar S, Selinger-Leneman H, Abgrall S, Pialoux G, Weiss L, Costagliola D. Prevalence and comparative characteristics of long-term nonprogressors and HIV controller patients in the French Hospital Database on HIV. *AIDS*. 2009; 23:1163–9. [PubMed: 19444075]
27. Deeks SG, Walker BD. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity*. 2007; 27:406–16. [PubMed: 17892849]
28. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, Martino L, Hallahan CW, Selig SM, Schwartz D, Sullivan J, Connors M. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A*. 2000; 97:2709–14. [PubMed: 10694578]
29. Emu B, Sinclair E, Hatano H, Ferre A, Shacklett B, Martin JN, McCune JM, Deeks SG. HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control. *J Virol*. 2008; 82:5398–407. [PubMed: 18353945]
30. Blankson JN. Effector mechanisms in HIV-1 infected elite controllers: highly active immune responses? *Antiviral Res*. 2010; 85:295–302. [PubMed: 19733595]
31. Blackburn DJ, Mackewicz CE, Barker E, Hunt TK, Herndier B, Haase AT, Levy JA. Suppression of HIV replication by lymphoid tissue CD8+ cells correlates with the clinical state of HIV-infected individuals. *Proc Natl Acad Sci U S A*. 1996; 93:13125–13130. [PubMed: 8917555]
32. Copeland KF, Leith JG, McKay PJ, Kelleher L, Smaill FM, Rosenthal KL. CD8+ T cell-mediated suppression of HIV long terminal repeat-driven gene expression is not associated with improved clinical status. *Aids*. 1997; 11:581–6. [PubMed: 9108939]
33. Lacey SF, Weinhold KJ, Chen CH, McDaniel C, Oei C, Greenberg ML. Herpesvirus saimiri transformation of HIV type 1 suppressive CD8+ lymphocytes from an HIV type 1-infected asymptomatic individual. *AIDS Res Hum Retroviruses*. 1998; 14:521–31. [PubMed: 9566555]
34. Overman RG, Llorens AL, Greenberg ML, Garcia-Blanco MA, Tomaras GD. Initiation of Human Immunodeficiency Virus Type 1 (HIV-1) Transcription is Inhibited by Noncytolytic CD8+ Suppression. *The Open Virology Journal*. 2007; 1:1–7. [PubMed: 19440453]
35. Connor RI, Chen BK, Choe S, Landau NR. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology*. 1995; 206:935–44. [PubMed: 7531918]
36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods*. 2001; 25:402–8. [PubMed: 11846609]
37. Gomez AM, Smaill FM, Rosenthal KL. Inhibition of HIV replication by CD8+ T cells correlates with CD4 counts and clinical stage of disease. *Clin Exp Immunol*. 1994; 97:68–75. [PubMed: 7518369]
38. Landay AL, Mackewicz CE, Levy JA. An activated CD8+ T cell phenotype correlates with anti-HIV activity and asymptomatic clinical status. *Clin Immunol Immunopathol*. 1993; 69:106–16. [PubMed: 8403538]
39. Su Y, Popik W, Pitha PM. Inhibition of human immunodeficiency virus type 1 replication by a Tat-activated, transduced interferon gene: targeted expression to human immunodeficiency virus type 1-infected cells. *J Virol*. 1995; 69:110–21. [PubMed: 7983701]
40. Brinchmann JE, Gaudernack G, Vartdal F. In vitro replication of HIV-1 in naturally infected CD4+ T cells is inhibited by rIFN alpha 2 and by a soluble factor secreted by activated CD8+ T cells, but not by rIFN beta, rIFN gamma, or recombinant tumor necrosis factor-alpha. *J Acquir Immune Defic Syndr*. 1991; 4:480–8. [PubMed: 1673156]

41. Baier M, Werner A, Bannert N, Metzner K, Kurth R. HIV suppression by interleukin-16. *Nature*. 1995; 378:563. [PubMed: 8524386]
42. Weissman D, Poli G, Fauci AS. Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor alpha and interleukin 6 induction of virus. *AIDS Res Hum Retroviruses*. 1994; 10:1199–206. [PubMed: 7848677]
43. Pal R, Garzino-Demo A, Markham PD, Burns J, Brown M, Gallo RC, DeVico AL. Inhibition of HIV-1 infection by the beta-chemokine MDC. *Science*. 1997; 278:695–8. [PubMed: 9381181]
44. Cocchi F, DeVico AL, Demo AG, Gallo RC. Autoresistance to X4 HIV Infection by soluble suppressor factors secreted from CD4+ T cells. *J AIDS*. 2009; 51
45. Oberlin E, Amara A, Bachelier F, Bessia C, Virelizier JL, Arenzana-Seisdedos F, Schwartz O, Heard JM, Clark-Lewis I, Legler DF, Loetscher M, Baggiolini M, Moser B. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature*. 1996; 382:833–5. [PubMed: 8752281]
46. Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev*. 1998; 12:599–606. [PubMed: 9499396]
47. Sun D, Ding A. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. *Nat Immunol*. 2006; 7:375–81. [PubMed: 16491077]
48. Saez-Cirion A, Lacabartz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, Barre-Sinoussi F, Delfraissy JF, Sinet M, Pancino G, Venet A. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci U S A*. 2007; 104:6776–81. [PubMed: 17428922]
49. Kaslow RA, Carrington M, Apple R, Park L, Munoz A, Saah AJ, Goedert JJ, Winkler C, O'Brien SJ, Rinaldo C, Detels R, Blattner W, Phair J, Erlich H, Mann DL. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med*. 1996; 2:405–11. [PubMed: 8597949]
50. Mackewicz CE, Ortega H, Levy JA. Effect of cytokines on HIV replication in CD4+ lymphocytes: lack of identity with the CD8+ cell antiviral factor. *Cell Immunol*. 1994; 153:329–43. [PubMed: 7907003]
51. Horuk R, Hesselgesser J, Zhou Y, Faulds D, Halks-Miller M, Harvey S, Taub D, Samson M, Parmentier M, Rucker J, Doranz BJ, Doms RW. The CC chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains. *J Biol Chem*. 1998; 273:386–91. [PubMed: 9417093]
52. Geiben-Lynn R, Kursar M, Brown NV, Addo MM, Shau H, Lieberman J, Luster AD, Walker BD. HIV-1 antiviral activity of recombinant natural killer cell enhancing factors, NKEF-A and NKEF-B, members of the peroxiredoxin family. *J Biol Chem*. 2003; 278:1569–74. [PubMed: 12421812]
53. Wilkinson J, Zaunders JJ, Carr A, Guillemin G, Cooper DA. Characterization of the phenotypic and lymphokine profile associated with strong CD8+ anti-HIV-1 suppressor activity (CASA). *Clin Exp Immunol*. 2002; 127:145–50. [PubMed: 11882045]
54. Amella CA, Sherry B, Shepp DH, Schmidtmayerova H. Macrophage inflammatory protein 1alpha inhibits postentry steps of human immunodeficiency virus type 1 infection via suppression of intracellular cyclic AMP. *J Virol*. 2005; 79:5625–31. [PubMed: 15827177]
55. Cocchi F, DeVico AL, Yarchoan R, Redfield R, Cleghorn F, Blattner WA, Garzino-Demo A, Colombini-Hatch S, Margolis D, Gallo RC. Higher macrophage inflammatory protein (MIP)-1alpha and MIP-1beta levels from CD8+ T cells are associated with asymptomatic HIV-1 infection. *Proc Natl Acad Sci U S A*. 2000; 97:13812–7. [PubMed: 11095721]
56. Creery D, Weiss W, Graziani-Bowering G, Kumar R, Aziz Z, Angel JB, Kumar A. Differential regulation of CXCR4 and CCR5 expression by interleukin (IL)-4 and IL-13 is associated with inhibition of chemotaxis and human immunodeficiency Virus (HIV) type 1 replication but not HIV entry into human monocytes. *Viral Immunol*. 2006; 19:409–23. [PubMed: 16987060]
57. Kedzierska A, Turowski G. HLA class I antigens in families with coeliac disease. *Med Sci Monit*. 2000; 6:957–63. [PubMed: 11208438]
58. Richardson RM, Tokunaga K, Marjoram R, Sata T, Snyderman R. Interleukin-8-mediated heterologous receptor internalization provides resistance to HIV-1 infectivity. Role of signal strength and receptor desensitization. *J Biol Chem*. 2003; 278:15867–73. [PubMed: 12594210]

59. Rollenhagen C, Asin SN. IL-8 decreases HIV-1 transcription in peripheral blood lymphocytes and ectocervical tissue explants. *J Acquir Immune Defic Syndr*. 2010; 54:463–9. [PubMed: 20577090]
60. Frade JM, Llorente M, Mellado M, Alcamí J, Gutierrez-Ramos JC, Zaballos A, Real G, Martínez AC. The amino-terminal domain of the CCR2 chemokine receptor acts as coreceptor for HIV-1 infection. *J Clin Invest*. 1997; 100:497–502. [PubMed: 9239395]
61. Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, Baggiolini M, Moser B. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med*. 1996; 184:963–9. [PubMed: 9064356]
62. Hercus TR, Thomas D, Guthridge MA, Ekert PG, King-Scott J, Parker MW, Lopez AF. The granulocyte-macrophage colony-stimulating factor receptor: linking its structure to cell signaling and its role in disease. *Blood*. 2009; 114:1289–98. [PubMed: 19436055]
63. Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A. Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor. *Proc Natl Acad Sci U S A*. 1990; 87:9655–9. [PubMed: 1702217]
64. Brizzi MF, Aronica MG, Rosso A, Bagnara GP, Yarden Y, Pegoraro L. Granulocyte-macrophage colony-stimulating factor stimulates JAK2 signaling pathway and rapidly activates p93fcs, STAT1 p91, and STAT3 p92 in polymorphonuclear leukocytes. *J Biol Chem*. 1996; 271:3562–7. [PubMed: 8631962]
65. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *EMBO J*. 1994; 13:1591–600. [PubMed: 8156998]
66. Chang TL, Mosoian A, Pine R, Klotman ME, Moore JP. A soluble factor(s) secreted from CD8(+) T lymphocytes inhibits human immunodeficiency virus type 1 replication through STAT1 activation. *J Virol*. 2002; 76:569–81. [PubMed: 11752148]

Fig. 1. Soluble CD8⁺ T-lymphocyte noncytolytic suppression of HIV-1 replication. Seronegative (NEG, triangles), virus controller (VC; circles), or HVS-transformed (JR-HVS; squares) CD8⁺ T-lymphocytes were cultured in a transwell microtiter plate with HIV-1 infected cells at a 0.5:1 effector to target ratio. Suppression of HIV-1 replication is shown as the mean log_{10} reduction in HIV-1 replication. Significant reduction of replication compared to seronegative CD8⁺ T-lymphocytes was determined by Student's *t*-test (** $p < 0.01$; *** $p < 0.001$).

Fig. 2.

Protein concentration of cytokines secreted by soluble suppressive CD8⁺ T-lymphocytes during noncytolytic suppression. CD8⁺ T-lymphocytes were cultured with HIV-1 infected cells at 0.25:1, 0.5:1, and 1:1 effector to target (E:T) ratios. Protein concentration in the cell culture supernatant was determined by luminex. HIV-1 infected target cells cultured alone (0 E:T ratio) serve as the baseline measurement. Mean protein concentration, standard error, and linear trend lines are shown for JR-HVS, HIV-1 seronegative and virus controller (VC) CD8⁺ T cell co-cultures. CD8⁺ T-lymphocytes with significant soluble suppression are classified as soluble suppressors (SS, solid lines), and those lacking soluble suppression are designated as non-soluble suppressor (NSS, dotted lines).

Fig. 3. Protein concentration of cytokines secreted only by JR-HVS or by none of the CD8⁺ T-lymphocytes during soluble noncytolytic suppression. Cell culture supernatants were analyzed as in Fig. 2. Protein concentration from infected target cells cultured alone is shown as the 0 E:T ratio. The mean protein concentrations with linear trend lines for seronegative, VCs, and JR-HVS CD8⁺ T-lymphocytes are displayed for the three indicated E:T ratios. CD8⁺ T-lymphocytes are designated as soluble suppressors (SS, solid lines) or non-soluble suppressor (NSS, dotted lines). (A) Cytokines secreted highly only by JR-HVS CD8⁺ T-lymphocytes. (B) Antiviral cytokines that are produced at indistinguishable low levels by all 8 CD8⁺ T-lymphocytes.

Fig. 4.

Cytokine concentrations at different levels of soluble noncytolytic suppression. Cytokine concentrations were determined in the media of soluble noncytolytic suppression assays by luminex. Percent suppression of HIV-1 replication was measured from the same co-cultures containing seronegative (NEG, blue triangles), VC (green circles), or JR-HVS (red squares) CD8⁺ T-lymphocytes. Infected targets cells served as the baseline measurement for cytokine concentration (IC, open diamonds). Symbols represent mean percent suppression and protein concentration in pg/mL. A nonlinear curve was fit to each plot using a Hill function as described in Section 2. Materials and Methods.

Fig. 5.

Histone hyperacetylation regulates the suppressive ability of CD8⁺ T-lymphocytes and expression of cytokines. (A) CD8⁺ T-lymphocytes were cultured for 16 h in growth media supplemented with 2.0 mM valproic acid (filled bars) or PBS (open bars) as a vehicle control. Subsequently, the CD8⁺ T-lymphocytes were used in a direct contact noncytolytic CD8 suppression assay. Mean log₁₀ reduction in HIV-1 replication and standard error are shown. Significance was determined with a Student's *t*-test (* *p* < 0.05; ** *p* < 0.01). (B) RNA was isolated from the valproic acid-treated or control CD8⁺ T-lymphocytes used in (A), and differences in gene expression of 164 cytokine and cytokine receptor genes were analyzed by real-time RT-PCR Array. Mean fold change in expression of each gene is displayed, with negative fold changes designating down-regulated genes, and positive fold changes signifying genes upregulated in valproic acid-treated cells compared to control cells. Genes within the area shaded in gray were considered unchanged (less than a 3-fold change in expression). Fold down-regulation of genes in JR-HVS (C) and VC18 (D) CD8⁺ T-lymphocytes after inhibition of histone deacetylation.

Fig. 6.

Genes downregulated by histone hyperacetylation are expressed higher in cells with noncytolytic suppression. The expression of genes regulated by histone hyperacetylation was quantified with real-time RT-PCR. Normalized gene expression was calculated by the $\Delta\Delta C_t$ method described in the Section 2. Materials and Methods. (A) Genes expressed higher in JR-HVS and VC18 than in seronegative CD8⁺ T-lymphocytes. (B) Genes only expressed higher in JR-HVS than seronegative CD8⁺ T-lymphocytes. (C) Genes not suppressed higher in either JR-HVS or VC18 compared to seronegative CD8⁺ T-lymphocytes. Student's *t*-test was used to test for significant increases in expression compared to seronegative CD8⁺ T-lymphocytes (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Table 1

Spearman correlation coefficients of protein concentration and percent suppression for CD8⁺ T-lymphocytes individually and combined

Gene Name	JR-HVS Suppression	VC18 Suppression	^a JR-HVS and VC18 Suppression Combined	^{b,c} All Cell types Combined
MIP-1 beta	0.788	0.905	0.858	0.891
MIP-1 alpha	0.758	0.912	0.783	0.867
IP-10	0.960	0.891	0.912	0.827
MIG	0.865	0.877	0.891	0.801
IL-1 alpha	0.744	0.812	0.736	0.799
Interferon gamma	0.721	0.859	0.792	0.799

^aProtein concentration and percent suppression for JR-HVS and VC18 was combined into one data set for analysis.

^bProtein concentrations and percent suppressions for VC3, VC9, VC14, VC18, JR-HVS and Seronegative CD8⁺ T cells were combined into one data set for analysis.

^c $p < 0.0001$ for the cytokines shown.

Table 2

Genes upregulated by more than 10-fold in JR-HVS and VC18 after inhibition of histone deacetylation.

JR-HVS		VC18	
Gene	Fold Upregulation	Gene	Fold Upregulation
BMP4	57.0	BMP4	31.5
IL1F6	41.1	TGFA	27.7
CCR3	17.1	IL1F6	26.5
IL2	13.2	IL2	19.7
CMKLR1	10.7	GDF9	18.1
		CMKLR1	15.2
		CMTM4	14.3
		CXCL13	13.6
		CCR10	12.4
		IFNK	10.9
		CCL18	10.5
		TLR2	10.6

Table 3

Genes Identified as potential mediators of noncytolytic suppression.

Gene	JR-HVS CD8 ⁺		VC18 CD8 ⁺	
	<i>a</i> High Secretion	Regulated by HDAC	<i>a</i> High Secretion	Regulated by HDAC
<i>β-chemokines</i>				
MIP-1 Beta	+	-	+	-
MIP-1 Alpha	+	+	+	+
MCP-1	-	+	-	-
I-309	+	+	-	-
<i>α-chemokines</i>				
BCA-1	-	+	-	-
IL-8	-	+	-	-
<i>Interferon Response</i>				
IFN Gamma	+	-	+	-
IP-10	+	+	+	-
MIG	+	-	+	-
I-TAC	+	+	+	-
<i>TNF Family</i>				
TNF-Alpha	+	-	-	-
LTA/TNF-Beta	-	-	-	+
LTB	NA	+	NA	+
<i>Colony Stimulating Factors</i>				
M-CSF	-	-	-	+
GM-CSF	+	+	-	-
IL-3	-	+	-	-
<i>Immunoregulatory Interleukins</i>				
IL-9	-	+	-	-
IL-1 Alpha	+	-	+	-
IL-13	+	+	-	-

^aHigh secretion is defined as 3-fold greater secretion than seronegative and VC3 CD8⁺ T-lymphocytes NA, Analyte not available in the Milliplex platform